Options for the Control of Influenza VIII

Cape Town, South Africa
5-10 September 2013

Abstracts

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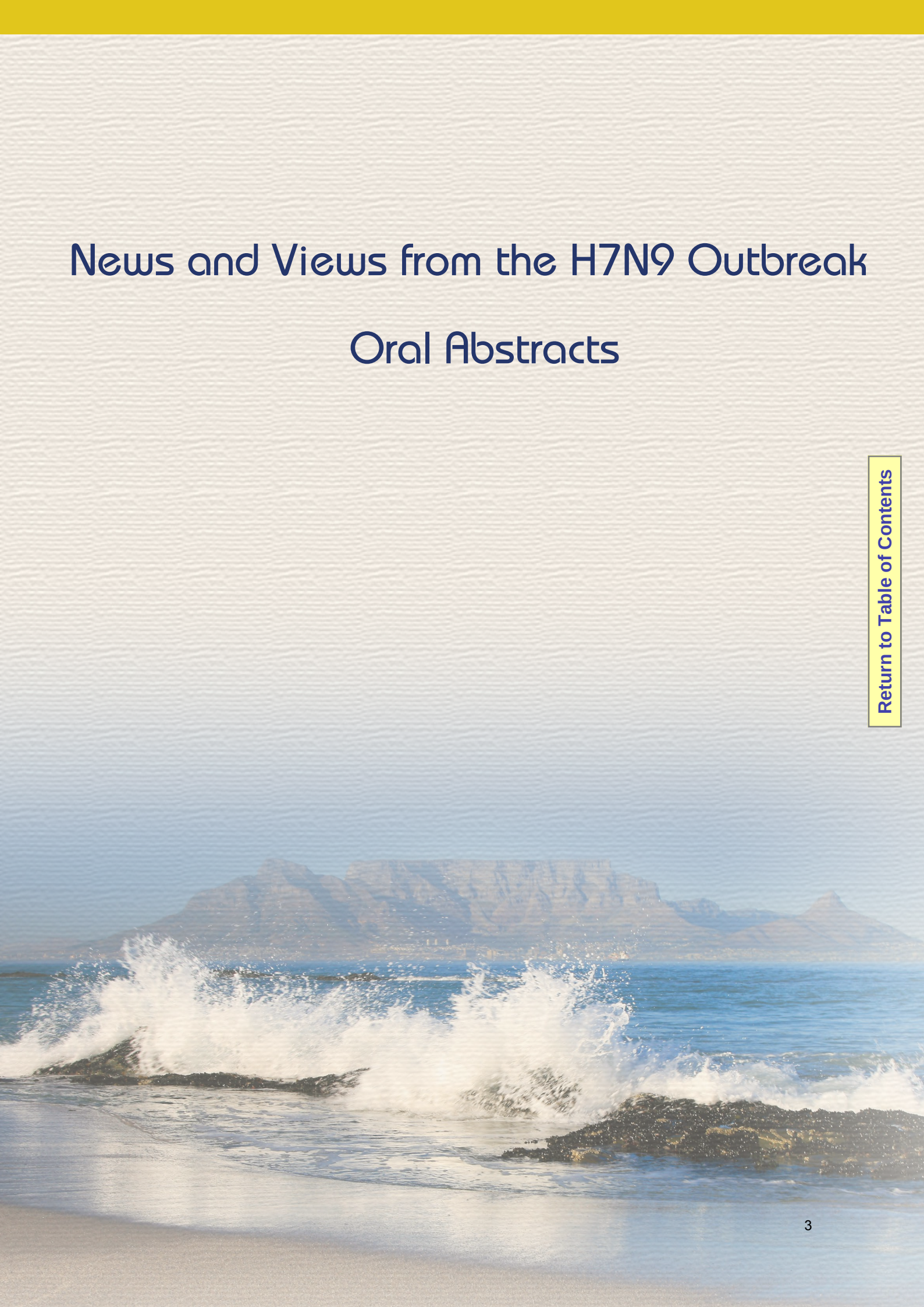

O-800

Comparative epidemiology of human infections with avian influenza A(H7N9) and A(H5N1) viruses in China

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Background: The novel influenza A(H7N9) virus recently emerged, while influenza A(H5N1) virus has infected humans since 2003 in mainland China. A striking feature of A(H7N9) is the relatively rapid accumulation of laboratory-confirmed cases in humans, even though phylogenetic and epidemiologic evidence points to predominantly zoonotic transmission. In contrast A(H5N1), similarly an exclusive zoonosis with very few exceptions, has caused only 43 laboratory-confirmed cases in China since 2003. We compared the epidemiologic characteristics of the complete series of laboratory-confirmed cases of both viruses in mainland China to date. Materials and Methods: An integrated database was constructed with information on demographic, epidemiologic, and clinical variables of laboratory-confirmed A(H7N9) and A(H5N1) cases that were reported to the Chinese Center for Disease Control and Prevention up to May 24, 2013. We described disease occurrence by age, sex and geography and estimated key epidemiologic parameters including the incubation period, onset to admission interval, onset to laboratory confirmation interval, and admission to death and discharge intervals using survival analysis. Results: Among 130 and 43 patients with confirmed A(H7N9) and A(H5N1), respectively, the median ages were 62 y and 26 y. Thirty three (77%) of the A(H5N1) cases occurred in the winter months November through February. In urban areas, 74% of cases of both viruses were male whereas in rural areas the proportions were 62% for A(H7N9) and 33% for A(H5N1). Among cases of A(H7N9) and A(H5N1), 75% and 71% reported recent exposure to poultry. Symptoms at illness onset were relatively similar between the two viruses, with fever and cough being the most frequently reported symptoms, but less frequently so for A(H5N1). The mean incubation periods of A(H7N9) and A(H5N1) were 3.1 and 3.3 days, respectively. On average, 21 and 18 contacts were traced for each A(H7N9) case in urban and rural areas, respectively; compared with 90 and 63 for A(H5N1). The hospitalization fatality risk was 35% (95% CI: 25%, 44%) for A(H7N9) and 70% (95% CI: 56%, 83%) for A(H5N1). Conclusions: The sex ratios in urban compared with rural cases are consistent with poultry exposure driving the risk of infection. However the difference in susceptibility to serious illness with the two different viruses remains unexplained, given that most A(H7N9) cases were in older adults while most A(H5N1) cases were in younger individuals. Our estimates of biological parameters, such as the incubation period and to some extent the hospitalization fatality risk, should generalize to other countries. If A(H7N9) follows a similar pattern to A(H5N1), it is possible that the A(H7N9) epidemic may reappear in the fall. This potential lull should be an opportunity for discussion of definitive preventive public health measures and optimization of clinical management, as well as capacity building in the region, given the possibility that A(H7N9) may spread outside China's borders.

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Mammalian adaptation markers in avian-origin H7N9 virus, a comprehensive investigation in isolates and clinical specimens from the H7N9 influenza affected area

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Background: An avian-origin H7N9 virus emerged in eastern China in February 2013 and has since caused 133 confirmed human infections (Gao R, et al. *N Engl J Med*. 2013;368:1888-1897). We have compared human isolates and avian viruses isolated from epidemiologically linked poultry markets and confirmed that the human infections were caused by direct transmission from a poultry source (Chen Y, et al. *Lancet*. 2013;381:1916-1985). In addition to the Q226L substitution in the HA, which may provide virus with some ability to bind to human-type receptors, what other adaptations has this virus gained to make it different from other avian influenza viruses? Materials and Methods: We characterized H7N9 and other related H7 and N9 subtype viruses isolated in April 2013 from local poultry markets that were associated with human infections. Genetic mutations, polymerases activity, growth kinetic in mammalian and avian cells and replication ability in mice were determined using reverse genetic versions of H7N9 virus. Results: Replication ability and growth kinetics of the avian H7 subtype influenza viruses were compared in avian and mammalian cell lines. Our data suggest that the reassortant H7N9 virus has adapted to, and may have become established in, land-based poultry. It is currently not clear if this virus may still be circulating in some poultry populations, continuing to evolve and posing a threat for further human infections. We studied the virus genome for mammalian adaptation markers by performing sequence analysis on virus isolates and RT-PCR products derived from samples obtained from 46 patients hospitalized in the First Affiliated Hospital of Zhejiang University Medical School, Hangzhou, China. Virus adaptation markers in the HA, NA and PB2 genes were analyzed in sequential samples. Multiple adaptation markers were identified in these genes of clinical isolates and serial respiratory samples. Our data showed that the avian-origin H7N9 has attempted to adapt to replicate in human cells using various mechanisms already displayed by other viruses. An in vitro assay showed that viruses with substitutions at these positions exhibit enhanced RNP polymerase activity, and a study of growth kinetics demonstrated that isolates carrying these adaptation markers replicate to a higher titer in mammalian cells. Replication efficiency of these clinical variants was also evaluated in mice. As neuraminidase inhibitors are used as the first line of antiviral drugs for the treatment of H7N9 infection, we also analyzed oseltamivir resistance-associated mutations in the NA genes of viruses shed in either serial nasal swab or sputum specimens obtained from 40 of the hospitalized patients. Conclusions: This study provides a comprehensive analysis of avian-origin H7N9 virus from poultry and in human infections. The novel H7N9 virus attempted multiple adaptive strategies for efficient replication in humans. Further characterization of this H7N9 avian influenza virus for understanding the mechanism of replication adaptation and the role in efficient human transmission is necessary.

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Pathogenesis and transmission novel influenza A (H7N9) virus in poultry

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Background: The recent and ongoing outbreak of H7N9 influenza in China has resulted in many human cases with a high fatality rate. Poultry have been suspected as the source of infection based on sequence analysis and virus isolations from live bird markets; however it's not clear which species of birds are most likely to be infected and shedding sufficient levels of virus to infect humans. In these studies we evaluated the potential role of different avian species in the epidemiology of H7N9 influenza, information that is vital for effective disease control. Materials and Methods: The pathogenesis of the human isolate A/Anhui/1/2013(H7N9) was determined in White Leghorn chickens (egg layer type), turkeys, Japanese quail, pigeons, Pekin ducks, mallard ducks, Muscovy ducks, and Embden geese, by intranasal inoculation with 10^6 mean embryo infective doses (EID_{50}) of the virus. In addition, three doses of virus were administered to groups of quail, pigeons and Pekin ducks (10^2,10^4, or 10^6 EID_{50}), and uninfected birds were added to each dose group to determine the transmission potential of the virus. Oropharyngeal (OP) and cloacal (C) swabs were collected at 2, 4, 6, 8, and 10 days post inoculation (dpi) from all birds to examine for virus shedding. Viruses recovered from infected birds were also sequenced. Results: Inoculation of the avian species examined with the H7N9 virus resulted in infection of all species but no clinical signs. Virus shedding in quail, chickens, turkeys, and Muscovy ducks was much higher and prolonged (≥ 10 dpi) than in the rest of the species (≤ 6 dpi). Quail effectively transmitted the virus to direct contacts but pigeon sand Pekin ducks did not. In all species, virus was detected at much higher levels from OP swabs compared with C swabs. The HA gene and part of the PB2 gene from selected virus-positive OP swabs collected from chickens and quail were sequenced to examine for changes in the virus after passage in these species. All 8 samples examined had lysine at position 627 of the PB2 gene, similar to the parent human isolate. However, 3 amino acid differences were observed in the HA compared with A/Anhui/1/2013: N123D, N149D, and L217Q. The inoculum used in these experiments already contained D at position 123 and 149, but maintained the L at position 217. Four different combinations were observed in the infected birds, indicating that most likely the inoculum had virus subpopulations that were selected after passage in birds. Conclusions: The H7N9 virus outbreak clearly has an important poultry component based on the sequence analysis and epidemiology of the virus, and these experimental studies corroborate that poultry species are important reservoirs of the virus. The high viral shedding from chickens, turkeys, quail and Muscovy ducks creates a likely source of infection for humans; however, under the right circumstances (stress, immunosuppression), all species examined could be a source of the virus. The high levels of viral replication in the upper respiratory tract and much lower levels in the intestinal tract are characteristic of poultry-adapted influenza viruses, and consequentially testing of bird species should preferentially be conducted with OP swabs for best sensitivity. As the H7N9 virus continues to circulate in poultry, changes—including both mutations and possible reassortment with other influenza viruses—are expected to occur. Continuous monitoring of influenza viruses in poultry is essential for prevention of future pandemics.

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Lessons learned from the first case of H7N9and H6N1 in Taiwan—public health implication from integrated surveillance from animals to humans

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Background: Avian influenza viruses (AIVs) donated viral genes to novel influenza viruses and resulted in past pandemics. In Asia, areas with high poultry densities, high population densities and wet markets, the interphase between animals and humans becomes the important source of spreading the newly reassorted influenza viruses. The emerging epidemics of the highly pathogenic avian influenza (HPAI) H5N1 virus since 1997, the pandemic influenza H1N1 virus in 2009 plus recent outbreaks of H7N9 in China all have highlighted the crucial importance of integrated surveillance and timely genetic analyses of the influenza viruses isolated from human and animal hosts. Materials and Methods: The first case of H7N9 outside China was reported in April in Taiwan. Field epidemiologic investigation started from the Centers for Disease Control and Prevention in Taiwan (Taiwan-CDC), and both throat and sputum specimens were collected and passaged to MDCK cells at the Clinical Virological Laboratory of National Taiwan University Hospital (NTUH) on April 22nd. Quantitative viral load was measured by real-time PCR. To verify the results, Taiwan-CDC took another sputum specimen on April 24th and cultivated the virus in embyonated chicken eggs. For better integrated surveillance, the two avian H7N9 strains and the selected 35 H7 and 12 N9 AI viruses isolated from wild birds in different years from February of 1998 to March of 2013 in Taiwan were sequenced, and their viral sequences were obtained and compared from the NCBI and GISAID gene banks for timely analyses. Furthermore, monthly serum samples collected from chickens in a wholesale live bird market (LBM) were tested for H6N1 for enhanced serologic surveillance, once a human H6N1 case was identified. Results: Epidemiologic investigation found that the laboratory-confirmed H7N9 patient did not have any contacts with avian species nor visited an LBM but worked in Jiangsu province and flew through Shanghai Airport. Since this case was very severe with dyspnea on April 19, 2013, and was put on extracorporeal membrane oxygenation on April 22nd, the viral sequences of both throat and sputum specimens from NTHU and Taiwan-CDC were compared, respectively, and identified amino acid variations plus heterogeneous populations of Tamiflu-resistant signatures. On the other hand, the avian H7N9, H7 and N9 viruses isolated from wild birds flying into Taiwan were quite different from Taiwan's human H7N9 isolates. In addition, the HA sequences of these wild bird isolates revealed temporal variations in various groupings of phylogenetic trees shown by different isolation years. All the NA segments of wild bird–derived N9 and H7N9 viruses did not have the five amino acid deletions as those human H7N9 viruses in China, implying that the NA gene of human H7N9 viruses probably went through further molecular adaptation from waterfowls to chickens or other unidentified hosts. For serologic surveillance of H6N1, seroprevalence of HI antibody against chicken H6N1 virus increased with higher serotiters, indicating the possible endemicity of this virus in chicken farms. Conclusions: The experiences in human cases of low pathogenic avian influenza (LPAI) H7N9 and H6N1 viruses in China and Taiwan have demonstrated that the increasing epidemic potential of these LPAI viruses in poultry cannot be ignored. Continuous surveillance and monitoring molecular changes and adaptation of AIVs in different host species is very important not only to understand the origin of viral genes but also to prevent large-scale epidemics of severe human influenza cases and possible emergence of novel influenza viruses leading to future pandemics.

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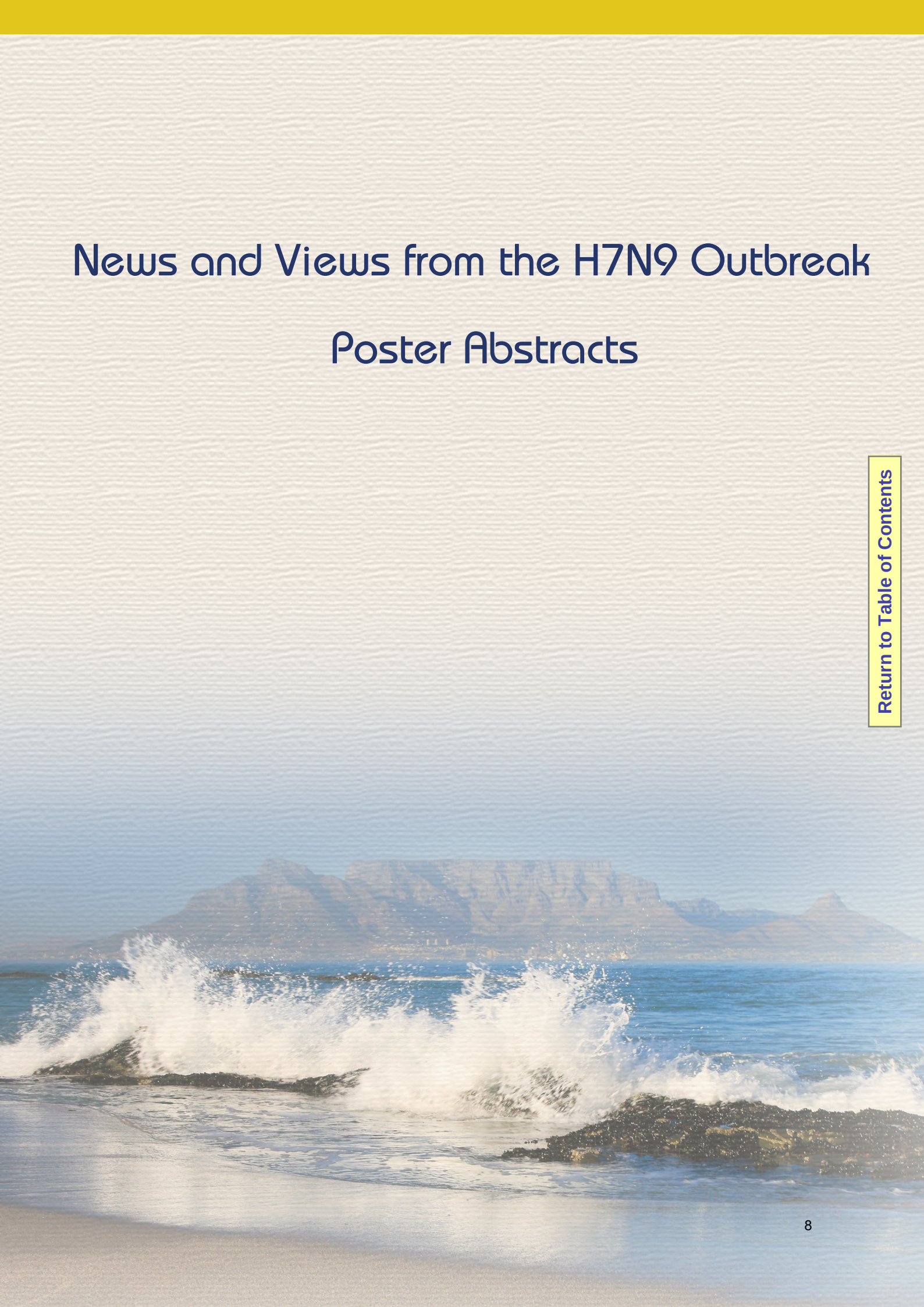
Poster Abstracts



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The hospitalization fatality risk and symptomatic case fatality risk of human infection with avian influenza A(H7N9) virus

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Background: Characterizing the severity of infection with influenza viruses of animal origin in humans is part of the process of assessing the risk for a pandemic and predicting the epidemiology of the disease. We assessed the clinical severity of human infection with the avian influenza A (H7N9) virus, which recently emerged in China. Materials and Methods: Among hospitalized patients with laboratory-confirmed cases of influenza A (H7N9) infection, we estimated the risk of death and the need for mechanical ventilation and admission to the intensive care unit based on censored data obtained during the current outbreak, allowing for the competing risks of severe outcomes versus recovery. We also used information on laboratory-confirmed cases detected through sentinel influenza-like illness (ILI) surveillance to estimate the number of symptomatic A (H7N9) virus infections to date and the fatality risk associated with symptomatic cases. Results: Of 123 hospitalized patients infected with the avian influenza A (H7N9) virus, 36 had died and 65 had recovered by May 19, 2013. Hospitalization was associated with an increased risk for death (35%; 95% confidence interval [CI]: 26%-45%), mechanical ventilation or death (70%; 95% CI: 61%-78%), and ICU admission or mechanical ventilation or death (84%; 95% CI: 77%-91%). The risk of these severe outcomes increased with age. Depending on assumptions about the coverage of the sentinel ILI network and healthcare–seeking behavior of individuals identified through the ILI who were infected with influenza A (H7N9), we estimated a symptomatic case fatality risk of 160 to 2800 per 100,000 symptomatic cases. Conclusions: Our estimate of a symptomatic case fatality risk of 160 to 2800 deaths per 100,000 symptomatic cases places the severity of influenza A (H7N9) below that of influenza A (H5N1) but above influenza A (H1N1)pdm09. We are not aware of comparative data on the symptomatic fatality risk associated with seasonal influenza viruses, but we speculate that they are of a similar order of magnitude as influenza A (H1N1)pdm09. As with seasonal influenza, the severity of influenza A (H7N9) increases with age. The estimated risk of death among patients hospitalized with an infection with this virus and among symptomatic individuals indicates the severity of this infection. The severity should not be affected by shifts over time in the probability of laboratory confirmation of mild cases and should inform the assessment of that risk.

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Pre-existing CD8$^+$ T-cell immunity to the novel reassortant A(H7N9) influenza virus

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Background: A newly emerged avian-origin A(H7N9) influenza virus spreads through sporadic poultry-to-human transmission and causes severe clinical symptoms in the infected individuals. To date, the World Health Organization has reported 132 laboratory-confirmed cases with 37 deaths, which represents a mortality rate of 28%. Given that the reassortant A(H7N9) virus is new to the human population, it is most likely that individuals of all ages will lack humoral immunity against this highly pathogenic virus. As T-cell immunity directed at conserved viral peptides promotes cross-strain protective immunity, limits disease severity and leads to a more rapid host recovery, we asked whether influenza-specific memory CD8$^+$ T cells established after exposures to seasonal influenza epidemics would recognise the novel A(H7N9) virus. Materials and Methods: To understand the potential of pre-existing T-cell immunity against the newly emerged influenza A(H7N9) virus, we first analysed the conservation of CD8$^+$ T-cell epitopes between the A(H7N9) strains and influenza viruses that had circulated in humans over the last century. Our focus was on CD8$^+$ T-cell peptides within the most immunogenic Influenza A proteins: nucleoprotein (NP) and matrix 1 (M1). Using this approach, we identified CD8$^+$ T-cell immunogenic regions that are conserved, unique or variable in A(H7N9), and we further dissected the immunogenicity of these regions by carrying out immunological assays. Additionally, we performed a structural analysis to explain why the newly identified HLA-A*0101-restricted NP_{44}-Y9N variant in A(H7N9) evades CD8$^+$ T-cell surveillance. Finally, we estimated the potential level of T-cell immunity toward the newly emerged A(H7N9) virus by calculating population coverage based on HLA restriction of the conserved and unique A(H7N9)-derived CD8$^+$ T-cell immunogenic peptides. Results: Our data show a substantial level of conservation (at 100% match) for the CD8$^+$ T-cell epitopes within immunogenic viral proteins, with 20/39 conserved epitopes in M1 and 8/56 in NP that collectively provide ≈ 70-80% coverage across different HLAs, depending on ethnicity. Despite this, the immunological data revealed that the novel A(H7N9) reassortant escapes some of the most immunodominant human epitopes, such as HLA-A*0101-restricted NP_{44} (a unique mutation at the anchor residue) with allele prevalence of 27% in Oriental populations. Furthermore, the NP_{418} variant (restricted to the large B7 family) within A(H7N9) was identical to the NP_{418} within the A(H1N1)-1918 virus and of similar structural homology to that found in A(H1N1)pdm-2009. This suggests that individuals exposed to A(H1N1)pdm-2009, although not recently circulating seasonal strains, would have protective immunity to H7N9-NP_{418}. Conclusions: Our study presents the most comprehensive immunological data published to date on epitope-specific CD8$^+$ T-cell immunity to influenza viruses circulating over the last century. Furthermore, our data on the high-level epitope conservation within the newly emerged A(H7N9) virus suggest that T cell–based vaccines have the potential to protect us against severe influenza infection and rapid viral spread by providing broad coverage against unexpected evolving influenza A strains.

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Molecular influenza surveillance with the FluSurver: H7N9 case study

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Background: Interest in new outbreaks as well as regular surveillance of circulating seasonal strains produce a constant flow of influenza sequences that need to be analyzed and interpreted for epidemiological and phenotypic features. Several steps in typical influenza sequence analysis can be automated, and we have been actively developing the free online analysis pipeline FluSurver over the last 4 years to facilitate identification and interpretation of mutations in influenza sequences. Materials and Methods: The FluSurver is implemented as a webserver (http://flusurver.bii.a-star.edu.sg/) where users can upload their sequences for fully automated analysis, which includes ultra-fast database searches with TACHYON, alignment with MAFFT, structural modeling with MODELLER, structure viewing with JMOL and several PERL scripts to link mutations to in-house derived databases. These include geographic and temporal frequency of occurrence as well as co-occurrence of mutations, curated literature annotations for > 200 known mutation effects, such as drug resistance, host receptor specificity, virulence, antigenic drift and antibody escape mutants. We also show the position of the mutation(s) in structural models and highlight if mutations are close to common drug, host receptor or antibody binding sites or if a glycosylation motif is lost or created through a mutation. Notable recent additions/changes are: updated reference strains, ultra-fast database searches using TACHYON, added city-level detail to map view, passage history bias information for selected mutations, integration into GISAID (beta), expansion of help and tutorial sections for guidance of using the results in publications and to avoid overinterpretation. Results: The FluSurver has already been instrumental in the discovery of new influenza strain variants with altered antiviral susceptibility, host specificity, glycosylation and antigenic properties. To showcase the usefulness and speed of analysis made possible with our tool, we use the recent H7N9 outbreak sequences deposited in GISAID as new case study. After submitting the sequences of the first 3 human cases, it only takes a few seconds to identify the critical HA-Q226L and PB2-E627K mutations that are known adaptations, facilitating infection of mammalian hosts. Further browsing the alignment results identifies absence of a multibasic HA cleavage site, suggesting low pathogenicity in birds, a small stalk deletion in NA of unclear significance, common resistance to amantadines through M2-S31N, normal neuraminidase drug-sensitivity at NA-H274, one isolated strain with NA-R292K, which is associated with drug-treatment–induced resistance in closely related avian N9 strains. Another common feature of the early H7N9 outbreak sequences is a truncation of NS1 through an early stop codon, resulting in removal of the PDZ-binding motif, which is normally used for binding and interfering with host proteins. When including the avian and environmental isolates in the analysis, one can see that while the closest avian precursor strains from 2011-2012 did not have the Q226L host specificity mutation, surprisingly, the recent outbreak-linked avian samples already had this mutation, which must therefore also be fit to circulate in birds to some extent. At the same time, PB2-E627K was only found in human samples, suggesting a stepwise acquisition of host adaptation factors. The FluSurver analysis also highlights unusually diverse strains, eg, A/Shanghai/1/2013 compared to A/Anhui/1/2013 and A/Shanghai/2/2013, which would indicate multiple introduction events from birds to humans instead of sustained human-to-human transmission in the beginning of the outbreak. Conclusions: The FluSurver allows researchers, clinician scientists and surveillance labs to rapidly screen their influenza sequences for potentially interesting mutations to identify candidates for phenotypic changes or special epidemiological relevance. In the case of H7N9, it can be used for a solid initial characterization as well as to continue monitoring if additional human host adaptation mutations would occur in the future.

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Serum antibody cross-reactivity to influenza A(H7N9) virus and impact of seasonal vaccination

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Background: In March 2013, the first human cases of severe respiratory disease associated with a novel avian influenza A (H7N9) virus were reported in China. Since then, over 100 cases of human A(H7N9) virus infection have been confirmed with a fatality rate of over 25%. Genetic analysis has indicated that the virus is a novel reassortant virus having acquired genes from avian origin H7N3, H7N9 and H9N2 viruses and possesses several molecular features associated with adaptation to mammalian species. It is reasonable to expect that the persons of all ages might be susceptible. At this time there is no evidence of sustained human-to-human transmission, and the outbreak is limited to China. However, for pandemic preparedness purposes, it is important to understand whether there is any preexisting serologic cross-reactive immunity to the A(H7N9) virus in humans. In this study, serologic assays were developed to detect antibodies to the A(H7N9) virus and used to detect the presence of antibody cross-reactivity to H7N9 virus that can be elicited in persons who received seasonal trivalent inactivated vaccine (TIV) or may be present in the US population at large. These studies will provide population immunity data to inform on the pandemic potential of H7N9 viruses. Materials and Methods: Two serologic assays were developed to assess the antibody cross-reactivity against H7N9 viruses: the microneutralization (MN) and hemagglutination inhibition (HI) assays. In this study, 30 pairs of sera (pre- and post vaccination) were selected from each of the 3 age groups: pediatric (6-35 mo), adult (18-49 y), and elderly adult (> 65 y) that received the 2012-2013 Northern Hemisphere seasonal TIV vaccine. The potential cross-reactivity of the sera was tested against 2 wild-type H7N9 viruses isolated from the first human cases in China: A/Anhui/1/2013 and A/Shanghai/1/2013, using both MN and HI assays. The antibody response to the seasonal vaccine H3N2 component A/Victoria/361/2011 was also tested in parallel as a control. Results: To improve the assay sensitivity for detection of antibody response to H7N9 virus exposures, the HI assay was further optimized using horse red blood cells and a modified sera treatment procedure. A total of 90 paired sera were tested from 3 age groups of US seasonal TIV recipients. In the HI assay, only one serum in the elderly adult group has a titer of 20 (3%) against A/Shanghai/1/2013 wild-type H7N9 virus post vaccination; none of the other sera tested in all 3 age groups (0%) had an HI titer above the detection limit of the assay (HI titer of 10) against either of the two H7N9 viruses pre- or post vaccination. In the MN assay, none of the sera (0%) tested in any age group had a MN titer equal to or higher than 20 against both H7N9 viruses either pre- or post vaccination. In contrast, antibody responses to A/Victoria/361/2011 (H3N2) virus were detected in all 3 age groups: 90%, 83%, and 73% of adult, elderly adult and pediatric age groups, respectively, had postvaccination HI titers equal to or greater than 40. Similarly, 97%, 90%, and 70% of adult, elderly adult and pediatric age groups, respectively, had postvaccination MN titers equal to or greater than 40. Conclusions: Our data indicate that vaccination with the current 2012-2013 TIV influenza vaccines induced no cross-reactive antibody response to the avian influenza A(H7N9) virus in any age group. Seasonal vaccination likely does not provide any protection against the H7N9 infection. These results emphasize the need to develop and evaluate H7N9 candidate vaccine viruses. A larger study to determine the age-specific rates of seroprevalence of cross-reactive antibody against A (H7N9) virus in the US population is ongoing.

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Evolution of H7 viruses in poultry: convergent and divergent mutations

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Background: Between 1999 and 2004 two avian influenza (AI) epidemics caused by different AIV subtypes (H7N1, March 1999-March 2001 and H7N3, October 2002-November 2004) affected the same poultry population in the northern part of Italy. Notably, while the low pathogenic (LP) H7N1 virus became highly pathogenic (HP) during the course of the epidemic, H7N3 did not. To prevent the reemergence of HP H7N1 and to control the H7N3 epidemic, two vaccination programmes were implemented from November 2000 (about 4 months before eradication of the first epidemic) and from January 2003 (about 23 months before eradication of the second epidemic). To identify genetic markers associated with the emergence of HPAI viruses and to determine the impact of vaccination on virus evolution in the field, we compared the evolutionary dynamics of the LP H7N3 and LP/HP H7N1 viruses, including substitution rates, selection pressures, and the emergence of amino acid mutations associated with a) the evolution of influenza viruses from low to high pathogenicity, b) antigenic changes, and c) virus adaptation to poultry. In addition, we assessed the intrahost genetic variability from the complete genome of H7 viruses using deep sequencing data from 30 clinical samples collected during the two epidemics. Materials and Methods: The complete genome of 176 H7 viruses representative of the two epidemics was phylogenetically analysed using the maximum likelihood method available in the PHYML package. Rates of nucleotide substitution per site and per year were estimated using the Bayesian MCMC approach. Site-specific selection was estimated for all genes using the fixed-effects likelihood and the fast unconstrained Bayesian approximation methods available in Datamonkey (online version of the Hy-Phy package). To assess virus population diversity, next-generation sequencing of the amplified genome was performed using an Illumina MiSeq on 30 H7 clinical samples (21 H7N1 and 9 H7N3). Results: Our phylogenetic analyses show that the H7 viruses collected during the two Italian epidemics fall within two well-supported monophyletic clades (H7N1 and H7N3), which evolve with similar rates of nucleotide substitutions (1 x 10^{-4} sub/site/year for the H7N1 and 8.4 x 10^{-3} sub/site/year for the H7N3). Within the H7N1 clade, the HPAI viruses form a distinct genetic group in all the eight phylogenies, while two major genetic groups of LPAI viruses (H7N1 LPAI-I and H7N3 LPAI-I) have been identified within the two major clades (H7N1 and H7N3). Interestingly, these two groups accumulated a high number of amino acid mutations along the course of the epidemics (14 in the H7N1 LPAI-I and 9 in the H7N3 LPAI-I). Notably, we found a total of 26 identical amino acid substitutions that have been acquired across the entire genome independently by both subtypes during their evolution. Most of these mutations (12 of

26) were observed in the HA protein and, in particular, 3 were located within antigenic sites, 6 were positively selected sites and 2 generated new additional glyscosylation sites. No evidence of gradual accumulation of mutations in the HA cleavage site of LPAI progenitor viruses resulted from our deep sequencing analysis; this might suggest that the multibasic cleavage site of the HPAI viruses has emerged suddenly. On the other hand, the LPAI viruses show a progressive accumulation of mutations in other genome regions with occasional fixation, suggesting that multiple genetic variants are being transmitted through the poultry population during the LPAI epidemic. Conclusions: This study contributes to a better understanding of the evolutionary dynamics of the H7 viruses at the epidemic level. Our deep sequencing data provide important information about the emergence of the HPAI H7 strain from the LPAI progenitor, suggesting the unpredictability of this event. However, our results should be interpreted with caution given the limited number of clinical samples suitable for this type of analysis. Interestingly, the convergent evolution observed in these two epidemics indicates that these commonly acquired mutations may confer a strong fitness advantage to H7 viruses in poultry.

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Inactivation of the novel avian influenza virus A (H7N9) under physical conditions or chemical agents treatment

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Background: During the spring of 2013, the emergence and spread of a novel avian-origin influenza A (H7N9) virus in humans in eastern China has raised concerns that a new influenza pandemic could occur. The potential effect of chemical agents and physical conditions on inactivation of the novel H7N9 virus has not been assessed. Materials and Methods: To determine the survival of the H7N9 isolates under various physical and chemical treatments, two H7N9 isolates, A/Anhui/1/2013 and A/Shanghai/1/2013, were treated by varied temperatures, ultraviolet light, varied pHs and disinfectants. The viruses with a concentration of $10^{7.7}$ EID50 were exposed to physical (temperature, ultraviolet light and pH) and commercial chemical (chlorine, Virkon S, and ethanol) agents. Harvested allantoic fluid from embryonated SPF chicken eggs inoculated with H7N9 treated virus was subjected to haemagglutination assay. Results: Both the tested viruses can tolerate 15-min exposure to 56°C but lost their infectivity totally when exposed to the temperature of 56°C for 30 min, and also readily inactivated by 65°C for at least 10 min, 70°C for 5 min or 100°C for 1 min. It was also observed the H7N9 viruses lost their viability after they were exposed to ultraviolet light radiation for 30 minutes or longer. Additionally, acidic pH 1-3 was virucidal after 0.5 h (pH1-2) or overnight (pH3) contact time. But, the virus retained infectivity after contacting pH 4-12 for 24 h. In addition, the virus would lose infectivity when exposed to chlorine, Virkon S and ethanol at recommended concentrations after only 5-10 min. Conclusions: The novel virus can be inactivated by general physical or chemical treatments, but presents a high tolerance on acidic or alkali conditions. The results provide support for public awareness of protection against the novel virus or the decontamination method of the novel virus at laboratory and field conditions.

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Pathogenesis of the novel avian-origin influenza A (H7N9) virus in human lower respiratory tract

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Background: As of May 2013, 131 laboratory-confirmed human infections with a novel influenza H7N9 virus had been reported from China. The source of human infection appears to be poultry. There is so far no evidence of sustained human-to-human transmission. Genetic analysis revealed that all eight gene segments of H7N9 were of avian origin; six internal gene segments from avian influenza H7N9 viruses, while hemagglutinin and neuraminidase genes were derived from influenza viruses circulating in ducks and wild ducks, respectively. The emergence of the H7N9 influenza virus catches global attention about whether the new virus could spark another pandemic. The majority of the infected patients were hospitalized and suffered from ARDS, with a fatality rate of about 37%. Our study aimed to determine the mechanism contributing to the pathogenesis of the H7N9 virus. A panel of proinflammatory cytokines and chemokines will be examined upon influenza H7N9 virus infection in alveolar epithelial cells in order to examine if these mediators were induced differentially when compared with the highly pathogenic avian influenza (HPAI) H5N1 and the 2009 pandemic H1N1 virus. Moreover, because cleaved caspase 3 is commonly employed as a marker for the indication of apoptosis, we further examined the extensiveness of cleaved caspase 3 in influenza virus infection in

human lung ex vivo cultures. Materials and Methods: Fresh biopsies of human lung tissue were obtained from patients undergoing surgical resection of lung tissues. Lung tissue fragments were cultured with F12K medium incubated at 37°C. For viral infection experiments, influenza viruses A/Shanghai/1/2013 (SH1, H7N9), A/Shanghai/2/2013 (SH2, H7N9), A/Hong Kong/483/97 (H5N1), and A/California/07 (Ca07, H1N1pdm) at a viral titer of 10^6 $TCID_{50}$/mL were used for ex vivo lung culture infection. Infected lung tissues were collected in 10% formalin at 24, 48, and 72 hpi for immunohistochemical staining. Costaining of cleaved caspase 3 and influenza virus nucleoprotein was carried out for the detection of apoptosis. Furthermore, primary culture of human alveolar epithelial cells was isolated from human lungs by mincing the lung, followed by filtration and centrifugation. Human alveolar epithelial cells were infected with the novel influenza H7N9, the HPAI H5N1, and the pandemic H1N1 virus. Virus replication was monitored by measuring infectious viral particles using $TCID_{50}$. mRNA and protein expression of proinflammatory cytokines and chemokines were quantified by real time qPCR and ELISA. Results: We found extensive apoptosis in influenza H7N9 (both SH1 and SH2) and H5N1, but not H1N1pdm infected ex vivo lung tissues, suggesting that both avian influenza viruses can induce apoptosis and cause severe cell death in human lung tissue. Furthermore, unlike HPAI H5N1 which induces dysregulated proinflammatory cytokine responses, the novel influenza H7N9 virus elicited poor proinflammatory cytokine responses, inducing type I and III interferon in ex vivo human lung explant cultures. The novel influenza H7N9 virus is an intrinsically more potent inducer of proinflammatory cytokine than the H1N1pdm virus but less than the H5N1 virus. Conclusions: The proinflammatory cytokine and chemokine responses may contribute modestly to the severity of human H7N9 disease, but it is likely that direct viral cytopathology is probably playing a more important role in pathogenesis of human H7N9 diseases. The recognition of the role of cleaved caspase 3 in severe human infection of avian influenza virus can provide insights on the development of novel therapeutic approaches for the preparedness of the future outbreak of pandemics.

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Antigenic and genetic evolution of low pathogenic avian influenza viruses (H7N3) following heterologous vaccination

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Background: Italy experienced H7N3 low pathogenic avian influenza (LPAI) outbreaks between 2002 and 2004 in a densely populated poultry area in the northeast. The H7N3 virus was first detected in October 2002 in poultry. This virus continued to circulate and the infection was controlled by August 2003 by stamping out, control of movements, improved bio-security, and heterologous vaccination. A vaccination program was designed based on a "DIVA" strategy and was carried out using an AI inactivated heterologous H7N1 vaccine and implemented from December 2002 to August 2003. In 2003, the virus circulated in unvaccinated and vaccinated poultry farms. In September 2004, approximately 1 year after the depopulation of the last LPAI affected flock, the AI virus of the H7N3 subtype re-emerged. This occurrence required the application of strict control measures in association with the rapid implementation of a booster immunization of the susceptible population. During the 2002-2004 Italian LPAI H7N3 epidemic, a longitudinal collection of H7N3 viruses was obtained and archived. To shed light on the occurrence, temporal pattern and genetic basis of antigenic drift for avian influenza (AI) viruses in absence and presence of heterologous vaccination, these viruses were subjected to genetic and antigenic characterization. Materials and Methods: H7N3 AI isolates were selected in this study according to the following criteria: 1) year of isolation; 2) vaccination status of the flock from which they were isolated. A total of 37 H7N3 isolates were selected. In particular, 16 H7N3 isolates were isolated during the 2002 epidemic (no vaccination campaign in place); 11 isolates collected in 2003 from unvaccinated (4 viruses) and vaccinated flocks (7 viruses); and 10 H7N3 isolates sampled from vaccinated farms during the 2004 epidemic were analysed, the latter were all available viruses isolated in 2004. Turkey sera were generated against representative H7N3 isolated

in 2002, 2003, and 2004 and cross HI tests conducted for the antigenic analysis. The haemagglutinin protein of the H7N3 isolates was sequenced and phylogenetic analysis was conducted to investigate the genetic basis of the viral antigenic profile. To investigate the impact of vaccinations on the evolutionary rate of HA gene of H7N3 viruses, an evolutionary analysis using Bayesian statistics was performed. The evolutionary rates of HA genes were estimated using Bayesian Evolutionary Analysis Sampling Trees (BEAST) version 1.7.1. Positive selection analyses were performed using PAML.
The antigenic cartography was used to analyze cross HI data generated using homologous and heterologous turkey sera against representative H7N3 isolates. Results: Our results showed that the HA genes of these H7N3 isolates belong to the same genetic group along with other Eurasian H7 lineage. However, our results demonstrated that there is a larger extent of genetic diversity among H7N3 isolates after vaccination than those during outbreaks. Computational analyses demonstrated that the evolutionary rate of the viruses after vaccination (1.1672×10^{-4} substitutions/nucleotide per year; standard error 9.3013×10^{-5}) was about twice of those before vaccination (6.8569×10^{-5}; 5.8367×10^{-5}). The antigenic cartography showed that these viruses were antigenically separated into two groups, one before and one after the vaccination campaign. Molecular analysis showed that viruses isolated after the implementation of vaccination acquired specific amino acid signatures: G133E (144, H3 numbering; antibody binding site A); A151T (160, H3 numbering; antibody binding site B), G177V (186, H3 numbering; antibody binding site B); Q201H (210, H3 numbering). Positive selection analysis demonstrated A151T was under positive selection. Conclusions: This study suggests that vaccination during 2002-2004 outbreaks affected H7N3 influenza evolution both antigenically and genetically. This is the first report on antigenic and genetic evolution of H7 LPAI viruses following the application of heterologous vaccination. Such findings may have impact on control strategies to combat AI infections in poultry based on vaccination.

P1-108

Genetic diversity in the internal genes of novel H7N9 influenza A viruses circulating in eastern China, 2013

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Background: This study was intended to better understand the genetic origins and evolutionary patterns of H7N9 viruses circulating in eastern China. Materials and Methods: We conducted a phylogenetic analysis of whole genome sequences from 40 H7N9 influenza virus strains isolated from human, poultry and free market environments. Results: Phylogenetic analysis revealed substantial genetic diversity in the internal genes; 17 different genotypes were identified from the H7N9 virus isolates. Conclusions: Our findings revealed that the H7N9 viruses are undergoing dynamic evolution in eastern China.

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A comparison of rapid point-of-care tests for the detection of avian influenza A(H7N9) virus

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Background: A(H7N9) avian influenza virus has caused at least 132 human cases and 37 deaths in China since its emergence in February/March 2013. This has led to significant concerns that it could in the future spread globally, causing the next influenza pandemic. Rapid testing and diagnosis of possible human influenza A(H7N9) virus infections are important diagnostic and public health tasks. An accurate diagnosis will allow the timely administration of antiviral therapy and may also enable quarantine of infected cases to prevent further spread of the virus. Point-of-care tests (POCTs) based on antigen detection are simple to use and are designed for use in a medical clinic or outpatient settings, enabling the rapid testing of patient specimens within 15 minutes. For public health purposes, it is important to determine whether POCTs can detect the novel influenza A(H7N9) virus, particularly as previous studies have found that some POCTs had poorer sensitivity in detecting avian influenza strains compared with circulating human seasonal influenza strains.
Materials and Methods: We evaluated six widely available POCTs (SD Bioline, BD Directigen EZ, BD Veritor, Binax Now, Clearview and Quidel Sofia) for their ability to detect the avian influenza A(H7N9) virus A/Anhui/01/2013, compared with three other recent low pathogenic avian influenza A(H7) viruses and two A(H3N2) and two A(H1N1)pdm09 human seasonal influenza viruses. All viruses were cultured in Madin-Darby Canine Kidney cells, standardised to an infectivity titre of 1 x 10^6 tissue culture infectious dose$_{50}$/mL ($TCID_{50}$/mL) and then diluted in phosphate-buffered saline in half-log_{10} dilutions. Real-time RT-PCR analysis was conducted on each virus dilution to determine a cycle threshold (Ct) value and RNA copy number. Each virus dilution was then tested in each POCT according to the manufacturer's instructions and a limit of detection (LOD), based on either the $TCID_{50}$/mL or the RNA copy number/µL, was determined. Four of the kits were read by eye, while two POCTs (Veritor and Sofia) utilised a mechanical reader. Results: Based on the $TCID_{50}$/mL, the LOD of five of the six POCTs for the A/Anhui/01/2013 influenza A(H7N9) virus ranged from 1 x 10^5 to 1 x $10^{5.5}$ $TCID_{50}$/mL, with the Sofia and Directigen EZ detecting virus at the lower limit. The Clearview POCT was unable to detect the A(H7N9) virus at any of the concentrations tested (1 x 10^6 $TCID_{50}$/mL or lower). In comparison, the LOD of the POCTs for the other influenza A(H7) and seasonal viruses tested was generally better than that seen with the A/Anhui/01/2013 virus. Comparison of POCT LODs based on RNA copy number/µL showed similar results to those based on $TCID_{50}$/mL for four of the kits (Binax Now, Clearview, Veritor and Sofia). These POCTs were less sensitive for the detection of the influenza A(H7N9) virus compared with the seasonal or other influenza A(H7) viruses. However, for the SD Bioline and the Directigen EZ tests, comparison of the LODs based on RNA copy number/µL showed that A(H7N9) was detected at a similar sensitivity to the other viruses. LODs based on RNA copy number/µL or Ct value allowed an estimate of the expected performance of the POCTs in detecting A(H7N9) virus in clinical samples. Comparison of the published matrix gene Ct values of clinical samples from patients with confirmed A(H7N9) infection suggested that five of the six POCTS would have detected only one of the four A(H7N9)-positive clinical specimens, with the other three specimens being outside the LOD of these assays. Conclusions: This study demonstrates that the sensitivity of at least four of the six evaluated POCTs is lower for the novel influenza A(H7N9) virus than for seasonal influenza viruses and the other avian influenza A(H7) viruses tested. Comparison with published Ct values for clinical specimens from A(H7N9) patients suggested that these POCTs may not detect the majority of A(H7N9) cases, particularly if samples are taken late in the course of disease. Therefore, real-time PCR remains the diagnostic test of choice for the testing of suspected A(H7N9) influenza cases.

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P1-110 SCHOLARSHIP Promising Investigator Scholarship

The emergence of the 2013 H7N9 and related viruses in China

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Background: The novel H7N9 influenza A virus first detected in March 2013 has caused more than 130 cases of human infection in China, resulting in 39 deaths. This virus is a reassortant of H7, N9 and H9N2 avian influenza viruses and carries some amino acids linked to mammalian receptor

binding, raising concerns of a new pandemic. However, neither the source populations of the H7N9 outbreak lineage nor the conditions for its genesis are fully understood. Materials and Methods: Following the initial reports of H7N9 influenza infection in humans, field surveillance was conducted during 4th-18th April in Zhejiang, Shandong and Guangdong provinces. Pairs of oropharyngeal and cloacal samples from chickens and other poultry, together with faecal and water samples from live poultry markets (LPMs), farms and wetlands were collected for virus isolation and whole genomic sequencing. H7, N9, N7 and H9N2 archived isolates, obtained during previous influenza surveillance between 2000-2013 in southern China, were also sequenced and phylogenetically analyzed to pinpoint the genesis of the H7N9 and a related H7N7 virus. The infectivity and pathology of H7N9 and H7N7 viruses were tested in a ferret model. Results: Through a combination of active surveillance, screening of virus archives, and evolutionary analyses, we found that H7 viruses have independently transferred from domestic ducks to chickens in China on at least two occasions. Subsequently they reassorted with enzootic H9N2 viruses to generate the H7N9 outbreak lineage, and a related but previously unrecognized H7N7 lineage. The H7N9 outbreak lineage has spread over a large geographic region and is prevalent in chickens at LPMs that appear to be the immediate source of human infections. In ferrets this virus caused a productive infection and pneumonia. Virus was shed via the nasal route and transmitted to physical contact and some airborne-exposed animals. Like the H7N9 virus, the H7N7 virus was also mainly isolated from chickens at LPMs and it could efficiently infect ferrets, be shed via the nasal and rectal routes, and cause severe pneumonia. Conclusions: These findings provide a clear picture showing how the current H7N9 human viruses emerged. Domestic ducks act as primary vectors to acquire and maintain diversified viruses from migratory birds, and facilitate different subtype combinations between H7 and N9 or N7 viruses and interspecies transmissions to chickens. After being introduced, the H7N9 or H7N7 viruses reassorted with enzootic H9N2 viruses and formed the current reassortant H7N9 or H7N7 viruses seen in chickens. This likely led to outbreaks in chickens, resulting in the rapid spread of the novel reassortant H7N9 virus through LPMs, which then became the source of human infections. Whether the H7N9 outbreak lineage will, or has, become enzootic in China needs further investigation. Our results also indicate that H7 viruses pose a broader threat than the current H7N9 virus. Continued prevalence of this family of H7 viruses in poultry could lead to further sporadic human infections, with an ongoing risk that the virus might acquire efficient human-to-human transmissibility.

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Virological characteristics and serological response of the human case of influenza due to novel avian-origin reassortant H7N9 virus in Taiwan

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Background: On 24 April 2013, the first imported case of H7N9 infection was confirmed in Taiwan in a patient who returned from China on 9 April. We described the clinical course and viral dynamics in the airway and blood, and the viruses isolated from the airway were studied for resistance mutations to neuramindase inhibitors and receptor specificity. Materials and Methods: Serial respiratory and blood specimens were collected from the case patient to determine the H7N9 viral load with the use of real-time RT-PCR, and the antibody titers in serial blood specimens were determined with the use of hemagglutination inhibition assay. The viruses isolated from the respiratory specimens were characterized for their ability to replicate in different cell lines and binding ability to α-2,3-linked orα-2,6-linked sialic acid; and sequencing of the virus genes was performed. Results: Despite treatment with oseltamivir that was begun at a daily dose of 150 mg on 17 April, the patient developed progressive pneumonia with respiratory failure. A more than 1-log reduction of viral load in the sputum specimen was detected 5 days after the change of oseltamivir daily dose to 300 mg, when the viral

load increased by 50-fold in the throat swab specimen. When a guinea pig's erythrocytes were used to determine the antibody titers of the case patient, an increasing trend of antibody titer was observed against the viruses isolated from the sputum and throat swab specimens, < 1:10 on 20 April to 1:40 and 1:80 on 7 May. The presence of R294K mutation that resulted in reduced susceptibility to oseltamivir and zanamivir was detected (IC50 > 50uM for oseltamivir; IC50 = 840 nM for peramivir). Two dominant virus populations were respectively amplified from in the upper and lower respiratory airways of the case patient and these viruses had a major difference at the residue 627 of PB2, with Lys-627 and Glu-627 respectively detected in the viruses isolated from sputum and throat swab specimens. These viruses exhibited similar binding affinity to both chicken and guinea pig's RBC with the use of hemagglutination assay. Conclusion: In this patient with progressive pneumonia due to H7N9, two dominant virus populations in the upper and lower respiratory airways was observed with a specific difference at the residue 627 of PB2.

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Distinguishing between market exposure and human-to-human transmission using H7N9 case data

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Background: The most important characteristic of spillover influenza strains such as H7N9 and H5N1 is R_0, the average number of secondary human cases generated by each initial case. If R_0 is less than 1, the virus will not cause a pandemic and its individual-level severity will only be of limited concern. The closer R_0 is to 1, the lower the hurdle that must be overcome by the virus to be able to persist within the human population. Although the outbreak of an avian strain of H7N9 in humans in China raises many immediate questions for the public health community, the cessation of cases following the closure of live bird markets (LBMs) implies that LBMs were a source of infection and that R_0 in humans is less than 1. Methods: To disentangle the role of market exposure and human-to-human transmission in the generation of human H7N9 cases, we fitted a model of LBM exposure and onward human-to-human transmission to publically available case cluster data. We assumed cases on a given day could arise because of market exposure or as a result of previous human cases, and that the temporal pattern of market exposure was not fully known. Using this framework, we estimated the value of R_0 and the change in market hazard over time in the three largest outbreaks thus far reported for H7N9 from China: Shanghai (33 cases), Zhejiang (46 cases) and Jiangsu (23 cases). Results: First, we used simulations to test whether our model could distinguish between human-to-human and market transmission when the basic reproductive ratio was significantly larger than zero, but still less than one. If we knew the drop in market hazard date, but nothing else about the shape or magnitude of the hazard, we were able to obtain unbiased estimates of R_0 from simulated case data. For the three largest outbreaks, we calibrated the market exposure portion of the model in the absence of human-to-human transmission, setting R_0 =0 and estimating a stepwise market hazard with arbitrarily many steps. Fitting these hazard functions to the case data, we found that models with three or four steps provided the most parsimonious explanation. Next, we added human-to-human transmission to the market spillover model with a three-step hazard function and known date for a decrease in hazard. For Shanghai and Jiangsu, the model with human-to-human transmission had greater statistical support than the market hazard-only model, whereas both models had a similar level of statistical support for Zhejiang. Conclusions: This methodology can be applied to future outbreaks of H7N9 and give a useful upper bound for R_0. The results illustrate how the ability to characterize the variation in market hazard over time can permit robust estimation of R_0 from relatively small outbreaks. Changes in typical human behaviour between the first two clusters (Shanghai and Jiangsu) and the most recent cluster (Zhejiang) may reflect a decrease in the transmission of the virus because of a change in human behaviour. Our approach needs to be generalized to account for asymptomatic infection.

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P1-115 SCHOLARSHIP Promising Investigator Scholarship

Novel avian-origin influenza A (H7N9) virus attaches to epithelium in both upper and lower respiratory tract of humans

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Background: In March 2013, an avian origin H7N9 virus emerged in China, causing severe pneumonia and acute respiratory distress syndrome in humans, with limited human-to-human transmission. Some of the human isolates contained a Q226L mutation in the HA, which is known to be associated with a switch in the receptor binding preference of HA from alpha-2,3-linked sialic acids to alpha-2,6- linked sialic acids. The receptor preference of influenza viruses is an important factor in the cell tropism. Previously, we studied the cell tropism of influenza viruses by attachment studies, which showed that the pattern of attachment is associated to both the pathogenesis and transmission efficiency. Viruses that transmit efficiently among humans (seasonal influenza viruses) attach abundantly to epithelial cells of the upper respiratory tract. Furthermore, viruses which cause a severe pneumonia (HPAI H5N1) attach to type II pneumocytes and alveolar macrophages in the alveoli. Therefore, the goal of this study was to determine the attachment pattern of A/Shanghai/1/13 or A/Anhui/1/13, the latter containing the Q226L mutation throughout the human respiratory tract. Materials and Methods: Human tissues from the nasal concha, trachea, bronchus, bronchiole, and alveoli were included to determine the attachment pattern of reassortant viruses consisting of seven gene segments of influenza virus A/PR/8/34 and the HA of either influenza virus A/Shanghai/1/13 or A/Anhui/1/13 using virus histochemistry. In addition, we determined the attachment pattern in the respiratory tract ferrets, mammals that are commonly used to study the pathogenesis of influenza. Results: In human respiratory tract tissues, both H7 viruses attached to tissues from the upper and lower respiratory tract. In the nasal turbinates, trachea, bronchi, and bronchioli the viruses attached to ciliated epithelial cells. In the alveoli, both viruses attached to type I and type II pneumocytes and alveolar macrophages. Interestingly, in the ferret respiratory tract, both H7 viruses attached to ciliated epithelial cells in the nasal turbinates, but rarely to ciliated epithelial cells of the trachea, bronchi and bronchioli. In the alveoli, both H7 viruses attached to type I and type II pneumocytes. Conclusion: The attachment pattern of these emerging H7 viruses in the human respiratory tract is different from the attachment pattern of other avian influenza viruses, which rarely attach to tissues of the upper respiratory tract. Interestingly, this pattern was independent of the Q226L mutation in the receptor binding site, as both viruses showed a similar pattern of attachment. The attachment pattern of H7 viruses in the ferret respiratory tract differed from that in humans. With the caveat that virus attachment is only the first step in the virus replication cycle, these results suggest that the emerging H7N9 virus has the potential both to transmit efficiently among people and to cause severe pneumonia.

P1-116

Mild to moderate influenza A(H7N9) infections detected through China's national influenza-like Illness sentinel surveillance system

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Background: The “clinical iceberg” phenomenon, where there are usually many more infected cases than is apparent symptomatically and even less so registered in the clinical setting, is a common feature of influenza disease. While this is certainly true for interpandemic influenza and the 2009 influenza A(H1N1) pandemic, this appeared to be less substantial for the Dutch A(H7N7) outbreak, and with A(H5N1) being an acknowledged exception. It remains unknown whether the “iceberg” applies to the influenza A(H7N9) virus that emerged in early 2013 in China. While the majority of laboratory-confirmed A(H7N9) cases presented with a severe clinical picture to a hospital, a small number of laboratory-confirmed cases have been identified through the sentinel influenza-like illness (ILI) surveillance system nationwide. The objective of our study was to describe the clinical characteristics of the complete case series of A(H7N9) cases as of May 15, 2013, that were identified through routine testing by the ILI sentinel surveillance system. Materials and Methods: ILI sentinel surveillance in China is conducted through a network of 554 hospitals across the country, with the total number of outpatient and/or emergency department visits and the number of patients fitting the WHO standard ILI case definition reported weekly online to the China CDC, and 10-15 nasopharyngeal swabs collected from ILI patients each week for routine laboratory testing and subtyping. All A(H7N9) cases detected through the ILI surveillance system by May 15, 2013, were identified by cross-referencing the laboratory-confirmed A(H7N9) line list with the routine sentinel ILI surveillance system. Demographic and epidemiologic data were extracted from field investigation records, and clinical and laboratory data were obtained from medical chart review. Results: Five (3.8%) of a total of 130 laboratory-confirmed influenza A(H7N9) cases reported as of May 28, 2013, were detected through the routine ILI surveillance system. Four (80%) of them were male. Mean age was 13 (range = 2-26) years and none had any underlying medical condition. Exposure history, geographic location and timing of symptom onset were otherwise similar to the general cohort of all laboratory-confirmed cases to date. All patients experienced only mild or moderate disease with an uneventful course of recovery. Among them three (60%) were managed only as outpatients and all quickly recovered after 3-5 days, with nasopharyngeal swabs tested positive for A(H7N9) only after their full recovery. Two patients (40%) were hospitalized for treatment. One was a 4-year-old child from Shanghai who presented initially as an outpatient with fever and mild rhinorrhea to a routine sentinel clinic, and was admitted on the next day for oseltamivir treatment after his nasopharyngeal swab was tested positive for A(H7N9). The other was a 26-year-old man from Jiangsu who presented initially with fever and productive cough to a sentinel clinic, being given ceftazidime without improvement. He was admitted 4 days later with radiologic evidence of left-sided pneumonia, and started on oseltamivir and moxifloxacin. Both remained clinically stable with quick resolution of symptoms within 10 days. Conclusions: Our complete case series of A(H7N9) cases detected through the routine ILI surveillance system provide contrasting clinical presentations to the generally much more severe clinical picture of the majority of laboratory-confirmed A(H7N9) cases detected otherwise. Our findings provide indirect evidence of a substantial proportion of mild disease and support the existence of a “clinical iceberg” phenomenon in influenza A(H7N9) infections. For the clinician, our findings reinforce vigilance to the diverse presentation that can be associated with influenza A(H7N9) virus infections. Our results also suggest that large-scale community surveillance networks can be useful as a population-based sampling tool to enhance understanding of the full spectrum of disease, especially in the early phase of an evolving epidemic.

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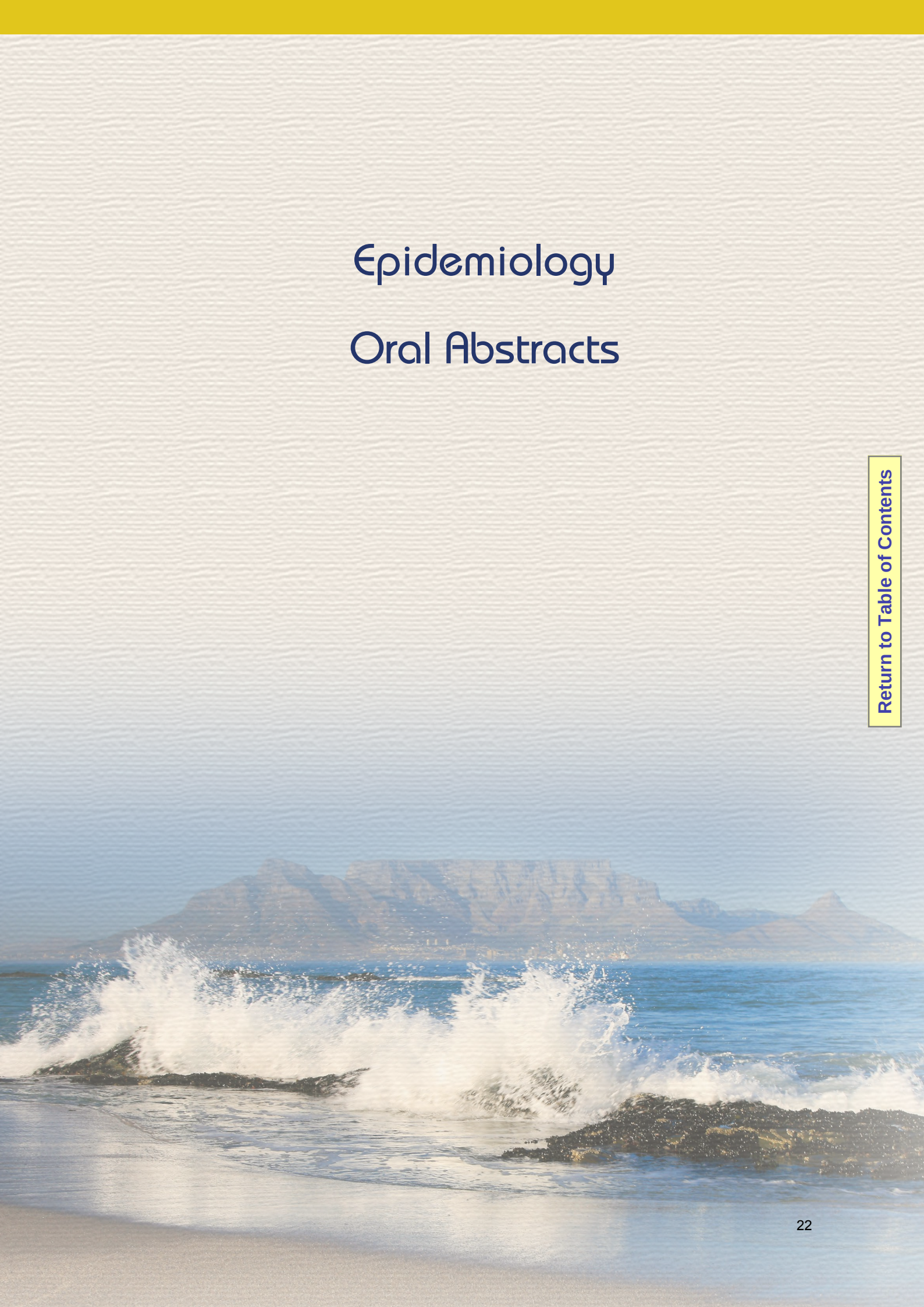

O-804 SCHOLARSHIP International Research Scholarship

The role of HIV in the household introduction and transmission of influenza in an urban setting, Nairobi, Kenya, 2008-2011

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Background: Little is known about how high HIV seroprevalence affects the transmission dynamics of influenza within household settings in sub-Saharan Africa. We used data gathered from a population-based household and clinic surveillance system from 2008 through 2011 to examine the association between the HIV status of household members and their risk of introducing influenza to the home. We also examined the association between the HIV status of laboratory-confirmed influenza index cases in homes and the risk of their household contacts developing influenza-like illness (ILI). Materials and Methods: We analyzed respiratory illness data from a Kenya Medical Research Institute (KEMRI) and Centers for Disease Control and Prevention-Kenya (CDC-K) population-based infectious disease surveillance (PBIDS) site in Kibera informal settlement, Nairobi, Kenya, from 2008 through 2011. ILI in a household member was defined as reported or diagnosed cough or sore throat with fever ≥ 38.0°C. Nasopharyngeal and oropharyngeal specimens were tested by rRT-PCR for influenza viruses. Index cases of influenza in households were defined as influenza rRT-PCR–positive individuals, with onset at least two weeks after the last prior household case of ILI to ensure they were the first case of influenza in the household. The secondary attack rate (SAR) was defined as the proportion of household contacts who developed ILI within 14 days after the laboratory-confirmed influenza index case was identified. HIV status was assessed via home-based testing and counseling in 2008 or at the local study clinic from 2008-2011. Log-binomial models using generalized estimating equations (GEE) to account for household clustering evaluated the association between laboratory-confirmed influenza index case status in the home and HIV status of the household members, as well as the association between secondary ILI status of household contacts and the HIV status of the influenza index case in the home. Covariates considered for inclusion in the multivariate models included age group of household members, household size, and year of index case incidence. Results: We identified 176 households with a laboratory-confirmed influenza index case. Among 874 household contacts of the index cases, 72 (8%) developed ILI. In comparison to persons over age 18 years, index cases of influenza were more likely to be children aged < 2 years (aRR 4.78; 95%CI 3.18-7.17), 2-4 years (aRR 3.35; 95%CI 2.20-5.09), and 5-17 years (aRR 2.56; 95%CI 1.78-3.67) when adjusted for HIV status, household size, and year of index case incidence. HIV status was not associated with influenza index case status among 1050 household members analyzed in the multivariate model (aRR1.34; 95%CI 0.68-2.66), when controlling for age group of the household member, household size, and year of index case incidence. However, the risk of developing ILI among 33 household contacts of HIV-positive index cases was more than twice the risk of developing ILI among 303 household contacts of HIV-negative index cases, when adjusted for age group of the household contacts (aRR 2.36; 95%CI 1.19-4.66). Additionally, the SAR was significantly greater in the 10 homes with an HIV-positive index case than in the 57 homes with an HIV-negative index case (19% vs 6%; *P* = .007).The association between the HIV status of index cases and secondary ILI case status was not modified by age of the index case. Conclusions: These results suggest that while children may be most likely to bring influenza into the homes in Kibera, HIV-positive influenza index cases may enhance transmission of influenza within the home. Prospective studies with larger sample sizes, laboratory confirmation of secondary cases, and additional measurement of immunosuppression and antiretroviral treatment are needed to further clarify the relationships between HIV and influenza. In addition to reducing disease burden in HIV-infected people, improving access and adherence to therapies for HIV and opportunistic infections may benefit the health of all people in areas of high HIV prevalence. Effective influenza vaccines are also needed to target HIV-positive individuals. Finally, HIV status should be considered when modeling influenza transmission at the household level in a region with high HIV seroprevalence.

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O-805

Investigating risk factors for severe acute respiratory infection (SARI) and influenza hospitalisation in New Zealand

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Background: The Southern Hemisphere Influenza and Vaccine Effectiveness Research and Surveillance (SHIVERS) Project was established in October 2011. The study operates in four hospitals that serve a population of 838,000 people in Auckland, New Zealand. There is a lack of knowledge about nonpharmaceutical interventions to reduce the population risk of influenza and other serious respiratory infections. One SHIVERS Project objective is to identify risk factors for influenza, particularly those that are modifiable. Materials and Methods: Year-round hospital-based surveillance is undertaken for:(i) severe acute respiratory infection (SARI) defined as onset of fever and cough within 7 days of hospitalisation, and (ii) respiratory infections caused by influenza and/or other respiratory pathogens. Data are collected from eligible patients using a standard case report form. Testing for respiratory pathogens is performed with nasopharyngeal swabs using a real-time polymerase chain reaction assay. Results: Between May and September 2012 (Southern Hemisphere winter), 4417 inpatients with suspected respiratory infections were assessed. Of these, 2023 (46%) met the SARI case definition, including 324 patients (23%) in whom influenza viruses were detected (70 influenza B, 122 A(H3N2), 93 A(H1N1)pdm09, and 41 A not subtyped). Both SARI and influenza hospitalisation rates were strongly associated with extremes of age, Māori and Pacific ethnicity, and residence within a more deprived neighbourhood. The overall SARI rate was 204/100,000 with peaks in those aged < 1 year (2722/100,000), 1 to 4 years (470/100,000) and 80+ years (821/100,000). The rate in Māori (315/100,000) and Pacific peoples (419/100,000) was markedly higher than in NZ European/others (152/100,000) and Asian peoples (98/100,000). The overall influenza rate was 34/100,000 with peaks in those aged < 1 year (282/100,000), 1 to 4 years (63/100,000) and 80+ years (144/100,000). The rate in Māori (45/100,000) and Pacific peoples (93/100,000) was markedly higher than in NZ European/others (21/100,000) and Asian peoples (19/100,000). There was a marked social gradient with the influenza rate in the most deprived 30% of neighbourhoods (32/100,000), greatly exceeding that in the least deprived 30% (12/100,000). Poor housing conditions were commonly reported by SARI cases, including 46% (823/1772) who reported that their home was usually colder than they would like; 39% (554/1426) that they were so cold that they shivered inside during the last month; 29% (412/1426) that their home smelled mouldy or musty; 26% (461/1772) that there was mould on the walls in bedrooms or living areas of their home; and 31% (556/1768) tha tthere were damp walls in the bedrooms or living areas of their home .In addition, 23% (413/1771) lived in social housing, 36% (645/1772) were living with someone who smoked, and 11% (186/1759) were with someone who smoked in a house or car in the past 7 days. Exposure to household crowding (a bedroom deficit of one or more for the size of the household) was common. Almost two-thirds (66%, 1161/1771) of the SARI cases reported contact with someone coughing and sneezing within the past 7 days, mostly at home (77%, 899/1161). Only 52 (3%, 52/1687) of the SARI cases had overseas travel within 7 days before hospital admission. Conclusions: Influenza is a common cause of severe respiratory disease in Auckland in the winter, affecting especially the very young, socially deprived and Polynesian ethnic groups. Adverse housing conditions, household crowding, and tobacco smoke exposure were found in many patients with SARI and influenza. A control population is needed to evaluate the importance of these potential risk factors and is being added to the SHIVERS study for its second year of operation. There may be potential to reduce the population risk of influenza and SARI, and health inequalities, through interventions to improve housing conditions and reduce household crowding. Understanding the role of such interventions is important

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for developing evidence-based strategies to reduce the impact of both seasonal and pandemic influenza, particularly for the most vulnerable populations.

O-806

Pandemic H1N1 virus transmission and shedding dynamics in houses of index cases detected by active surveillance: the Ha Nam cohort

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Background: Reliable estimates of influenza transmission and the underlying determinants, such as case infectiousness (virus shedding, symptoms) and demographics, and contact susceptibility and demographics, are required to develop interventions. Numerous H1N1 2009 pandemic transmission studies recruited houses after identifying cases presenting to health care facilities (case ascertainment design), but yielded variable estimates of secondary infection risk (SIR). We used a longitudinal household-based influenza surveillance study to obtain unbiased estimates of SIR and risk factors, and virus shedding dynamics. Methods: Participants were part of an influenza cohort study based in Ha Nam, Viet Nam. A total of 945 members of 270 randomly selected households have been enrolled since 2007 and under active surveillance for influenza-like illness (ILI) defined as temperature > 38ºC with cough, sneeze or running nose. The first pandemic H1N1 2009 case was detected in September 2009. Health workers examined all members of confirmed H1N1 case households. Nose and throat swabs were collected daily until 15 days after the last household case was detected, and used for diagnostic and quantitative real-time RT-PCR, and virus full virus genome sequencing and isolation. Mouth temperature, symptom score and antiviral use were recorded for 10 days. HI assays were performed on paired pre- and postpandemic sera collected June 2009 and April 2010. Household SIR was defined as the proportion of contacts with infection commencing 1-8 days after symptom onset in the index case. Results: Twenty-two index cases were detected in twenty households comprising 81 people. Sixty-eight of 69 that provided prepandemic blood had HI titers < 10 against H1N1 2009 reference strain. The SIR in 59 contacts was 18.6% (95% CI 10.7–0.4%). Five (45%) of eleven infected contacts were asymptomatic. Virus sequences indicated that secondary infection occurred in the house. Index and secondary cases were evenly distributed between mothers (n = 9/20), sons (n = 11/21) and daughters (n = 10/19) but only 1 of 15 fathers was infected (index case). The proportion infected was higher for children (16/30, 53.3%) than adults (16/48, 33.3%). The median serial interval was 2 days. Three of six symptomatic secondary cases shed virus before symptoms developed. In most cases shedding peaked on day 2 of illness and ceased by day 7 to 9, and symptoms peaked on day 2 and ceased by day 8. The duration of shedding was similar across ages, in index and secondary cases, and in cases with and without symptoms or antiviral treatment, but the amount of virus shed was lower in asymptomatic. Symptom scores were similar across age groups. Significant risk factors for household transmission included wet cough in the index case (*P* =.002) but not viral load. Conclusions: This study examined pandemic H1N1 transmission in randomly selected households. Participants overwhelmingly lacked HI antibodies indicating susceptibility. Viral sequencing confirmed that transmission occurred within households. Unlike case ascertainment studies, index cases were similar to secondary cases suggesting that selection was unbiased, a relatively high proportion of cases were adults, and fathers were spared from infection, as in cohort studies in the 1950s and 1970s. Asymptomatic and presymptomatic shedding were relatively common but may contribute little to transmission, because only wet cough was significantly associated with transmission. Household cohort studies are resource intensive but may be worthwhile on a large scale because SIR estimates vary dramatically between case ascertainment studies.

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O-807

Severe influenza-associated lower respiratory tract infection in a high HIV-prevalence setting-South Africa, 2009-2011

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Background: Knowledge on influenza morbidity and mortality in HIV-infected individuals is limited. Influenza vaccination has been shown to be efficacious in HIV-infected adults in South Africa. While influenza vaccination is recommended for persons falling into established risk groups (including HIV) in South Africa, coverage is extremely low. We aimed to characterise the epidemiology of influenza-associated acute lower respiratory-tract infection (ALRI) in HIV-infected and -uninfected individuals in South Africa. Methods: During 2009-2011, we prospectively enrolled patients hospitalised with ALRI (defined according to World Health Organisation case definitions). Using polymerase chain reaction, respiratory samples were tested for ten respiratory viruses and blood samples were tested for pneumococcal DNA. Influenza-associated ALRI incidence was estimated at one hospital. Results: Influenza was identified in 1056 (9%) of 11,925 patients enrolled. HIV infection status was available for 819 (78%) patients. The HIV prevalence among influenza-infected patients was 44% (358/819), ranging from 10% (16/164) in infants to 84% (212/251) in adults aged 25-44 years (P < .001). The overall age-adjusted incidence of influenza-associated hospitalisation for ALRI ranged between 49 and 78 cases per 100,000 population from 2009-2011. The incidence was highest in children aged 0-4 years (range 153-336/100,000 for 2009-2011) with highest incidence in those aged < 1 year (300-469/100,000 for 2009-2011). Incidence was lowest in individuals aged 5-24 years (4-14/100,000 for 2009-2011) and then increased in those aged ≥ 25 years (10-32/100,000 for 2009-2011). The age-adjusted incidence of influenza-associated hospitalisation for ALRI was 4-8 times greater in HIV-infected (186-228/100,000) than HIV-uninfected (26-54/100,000) individuals. In multivariable analysis controlling for age, race and sex, HIV-infected patients, compared with HIV-uninfected patients, were more likely to have pneumococcal coinfection (11%, 37/345 vs 4%, 17/389; odds ratio [OR] 2.3, 95% confidence interval [95% CI] 1.0-5.0), influenza type B than type A (41%, 148/358 vs 29%, 133/461; OR 1.6, 95% CI 1.0-2.4) and longer hospitalisation duration (median [interquartile range]: 6 [4-8] vs 3 days [1-6], P < .001). In multivariable analysis comparing patients who died with those who did not, HIV infection (OR 4.3, 95% CI 1.4-13.4), tuberculosis coinfection (OR 3.9, 95% CI 1.5-10.0), underlying medical conditions (OR 3.2, 95% CI 1.1-9.6), pneumococcal coinfection (OR 3.4, 95% CI 1.2-9.4) and intensive-care admission (OR 15.4, 95% CI 1.1-208.0) were independent risk indicators for death. No influenza influenza-infected patient reported having received influenza vaccination. Conclusion: HIV-infected individuals experience substantially elevated incidence of influenza-

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associated hospitalisation, have increased risk of in-hospital mortality and should be prioritised to receive interventions to prevent influenza such as vaccination.

O-808

Variation in burden of influenza-associated hospitalization between two rural communities in India: results of a population-based study, 2010-2012

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Background: The global burden of influenza is increasingly recognized. However, data on influenza burden in India, the second most populous country, remain sparse. Due to India's size and geographic, climatic, and sociocultural diversity, estimation of disease burden in India requires a multisite approach. We conducted a study to estimate burden of influenza-associated hospitalization in two rural communities in India using a standardized protocol for 3 consecutive years during 2010-2012. Methods: Population-based surveillance for all acute medical hospitalizations was conducted at selected primary- and secondary-level health facilities serving the communities of two demographic surveillance sites (DSS) in Ballabgarh (pop. 89,187) and Vadu (pop. 120,586). Hospitalizations for trauma, elective surgery, and obstetric reasons were excluded. Enrolled patients had data collected on medical history and clinical course and had nasopharyngeal swabs collected within 24-48 hours of hospitalization for influenza virus testing by real-time reverse transcription polymerase chain reaction. Health care utilization surveys (HUS) were also conducted in both communities. Incidence rates of influenza-associated hospitalization were calculated using population denominators from DSS census data and adjusted for the proportion of hospitalizations reported on the HUS to occur at nonstudy facilities. Results: Based on the HUS, study facilities captured 55% of hospitalizations in the Ballabgarh population and 67% in Vadu. During 2010-2012, 6012 patients with acute medical illness were enrolled through facility-based surveillance, including 4276 from Vadu and 1736 from Ballabgarh. The median age of enrolled patients was similar in Ballabgarh and Vadu (25 vs 23.7 years), but the proportion of patients with ≥ 1 underlying medical condition was higher in Ballabgarh than in Vadu (16.8% vs 1.9%). Also, the median time from symptom onset to hospitalization was higher among patients in Ballabgarh than in Vadu (4 vs 3 days). The proportion of patients with laboratory-confirmed influenza was higher in Vadu than Ballabgarh during all study years (21% vs 5%, $P < .05$ in 2010; 18% vs 5%, $P < .05$ in 2011; 23% vs 5%, $P < .05$ in 2012). Influenza A and B viruses were detected among patients at both sites each year with a predominance of influenza A/H1N1pdm09 and B during 2010; A/H3N2 and B during 2011; and A/H1N1pdm09 and B during 2012. Annual acute medical illness hospitalization rates were similar at both sites (Vadu: 101.3 to 224.7 per 10,000; Ballabgarh: 86.0 to 126.2 per 10,000), whereas annual influenza-associated hospitalization rates were 5- to 9-fold higher in Vadu (20.3-51.6 per 10,000) compared with Ballabgarh (4.4-6.3 per 10,000). Peak monthly influenza virus detection coincided with the rainy seasons in both Vadu (July-September) and Ballabgarh (July-September), although the period of influenza virus detection was generally more prolonged in Vadu. Conclusions: Although influenza contributed to hospitalizations for acute medical illness annually in our study communities, rates of influenza-associated hospitalization varied substantially between communities. Differences in influenza-associated hospitalization rates between communities likely resulted from differences in virus circulation and possibly propensity to seek early care. Further analysis is warranted to evaluate factors that influence influenza virus circulation and hospitalization rates. This study was supported in part by cooperative agreement U01 IP000206 from the Centers for Disease Control and Prevention, Atlanta, Georgia, United States.

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O-809

Environmental correlates of H5N2 low pathogenicity avian influenza (LPAI) outbreak heterogeneity in domestic poultry in Italy, 2010-2012

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Background: Since 1997, Italy has experienced recurrent incursions of H5N2 avian influenza (AI) viruses in different geographic areas and varying sectors of the domestic poultry industry despite significant prevention and control efforts implemented. This is likely to be influenced by dynamic interactions between poultry and the environment where these viruses circulate, including both biotic and abiotic factors, and these interactions may vary along ecologic gradients in a more or less structured way. Understanding such interactions would support us in generating hypotheses about possible ecologic pressures of H5N2 LPAI outbreak differentiation (heterogeneity) over time, space and affected poultry populations to identify possible control strategies that are also ecologically grounded. We referred to heterogeneity among outbreaks in eco-epidemiologic terms as the increased difference in their spatiotemporal and flock-related characteristics (ie, location and detection date of the affected flocks, flock type, number and species of birds therein), which may be associated with specific environmental variables able to disentangle the pattern of (dis)similarities among the outbreaks. Materials and Methods: Twenty-seven outbreaks of H5N2 LPAI were detected in domestic poultry in Italy between 2010 and 2012. Following a recently proposed analytic approach (Carrel MA, et al. *Health Place*. 2012;18:1122-1131), nonmetric multidimensional scaling (NMDS) was used as an ordination technique to explore the underlying pattern of (dis)similarities among the 27 H5N2 LPAI outbreaks, and to further relate this pattern to a set of 35 environmental variables (about human, poultry and wild waterfowl population densities, elevation and land cover/use) hypothesized to be related to outbreak differentiationin a 10-km radius around each of the outbreaks. Firstly, the outbreaks were scaled using NMDS according to their spatiotemporal and flock-related characteristics, and then each of the environmental variables was aligned in the NMDS-ordinated space. We thus obtained NMDS scores, which reflected the amount of difference in the spatiotemporal and flock-related characteristics among the outbreaks. NMDS scores were then regressed on the environmental variables to study their association with the heterogeneity among the outbreaks: a positive correlation indicated that the environmental variable was associated with increased outbreak heterogeneity, whereas a negative correlation indicated that the environmental variable was associated with decreased outbreak heterogeneity (ie, outbreaks that are more similar to each other in ecologic dimension). Results: Most of the environmental variables had their axes of differentiation with the same alignment in the ordination space. Variables mostly associated with the ordination (as reflected by their larger R^2) were those for densities of broiler and turkey breeders, ducks and geese, amounts of arable lands, permanent crops, maritime waters and maximum elevation. Outbreak heterogeneity was positively associated with increased densities of broiler and turkey breeders, ducks and geese, increased amounts of mine, dump and construction sites, permanent crops, scrub/herbaceous vegetation, maritime waters and maximum elevation, whereas it was negatively associated with increased amounts of arable lands and densities of poultry other than broilers, layers and turkeys. This means that where the positively associated variables were more represented, outbreaks tended to occur more unpredictably in time, space and affected poultry populations, and vice versa for the negatively associated variables. Conclusions: Although it was not possible to infer any relationship of causation, results indicated that the pattern of (dis)similarities among the H5N2 LPAI outbreaks entail an underlying structuring that might be the outcome of possible large-scale, environmental interactions in ecologic dimension. Exploring the distribution of AI viruses over time, space and affected poultry populations by investigating their fundamental environmental correlates through the combined application of multivariate data analysis and classical regression modelling may well complement traditional experiments and hypothesis testing as to frame a disease ecology perspective using, for instance, data obtained from ongoing surveillance activities. This allows the findings to be informed by hypotheses about how ecologic interactions among poultry

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populations, viruses and their environments can be related to the observed patterns of AI infections, which in turn allows us to enhance any intervention by matching the specific (ecologically grounded) strategy.

O-822

The dynamics of pandemic and seasonal influenza viral shedding and clinical illness in naturally acquired infections

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Background: Understanding the dynamics of viral shedding associated with influenza virus infection is important. Viral shedding is generally accepted to be a proxy for infectiousness, thus the patterns of influenza viral shedding over time can inform national and international public health policies designed to mitigate and control outbreaks of interpandemic and pandemic influenza. Here we report the characteristics of viral shedding and illness in naturally acquired influenza virus infections among persons exposed to infection in households and tested by RT-PCR regardless of illness. Our report is an update of earlier data (Lau L, et al. J Infect Dis. 2010;201:1509-1516) now including cases of A(H1N1)pdm09 virus infection. Materials and Methods: Household transmission studies were conducted in Hong Kong, in which index cases were recruited from outpatient clinics and screened with a rapid antigen test. Those with a positive rapid test result, and their household contacts, were followed up for 7-10 days to identify secondary infections. All household contacts kept symptom diaries during follow-up, and provided nose and throat swabs at three-day intervals for testing by quantitative RT-PCR and quantitative viral culture. The present analysis focuses on PCR-confirmed influenza virus infections among the household contacts. Results: In total, 2899 individuals from 709 households were recruited. NTS specimens were successfully collected from 1864 household contacts and 294 (16%) tested positive for the influenza virus by either RT-PCR or TCID50. In 2008 and 2009, 135 and 126 secondary cases were identified, respectively, and between 2010 and 2012, 33 secondary cases were identified. Over the five years, 17 cases were found to be positive for pandemic A(H1N1), 112 cases for seasonal A(H1N1), 80 cases for seasonal A(H3N2), and 68 cases for seasonal B virus infections. In addition, 17 positive cases could not be subtyped and were excluded from the analysis in this study. Febrile illness, or ILI, was reported in 31% to 53% of the cases. Confirmed infections were asymptomatic 6% to 19% of the time, and pauci-symptomatic confirmed infections reporting only one symptom made up 7% to 31% of infections according to subtype. Among the household contacts reporting at least two symptoms, peak mean viral shedding assessed by RT-PCR for influenza A(H1N1)pdm09, seasonal A(H1N1), and seasonal A(H3N2) were observed to peak on the day of ARI onset, or on the day following ARI onset. The pattern of viral shedding then steadily decreased, resolving to undetectable levels approximately 6 to 7 days after ARI onset. The pattern of viral shedding for influenza B cases differed, instead of a clear peak, viral shedding rose three to four days prior to ARI onset and persisted for 6 to 7 days. For seasonal influenza A(H1N1) and A(H3N2), the time of peak shedding was comparable, however the duration of shedding according to $TCID_{50}$ appeared shorter, 4 to 6 days instead of 9 to 10 days. Presymptomatic viral shedding was detected by RT-PCR in 20% to 37% of cases by subtype. The patterns of mean scores for respiratory signs and symptoms were largely comparable across all influenza A and B virus infections, peaking on the day of ARI onset and steadily decreasing over 3 to 6 days. Systemic signs and symptoms were observed to decline more rapidly than upper and lower respiratory symptoms, with lower respiratory symptoms appearing to be the most prolonged. Conclusions: This study provides a description of viral shedding dynamics in 132 cases of naturally acquired influenza virus infections spanning five years. The duration and patterns of viral shedding observed in these cases of influenza A(H1N1), A(H3N2) and A(H1N1)pdm09 virus infections were comparable, rapidly rising and peaking on the day of or on the day following illness onset before declining, whereas in cases of influenza B virus infection, viral shedding followed a different pattern.

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O-823

Divergent patterns of circulating influenza viruses in two regions of Northern India: When does equator not define hemisphere?

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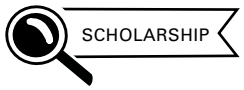
Background: Distinct patterns of pandemic 2009A/H1N1 (pH1N1) influenza were observed recently in the two northern regions of India (500 km apart), with Srinagar having peaks of influenza activity in the winter season (Jan-Feb 2009) and the Delhi region in monsoon times (July-Aug 2010). We sought to reassess seasonality of influenza in these two cities in north India to better define the seasonality of influenza in this region, and allow better recommendation of timing of vaccinations best suited for these cities in India. Methods: A total of 4652 patients presenting with influenza-like illness in an outpatient facility at the tertiary care centers of SKIMS, Srinagar (n = 2126) or AIIMS, New Delhi (n = 2526) were enrolled from January 2011 to December 2012. Nasopharyngeal swabs were tested by real-time PCR for seasonal and pandemic influenza viruses. Meteorologic parameters between Delhi and the Srinagar area were compared. Results: Influenza positivity was higher in Srinagar (375/2126; 17.6%) than in Delhi (239/2526; 9.46%). Distinct seasonality and subtype patterns were observed in these two cities. Srinagar had peaks in January-March with predominant strains being A/H1N1pdm09 in 2011 and A/H3N2 in 2012, followed by resurgence of A/H1N1pdm09 in November-December 2012. In contrast, Delhi had peak influenza circulation in July-September with co-circulation of A/H3N2 and influenza B in 2011 and A/H1N1pdm09 in 2012. For both years we observed that a co-circulating subtype in the Delhi area precedes that of the Srinagar area. In addition, influenza seasonality in Srinagar (34.09°N) matched the pattern observed in a temperate climate, whereas Delhi with monsoons in July-September, latitude of 28.66°N and just 500 km from Srinagar revealed influenza during summer months (July-September). Conclusions: Our data show that two regions in northern India have distinct influenza seasonality and circulating viruses. Thus India, though physically located in the northern hemisphere, has distinct seasonality related to the latitude location and environmental factors such as monsoons, temperature and humidity of the cities. While cities with temperate seasonality will benefit from vaccination in October-November, cities with peaks in the monsoon season in July-September will likely benefit from vaccination in May-June. Continued surveillance globally will help define regional differences in influenza seasonality and types and subtypes, as well as determine optimal periods to implement influenza vaccination programs among priority populations. Study supported in part by cooperative agreement 5U51IP 000333 from the Centers for Disease Control and Prevention and Indian Council of Medical Research, New Delhi, India.

O-824



Promising Investigator Scholarship

Higher incidence of pandemic influenza natural infection in the third wave compared to the second wave in an adult community cohort in the UK

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Background: Case-based population-level surveillance and cross-sectional serologic surveys used to estimate the incidence and patterns of influenza infection are limited by a lack of accurate

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denominator data, the inability to account for subclinical infections, difficulties in distinguishing antibodies induced by natural infection or vaccination, and the use of samples often originating from high-risk groups. For these reasons, community-based longitudinal cohorts, following the same individuals over time, offer the ideal design to estimate the incidence of infection and spectrum of illness. However, studies of this design describing the 2009 H1N1 pandemic (H1N1pdm09) are reported only from Hong Kong, Singapore and Vietnam, and examine only the first two, but not the third, pandemic wave. We report results from a community-based longitudinal cohort study undertaken in England from 2009-11 comparing the epidemiology of natural infection over the second and third pandemic waves. Methods: A total of 342 healthy adults (> 18 years of age) recruited between September and November 2009 after the first UK pandemic wave were followed from 2009-11. Samples were collected at the start and end of each influenza season. Infection was defined by a four-fold rise in haemagglutination inhibition titre and/or detection of virus by RT-PCR in nasal swabs among unvaccinated individuals. Development of any symptoms was recorded on a Web-based questionnaire e-mailed every three weeks. Among infected individuals, symptoms associated with the illness episode were categorised as acute respiratory infection (ARI, episode with any symptoms), influenza-like illness (ILI, episode with fever plus cough or sore throat) and fever (recorded temperature ≥ 38°C) alone. Statistical analysis was undertaken using Stata with the chi-square test and the *t* test to compare proportions and means, respectively. Results: At recruitment, after the first pandemic wave, seroprevalence of H1N1pdm09 antibodies with HI titre ≥ 1:32 was 26% (95% CI 21.39-31.19) with seroprevalence significantly higher in 18-25 year olds compared with older age groups. We calculated cumulative incidence of infection in our cohort over the first wave by subtracting published prepandemic age-specific seroprevalence for England. The overall cumulative incidence during the first wave was 12.74% (95% CI 7.1%-18.36%), 26.56% (95% CI 15.32%-37.81%) among 18-25 year olds with no increase in older age groups. The incidence rate of natural infection during the third wave (August 2010-April 2011) was significantly higher (P = .02) compared with the second pandemic wave (September 2009-April 2010). Age-specific incidence rate was significantly higher (P = .01) in the third wave compared with the second wave among the 26-40 year olds (third wave: 25.35%, 95% CI 15.23-35.47; second wave: 10.91%, 95% CI 5.08-16.74) but not the other age groups. Multivariate logistic regression adjusting for sex and age found each doubling increase in baseline HI titres measured at the start of each influenza season was associated with a significant lowering in risk of infection (odds ratio 0.92; 95% CI 0.86-0.99; P = .04) during the third but not the second pandemic wave. During an illness episode, 20% of infected individuals reported fever or ILI, 17% visited their general practitioner and none visited a hospital. Conclusions: A community cohort study of healthy adults in the UK revealed a substantial proportion of subclinical pandemic H1N1 infection, significantly higher incidence of natural infection in the third versus second pandemic wave, and higher risk of infection during the third wave among individuals with lower preinfection antibody titres.

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O-825

Comparative incidence and severity of infection with currently endemic human influenza A viruses: a longitudinal study

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Background: Novel strains of influenza emerge periodically into the human population with initial waves of infection characterised by increased incidence of infections and, sometimes, increased impact on morbidity and mortality. While the first wave of A(H1N1)pdm09 has been studied in detail in many countries, relatively little has been published on the postpandemic period. In addition, assessment of the severity of A(H1N1)pdm09 has been hampered by the lack of comparative data on seasonal influenza viruses. The objective of our study was to characterise the incidence and severity of infections with influenza A(H1N1)pdm09 and A(H3N2) viruses in the pandemic and postpandemic period. Materials and Methods: We collected four blood samples from a representative cohort of

individuals in Hong Kong before and after epidemics of A(H1N1)pdm09 and A(H3N2) in 2009-11. Serum from each individual was tested in parallel by hemagglutination inhibition assays against A/California/7/2009 (H1N1)pdm09 and A/Perth/16/2009-like (H3N2) using standard methods. We used the serologic data to estimate the incidence of influenza virus infections during each epidemic period, defining a seroconversion (indicating recent infection) as a 4-fold or greater rise in antibody titer against a specific virus across a period of time. We used population-level surveillance data to estimate the number of outpatient illnesses and excess respiratory deaths during each epidemic and thereby to characterize the severity profile of each virus. Results: We analysed quadruples of sera from 319 individuals who did not report potentially confounding vaccination histories between July 2009 and December 2011, a period that included three influenza virus epidemics. Cumulative incidence of infection was very high among children in the first epidemic of A(H1N1)pdm09 as previously reported. There was a change in age distribution of infections with A(H1N1)pdm09 from the first to the second epidemic, with a similar age pattern of infections in the second epidemic of A(H1N1)pdm compared with the epidemic of A(H3N2). The risks of excess all-cause and respiratory mortality were higher for A(H3N2) than for A(H1N1)pdm09, with relative risks of 2.1 (1.4,3 .2) and 1.3 (0.8, 1.9), respectively (95% confidence intervals). Conclusions: Despite a very high incidence of infection in children in 2009, the A(H1N1)pdm09 virus returned within a year to cause a second epidemic with substantial impact, implying that herd immunity among children may not be sufficient to prevent large epidemics. We have presented the most robust comparative estimates of infection-based severity of the two currently endemic human strains of influenza A. Infection-based estimates of individual-level severity, both relative and absolute, have a role in the assessment of emerging strains and the optimization of vaccination programs, especially in resource-limited settings.

O-826

Evolution of an influenza pandemic in 13 countries on 5 continents monitored by protein microarray from neonatal screening blood spots

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Background*:* In April 2009 a novel influenza virus, A(H1N1)pdm09, emerged from Mexico, and quickly spread all over the world causing a pandemic. It was estimated that the pandemic affected tens of millions of persons, but such estimates are difficult to obtain. Population-based serologic surveys can be helpful to get a better picture of the attack rate of an outbreak or widespread epidemic (pandemic). Comparative analysis of age-structured seroprevalence with notification based on clinical parameters has helped determine population impact across age groups. However, international comparison is challenging due to variability in laboratory methods. Therefore, we compared seroprevalence in women to a range of influenza virus subtypes by protein microarray, by measuring maternal antibodies in neonatal screening dried blood spots collected October 2008 through August 2010 in 13

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countries on 5 continents. Materials and Methods: Heads of 15 neonatal screening laboratories agreed to participate and sent 10 randomly collected dried blood spot punches per week. All 10 dried blood spots were collected in a single tube per week and were fully anonymized, in concordance with policies of local ethical committees. Samples were stored at minus 20 degrees Celsius until testing, which was done by protein microarray as previously described (Koopmans M, et al. *Clin Microbiol Infect*. 2012;18:797) against 11 influenza virus type A haemagglutinin 1 (HA1) antigens of H-subtypes 1, 2, 3, 5, 7 and 9. An international standard serum against A(H1N1)pdm09 (NIBSC) was used to harmonize test results throughout the study. Results: The proportion of sera with reactivity above cut-off for the HA1 of influenza virus A/California/6/2009A(H1N1) pdm09 was 2% in the prepandemic period (samples collected before April 2009). This proportion ranged from 1%-7% in the early pandemic phase April 1st through June 11th when the pandemic alert was given. It ranged from 1%-27% in the period June 12th through December 2009, and from 4%-64% in 2010. As expected, a gradual increase in the proportion of seropositives coinciding with reports of increased influenza-like illness activity was observed in late 2009 (autumn) in most countries from the Northern Hemisphere, whereas virtually no increase in seropositivity rate was observed in samples collected in India, Lebanon, and South Africa in 2009. Remarkably, the data from Mexico (2 sites) showed a similar no-increase trend in 2009 despite high numbers of A(H1N1)pdm09 detections, whereas samples from Argentina reflected a high seropositivity rate starting July 2009. This was not explained by increased levels of antibodies to other seasonal influenza virus antigens, but seemed to reflect a preceding peak of A(H1N1)pdm09 virus detections in Argentina. The overall results showed good correlation with data from published serosurveys. Conclusions: We demonstrate that international comparative serosurveys can be performed in a highly standardized way through the use of prospectively collected neonatal screening dried blood spot samples and protein microarray. In addition, protein microarray allowed the use of a broad range of relevant antigens without impact on hands-on time and needed serum volume. Our data reflect the known trends for pandemic influenza A(H1N1)pdm09 in most countries with the exception of Mexico, where compared with virus detections a late rise in seroprevalence was observed in two sites. In contrast, our data suggest that Argentina had an early wave of pandemic influenza, starting June/July 2009.

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O-827

Influenza seasonality in Madagascar: the mysterious African free-runner

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Background: It has been proposed that nonenvironmental factors may dictate the seasonality of influenza in tropical settings where environmental forcing is limited, perhaps via population travel with regions that experience seasonal outbreaks. Madagascar is a particularly interesting case study because it is an island comprising a diversity of climatic zones, which may experience relatively limited external virus seeding relative to the continent. In this study, we aim to disentangle the contribution of environmental forcing and population travel on influenza transmission in Madagascar. We assess the association between influenza activity and local climate in Antananarivo (Madagascar's capital) and compare influenza activity patterns in countries in close proximity or with high connectivity to Madagascar. Materials and Methods: Influenza virus activity was based on laboratory testing of respiratory specimens contributed by sentinel health care centers participating in influenza-like-illness surveillance in Antananarivo. We compiled the weekly number of influenza A/H3, A/H1, A/H1N1pdm and B positive specimens for the period 2002 to 2012. We obtained similar data from WHO FluNet to study viral activity patterns in countries in the sub-Saharan African region and those contributing most foreign travelers to Madagascar (including France, Germany, and the Americas). Weekly climate indicators were compiled for the study period. Fourier analysis, Spearman cross-correlation and hierarchical clustering were used to assess statistical association between time series. Results: Viral detection in Antananarivo did not reveal a clear seasonal pattern, with an almost flat periodic annual signal with a slight trimodal pattern. Antananarivo generally experienced two influenza peaks in the year (roughly one in the middle of each semester), but those rarely coincided with winter peaks in the Northern or Southern Hemisphere. Overall in the interpandemic period,

influenza circulation in Antananarivo led viral activity in the Northern Hemisphere winter (in particular, France), and presented poor temporal matching with viral activity in other African countries. In contrast, the onset of widespread A/H1N1pdm virus activity in Antananarivo coincided with the timing of the second (and major) pandemic wave in the Northern Hemisphere countries in the fall of 2009, but this coincidence disappeared in the following years. Influenza detection was not associated with any climatologic signal in Antananarivo. Conclusions: Our analyses indicate that the timing of circulation of influenza strains in Antananarivo is not driven by seeding from countries that contribute the most intense travel volume to Madagascar, nor does it fall in the months with the most intense travel influx to the island. Influenza circulation in Antananarivo does not coincide with influenza seasonality in South Africa or with local climate factors either. Therefore, the intriguingly irregular circulation of influenza in Madagascar may obey unidentified internal factors that are highly stochastic, rather than "echo" patterns from more connected locales. This "free running" attitude opens fresh questions regarding the drivers of influenza seasonality in the tropics and confirms the importance of conducting robust year-round surveillance in this region to guide intervention strategies.

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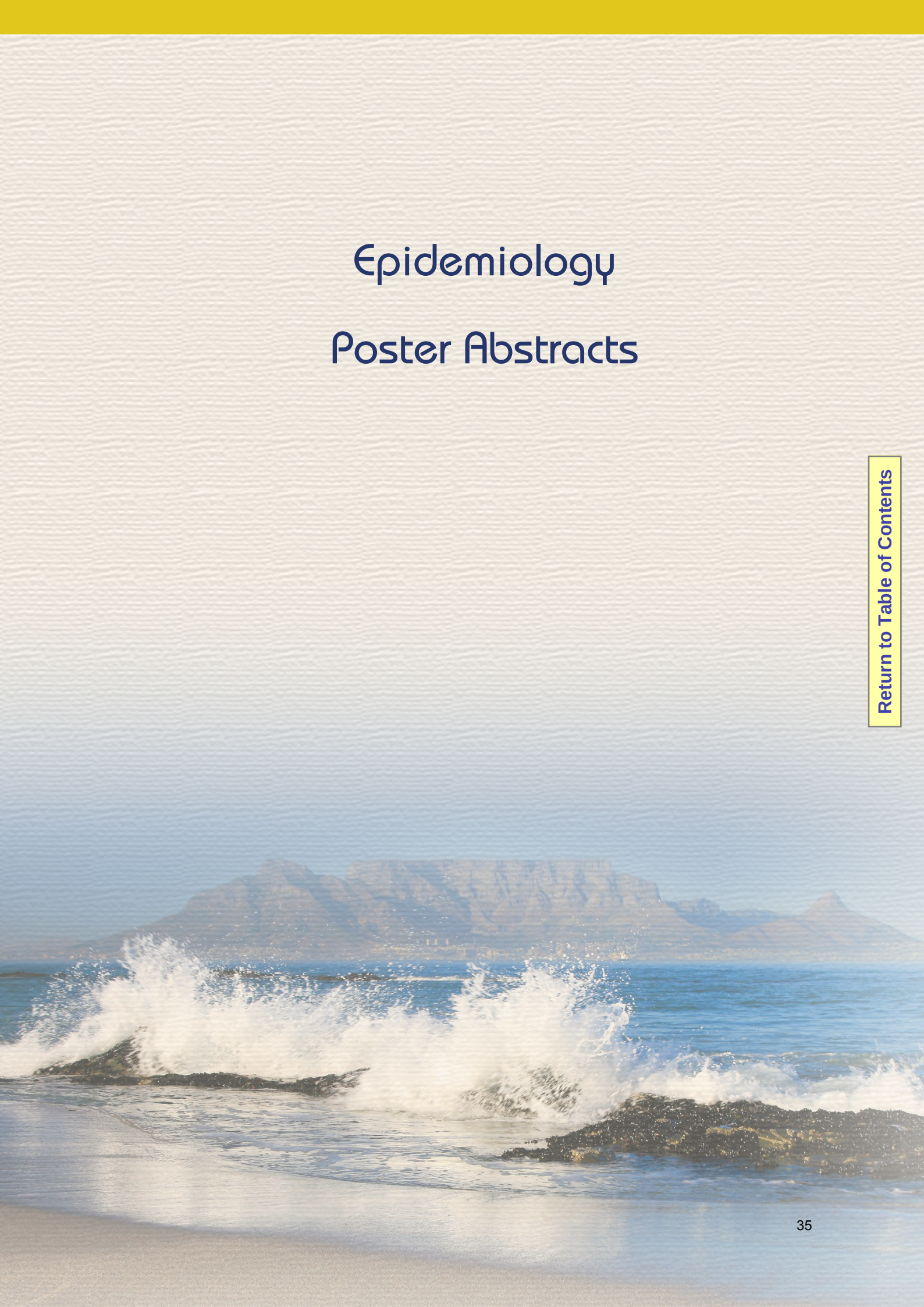
Epidemiology

Poster Abstracts



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P1-119 SCHOLARSHIP Promising Investigator Scholarship

The Nicaraguan Influenza Birth Cohort Study: methods and results from the first year

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Background: Influenza is a major public health problem worldwide; however, relatively little is known about influenza in tropical regions, especially in very young children. Materials and Methods: In September 2011, the Nicaragua Influenza Birth Cohort Study was established in Managua, Nicaragua, to study the burden of influenza in infants and toddlers. The study design allows for the identification of repeat infections, characterization of the immune response, and investigation of determinants of disease severity. We plan to enroll infants into the study for three years, with 250 infants recruited each year and followed until 2 years of age, for a total sample size of 750 infants. Approximately 21 newborns, aged 4 weeks or less, are enrolled in the study each month to maintain the age structure. Families of infants are contacted on a weekly basis by study personnel, and data on daily symptoms are collected using diary cards. Children are provided with all primary medical care through the study, and 189 variables are collected at each medical visit. All participants presenting with fever or reported fever are tested for influenza by RT-PCR. Respiratory samples are collected at the participant's home in the event that the family does not bring the infant to the health center for a consultation. Yearly blood samples are collected beginning at 6 months of age to enable detection of asymptomatic influenza infections and to evaluate nutritional status. Socioeconomic, household risk factor, and breastfeeding data are collected at enrollment and on a yearly basis. Numerous information and communications technologies are employed to manage study data, track samples, and maintain quality control, including smartphones, tablets, barcodes, geographic information system (GIS), and scannable forms. Results: To date, acceptance into the study has been high (96.2%), and loss to follow-up/withdrawal has been low (6.5% per year). As of May 2012, 472 infants have been enrolled, with a median age at enrollment of 14 days. At enrollment, a majority of infants were breastfed (96.1%); however, only 48.6% were exclusively breastfed. The participants attended more than 5008 medical visits at the study health center. Among the 244 infants who participated in the first year of the study, the incidence of influenza was 15.0 cases per 100 child-years (95% CI: 9.9, 22.8). A majority of influenza infections occurred in infants aged 6 months and older. A total of 71 participants were transferred to the hospital. Forty-four percent of the transfers to the hospital were for respiratory illness; one infant was transferred for bronchitis, one for bronchial hyperactivity and 29 for pneumonia. Two infants died of pneumonia. Conclusions: Infants in Nicaragua experience a high incidence of influenza with a majority of cases occurring in infants aged 6 months and older. This study will provide crucial information about the burden of influenza and the incidence and characteristics of sequential influenza infections in infants.

P1-120 G FELLOWSHIP GATES Fellowship

Influenza virus infection in Kenya

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Background: The World Health Organization estimates that acute lower respiratory infections cause nearly four million deaths per year, a rate of more than 60 deaths/100,000 population. Rates are even higher in developing countries, where pneumonia is responsible for an estimated 10%-25% of all deaths among children under 5 years of age. Although influenza has been widely studied in developed countries, little is known about its epidemiology, seasonality and burden in developing

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countries. Methods: A review of journal was done on papers for influenza virus infection in Kenya. A PubMed-based Web search was done on papers published before Jan 1, 2012. Results: The 2009 pandemic influenza A (pH1N1) virus was responsible for at least 20,000 laboratory-confirmed deaths globally. The first recognized case of laboratory-confirmed pH1N1 in Kenya was identified on June 29, 2009, and by September 2009 a majority of influenza cases in the country were caused by pH1N1. The rates for most viruses peaked from November to February. In one population-based study, of the 2079 patients with tested swabs, infection with influenza virus was confirmed in 204 (10%); 176, 27 and 1 were found to be RT-PCR–positive for influenza A virus only, influenza B virus only, and both influenza A and B viruses, respectively. The case fatality rate among admitted patients with PCR-confirmed infection with influenza virus was 2.0%.The annual rate of hospitalization (per 100,000 population) among patients with influenza ranged from 3-72 in different studies. The annual crude rate of hospitalization was highest in children < 1 year old (156 per 1000 children per year). Virus-positive hospitalization crude rates per 1000 child-years for children < 1 year old ranged from 2 to 35. The rates of RSV associated hospitalization were highest during November to February, with a minor increase in June. Conclusions: Respiratory viral infections, particularly RSV and AdV, were associated with high rates of illness and made up a substantial portion of respiratory infection. The rate of influenza-associated hospitalization was highest among children aged < 5 years. There are no adequate prevalence studies to conclude with figures. The magnitude of the disease prevalence needs to be studied with further research to reach those desperate patients.

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Update from the Consortium for the Standardization of Influenza Seroepidemiology (CONSISE)

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[†]Additional members of the Steering Committee include: Anthony Mounts, Wenqing Zhang from WHO; Eeva Broberg from ECDC, Jackie Katz and Tim Uyeki from US CDC; Karen Laurie from the WHO Collaborating Centre for Reference and Research on Influenza, Melbourne, Australia; Peter White and Steven Riley from the MRC Centre for Outbreak Analysis and Modelling, Imperial College London, UK; Benjamin Cowling and Malik Peiris from the University of Hong Kong, School of Public Health and Department of Community Medicine, Hong Kong; Katja Hoeschler and Richard Pebody from Public Health England, London, UK; Peter Horby from the Oxford University Clinical Research Unit in Hanoi, Vietnam; Monique St-Laurent from Public Health Agency Canada; Marianne van der Sande from National Institute for Public Health and the Environment (RIVM) and Olav Hungnes from the Norwegian Institute of Public Health, Oslo, Norway

Background: CONSISE, the Consortium for the Standardization of Influenza Seroepidemiology, is a global partnership to develop influenza investigation protocols and standardize seroepidemiology to inform health policy. This international partnership was formed in 2011 and was created out of a need, identified during the 2009 H1N1 pandemic, for timely seroepidemiologic data to better estimate pandemic virus infection severity and attack rates to inform policy decisions. Methods and Materials: CONSISE has developed into a consortium of two closely interlinked working groups (Working Group 1: Epidemiology and Working Group 2: Laboratory) and a steering committee composed of individuals from several organizations including the World Health Organization, the US Centers for Disease Control and Prevention, the European Centres for Disease Control, Public Health England (Formerly the UK Health Protection Agency), Imperial College London, the WHO Collaborating Centre for Reference and Research on Influenza (Melbourne, Australia), University of Hong Kong, Oxford University Clinical Research Unit in Hanoi, and Public Health Agency of Canada. CONSISE has held three international meetings (February 2011 in Ottawa, Canada; December 2011 in Stockholm, Sweden; and January 2013 in Hong Kong, SAR) and a fourth will be a satellite meeting immediately

preceding Options VIII in September 2013. Results: CONSISE's work plan is divided into epidemiology and laboratory working groups. The epidemiology working group is developing seven comprehensive epidemiologic, virologic and serologic investigation protocols that can be used in pandemic outbreak settings and for routine serologic collection during nonpandemic seasons. The epidemic/pandemic protocols under development include: 1) longitudinal cohort study of influenza virus infection during epidemic periods; 2) cross-sectional seroprevalence study of a novel influenza virus infection prior to- and postepidemic periods; 3) household transmission studies for pandemic influenza; 4) closed setting outbreak investigation protocol for pandemic influenza; 5) assessment of health care personnel in contact with infected influenza patients; a seasonal influenza protocol: 6) seroepidemiology of human influenza virus infection using residual sera/convenience samples for establishing baselines and/or monitoring trends over time; and a zoonotic influenza protocol; and 7) outbreak investigation of zoonotic influenza virus infection in humans exposed to a confirmed animal source of influenza virus. The protocols are specifically designed for influenza viruses, but may be adapted for other respiratory pathogens. Three of these protocols have recently been adapted for human novel coronavirus and for H7N9 investigations. The Laboratory Group has been working on a number of projects within the three themes identified at the Stockholm meeting: serologic assay standardization; quality assurance and assessment; and cooperation. As microneutralization (MN) assays using a two-day and a three-day format are both being used for influenza serology, they have been evaluated in a collaborative study of antibody to A(H1N1)pdm09 virus involving 14 laboratories from eight countries. Preliminary analysis concluded that there was no underlying reason why the two assay formats were not comparable. Further assays using H3N2 and H5N1 viruses are planned for the coming months. There are many differences in assay protocols for the haemagglutination inhibition (HI) serology assay, so HI assay protocols from different laboratories are being compared with a view to developing a consensus HI protocol. The next step will be a comparative evaluation of consensus HI protocol and local HI protocols. It is considered premature to implement an external quality assessment (EQA) scheme at the moment while standardised assay protocols are still being developed. Laboratories are encouraged to evaluate the enzyme-linked lectin assay (ELLA) neuraminidase inhibition assay, which has been developed by Dr Maryna Eichelberger (FDA, USA). Conclusions: CONSISE is playing a role in public health evaluation and management of newly emerging respiratory agents and has been called upon for nCoV and novel influenza virus investigations. Our materials will be open access and shared on the Global Health Network (http://CONSISE.tghn.org/) Web site by summer 2013. Validation of the CONSISE influenza protocols is planned for late 2013 to early 2014. We seek additional members from public health agencies, academic institutions and other interested parties.

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Factors influencing infection by pandemic influenza A(H1N1)pdm09 over three epidemic waves in Singapore

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Background: Previous influenza pandemics had second and on occasion third waves in many countries that were at times more severe than the initial pandemic waves. Several questions about the epidemiology of successive epidemic waves remain unanswered—are individuals infected in the initial epidemic wave protected in subsequent waves? Are there changes in age distribution and other risk factors between epidemic waves? Do these risk factors explain the differences in infection rates across waves? It is therefore important to explore the epidemiology of H1N1pdm09 infections after the first epidemic wave. Materials and Methods: This study aimed to determine the seroepidemiology of successive waves of H1N1pdm09 infections in Singapore, and the overall risks for infection. We

performed a cohort study among 838 adults, with blood samples provided on recruitment and at 5 additional points from 2009 to 2011 and tested by hemagglutination inhibition (HI) with A/California/7/2009 (H1N1pdm09). Surveys on key demographic and clinical information were conducted at regular intervals, and associations between seroconversion and these variables were investigated. Results: After the initial wave from June to September 2009, second and third waves occurred from November 2009 to February 2010, and April to June 2010, respectively. Seroconversion was 13.5% during the first wave and decreased to 6.2% and 6.8% in subsequent waves. After adjusting for effect of other covariates, participants had an overall lower odds of seroconversion in the second and third waves than the first wave. Across the three waves, the elderly and those with higher starting HI titres were at lower risk of seroconversion, while those with larger households were at greater risk. Females had significantly lower odds of seroconversion than males, and participants in larger households were associated with higher odds of seroconversion. Those with higher starting HI titres were also less likely to have an acute respiratory infection. Conclusions: The second and third waves in Singapore had lower serologic attack rates than the first wave. This may be due to the substantial number of individuals who were infected and developed immunity in the initial wave, and were hence less likely to be infected in subsequent waves, with the lower resultant force of infection on transmission of influenza in the population. The elderly and those with higher HI titres had lower risk while those in larger households had higher risk of seroconversion.

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A nation-wide study of the incidence of acute respiratory infections in Australia

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Background: The sheer number of people infected with acute respiratory infections (ARI) in a given year means that the nonhealth impacts are significant, although the burden remains unknown. We estimate the incidence, and describe the epidemiology of ARI in the Australian community. Materials and Methods: In 2008-2009, 7578 people across Australia were surveyed about common illnesses. ARI was defined as a person experiencing cold or flu with at least 1 of the following symptoms: fever, chills, sore throat, running noise, or cough in the past 4 weeks. Period prevalence of ARI in the 4 weeks prior to the survey was examined by season and state of residence. Incidence of ARI was obtained by weighting to the Australian population. We used Stata 12.1 to analysis data. Results: There were an estimated 68.9 million cases (95% CI 65.1-72.7 million) of ARI in Australia annually, equating to 3.2 (95% CI 3.0-3.4) episodes per person per year. Most cases occurred in winter with a 4-week period prevalence of 34.3% (95% CI 31.4-37.2), following by autumn (24.9%, 95% CI 22.0-27.8) and spring (24.6%, 95% CI 21.8-27.4). The incidence of ARI was highest in children and women of childbearing age. Incidence decreased with increasing age of survey respondent. Conclusion: ARI imposes a significant burden across Australia. Understanding the burden of ARI can assist in targeting interventions to reduce the incidence in certain groups.

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Sentinel severe acute respiratory infection (SARI) surveillance in 10 provinces of China, December 2009-April 2013

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Background: In December 2009, a sentinel SARI surveillance for inpatients was initialized in 10 sentinel hospitals, 4 of them located in the north of China and 6 of them located in the south of China. Materials and Methods: Inpatients who matched the SARI case definition were registered, and uniform case report form (CRF) information, including demographic, clinical symptoms, medical history, complications, treatment and outcomes, was collected. Respiratory specimens of the cases were collected and transferred to the influenza network laboratories to determine influenza subtype. We analyzed the surveillance data from December 2009 to April 2013. Results: A total of 5890 SARI cases were registered from December 2009 to April 2013 and had information collected. Of them, 551 (10.7%; 551/5890) SARI cases were influenza positive. The age median of SARI cases and influenza-positive cases was 12 and 29 years old, respectively. In terms of age of cases, 40.6% of SARI cases and 28.9% influenza-positive cases were under 5 years old, and 19.5% of SARI cases and 24.9% influenza-positive cases were ≥ 65 years old. The age distribution of SARI cases and influenza positive cases was different (P < .001). A majority of SARI cases (58.3%; 3433/5890) and influenza-positive cases (58.8, 324/551) were male, and there was no statistical significance in gender distribution between SARI cases and influenza-positive cases (P = .128). Of 551 influenza-positive cases, 329 (59.7%) cases were flu A positive, and 171 (31.0%) cases were flu B positive. The most common medical histories of adult SARI cases were hypertension, coronary heart disease, diabetes, COPD and chronic bronchitis. The most common medical histories of adult SARI cases were asthma and cancer/tumor; about 95% of child SARI cases had no medical history. There was a total of 124 fatal cases; of them, 22 cases were influenza positive, 80 (64.5%) deaths were in patients aged ≥ 65 years, and only 5 (4.0%) deaths occurred in children under 5 years of age. Conclusions: For the SARI% and number of SARI cases and influenza-positive rate, there is a regular winter peak in the 4 northern sentinel hospitals, which was similar with the findings of sentinel ILI surveillance. The highest burden of hospitalization for SARI cases and influenza- positive cases was in children under 5 years of age, but the highest burden of death for SARI cases and influenza-positive cases was in adults aged ≥ 65 years. Our findings could provide support for influenza vaccination policy.

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Inferring influenza infection attack rate from seroprevalence data

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Background: Seroprevalence studies are crucial for estimating infection attack rate and disease severity in an epidemic such as influenza. These studies typically entail selecting a titer threshold for seropositivity (eg, microneutralization [MN] 1:40) and assuming the probability of seropositivity per infection (infection-seropositivity probability, ISP) among community infections is 100% or similar to that among clinically overt cases. Different thresholds applied to the same seroprevalence data may give different IAR estimates if serologic responses of clinical cases is not representative (eg, clinical cases have higher ISP). Materials and Methods: To illustrate this, we fitted a transmission model to the 2009 pandemic influenza A/H1N1 (pdmH1N1) hospitalization and seroprevalence data in Hong Kong. Results: We estimated that while almost all pdmH1N1 infections became $MN_{1:20}$ seropositive, only 72%, 65%, 58% and 34% of infections among ages 3-12, 13-19, 20-29, 30-59, respectively, became $MN_{1:40}$ seropositive, much lower than the 90%-100% observed among clinical cases. The fitted model was consistent with prevailing consensus on pdmH1N1 transmission characteristics (eg, initial reproductive number of 1.28 and mean generation time of 2.4 days), hence our ISP estimates were consistent with the transmission dynamics and temporal buildup of population-level immunity. Conclusions: Incidence of pdmH1N1 in Hong Kong and other countries might have been previously underestimated, especially among adults, due to overestimations of ISP. Because antibody kinetics among asymptomatic or mild community infections will likely be lacking during a pandemic, real-time seroprevalence studies should evaluate the consistency of IAR estimates across multiple thresholds to provide timely and robust estimates of disease burden and severity.

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Epidemiological and virological characteristics of influenza-like illness outbreaks during 2012/13 influenza season in the UK

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Background: The UK has an established outbreak surveillance system for influenza-like illness (ILI) in closed settings to monitor activity of circulating influenza and other respiratory viruses. This abstract reports findings from the 2012/13 winter season and compares recent seasons in 20110/11 and 2011/12. Materials and Methods: A respiratory disease outbreak is defined as two or more cases with acute respiratory infections occurring in an institution with onset time within 7 days of each other. ILI outbreak information collected include outbreak onset date, reporting date, outbreak setting type, affected population, severity of cases, laboratory test results, influenza vaccination–related information and antiviral use. Information was collected using a standard outbreak reporting form. Epidemiologic and virologic characteristics of outbreaks reported in the period from week 40/2012 to week 16/2013 were described in terms of time, location, setting and virus. Comparisons were made with other ILI surveillance systems and the previous two season's data. Results: A total of 423 ILI outbreaks were reported during the data collection period between weeks 40/2012 and 16/2013 in the UK. Of these, 48.2% (204/423) occurred in care homes for the elderly, 39.2% (166/423) in schools, 9.2% (39/423) in hospitals and the remaining 3.3% (14/423) in other settings. ILI outbreaks were first reported in week 41/2012 (early October 2012). The number of reported outbreaks started to increase notably from week 47/2012 with first peak observed in week 51/2012 with 61 outbreaks mainly from school settings dominated by influenza B infection, followed by a prolonged period of activity with a lower, second peak reported in week 11/2013 (mid-March 2013) with 39 outbreaks, mainly from care home settings, dominated by influenza A (mainly H3N2 subtype). Sixty-four outbreaks in care homes reported severe cases being hospitalised with 1-8 hospitalised cases per outbreak, and the median case hospitalisation ratio was 12.5% (range 2.7% to 100%). Thirty-six outbreaks reported associated fatal cases (22 of these outbreaks were due to influenza A), with fatal case numbers ranging from 1 to 8 (case fatality ratio 0.5% to 11.1%). Of the 200 outbreaks with laboratory test information available, 66% (132/200) were due to influenza A, 22% (44/200) influenza B, 4.5% (9/200) with both influenza A and B, and the remaining 7.5% (15/200) were due to other respiratory viruses (RSV, rhinovirus and hMPV). Influenza vaccine uptake varied in care home populations (median 86.5%, range 11.5% to 100%) with information available from 165/204 care homes. Antiviral use information was recorded in 76 outbreaks in care homes, with antivirals (mostly oseltamivir) used in 43 (57%) outbreaks. This season's ILI outbreak pattern contrasts with outbreaks reported during the 2011/12 influenza season, which was dominated by influenza A(H3N2) and very low levels of influenza B activity. Activity of both co-circulating influenza A(H3) and B were higher in 2012/13 than that in 2011/12 season, and a much greater number of outbreaks were reported in 2012/13 (compared with 2011/12 (n = 174) for the same time period between week 40 and week 16 of both seasons. This season's pattern is also different from the outbreaks observed in 2010/11 season when A(H1N1)pdm09 and influenza B were co-circulating with outbreaks reported, mostly (74%) from schools. Conclusions: An unusual two-wave and prolonged epidemic pattern in ILI outbreaks was observed in the UK during the 2012/13 season. This pattern was consistent with that observed from other epidemiologic and virologic surveillance systems. The late-season H3N2 activity in often highly vaccinated care home settings and AVs not used in significant proportion was similar to that observed during the 2011/12 season and may have resulted from a number of factors including poor response to vaccine and waning immunity in elderly. These findings suggest that ILI infection control measures including vaccination and antiviral use in closed settings need to be strengthened. These findings also support that outbreak surveillance is an integral part of the ILI surveillance system, providing important additional intelligence on influenza activity in the community.

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The second and third waves of A (H1N1)pdm09 virus attacks in Mongolia

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Background: Detailed analysis of pandemic influenza in every country of the globe will contribute to the understanding of the real nature of influenza pandemics. We have reported the first wave of the A (H1N1)pdm09 in Mongolia to the Options-VII, and an article based on it has been published

elsewhere (Nymadawa P, et al. *IRV.* 2011;5(suppl 1):163-165). Here, we report the descriptions of the 2nd and 3rd waves of the pandemic A (H1N1)pdm09 in Mongolia. Materials and Methods: The epidemiologic and virologic data collected through the influenza sentinel surveillance system of National Influenza Center (NIC), Mongolia, were used for the analysis. For the epidemiologic analysis we used routine epidemiologic procedures, like calculation of weekly incidence rate of influenza-like illnesses (ILIs) per 10,000 population, tolerant limits based on the past 10 years' morbidity, etc. Influenza viruses were detected in nasopharyngeal samples from ILI patients by rt-RT-PCR with Applied Biosystems Fast Real Time PCR System 7500 using primers and instructions supplied by the CDC, USA. Influenza viruses were isolated by inoculation of rt-RT-PCR-positive samples of MDCK cell culture according to the standard protocol. Results: After forty-four weeks' interval from the end of the first wave, A (H1N1)pdm09 virus began to circulate from the 1st week of 2011. During the second wave of the pandemic, ILI activity was mild and it exceeded the upper threshold limit from the 1st to 4th week of 2011. Indeed, pandemic virus continued to be a causative agent in the community approximately ten weeks in total. The second wave of the pandemic lasted 2.8 times shorter in duration, peak morbidity was 3.2 times lower and outpatient visits were fewer by 2% in comparison to the first wave. Also virus spread was sporadic covering local communities. In the hospital-based sentinel sites 5406 patients were admitted with severe acute respiratory infections (SARIs) and there were 14 fatal cases (85.7% in children < 4 years old). The positive rate for influenza A (H1N1)pdm09 in 2010/11 decreased by 56.5% and reached 24.2%. The 2011/12 influenza season was dominated by influenza A (H3N2) virus between February and April 2012, and thereafter influenza A (H1)pdm09 virus was introduced simultaneously, causing the third wave of A (H1N1)pdm09 virus. The virus caused a relatively mild epidemic, not exceeding the threshold limit, and lasted approximately five weeks. During that period there were 1749 patients registered with SARI and 2 fatal cases due to SARI. In the third wave the positivity rate for A (H1N1)pdm09 virus was 8.0%. Conclusions: In Mongolia, the magnitude of A (H1N1)pdm09 virus attacks decreased from the first to the subsequent waves. The first wave of A (H1N1)pdm09 virus attack was replaced by influenza B virus, but in the last two waves, pandemic virus was followed by A (H3N2) virus intensive circulation in the community.

P1-129

Analysis of potential changes in seriousness of influenza A and B viruses in Hong Kong from 2001 to 2011

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Background: Continued monitoring of the seriousness of influenza viruses is a public health priority. The infection fatality risk (IFR), defined as the risk of death among people infected by a virus, is a useful measure of seriousness of influenza virus infections. When estimating excess influenza mortality via linear regression modeling, the regression coefficient for influenza activity is in theory directly proportional to the IFR. We investigated drifts over time in the regression coefficients for influenza activity to assess potential changes in the association between mortality rates and influenza activity in Hong Kong. Materials and Methods: We applied time series regression models to data on cardiorespiratory mortality rates in Hong Kong from 2001 through 2011. We used surveillance data on outpatient consultations for influenza-like illness, and laboratory detections of influenza types/subtypes to construct proxy measures of influenza activity (Wu P, et al. J Infect Dis. 2012). In the regression model we allowed the regression coefficients for influenza to drift over time, lagged by 1 week to allow for a delay between infection and death, and adjusted for temperature and humidity. We selected specific potential change points in influenza A that occurred before and after at least three influenza seasons in our study period, namely A(H3N2) in 2005 and 2007, and A(H1N1) in 2008, and used the Akaike Information Criteria as a measure of goodness-of-fit between models with different combinations of change points. Results: There were 14 influenza seasons in Hong Kong from 2001 through 2011(excluding the year 2003). The estimated regression coefficients for A(H3N2) remained constant in 2001-04, there was a substantial step increase at the beginning of 2005 and the coefficient remained fairly stable thereafter. The regression coefficients for A(H1N1) remained fairly

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stable from 2001 through 2011, while there was a suggestion of an increase in the coefficient at the end of the period during the second epidemic wave of A(H1N1)pdm09 in 2011. The regression coefficient for influenza virus B slowly increased over time with no rapid changes. Conclusions: Our model suggested an increase in seriousness of A(H3N2) that could be associated with the appearance of the A/Fujian/411/2002(H3N2)-like virus. However, an apparent increase in the regression coefficient in A(H1N1)in early 2011 could be an artifactual change because the A(H1N1)pdm09 virus was associated with a higher cumulative incidence of infection in children than seasonal viruses. Ongoing monitoring of mortality and influenza activity could permit identification of future changes in seriousness of influenza virus infections.

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Epidemiological analysis of influenza-like illness (ILI) and severe acute respiratory infections (sARI) in Mongolia, 2007-2012

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Background: Mongolia's Health Service started influenza surveillance in the 1970s when the country has experienced population growth and urbanization. Initially, surveillance included influenza-like-illness (ILI) counts with some laboratory confirmation, which was conducted only in the capital city, Ulaanbaatar. Over time, this surveillance became more comprehensive by expanding ILI and sARI and has involved all aimags (provinces) of the country since 2009. We involved the surveillance data from 2007/08 to 2011/12 season because our surveillance data on ILI and sARI became more completed in the selected areas in that period. Materials and Methods: We studied the epidemiology of influenza through an analysis of both ILI and sARI surveillance data from the 2007/08 season to 2011/12 season. Incidence rates of ILI and sARI were calculated as the proportion between the number of ILI and sARI and the total population in the areas covered by surveillance for each year. We calculated a tolerant limit by Serfling method (cyclic regression model) using nationwide data on ILI morbidity per 10,000 population from 2000 to 2011 year in Mongolia, and the peak period of influenza season is defined as an interval when the number of ILI cases exceed above the upper tolerant limit and a proportion of influenza virus positives exceed 15% or more. We used the real-time reverse transcriptase polymerase chain reaction (rtRT-PCR) assays for detecting influenza viruses according to the standard protocol developed by the Centers for Disease Control and Prevention (CDC), United States, and primers were provided by WHO Collaborating Centers. Results: During the five influenza seasons, there were 14.0 ILI and 0.8 sARI episodes per 100 populations per year on average. The highest ILI incidence of 22.2 was observed in 2009/10 season, which was 1.6 times higher than the average. The highest sARI incidence of 1.0 was also observed in the 2009/10 season, which was 1.3 fold higher than the average. Overall the ratio of sARI episodes to ILI episodes was calculated as 0.054. With age-stratified incidences of ILI and sARI, the highest incidence was observed among children aged 0-4 years. The number of ILI cases showed a clear seasonality generally peaking between December and February, except for the 2009/10 season when an early peak in October and November was observed, which was caused by A (H1N1)pdm09. In contrast, sARI incidence showed slightly different patterns from ILI. There were generally two peaks in each season; the first peak in October to December and the second one in January to February. During the study period at the selected sites, there were a total of 65,392 sARI hospitalization (7.8% of all hospitalizations) with 181 fatal cases (0.3% of all sARI hospitalization). Mainly fatal cases were registered during the epidemic period of each season. Children < 4 years old accounted for 77.1% of the hospitalized pneumonia and 84.0% of all mortality cases. Dominant influenza types/subtypes were also different year by year. Influenza B was dominantly detected in 2007/08 and 2011/12, influenza A (H3N2) was in 2010/11 while seasonal A (H1N1) was in 2008/09 season. In the 2009/10 season a predominant virus was A (H1N1)pdm09. Total positive rates of influenza were 2.2% in 2007/08, 4.2% in 2008/09, 25.6% in 2009/10, 10.5% in 2010/11 and 11.2% in 2011/12. Conclusions: Collecting epidemiologic surveillance data is needed to gauge the timing and peak of the influenza season and is an important pandemic preparedness activity. The recent pandemic A(H1N1)pdm09 influenza

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outbreak has also highlighted the importance of timely surveillance data to monitor epidemiologic trends for guiding public health control measures.

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Investigation of human infections during H5N1 crow outbreaks in Jamshedpur, India

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Background: Highly pathogenic avian influenza (HPAI) H5N1 emerged in China in 2003. Since then, it caused 628 cases including 374 deaths (59.55%) as reported to the World Health Organization (WHO) as of 26 April 2013. H5N1 virus continues to pose a serious threat to global public health. In spite of many outbreaks since the first poultry outbreak in January 2006, India has not reported any confirmed human cases. However, neighboring Bangladesh has reported 7 cases and 1 death since 2008. Importantly, H5N1 strains from Bangladesh are genetically similar to those isolated from West Bengal, Assam and Tripura states in India. Outbreak response activities during H5N1 poultry/wild bird outbreaks mainly focus on respiratory illnesses among occupational risk groups. Serosurveillance studies are usually undertaken following the confirmation of human H5N1 disease. These are mostly limited to detect asymptomatic infections among contacts of confirmed cases. There is limited literature on the need and usefulness of serosurveillance studies in the absence of laboratory-confirmed clinical H5N1 disease. The similar efforts in India earlier did not yield any asymptomatic infections. In November 2011, an unusual high mortality among crows from Jamshedpur (Jharkhand) was confirmed as the H5N1 virus. Crow mortality was reported in Jamshedpur from 28th August 2011, peaked in September 2011 and continued until March 2012. Five poultry samples also showed the presence of H5N1 virus. The virus belonged to the 2.3.2.1 clade. However, no human illnesses were reported. The recent affliction of crows during the H5N1 virus outbreak implied an increased risk of human infections. This prompted us to explore the possibility of asymptomatic human infections during the recent H5N1 crow outbreaks in Jamshedpur, India. Materials and Methods: Serologic survey was undertaken between the 10th and 18th of March 2012, approximately 3-4 months of reported peak activity. Outbreak investigations confirmed H5N1 virus as the etiology in November 2011 with the infected poultry hypothesized to be the probable source. Occupational risk groups were investigated for exploring the possibility of asymptomatic infections and understanding the behaviors and practices likely to transmit infections. Sample-size calculation was not attempted as it was designed as an exploratory pilot study. The study protocol was approved by the institutional human ethics committee. Written informed consent was obtained from each study participant. A total of 128 human subjects involved in outbreak response activities were interviewed using the questionnaire for the list of activities undertaken and likely exposures. The occupational exposures were listed and categorized as either direct or indirect. The blood samples (5 mL) were collected in sterile evacuated gel tubes, centrifuged, sera separated and transported on cold-chain for storage at –20ºC until testing. Sera were screened for antibodies against HPAI H5N1 virus by hemagglutination inhibition (HAI) and microneutralization (MN) assays. HAI assay was performed using horse and turkey red blood cells as per the protocols recommended by World Health Organization. MN assays were undertaken in biosafety level 3 laboratories. HI and MN assays were performed using H5N1 virus similar to the 2.3.2.1 clade obtained from the Centers for Disease Control, USA. Antibody titers of ≥ 1:40 for HAI and ≥ 1:80 for MN assays were considered positive.Results: All veterinary (poultry) workers were exposed directly during the outbreak response procedures like slaughtering, defeathering and handling of dead crows or cleaning of infected premises. All of them reported use of personal protective equipment (PPE). All sera were negative for H5N1 specific antibodies by both HAI and MN assays. Conclusions: Asymptomatic human infections were not detected in occupational risk groups during H5N1 virus crow outbreaks. The use of PPE might have reduced the exposure risks. The serosurveillance studies need to be evaluated for early detection of infections during the poultry or wild bird outbreaks.

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Use of real time RT-PCR to estimate influenza A (H3N2) virus shedding: Association of cyc

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Background: Real-time reverse transcriptase polymerase chain reaction (rRT-PCR) is widely used for influenza diagnosis. Cycle threshold (Ct) values have been used as a proxy for the amount of influenza virus nucleic acid in a given respiratory sample. Ct values are inversely related to the amount of nucleic acid: low Ct value indicates a high viral load. However, Ct values are not standardized, and the relationship between Ct value and clinical characteristics of influenza is largely unknown. We performed an exploratory analysis to examine the association between Ct value and demographic or clinical characteristics among patients with influenza A (H3N2) infection who were enrolled in a vaccine effectiveness study during the 2011-12 and 2012-13 influenza seasons in Wisconsin. Materials and methods: Adults and children seeking outpatient care for acute respiratory illness lasting ≤ 7 days were prospectively enrolled, and combined nasal and oropharyngeal swabs were obtained from consenting individuals for influenza testing. Clinical and demographic data were obtained from the enrollment interview and medical record. Influenza was confirmed by RT-PCR using the LightCycler Real-Time PCR System (Roche Diagnostics); PCR primers and probes were provided by CDC. Samples with Ct value < 40 were defined as positive for influenza. A human RNase P gene primer and probe set was used as a positive control for the presence of human nucleic acid. Mean Ct values were compared by t-test or ANOVA. A linear regression model assessed clinical and demographic factors associated with higher Ct value. Influenza vaccination status was determined using a validated immunization registry, and participants who received one or more doses were considered vaccinated. The analysis was restricted to participants with a positive RT-PCR for influenza A (H3N2). Children < 2 years old were excluded because sample collection procedures differed in that age group. Results: There were 523 patients with influenza A (H3N2) infection in the analysis, including 159 enrolled in 2011-12 and 364 in 2012-13; 232 (44%) were men and the mean (±SE) age was 33.1 ± 1.1 years. The mean (±SE) Ct value was 26.6 ± 0.2. In univariate analyses, Ct values did not differ significantly by gender or self-rated general or current health status. Mean (±SE) Ct values were 24.8 ± 0.4 for children 2-8y, 27.5 ± 0.5 for children 9-18y, 27.3 ± 0.4 for adults 19-49y, 27.5 ± 0.5 for adults 50-64y, and 25.4 ± 0.6 for adults ≥65y after controlling for interval from illness onset to swab date, termed “swab interval” ($p < 0.0001$). The mean (±SE) Ct value was 25.7 ± 0.3 for individuals with a swab interval 0-3 days and 28.5 ± 0.4 for a swab interval 4-7 after controlling for age group ($p = < 0.0001$). The mean (±SE) Ct value was 25.8 ± 0.3 among patients who were vaccinated, and 27.2 ± 0.3 among those not vaccinated, after controlling for age group and swab interval ($p= 0.001$). This association was also seen after excluding 115 children under 9 years old ($p = 0.008$). In a linear regression model, Ct was associated with age group (β ±SE: 0.4 ± 0.2 for each increase in age group, $p = 0.02$), swab interval (β ±SE: 2.7 ± 0.5, $p < 0.0001$), receipt of influenza vaccine (β ±SE: -1.6 ± 0.4, $p = 0.0004$), and male gender (β ±SE: -0.8 ± 0.5, $p = 0.05$). Conclusions: Lower Ct values (i.e., higher viral load) were associated with shorter swab intervals, consistent with other studies that have demonstrated reduced viral shedding over time. Lower Ct values were also associated with younger age, consistent with existing evidence that viral shedding is higher in children. In an unexpected finding, Ct values were significantly lower in persons who received at least one influenza vaccine after adjustment for potential confounders, suggesting greater shedding of virus nucleic acid in persons with vaccine failure compared to unvaccinated persons. The potential biological mechanism is unknown; this finding requires confirmation in other seasons and populations.

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The immunogenicity of influenza A(H1N1)v pandemic virus studied in different models

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Background: The new influenza pandemic announced by WHO in 2009 was caused by the influenza A(H1N1)v virus, which resulted from multiple gene reassortment occurring between swine, avian and human viruses. Its antigenic structure was rather new for humans; thus studying the A(H1N1)v virus's immunogenic properties is important for the spread control and specific prophylaxis, and mainly for the development of vaccines. The aim of this study was to access the immunogenic properties of pandemic influenza virus A (H1N1)v in different population groups. Materials and Methods: Several antigens were studied in the HI test: A/Brisbane/59/07 (H1N1), A/Brisbane/10/07 (H3N2), A/Perth/16/09 (H3N2), A/California/07/2009 (H1N1)v, B/Brisbane/60/2008-like (Victoria Lineage), B/Florida/4/2006-like (Yamagata Lineage) taken from Kit-WHO. Seven virus strains (various isolations of the Moscow 2009 (H1N1) pandemic virus) were studied in the microneutralisation assay: A/Moscow/IIV01;02;03;09;11;90;92/2009 (H1N1)swl. Monovalent pandemic vaccines included A/California/07/2009 (H1N1)v virus. Trivalent seasonal vaccine included A/Brisbane/59/07 (H1N1), A/Brisbane/10/07 (H3N2) and B/Brisbane/60/2008-like (Victoria Lineage) viruses. Comparative study of vaccines of different type (trivalent seasonal and monovalent pandemic ones) was performed on animal models. Three various vaccines were included into this investigation, which differed by their type (split or polymer-subunit) and HA content (15 µg or 5 µg). The rats were vaccinated three times in dose of 0.5 mL IM. The titers of specific antibodies were accessed in HI test and microneutralisation assay. Investigation of the population immunity with respect to pandemic A(H1N1)v virus was performed in four different age groups: 1) children vaccinated by seasonal trivalent vaccine before pandemic (2008-2009); 2) medical workers possessing a high risk to be infected during pandemic (2009); 3) patients with PCR-confirmed A(H1N1)v and with PCR-unconfirmed influenza, and also pregnant females (2009-2011); 4) schoolchildren and teachers of residential care after the first wave of pandemic (spring of 2010). Paired sera were studied in HI. Results: Specific antibodies to the pandemic influenza A (H1N1)v virus were found only in those rats that were vaccinated by monovalent pandemic vaccines. In rats vaccinated by the seasonal vaccines, only antibodies reactive with the vaccine composition strains A(H1N1), A(H3N2) and B virus, and no antibodies reactive with the pandemic A(H1N1)v virus, were found. The microneutralisation test revealed that the high titers of specific antibodies generated by the monovalent pandemic vaccine reduced the virus reproduction in MDCK cells. Results of HI test and microneutralisation assay differed with respect to detection of specific antibodies for pandemic A(H1N1)v virus in the compared groups. In children who were vaccinated with the seasonal vaccine, only the antibodies reactive with the seasonal strains were found, but not ones reactive with the pandemic strain even when high titers of seasonal A(H1N1) antibodies were detected. Only 10% of evaluated population of medical workers possessed antibodies to the A(H1N1)v virus at a protective level by the end of 2009. About 78% of assessed schoolchildren in a boarding school and 30% of teachers, which were in close contact during pandemic spreading, had antibodies to the pandemic strain by the spring of 2010; in paired sera increased levels of specific antibodies both to pandemic A(H1N1)v and seasonal A(H1N1) viruses were detected in the group of hospitalized patients infected with the A(H1N1)v virus. Conclusions: Immunogenicity of monovalent vaccines depended on their type, HA content and strain composition. The seasonal vaccine was not protective against pandemic A(H1N1)v virus infection. Monovalent pandemic vaccines had high immunogenicity resulting in production of specific antibodies that neutralized virus reproduction in MDCK cells. The buster effect of antibodies' titer increasing in relation to a seasonal A(H1N1) virus after the infection with pandemic A(H1N1)v virus was also found. We detected decreased antibody titers in pregnant females.

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Self-reported severity and impact of illness of influenza and noninfluenza acute respiratory illnesses among pregnant women in the United States, 2010-12

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Background: Although pregnant women are at increased risk of complications from influenza virus illness, little is known about the clinical characteristics and severity of influenza illness among nonhospitalized pregnant women or about how influenza illness differs from noninfluenza acute respiratory illness (ARI) in pregnant women. Methods: The study took place in two metropolitan areas on the west coast of the United States over two influenza seasons (2010–2011 and 2011–2012). Eligible women were pregnant and attended at least one prenatal visit. Potential ARIs were identified by daily surveillance of medical visits (using *ICD-9* codes 460–466 and 480–488) during both seasons and by weekly Internet- and/or telephone-based surveillance among a prospective cohort enrolled in the first season only. Respiratory specimens were collected using nasopharyngeal swabs from women reporting fever/feverishness/chills and cough within 7 days of illness onset and tested for influenza viruses using real-time reverse transcription polymerase chain reaction assay. At specimen collection, women rated the severity of 8 illness symptoms (0–3; absent to severe), and these were summed as a severity score (0–24). Women also rated the extent to which the illness interfered with their daily activities from 0 (unable to perform) to 9 (able to perform usual activities). Illness duration, duration of fever, and use of prescription or over-the-counter medications were assessed in a follow-up phone interview 10–14 days after swab collection. Highest recorded temperature was taken from the follow-up interview, or from the medical record for visits that occurred during the illness. Chronic medical conditions, dispensed prescriptions, and subsequent medical encounters were extracted from medical records. Results: Of 292 women interviewed and swabbed, 100 (34%) tested positive for influenza (45 A[H1N1]pdm09, 33 A[H3N2], 1 influenza A unsubtyped, or 21 influenza B viruses) and 192 (66%) were influenza negative. These groups (influenza positive vs negative) did not differ in demographic characteristics or trimester of pregnancy; most women were in their second (43%) or third trimester (42%) at the time of illness. However, influenza-positive women were less likely than influenza-negative women to have a chronic medical condition (17% vs 30%; $P < .05$) and less likely to have a prepregnancy body mass index (BMI) ≥3 0 (15% vs 31% obese; $P < .05$). Women with influenza rated their symptoms as more severe (mean ± standard deviation = 14.1 ± 3.5) than those with noninfluenza ARI (12.7 ± 3.9) ($P < .01$); specifically, cough, myalgia, feverishness, and chills were rated as more severe for influenza illnesses ($P < .05$). Women with influenza were also less able to perform daily activities (3.1 ± 1.9 on a scale of 0–9) than women with noninfluenza ARI (4.2 ± 2.3) ($P < .001$). Differences between influenza and noninfluenza illnesses in symptom severity and interference with activities remain statistically significant after adjusting for site, season, age, chronic medical conditions, BMI, and days between illness onset and specimen collection. Most influenza illnesses (63%) were associated with a fever (≥ 100°F), compared with only 25% of noninfluenza ARI ($P < .001$). In a multivariate model, adjusting for site, age, season, chronic medical conditions, BMI, and days since illness onset, high fevers (≥ 102°F) were 8-fold more likely (adjusted odds ratio [AOR] = 8.09; 95% confidence interval = 3.32–19.55) and prolonged fevers (≥ 2 days) were twofold more likely (AOR = 2.61; 2.32–2.96) among women with influenza compared with noninfluenza ARI. The duration of illness, number of medical encounters, and number of antibiotics dispensed did not differ for influenza versus noninfluenza ARI. However, women with influenza were more likely to report using over-the-counter medication and antiviral medication (92% and 19%, respectively) than women with noninfluenza ARI (81% and 3%) ($P < .05$). Conclusions: Pregnant women with influenza described their illnesses as more severe and more disruptive to their daily activities than women with

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noninfluenza ARIs. Most notably, influenza illnesses were substantially more likely to be associated with high and prolonged fevers, which is of particular concern given that febrile illnesses may increase the risks of adverse fetal outcomes. These findings highlight the need for all countries with influenza vaccine programs to vaccinate pregnant women, as recommended by the World Health Organization.

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New approaches to safety surveillance of seasonal trivalent influenza vaccine in pregnant women: experiences from FASTMum 2013

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Background: Pregnant women are at increased risk of morbidity and mortality due to serious influenza-related complications. Antenatal vaccinations with seasonal trivalent influenza vaccine (TIV) can reduce morbidity among pregnant women, and the available data suggest a protective effect among infants born to vaccinated mothers. For these reasons, in 2012, the World Health Organisation's Strategic Advisory Group of Experts on Immunization stated that pregnant women are the highest priority for seasonal influenza vaccination. Influenza vaccination coverage rates among pregnant women remain low in a number of countries. One of the reasons for this low coverage may be related to pregnant women's perceptions of influenza vaccination. Although the safety of influenza vaccination administration during pregnancy has been well established, half of antenatal patients who refuse vaccination cite safety concerns as their primary reason. Ongoing and timely safety surveillance could help reassure pregnant women and promote vaccine uptake. In 2013, the Western Australia Department of Health modified its TIV safety monitoring system FASTMum (Follow-up and Active Surveillance of TIV in Mums) to collect information regarding the safety of TIV in pregnant women using SMS technology. Materials and Methods: In Western Australia, seasonal antenatal influenza vaccinations are reported to the Western Australia Department of Health by vaccination providers. In 2013, SMS technology was introduced as a semiautomated, rapid method to capture adverse events following immunization in a timely manner. Of the 2203 antenatal vaccination reports received between 01 March and 15 May 2013, 1852 (84.1%) of these women consented to SMS follow-up and provided a mobile telephone number. One week post vaccination, the Western Australia Department of Health sent a SMS message to these women asking if they had experienced any reactions following the 2013 TIV. Women who responded "yes" to this message were followed-up with a telephone questionnaire. Results: On average, complete information regarding adverse events following TIV was obtained within one month (28 days) of vaccine administration. Of the 1425 women who responded to SMS in the first two months of influenza season, one in ten (10.8%) pregnant women reported a nonserious adverse event one week following receipt of the 2013 TIV. Of these women, 52 (3.6%) reported a local reaction at the site of injection, 29 (2.0%) reported fatigue, 28 (2.0%) reported fever, 27 (1.9%) reported respiratory symptoms (eg, coughing, congestion, sneezing), and 24 (1.7%) reported headache. Of the 427 women who did not respond to SMS, 333 (88.0%) were followed-up with a telephone interview. With the exception of injection-site reaction, the rate of adverse events was consistent with that of women who responded to SMS. No serious reactions related to the influenza vaccine were identified. Conclusions: The results from FASTMum 2013 are promising and provide timely evidence of 2013 TIV safety in pregnant women in the early months of influenza vaccination season in the southern hemisphere. These results demonstrate the feasibility of using mobile phone technology to monitor vaccine safety in near real-time. Information resulting from this surveillance effort is important for addressing lingering concerns among providers and antenatal patients about the safety of influenza vaccine for pregnant women.

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Epidemiology and clinical presentation of influenza types and subtypes among patients hospitalized with acute lower respiratory illness in South Africa, 2009-2012

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Background: The frequency of types and subtypes of influenza viruses that circulate in humans varies each year. Clinical and epidemiologic differences between types and subtypes have been reported, for example, the younger age distribution of the first wave of 2009 pandemic influenza A(H1N1) strain [A(H1N1)pdm09] compared with previously circulating types and subtypes. However, reports are conflicting, and little or no data have emerged from Africa. We sought to compare the demographic and clinical characteristics, risk factors, and severity among case-patients hospitalized in South Africa with severe acute respiratory illness associated with (1) influenza A(H1N1)pdm09, influenza A(H3N2), and influenza B from 2009-2012, and (2) influenza A(H1N1)pdm09 in the first wave in 2009 and second wave in 2011. Materials and Methods: Children and adults of all ages with influenza-confirmed severe acute respiratory illness (SARI) were identified through active, prospective, sentinel hospital-based surveillance system in four provinces in South Africa. Respiratory specimens were tested by multiplex real-time reverse transcription polymerase chain reaction (PCR) assay for influenza; influenza A–positive specimens were further subtyped by PCR. Blood specimens were tested by PCR for pneumococcus and HIV. Clinical and epidemiologic information was gathered by patient interview and medical record review. Multinomial and logistic multivariable regression models were used to identify factors associated with SARI caused by each influenza type and subtype and first and second wave of A(H1N1)pdm09, respectively. Results: From 2009 to 2012, 1238 (8.0%) of 15,540 SARI patients tested positive for influenza. Of these, 463 (37.4%) were influenza A(H3N2), 336 (27.1%) were influenza A(H1N1)pdm09, 419 (33.8%) were influenza B, and 20 (1.6%) were not subtyped. The predominant type or subtype each year was as follows: A(H3N2) (192/375, 51.2%) in 2009, B (164/273, 60.1%) in 2010; A(H1N1)pdm09(139/361, 38.5%) in 2011; and B (105/204, 51.5%) in 2012; in addition, the first wave of influenza A(H1N1)pdm09 (159/375, 42.4%) occurred in 2009. Case-patients with influenza B were older than those with other types and subtypes (B median age 23.7 years [5%-95%ile 0.2-63.2], A(H3N2) median age 2.8 years [5%-95%ile 0.2-64.6], A(H1N1) median age 5.1 years [5%-95%ile 0.3-60.6)]; $P < .001$ by Wilcoxon rank sum test). HIV testing results were available for 955 (77.1%) of influenza cases; of those, 399 (41.8%) tested positive. On univariate analysis, case-patients with influenza A(H1N1)pdm09 were less likely to be coinfected with any respiratory virus other than influenza (crude relative risk ratio [cRRR] 0.6; 95% CI 0.4-0.8). Case-patients with influenza B were more likely than case-patients with influenza A(H3N2) to be HIV infected (cRRR 1.6, 95% CI 1.2-2.3) and hospitalized for ≥ 2 days (cRRR 1.9; 95% CI 1.4-2.6) compared with < 2 days). Multivariable logistic regression, controlling for year, found case-patients with influenza B were more likely to be HIV infected (aOR 1.4; 95% CI 1.01-1.8) than patients with influenza A. The median age of case-patients infected during the second influenza A(H1N1)pdm09 wave was higher than those infected during the first influenza A(H1N1)pdm09 wave (2009 median age 3.6 years [5%-95%ile 0.3-59.0] vs 2011 median age 19.2 years [5-95%ile 0.2-59.0]; $P < .001$ by Wilcoxon rank sum test). In multivariable logistic regression, controlling for age and surveillance sites as categorical variables, case-patients infected during the second influenza A(H1N1)pdm09 wave were less likely to have pneumococcal coinfection (aOR 0.3; 95% CI 0.1-0.8) than case-patients

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infected during the first pandemic wave. There was no difference in the case-fatality rate between types and subtypes nor between the first and second wave of A(H1N1)pdm09. Conclusions: Clinical disease in patients hospitalized with SARI in South Africa differed by infection with different influenza types and subtypes, particularly in regard to year, age distribution, and coinfection with HIV and pneumococcus. Patients with influenza B and the second wave of influenza A(H1N1)pdm09 were older. No increase in lethality was noted between the first and second waves of influenza A(H1N1)pdm09. Influenza B may be associated with HIV infection compared with influenza A.

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The burden of influenza in young Kenyan children

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Background: Influenza-associated disease burden among children in sub-Saharan Africa is not well established. Annual estimates of influenza-associated hospitalizations in children from 2008-2011 in the US states of Oregon and Michigan ranged from 0.2-2.3 per 1000 children < 5 years old. Of US children with laboratory-confirmed influenza, only 28% of inpatients and 17% of outpatients were clinically diagnosed with influenza by their health care provider. Among 14 African countries during 2006-2010,10% of children 0-4 years old hospitalized with severe acute respiratory illness (SARI) were influenza positive by rRT-PCR, but these surveillance data cannot assess the rates of disease needed to understand the burden of influenza. We estimated the burden of influenza in children < 5 years old through population-based influenza-like illness (ILI) surveillance in 2 communities in Kenya: Kibera, an informal urban settlement of Nairobi, and Lwak, a rural community in western Kenya. Materials and Methods: From January 2008 to December 2012, household surveillance for ILI was conducted every 2 weeks to capture all persons with ILI in the household regardless of whether or where they sought care. The ILI case definition was temperature of ≥ 38°C plus cough or sore throat with onset in the past 7 days. Household surveillance was conducted weekly from September 2009 to January 2010 when the influenza A (H1N1)pdm09 strain was introduced. Information was also gathered on hospitalization from SARI in both sites, although laboratory confirmation of hospitalized patients was only available in Lwak. Nasopharyngeal and oropharyngeal specimens were obtained from patients who met the ILI or SARI case definition and attended the study outpatient clinic (both sites) or who met the SARI case definition and were admitted to the study hospital in Lwak. Specimens were tested by rRT-PCR for influenza. The case definition for SARI was based on a modified WHO Integrated Management of Childhood Illness (IMCI) severe pneumonia definition: cough or difficulty breathing with presence of a danger sign or oxygen saturation ≤ 90% on room air. Population-based rates of influenza-associated hospitalization, medically attended SARI (inpatient and outpatient), and medically attended ILI were calculated for children < 5 years. Crude rates were adjusted for health-seeking outside the study clinics, and the proportion of influenza-positive among sampled children was applied to those without influenza test results. Results: The average rate of influenza-associated hospitalization (inpatient SARI) was 3.6 (95% CI 2.3-75.5) per 1000 person-years in children < 5 in Lwak. The average rate of influenza-associated medically attended SARI (inpatient and outpatient) in children < 5 was 13.6 (95% CI 10-18.5) per 1000 person-years in Lwak and 20.2 (95% CI 16.4-24.9) per 1000 person-years in Kibera. The average rate of influenza-associated medically attended ILI in children < 5 was 67.5 (95% CI 59.9-76.1) per 1000 person-years in Lwak and 45.5 (95% CI 39.3-52.6) per 1000 person-years in Kibera. Few children who had laboratory-confirmed influenza were diagnosed with influenza by the treating clinician in the inpatient (0%) or outpatient (2.5%) settings. In addition, 55% of children in Lwak and 99% of children in Kibera meeting the SARI case definition were treated as outpatients. Outpatient influenza-associated SARI cases in Lwak and Kibera were as likely as hospitalized influenza-associated cases to be hypoxic (P = .48) and to be diagnosed with pneumonia (P = .11), but hospitalized influenza-associated SARI cases were more likely to be diagnosed with malaria (P = .01). Conclusions: The burden of influenza-

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associated hospitalization in Kenyan children from 2008-2012 was 1.2-10 times higher than estimates of influenza-associated hospitalizations in the US states of Oregon and Michigan from 2008-2011. Many children who had an IMCI danger sign were not hospitalized and the SARI case definition applied was fairly stringent, which may indicate that the true burden of influenza-associated severe disease in Kenyan children is much higher than current estimates suggest. Few clinicians diagnosed children with influenza despite the presence of a global pandemic during the reporting period. Influenza-associated disease remains underrecognized in Kenyan children.

P1-140

Influenza-associated excess mortality model incorporating temperature data; Germany, 2001/02-2009/10

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Background: Excess mortality is being monitored through the European Mortality Monitoring (Euro MOMO) system. Recent discussions have taken place with the aim to develop models to calculate influenza-associated mortality (IAM) at three time points: (i) during the season, (ii) at the end of each season (using all-cause data), and (iii) two years after the season (when final cause-specific data are available). In addition to outcome-specific issues (all cause vs cause specific), the question how to include temperature and what kind of lag time has been controversial. Methods: We used cause-specific, age group (0-64, 65-74, 75+), and week of death aggregated mortality data from 2001 until 2010 obtained from the “Research Data Centre of the Statistical Offices of the Länder.” To simulate the difference between options (ii) and (iii), we calculated excess mortality for (a) deaths of any cause, (b) cardiovascular deaths (CAR), (c) respiratory deaths (RES), and (d) their sum. We parameterized the model as follows: indicator for influenza activity: (1) number of influenza cases reported through the mandatory notification system, and (2) temperature: weekly mean of four cities representing four geographical regions in Germany. For the model we used a penalized spline of the temperature with 4 degrees of freedom to model its impact on mortality. In addition a sine/cosine curve simulated seasonality. We allowed a different parameter for the impact of influenza activity in each season. To investigate the need for possible lags between influenza and mortality we analysed case-based data on the interval from symptom onset to death (using data from the mandatory reporting system). Results: The variables sine/cosine and temperature fitted the course of the overall mortality very well. Heat waves, but not cold spells, were associated with excess mortality. The temperature splines showed a low in mortality between approximately 5°C and 15°C with a smaller rise below and a higher rise (≈ 10%-20% increase) above. For calculation of the interval from symptom onset to death, most data were from the pandemic season 2009/10. The mode was in the first week after symptom onset. In six of the nine seasons examined, including the pandemic season 2009/10, the model yielded negative, no or low (< 1000) numbers of influenza-associated excess deaths. In 2002/03, 2004/05 and 2008/09, total numbers of IAM of any cause ranged between 11,700 and 19,400 deaths. Cardiovascular mortality attributed to approximately half, respiratory deaths to approximately a quarter of these, and their sum to about three quarters. Conclusions: In Germany, the association of temperature and overall mortality is generally well fitted by a penalized spline. However, cold spells do not seem to play a major confounding factor for the calculation of influenza-associated deaths. Statistically significant “negative” excess mortality as well as the role of RSV epidemics still needs to be tackled. Incorporation of a lag time does not seem to be necessary. Using all-cause deaths (as available immediately at season’s end) would “overestimate” cardiovascular + respiratory deaths by ≈ 25%.

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Results from the first five years of national sentinel surveillance for influenza in Kenya, July 2007-June 2012

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Background: While seasonal influenza has been long known to cause morbidity and mortality in developed countries with temperate climates, recent studies have shown that influenza causes a significant burden of disease in countries throughout the tropics as well. In 2007, the Kenyan Ministry of Health, together with the Centers for Disease Control and Prevention-Kenya (CDC-K), established a national sentinel surveillance system for influenza to describe the epidemiology and burden of influenza in Kenya, to identify circulating influenza strains, and to provide an early warning system for pandemic influenza. Materials and Methods: We conducted surveillance for influenza-like illness (ILI) and severe acute respiratory illness (SARI) in 11 health care facilities in Kenya. For children < 5 years old with SARI, a modified version of the World Health Organization's Integrated Management of Childhood Illness (IMCI) definition for pneumonia was used. For patients ≥ 5 years old, SARI was defined as axillary temperature ≥ 38°C plus cough, sore throat or shortness of breath. Hospitalization was a required for all SARI cases. We defined ILI as axillary temperature ≥ 38°C and cough or sore throat in an outpatient of any age. From July 2007-December 2011, at all 11 hospitals, for every person with SARI, and for the first three ILI patients per day at each site, a two-page questionnaire with questions about demographics, signs and symptoms, and exposures was administered, and a nasopharyngeal (NP) swab and oropharyngeal (OP) swab was obtained. From January 2012-June 2012, surveillance was conducted at 9 hospitals only and was limited to SARI in all but 2 locations. Beginning in 2008, we conducted follow-up for SARI patients to determine final outcome (discharge or death). NP and OP swabs were placed into a single cryovial with viral transport medium, refrigerated at 2-8 degrees Celsius, and transported to the National Influenza Center (NIC) in Nairobi, where they were immediately placed in a –80 degrees Celsius freezer. At the NIC and the Kenya Medical Research Institute (KEMRI)/CDC-K laboratory, specimens were tested by real-time reverse transcriptase polymerase chain reaction (rtRT-PCR) for influenza A and influenza B. All specimens positive for influenza A were subtyped for seasonal H1, H3, H5, and (beginning in May 2009) pH1N1 using rtRT-PCR. Results: From July 1, 2007, through June 30, 2012, we enrolled 21,467 SARI patients and 13,019 ILI patients. Of SARI and ILI patients, 44% and 47%, respectively, were female; 1 year was the median age for both, and 91% of SARI patients and 88% of ILI patients were < 5 years old. Of SARI and ILI patients, 7% and 3%, respectively, had chronic illness; 62% and 75%, presented within 3 days of symptom onset; and 1% of all patients reported having been vaccinated against influenza in the past year. Overall, 10% of SARI cases were positive for influenza, compared with 16% of ILI cases (P < .001). The highest percent positive for influenza occurred in 2009 (13% for SARI; 19% for ILI), when pandemic influenza A (H1N1) first circulated in Kenya. Influenza A comprised 81% of all influenza-positive specimens, and predominated in all years except for 2007. Influenza circulated year-round and was detected during every month of the five-year period, but influenza activity peaked during April-November of most years. We collected follow-up information for 6641 patients. The case fatality ratio for all SARI patients was 2%. The percentage of SARI patients who died in the hospital and tested positive for influenza was lower than the percentage of patients discharged alive who had influenza (3% vs 9%, P = .015). Conclusions: During five years in Kenya, influenza was an important contributor to outpatient and inpatient respiratory illness and circulated throughout the year with peaks during the colder months. Most influenza-associated SARI and ILI cases were in children < 5 years old; interventions to reduce the burden of influenza, such as vaccine, should initially target this age group.

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P1-142

Characterization of the severe influenza infection cases in the Czech Republic during the epidemic season 2012/2013

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Background: Influenza A viruses represent one of the major global health problems in the human population. Clinical forms of infection vary from mild to severe and even life threatening. The virus is variable and genetically unstable. Currently, three subtypes of influenza A virus circulate in the human population: H3N2, H1N1 and since 2009 the so-called pandemic H1N1 (H1N1pdm). The objective of our study was to explore the genetic characteristic of the influenza A virus strains that caused severe clinical manifestation in the Czech Republic (CR) during the epidemic season 2012/2013. Materials and Methods: The epidemiologic and virologic surveillance system of influenza is active throughout the year and uses the European Union case definition for influenza. Data are collected weekly and analyzed at the national level. The A influenza strains studied were isolated on cell culture (Madin-Darby canine kidney cells) from samples taken from patients with severe influenza-like illness (ILI) symptoms or from postmortem samples that were positive for H1N1pdm virus by RT-qPCR. The entire coding genomes of the representative group of H1N1pdm strains were sequenced, and the phylogenetic relationships between each of the genome segments were estimated. Subsequently, neighbour-joining or maximum likelihood trees were calculated for each genome segment using the PHYLIP 3.63 package. Trees were drawn by the TreeExplorer tool in the MEGA4 program. Results: A sudden increase in severe influenza cases has been registered in the CR since the end of 2012, with 528 cases requiring intensive care, including 123 deaths. The morbidity gradually increased with the epidemic wave culmination in the 6th calendar week (CW). The incidence of severe ILI cases persisted from the 52nd CW of 2012 until the 12th CW of 2013. Most patients had at least one risk factor for severe influenza infection: cardiovascular disease, obesity, smoking, chronic pulmonary disease, diabetes mellitus, epilepsy and/or hematologic disease. From the etiologic point of view, the H1N1pdm subtype was confirmed in 60% of patients, H3N2 in 9% and influenza B (Yamagata lineage) in 7%. In 24% of cases the influenza A virus subtype could not be determined due to low sample positivity. As the H1N1pdm virus was the predominant strain, we presume that the majority of unsubtyped influenza A viruses also belonged to this subtype. Analysis of the H1 sequences of 24 influenza A H1N1pdm isolates representing the 2012/2013 influenza season indicated high sequence similarity both at the nucleotide and amino acid level, with the 1310 H1 sequence as the most diverse. This was also reflected in the H1 phylogenetic tree, where all the 24 sequences belonged to the H1 subclade 6 defined by the A/St.Petersburg/27/2011 reference strain. Currently, subclade 6, along with subclade 7, represents the predominating phylogenetic lineages of H1N1pdm influenza virus subtype in Europe. This group, along with H1N1pdm09 group 7, represents the currently predominating phylogenetic lineages of this influenza virus strain in Europe. Conclusions: The entire influenza season 2012/2013 in the CR was atypical regarding the number of severe cases requiring in-hospital and even intensive care, the death rate and the duration of the epidemic. Each of these was significantly higher than in previous seasons. The impact of this epidemic has been comparable with the pandemics in 2009/2010. Influenza H1N1pdm and unsubtyped influenza A viruses were found in almost all cases of influenza ICU patients. Most deaths were linked primarily to rapidly developing respiratory failure due to progressive pneumonitis, as previously described (Chowell G, et al. *N Engl J Med*. 2009;361:674-679). These complications caused by the H1N1pdm virus are difficult to explain because the virus is neither shown to have markedly increased virulence over other seasonal influenza viruses, nor is it a particularly strong cytokine inducer in vitro. The potential for increased pathogenicity could be a result of the combination of other genetic markers.The study was supported by grant IGA MZ CR NT/12493-3.

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Epidemiological characteristics analysis for influenza-associated hospitalization in Jingzhou from 2006 to 2008

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Background: We analyzed the epidemiologic characteristics of influenza-associated hospitalized patients in Jingzhou, Hubei province, from 2006 to 2008. Methods: We conducted a retrospective study for hospitalization cases admitted to all health facilities in Jingzhou urban area from 2006 to 2008. Information from medical records were collected, based on the *International Classification of Diseases, Tenth Revision codes* for influenza-associated disease. We analyzed the epidemiologic characteristics and calculated the hospitalization rate. Results: 38,020 hospitalization cases admitted to 27 medical facilities for influenza-associated disease were extracted from Jan 1, 2006 to Dec 31, 2008. Our study showed that the mean annual hospitalization rate was 11‰, and increased year by year. The annual hospitalization rate was highest in the < 1-year-old group (335‰), and the ≥ 65-year-old group reached the peak of the case fatality of influenza-associated disease (1.8%). There were a few more males with influenza-associated disease than females. While the influenza-associated disease was present throughout the three years, cases were mostly in March, with a spring peak. Conclusions: The influenza-associated hospitalized burden in the study area was relative high, especially in infants and elderly people, who would be the high-risk group. These findings highlight the need for more efforts on influenza prevention for both infants and elderly people.

P1-144

Viral and clinical risk factors for hospitalizations and severe pneumonia in children under 5 years in Western Kenya

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Background: With the global expansion of vaccine use for bacterial respiratory pathogens, respiratory viruses, including influenza and respiratory syncytial virus (RSV), are a growing relative contributor to severe pneumonia. To reduce mortality in children, WHO introduced the Integrated Management of Childhood Illness (IMCI) guidelines that define pneumonia and severe pneumonia to assist health care providers in determining whether patients should be treated as outpatients or inpatients. However, countries in sub-Saharan Africa report poor compliance with IMCI guidelines undermining efforts to reduce child mortality. Poor IMCI compliance may also result in a significant burden of severe pneumonia that is not hospitalized. We sought to determine the proportion of children with severe pneumonia who were not hospitalized, and the contributions of influenza and RSV to both hospitalized and nonhospitalized severe pneumonia in western Kenya. Methods: Between Jan 2008 and Dec 2012, we conducted population-based surveillance for inpatient and outpatient respiratory illness at Lwak Hospital. Children from this surveillance system aged < 5 years, and who met the IMCI case definition for pneumonia (cough or difficulty breathing with age-specific tachypnea) or a modified IMCI case definition for severe pneumonia (cough or difficulty breathing with hypoxia or one or more danger signs [lethargy/unconsciousness, unable to drink or breastfeed, vomiting everything and convulsions]) were included in this analysis. Nasopharyngeal and oropharyngeal specimens were collected and tested by rRT-PCR for influenza viruses during the entire study period, and for RSV during Jan 2008-Feb 2011. We described the distribution of influenza and RSV infection among tested hospitalized and nonhospitalized patients with severe pneumonia. We conducted bivariate and multivariate analyses using logistic regression analyses to evaluate i) if children with influenza or RSV

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infections were at greater risk of hospitalization, and ii) if outpatients with influenza or RSV infections were at greater risk of severe disease. Multivariate analyses included possible confounders of HIV status, malaria status and low weight-for-age Z scores (< –2) if they were associated with primary outcomes at $P < .2$ in bivariate models. HIV, malaria, and low weight-for-age Z scores were included in the final model predicting hospitalizations; and malaria and low weight-for-age Z scores were included in the final model predicting severe pneumonia. Results were stratified by child's age category (0-24 and 25-59 months). Results: Among 4314 children aged < 5 years with pneumonia, 2280 (53%) were < 24 months and 2297 (53%) were males. Of the 4314 children, 3621 (84%) and 693 (16%) were seen as outpatients and inpatients, respectively. In addition, 726/4314 (17%) had severe pneumonia of whom 414/726 (57%) were seen as outpatients. Among outpatients, 1118/3621 (31%) and 660/2390 (28%) were tested for influenza and RSV, respectively. Of those tested, 85/1118 (8%) had influenza infection (41/514 [8%] aged 0-23 mo and 44/604 [7%] aged 24-59 mo) and 117/660 (18%) had RSV infection (65/306 [21%] aged 0-23 mo and 52/354 [15%] aged 24-59 mo). Among hospitalized patients, 241/693 (35%) and 183/594 (31%) were tested for influenza and RSV, respectively. Of those tested, 12/241 (5%) had influenza (8/141 [6%] 0-23 mo and 4/100 [4%] 24-59 mo) and 46/183 (25%) had RSV (35/111 [32%] 0-23 mo and 11/72 [15%] 24-59 mo). Among outpatients with severe pneumonia, 122/414 (29%) were tested for influenza and 58/242 (24%) tested for RSV; 11/122 (9%) had influenza infection and 11/58 (19%) had RSV infection. In multivariate analysis, detection rates of influenza (aOR 0.58, P =.09, 95% CI 0.31-1.09) and RSV (aOR 1.18, P = .44, 95% CI 0.77-1.81) were not different between inpatients and outpatients, or among those with and without severe pneumonia (influenza aOR 0.81, P = .57, 95% CI 0.41-1.64) and (RSV aOR 1.11, P = .64, 95% CI 0.72-1.72). Of the 41 deaths occurring within one month of follow-up, 12 (29%) deaths had been seen as outpatients only, 5/12 (42%) of these deaths had at least 1 IMCI danger sign. Conclusions: A significant portion of persons with severe respiratory disease are not hospitalized in this context. Monitoring hospitalizations alone may underestimate respiratory viral contributions to severe respiratory disease. Over half of patients with severe pneumonia were seen as outpatients. Further adherence to IMCI standards for hospital admission might reduce morbidity and mortality due to respiratory disease.

P1-145

Risk factors for seropositivity to 2009 pandemic influenza A/H1N1 virus in the United States before and after the 2009 pandemic

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Background: Since its initial detection in April 2009, pandemic influenza A/H1N1pdm09 caused a global pandemic with widespread illness. Estimating the incidence of infection following the virus' emergence and identifying factors associated with increased risk of infection are important to understanding the spread of H1N1pdm09. Clinical surveillance underestimates the community incidence of influenza, as it may not capture asymptomatic infections or many mild illnesses. In addition, surveillance among persons seeking medical care in outpatient or hospital-based settings may be biased by factors associated with the severity of illness or access to medical care. Population-based serologic surveys can complement clinical surveillance and provide a fuller measure of the incidence of H1N1pdm09 infection over time and also identify risk factors for infection that are unbiased by the propensity to seek care. We conducted a series of cross-sectional serologic surveys in the United States to better understand the overall and age-specific incidence of infection with H1N1pdm09 following the spring and fall waves of the pandemic and to identify risk factors that may be associated with an increased risk of infection with H1N1pdm09. Materials and Methods: We conducted three cross-sectional serologic surveys for pH1N1. Two of these surveys were among participants in the National Health and Nutrition Examination Survey (NHANES), an ongoing population-based survey conducted nationwide in the United States. We obtained a probability-based sample of banked serologic specimens from participants during each of two enrollment periods: the years 2007-08 prior to the pandemic, and the year 2010 following the spring and fall waves of the pandemic. An additional serosurvey was conducted using leftover serum specimens submitted to clinical laboratories from December 2009-January 2010 in ten US states, one from each of ten

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geographic regions of the country. Each serum specimen was tested in triplicate using the hemagglutination inhibition (HI) assay against A/California/7/2009; a specimen was considered seropositive if the geometric mean HI titer was ≥ 40. The increase in seroprevalence was calculated as the difference in the proportion of participants seropositive for H1N1pdm09 between two surveys. For NHANES samples, titer results were linked to data collected for each participant, including demographic and social factors, medical history, access to health care, and a routine physical exam. For the 10-state sample, only patient age and date of collection were available. Results were analyzed in SUDAAN to accommodate the NHANES sampling frame and sample weights. Results: A total of 1142 NHANES participants were included from 2007-08 and 3334 participants from 2010. Prepandemic seropositivity varied by age, with the highest prevalence among adults over age 80 years (28%; 95% CI: 13%–43%), and lowest in children aged < 12 years (1.0%; 95% CI: 0%–2.2%). After adjusting for age, persons born outside the US were less likely to have prepandemic cross-reactive antibody to pH1N1 (OR = 0.38, 95% CI: 0.21–0.69), but there was little variation by other demographic or socioeconomic factors. An additional 2759 persons were included from ten states at the end of 2009. The age-adjusted increase in seroprevalence to pH1N1 from baseline to the end of 2009 in the ten-state sample was 26.2% (95%CI: 23.5%–28.8%). The increase in seroprevalence was highest in children aged 5-17 years (52.7%; 95% CI: 46.6–58.8). Further analysis will include comparing titers and survey data among the second NHANES sample to identify subgroups with greater increases in seropositivity following the pandemic. In addition, while influenza vaccine status was not known for each participant, we will incorporate data on pH1N1 and seasonal vaccine coverage over the same time periods. Conclusions: Understanding better how an influenza pandemic affects the population and which subpopulations are at greater risk for infection informs future planning and prevention policies. Approximately one-fourth of the US population, including half of children, developed seropositivity to H1N1pdm09 by the time activity waned. Ongoing analyses comparing increases in seropositivity by demographic and socioeconomic factors and underlying health history will identify additional factors associated with the risk of infection during the 2009 pandemic in the United States.

P1-146

Antibody responses in a predominantly young cohort to naturally acquired influenza infection

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Background: Studies on the antibody responses during natural influenza virus infection in humans are lacking, especially in younger individuals. Here we present the pattern of antibody responses in a predominantly young cohort of influenza patients from two influenza seasons. Materials and Methods: We assessed the pattern of serologic and site-of-infection antibody responses to homologous and heterologous hemagglutinins by neutralization or enzyme-linked immunosorbent assays. Results: In the nasal compartment, influenza virus-specific IgA and IgG levels were detectable as early as on enrollment and showed age-specific trends in the antibody-isotype response, with a positive correlation between age and speed of response. Infants had a more robust influenza virus-specific IgA profile compared with IgG, whereas the inverse was true in older children and adults. We also identified a subset of individuals who seroconverted to the H3 hemagglutinin after infection with H1N1 virus and vice versa, suggesting that influenza virus infection can induce a cross-subtype antibody response within the population. Conclusion: We showed the age-specific trends of influenza antibody responses during naturally acquired influenza virus infections in the serum and nasal compartments.

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Influence of preexisting hemagglutination-inhibition titers against historical influenza strains on antibody response to inactivated trivalent influenza vaccine in adults 50-80 years of age

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Background: A better understanding of the serologic response to influenza vaccination is needed due to concerns about the effectiveness of influenza vaccine in older persons and the phenomenon of higher antibody titers being produced in response to influenza strains encountered earlier in life. Methods: Antibody responses against the three viruses in the 2011-2012 trivalent influenza vaccine before (Day 0) and 21 days post vaccination (Day 21) were analyzed in 272 individuals 50-80 years old. Sera were tested for hemagglutination-inhibition activity at Days 0 and 21 against vaccine strains and at Day 0 against a panel of historical seasonal influenza strains. Seroprotection was defined as a titer of ≥ 1:40 and seroconversion as a 4-fold rise in titer from Day 0 to Day 21. Results: The proportion of subjects whose postvaccination titers were seroprotective at Day 0 and 21, respectively, was 37% versus 66% for A (H1N1), 29% versus 63% for A (H3N2), and 29% versus 42% for B. Tests for trend demonstrated that the percent of seroprotected subjects for each strain rose with an increasing number of seroprotective responses against historical strains ($P < .01$) for A (H1N1) and A (H3N2) but not B, among those seronegative at Day 0 for vaccine strains. In multivariable logistic regression analyses that controlled for age, sex, race, site and diabetes status, seroprotection at Day 21 for the A strains was significantly more likely as the number of seroprotective responses against historical strains increased (A (H1N1) odds ratio [OR] 1.43, 95% confidence interval [CI] = 1.10-1.85; H3N2 OR = 1.29, 95% CI = 1.05-1.59; B OR = 1.28, 95% CI = 0.96-1.69). Likelihood of seroconversion was significantly higher with number of responses to historical strains for A (H3N2) only (OR = 1.23, 95% CI = 1.00-1.51). Seroconversion for all three strains was significantly less likely as Day 0 vaccine strain titers increased. Conclusion: Seroprotection against influenza increased as seroprotection to historical strains increased, with antigenic cross-reactivity demonstrated for influenza A.

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Age synchrony in seasonal variations of influenza and respiratory syncytial virus in a subtropical city

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Background: It is well known that influenza and respiratory syncytial virus (RSV) tend to infect more children and elderly than younger adults. However, it is not clear whether epidemic curves of influenza had different seasonal patterns across age groups. No such studies have been conducted so far in subtropical/tropical regions where the seasonality of these viruses still remains poorly understood. The potential interference effect of the 2009 influenza pandemic on seasonal fluctuations of other viruses is also unknown. Materials and Methods: A total of 7080, 1060 and 3540 cases tested positive for influenza A (subtyped into seasonal sH1N1, H3N2, and pandemic virus pH1N1), influenza B and respiratory syncytial virus (RSV), respectively, from 2004 to 2010 in Hong Kong Island. We

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examined the weekly age-specific epidemic curves for the age groups of 0-4, 5-17, 18-64 and 65+ years. The synchrony and time lag across age groups were assessed by cross-correlation. Age-specific proportions of specimens positive for each virus (positive proportion) were also compared between the 2009 pandemic for influenza H1N1 and the interpandemic period. Results: The epidemic curves of sH1N1, H3N2 and influenza B were highly synchronized across age groups during the interpandemic period, with the highest correlation coefficient consistently found at lag 0. A few exceptions were found, including a lead time of two weeks in the 5-17 age group for H3N2 and a lag time of one week in the 18-64 age group for influenza B. The 2009 pandemic period was characterized with decreased activity of most viruses other than pH1N1, compared with the interpandemic period. This change was found in all age groups with the exception of a small increase in RSV in the 0-4 age group. Highly synchronized virus activity was found in all age groups with pH1N1 and most age groups of H3N2 during the pandemic. But a lag of up to four weeks was observed in the 65+ age group with H3N2. RSV virus activity became more synchronized during the 2009 pandemic, despite that no age synchrony was found in the interpandemic period. Conclusions: Influenza demonstrated a highly synchronized virus activity across age in the subtropics. Our findings of lower positive proportions and varied age synchrony in nonpandemic respiratory viruses during the 2009 pandemic need further investigation.

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The association of benzodiazepines with influenza-related mortality

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Background: Influenza has a huge impact on morbidity and mortality worldwide. One potentially important strategy to reduce this is to determine whether commonly prescribed drugs modify the risk of acquiring and mortality from influenza infection. The benzodiazepines, a class of psychoactive drugs, have recently been suggested as having detrimental effects on immune response to infection, predisposing its users to increased risk of infection and mortality. This study aims to investigate whether benzodiazepines modify the risk of influenza-related mortality. Methods: Data from the Clinical Research Datalink (CPRD), a UK-based longitudinal database of anonymised computerised general practice patient records, were used. Data on patients diagnosed with influenza were extracted for 2010 and 2011. Death within 30 days of influenza diagnosis was considered as influenza-related mortality. Study subjects were considered to be exposed to benzodiazepines if they had ever had a benzodiazepine prescription. Crude Cox regression analysis has been conducted and the multivariable analyses are under way. Factors to be considered as potential confounders include age, sex, Charlson comorbidity index score, depression, psychosis, statins, opioids, corticosteroids, alcohol, smoking and deprivation. Results are presented as unadjusted hazard rates (HRs) and 95% confidence intervals (CIs). Results: Of the 256,641 study subjects with influenza, 173 (0.07%) died within 30 days of their influenza diagnosis. The use of benzodiazepines was associated with a nearly six-fold increase in influenza-related mortality (unadjusted HR 5.73; 95% CI 3.99-8.21). Conclusions: Preliminary unadjusted analysis suggests an association between benzodiazepines and increased mortality following influenza infection. Further adjusted analyses are being undertaken to determine the independent effect of this class of drugs on the risk of mortality from influenza.

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Influenza surveillance network in Côte d'Ivoire from 2007 to 2011: what have we learned?

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Background: In 2006, after the detection in poultry of several avian influenza outbreaks, caused by influenza A (H5N1) in Abidjan, the Ivorian economic capital, a sentinel surveillance of human influenza has been established in the Ivory Coast by the Ministry of Health, for case detection and the control of the disease. After five years of operation, marked, among other things, by the episode of the influenza pandemic of 2009, what conclusion can be drawn? The present work aims to assess the epidemiologic surveillance of influenza. Materials and Methods: A review of the data and documents (protocol and monitoring tools) of the influenza epidemiologic surveillance unit of the National Institute of Public Health, for 2007 to 2011, was performed. It was to collect information on the simplicity (organization, case definition), acceptability (participation rate and refusal completeness of information), responsiveness (time between receipt of information and response) and flexibility (monitoring of pandemic influenza) of the influenza epidemiologic surveillance system. Results: The influenza sentinel surveillance was made up of 19 sentinel sites including 11 ILI sites and 8 SARI sites. The algorithm sample of patients consisted of three levels of performance (recording, verification of clinical signs, nasal pharyngeal samples). ILI case definition was based on two items (date of onset and clinical signs), while for SARI, it was based on four items (onset, clinical and laboratory findings, age). Regarding the acceptability of the monitoring system, the participation rate was 95% (18/19 sites), the completeness of notification reports for three main variables (age, sex, vaccination status) was 100% for 12/19 (63.2%) sentinel sites in 2011. A total of 56 health care professionals were involved in the operation, consisting of 23 (41.1%) physicians, 16 (28.6%) nurses and 17 (30.4%) laboratory technicians. A total of 3914 nasopharyngeal samples were collected, including 1328 (33.9%) in 2009, the year of the pandemic influenza occurrence. The ILI accounted for 97.6% (n = 3820) and SARI 2.4% (n = 94). Among ILI, 994 (26%) samples were positive, including 671 (67.5%) influenza A, 300 (30.2%) influenza B and 23 (2.3%) coinfection influenza A and B. Influenza A subtypes detected were H1N1 (12.7%, n = 85), H3N2 (29.7%, n = 199), and pH1N1 (19.7%, n = 132). On the reactivity of the monitoring system, the response time (investigation, care) after the detection of the first cases of influenza A (H1N1) pdm was less than 24 hours. In addition to the seasonal flu, the network managed to adapt to the new influenza A (H1N1) pdm. Conclusions: The sentinel surveillance system for influenza allowed, during the five years, to confirm the circulation of influenza viruses in Côte d'Ivoire and the occurrence of severe acute respiratory infections related to these influenza viruses, and to detect the emergence of a new phenomenon such as influenza A (H1N1) pdm. This system was simple, fair, responsive and flexible.

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Comparative analysis of the epidemiological and clinical profiles of influenza infection due to pandemic H1N1 (2009), H1N1, H3N2 and B in Abidjan, Cote d'Ivoire, 2007-2010

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Background: Influenza infection, whatever the responsible virus, may have diverse epidemiologic and clinical characteristics. Following the detection of avian influenza outbreaks in Abidjan, Cote d'Ivoire, in April 2006, the Ministry of Health of Cote d'Ivoire set up a new influenza surveillance network in 2007 to monitor influenza caused by highly pathogenic avian influenza A (H5N1) in addition to

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seasonal strains. This study aimed to compare the epidemiologic and clinical profiles of influenza-related infections with pH1N1, H1N1, H3N2, and B viruses within the Abidjan population. Materials and Methods: We conducted a review of the influenza surveillance network data from the National Institute of Public Hygiene (INHP) from 2007 to 2010. We considered data from 15 sentinel sites in Abidjan, which are part of the INHP influenza surveillance network. The proportions of socioepidemiologic characteristics and clinical symptoms were compared according to the different influenza viruses using the χ^2 test. Differences in the epidemiologic and clinical profiles were determined by using simple linear regression and sequential logistic regression models. All data analyses were performed with Stata 12.0 software. Results: Influenza viruses were isolated from a total of 921 patients, of whom 60 (6.5%) tested positive for H1N1, 177 (19.2%) for H3N2, 34 (3.7%) for pH1N1, 392 (42.6%) for unsubtyped influenza A, and 258 (28.0%) for influenza B. The characteristics of patients who tested positive for unsubtyped influenza A strains were not evaluated. The proportion of patients 5-14 years of age (37.5%) affected by pH1N1 was significantly higher than those with influenza B (9.9%) ($P < .05$). The proportions of patients aged 5-14 and 45-64 were also significantly different according to the type and subtype of influenza viruses ($P = .002$ and $P = .01$, respectively). Compared with cases of influenza B, those infected with H1N1 were more likely to be 5-14 years of age ($P = .044$) or 45-64 years of age ($P < .000$). Both the incidence of fever (T > 38°C) and the mean body temperature were higher in patients with H1N1 ($P = .005$ and $P = .001$, respectively) and lower in those with pH1N1 ($P = .005$; $P = .001$). The proportion of myalgia/arthralgia was also higher in patients with the B strain (55.4%) and lower with pH1N1 (18.2%) ($P = .012$). The age of patients was inversely associated with body temperature with influenza B virus ($P < .05$) and with the H3N2 virus ($P < .05$). Patients infected by pH1N1 strain were 10.5 times more likely to have a cough compared with those infected by influenza H1N1. Those infected with pH1N1 were also three times more likely to be aged 5-14 years compared with those with influenza B. The odds of being male and aged 15-44 years were 2.85 and 2.77, respectively, given infection with influenza B compared with H3N2. Compared with those infected with H1N1, patients with influenza B were approximately 8.2 times and 6.2 times more likely to respectively be 15-44 years of age and to have experienced a cough. Patients positively tested for H3N2 were 6.5 and 2.5 times more likely to have a cough and experience myalgia/arthralgia compared with those who tested positive for H1N1. Conclusions: In the Abidjan population, influenza viruses present different clinical and epidemiologic profiles in different age groups that were included in our data. Based on the current strains circulating, public health actions, such as vaccination campaigns, education and advice on measures to avoid influenza infection, should be targeted at the populations most at risk. Our study demonstrates the variability in symptoms of influenza patients, and it is likely that some cases may be misdiagnosed if clinicians strictly follow the recommended World Health Organization case definition of suspect influenza cases. Thus, in the context of an influenza infection, apyrexia and absence of cough should not lead clinicians to automatically exclude influenza.

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Influenza-associated severe acute respiratory illness deaths in the WHO African Region: a case-series from 8 countries

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Background: Influenza virus infections cause 250,000 to 500,000 deaths each year. Global mortality from the 2009 H1N1 pandemic was estimated to be nearly 300,000 deaths in the first 12 months after virus introduction, and more than half of these deaths occurred in Southeast Asia and Africa. Surveillance from 2006-2010 in 14 African countries found that 8.9% of hospitalizations meeting the severe acute respiratory illness (SARI) case definition were positive for influenza. Specific data on influenza mortality are needed to help inform vaccine policy, but data on influenza deaths in the WHO African Region are extremely limited. Hospital-based influenza surveillance has increased dramatically in Africa, and we aimed to assess if the current systems could provide insights into seasonal influenza mortality among persons hospitalized for SARI in Africa. Materials and Methods: From January to April 2013, representatives from 28 (59%) of the 46 WHO African Region Member States, including all 24 countries who reported data to WHO's FluNet in 2012, were invited to participate in a review of influenza-associated deaths in the region. A standard template was provided to countries to report SARI surveillance data collected in 2009 through 2012. Collected variables included number enrolled, age, gender, pregnancy status, underlying medical conditions, results of influenza testing by rRT-PCR, results of testing for other respiratory c-infections and outcome of hospitalization (discharge or death). Results: Twenty-three (82%) of 28 countries provided information about their SARI surveillance. Of these, 11 (48%) did not collect outcomes data, 9 (38%) collected SARI mortality data systematically either prospectively or retrospectively, 2 (8%) received reports of SARI deaths from sentinel sites but not systematically, and 1 (4%) had less than 1 year of surveillance data. Of the 11 countries that collected mortality data, 8 (73%) provided data for this analysis: Democratic Republic of the Congo, Kenya, Madagascar, Malawi, Rwanda, South Africa, Tanzania and Uganda. From 2009-2012, in the 8 countries that reported SARI deaths, data were collected from 40,184 subjects meeting the SARI case definition and enrolled in routine surveillance, and a total of 1222 deaths were reported (SARI CFR = 3.2%, range by country 0.1-5.5%). Overall, 37,722 SARI (93.9%) were tested for influenza and 3514 (9.3%, range by country 5.1%-41%) tested positive for influenza. A total of 1073 deaths were tested for influenza and 58 (5.4%) tested positive for an influenza-associated CFR of 1.7% (58/3514) compared with 3.0% (1015/34,208) of influenza-negative SARI (P = .02). The median age of influenza-infected deaths was 32 years (interquartile range 1-56) compared with 28 years (interquartile range 1-44) in SARI deaths without influenza infection (P = .05). Information on underlying medical conditions was available for 801 (66%) of SARI deaths including 44 (76%) of 58 with influenza. Thirty-one (70%) of 44 influenza-positive deaths had an underlying medical condition, compared with 501 (66%) of 758 without detected influenza (P = .53). HIV status was reported for 580 deaths (47%) among whom 419 (72%) were HIV infected

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including 23 (70%) of 33 influenza-associated deaths. Of the 256 deaths in women of childbearing age, pregnancy status was provided for 237 (93%) among whom 7 (3%) were pregnant at the time of death. None (0/7) of the pregnant women with SARI identified in routine surveillance who died were influenza infected. Conclusions: Although most countries in the WHO African Region are conducting SARI surveillance, few are systematically collecting data on outcomes of hospitalization, and available information on deaths is sparse and incomplete. Among deaths identified through routine SARI surveillance, more than 5% were influenza associated. There is considerable variation in the proportion of SARI cases who died by country and the proportion of SARI deaths with influenza. This may reflect differences in case definitions, criteria for hospitalization, quality of hospital care or the quality of surveillance. The low number of deaths in pregnant women is unexpected given their increased risk of influenza-associated death during the pandemic, and may reflect inadequate surveillance in antenatal clinics and maternity wards. Surveillance for influenza-associated deaths should be strengthened in Africa.

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Development of an online, real-time information retrieval system for influenza surveillance data collection and sharing in Mongolia

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Background: Information gathering, analysis and utilization of the epidemiologic and virologic data from the influenza surveillance sites by traditional methods become challenging task for National Influenza Centers (NIC) in resource-poor countries with the increase of sentinel site numbers. Here, we report the results of development, testing and everyday utilization of a modern IT-based information retrieval system for influenza surveillance data collection and sharing in Mongolia. Materials and Methods: A software for influenza surveillance data collection and sharing for NIC, Mongolia has been developed named FIS (Flu Information System) and FluLab in January-March 2010 on the platform MySQL and ORACLE 10g using programming tools PHP and Borland Delphi 7. Retrospective and current influenza surveillance data of Mongolia in NIC, Mongolia have been used for the testing and building up online open database. Results: A server with the FIS software at NIC, Mongolia has been established in April 2013 and trained the first 15 trainers for FIS. In April-May 2010, 15 trainers have been formed 5 teams and visited all influenza sentinel surveillance sites (ISSSs) of NIC, Mongolia in the Capital City-Ulaanbaatar and 21 aimaks (provinces of Mongolia) and made an FIS and FluLab readiness assessment of 186 units: outpatient and hospital ISSSs, Province Health Departments and trained 370 epidemiologists and statisticians how to use FIS and FluLab and connected ISSSs' computers to the NIC, Mongolia server negotiating with local internet server companies. After this broad testing of FIS software with all ISSSs, some refinements have been done to the software and in September 2010 NIC, Mongolia has organized a training for all responsible officers for FIS and FluLab in all ISSSs and NIC, Mongolia. From October 1, 2010, it has been started o-line electronic reporting of epidemiologic and collected sample data and feedback from/to ISSSs countrywide parallel with the old system (phone information and faxing) and begin to publish the weekly surveillance data real-time online in form of maps and graphics using NIC, Mongolia Web sote www.flu.mn in Mongolian and English parallel. It has been started also building a database back to the 40th week of 2008 week by week. This testing has been supported by monthly audioconferencing about the data quality and corrections using Skype. Starting October 2011 the reporting become only online and the system is now functioning well with online, real-time sharing of current weekly epidemiologic and virologic data and database covering weekly information since the 40th week of 2008. Information sharing and surveillance data utilization have been improved enormously and the speed of communication and analysis have been promoted also greatly. Conclusions: Modern online information collection and sharing of influenza surveillance is possible with a relatively small investment and budget in resource-poor countries and the financial and technical returns are encouraging.

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Quantifying the respective roles of exposure and susceptibility to H1N1pdm infection: a structural equation modeling approach

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Background: Infection with H1N1pdm influenza results from a complex combination of biological characteristics of the virus and the host, individual and collective behaviors, social interactions (exposure) and environmental context. Numerous studies have been published since 2009 to explore associations between H1N1pdm infection and some of these risk factors. However, none tried to quantify the respective roles of variables related to exposure such as risk perception or intensity of contacts, and individual susceptibility. Using data from the CoPanFlu study, a large, nationwide representative cohort dedicated to investigating risk factors of H1N1pdm infection, we used a structural regression framework to model causal relationships between observed and latent variables and infection with H1N1pdm influenza. Structural equation models have the advantage over regression models to estimate direct and indirect relationships between observed or latent variables, and to quantify their independent effect on the outcome, simultaneously. Materials and Methods: In the CoPanFlu study, enrolled households were actively followed over three consecutive winters between 2009 and 2012.Questionnaires exploring risk perception and intensity of contacts were administered yearly, and annual serologic collection allowing pre/post influenza HAI titration. We limited our analysis to the 2010-11 season during which H1N1pdm was circulating for the second year in France. A H1N1pdm infection was defined as either a positive H1N1pdm RT-PCR or a seroconversion (4-fold increase of HI titer) during the follow-up period. We hypothesized influenza infection to result from two unobserved phenomena, exposure to the virus and individual susceptibility to influenza infection. Exposure to H1N1pdm virus was modeled using 3 latent variables, contacts with individuals at risk (3 indicators), protective measures against infection taken by the individual and by the other subjects of the household (4 indicators), and perception of efficient measures to avoid being infected (3 indicators). Susceptibility to influenza infection was modeled with a single indicator, the preseasonal HAI titer. Factors associated with high preseasonal HAI titer such as influenza vaccination were also studied. As age has been shown to be a major determinant of influenza infection, associated with variable intensity of contacts and differential susceptibility, we divided our sample into 2 subpopulations, individuals aged less than 15 years (N = 220) and individuals aged between 15 and 50 years (N = 494). Standardized path coefficients (β_{std} ranging from –1 for a complete negative effect to 1 for a complete positive effect) were used to assess the relative effects of the variables within the structural model. Results: The susceptibility to influenza infection was positively and significantly associated with H1N1pdm infection with both populations (β_{std} = 0.39 for child model and β_{std} = 0.31 for adult model) and had the most important effect on H1N1pdm infection in the adult model. The latent variables “protective measures against infection taken by the individual and by the other subjects of the household” and “perception of efficient measures to avoid being infected” were negatively associated with H1N1pdm infection in the child model (β_{std} = –0.36 and β_{std} = –0.34) but were not significant in the adult model. Finally, the latent variable “contacts with individuals at risk” showed a nonsignificant positive standardized coefficient in the child model (β_{std} = 0.31, *P* =.11) and a negative significant coefficient in the adult model (β_{std} = –0.26). The preseasonal HAI titer was found to be associated with age and preseasonal pandemic vaccination in the adult model (β_{std} = –0.10 and β_{std} = 0.27, respectively). The proportion of total variation of the outcome (*R2*) for the adult and the child models were 0.170 and 0.415. Conclusions: Using a structural equation model approach, we showed that protective measures against infection, taken by individuals and by their household members, and the perception of efficient measures to avoid being infected, were important factors driving the risk of H1N1pdm infection in children. Susceptibility to the virus is the leading factor to explain H1N1pdm infection in adults; however, there is a great part of variability that remains unexplained and deserves further investigation.

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An international laboratory comparative examination of influenza 2 day ELISA and 3 day hemagglutination consensus microneutralization assays conducted by the Consortium for the Standardization of Influenza Seroepidemiology (CONSISE)

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Background: The Consortium for the Standardization of Influenza Seroepidemiology (CONSISE) is a global partnership formed in 2011 to develop influenza investigation protocols and standardize seroepidemiology to inform health policy. The activities of CONSISE are performed by two interactive working groups, laboratory and epidemiology. Following infection with novel influenza virus, most people develop specific antibodies that persist for months. Virus-specific antibodies are typically quantified using two main assays, the microneutralization (MN) assay and/or the hemagglutination inhibition (HI) assay. The MN assay is comprised of two main steps: an initial incubation of virus with antisera, followed by inoculation of a permissive cell line with the virus/antisera mix and detection of virus infection. For the purpose of seroepidemiology, two main detection protocols for the MN assay are conducted: the 2-day ELISA protocol and the 3-day red blood cell agglutination (HA) protocol. To enable serologic data to be compared and combined on emergence of a novel influenza virus, it is essential to understand the impact of MN protocol variation and reproducibility, and interlaboratory variability. Thus an international within-laboratory comparison of the 2-day ELISA and 3-day HA MN protocols, using A(H1N1)pdm09 virus vaccine strains, was performed by members of the CONSISE Laboratory Working Group. Materials and Methods: The 2-day ELISA protocol is published by the World Health Organization. Specific guidelines for the 3-day HA protocol have not been formerly defined, hence more laboratory-to-laboratory variation exists. Ten laboratories worldwide shared their protocols and from these submitted protocols, a 3-day HA consensus MN protocol was developed. Specific parameters were required for dilutions, calculations and incubation times for the 3-day HA MN assay. Cell culture conditions were recommended as those used routinely for each laboratory. Twelve laboratories from eight countries participated in the laboratory evaluation of the 2-day ELISA (WHO) and 3-day HA consensus protocols. All laboratories were required to perform both MN assays to enable a within-laboratory comparison of the protocols. The 2-day and 3-day assays were performed at least three times in each laboratory, on different days. Each laboratory provided their own wild-type or reassortant A(H1N1)pdm09 virus and panel of 10 human or animal sera with a spectrum of titres. Two International Standards for antibody to influenza A(H1N1)pdm09 were recommended for inclusion. Results: There were differences in the sensitivity of the assays between laboratories and between the MN assay methods. However, the ratio of titres between the 3-day and 2-day assay was similar for the International Standard that was included in the study and the in-house serum samples. Overall, in most laboratories, there was good correlation between the results obtained using the two assay protocols. Conclusions: Our results indicate that the 2-day ELISA (WHO) and 3-day HA consensus protocols for MN assays may be considered interchangeable for assays of antibodies to the influenza A(H1N1)pdm09 virus. Further comparative evaluations of the MN assays are under way using A(H3N2) and A(H5N1) viruses and corresponding antisera. A standardised HI assay protocol is also under development and will be compared in 2014.

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Influenza-like illness surveillance in the population of the United Kingdom during the 2012-13 season using telephone survey methodology

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Background: One of the key lessons learned during the 2009 influenza pandemic was the need to rapidly assess the severity of a novel influenza virus to inform optimal prevention and control activities. A number of influenza surveillance systems were used during the pandemic that relied on clinical and virologic data, derived primarily from patients in contact with the health care system. The relative mildness of the epidemic meant that many symptomatic cases did not seek medical care, creating uncertainty about the rates of influenza-like illness (ILI) attributable to the H1N1 virus in the population. Telephone surveys have been used in a number of countries as a surveillance tool to estimate ILI in the population. A telephone survey was conducted during the 2012-13 influenza season with the objective of developing a mechanism to collect epidemiologic information at key points during the season. The survey was also used to assess the willingness of participants to provide biological samples (serum, oral fluid and dried blood spot) for seroepidemiology and the development of new influenza assays. Materials and Methods: A population-based two-stage household telephone survey was conducted during the 2012-13 influenza season in England, Scotland and Wales. Quota sampling was used with quotas for gender, age, region and work status. Households were recruited using random digit dialling during November 2012 (preseason) and participants were recontacted during January-February 2013 (post peak). Information collected at baseline included demographic characteristics, chronic health conditions, pregnancy, influenza vaccination status, and household composition. In the postseason survey, influenza vaccination status was updated and participants asked whether they had experienced clinical symptoms such as fever and cough and whether they had sought any health care as a result. Data on demographic, vaccination and chronic disease characteristics of participants who took part in both surveys were compared to external national census data. Self-reported chronic health conditions were used to determine whether respondents were in one of the clinical risk–group categories that are targeted in seasonal influenza vaccination programmes. World Health Organization (WHO) and European Centre for Disease Control (ECDC) case definitions for ILI were used to categorise respondents' ILI status using their self-reported clinical symptoms. Results: A total of 1203 participants and their household were recruited in the preseason survey that took place between 1 and 12 November 2012. During the postseason survey, which took place from 25 January-6 February 2013, 960 participants were successfully recontacted. Demographic characteristics of the study participants were comparable to census data; although males and the youngest age group (18-25 years) were slightly underrepresented. The seasonal influenza vaccination rate in those aged 65 years and older was 76.9% and 57.1% in the under 65 years clinical risk groups; these estimates were comparable to the national vaccine coverage for these groups. The cumulative clinical attack rate for the WHO and ECDC ILI case definitions were 5.7% and 13.5%, respectively. The cumulative clinical attack rates in those aged 65 years and older were 2.9% and 9.2% for the WHO and ECDC ILI case definitions, respectively. Twenty percent of the WHO and 16.9% of the ECDC ILI cases reported that they visited their GP; 41.9% of the 1203 participants agreed to be further contacted regarding biological sampling. Conclusions: A population telephone survey conducted during the 2012-13 influenza season provided useful data on seasonal vaccination, ILI and health care–seeking behaviour from a sample that was broadly representative of the general population of England, Scotland and Wales. Over 40% of those surveyed were willing to provide biological samples that could be used to estimate seroprevalence and seroincidence rates for influenza viruses. Telephone survey methodology can be established as a routine annual system to provide key epidemiologic data during seasonal influenza. In the event of an

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influenza pandemic, the system could be modified to provide timely data on the extent and severity of illness in the population, which in turn could be used to inform preventative and control measures.

P2-438

Influenza virus infection is associated with increased risk of death amongst patients hospitalized with confirmed pulmonary tuberculosis in South Africa

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Background: Data on the association between influenza and tuberculosis are limited. Underlying tuberculosis infection may have contributed to the elevated mortality observed in young adults in the 1918 influenza pandemic. We describe the characteristics of patients with laboratory-confirmed tuberculosis, influenza and tuberculosis-influenza coinfection among patients hospitalised with severe respiratory illness in South Africa. Materials and Methods: Patients hospitalized with severe respiratory illness were enrolled prospectively at 6 hospitals in 4 provinces in South Africa. Nasopharyngeal specimens were tested for influenza by reverse-transcription real-time polymerase chain reaction (RT-PCR). Tuberculosis testing was conducted as part of clinical management. A laboratory-confirmed tuberculosis case was defined as an individual with a result positive for *Mycobacterium tuberculosis* on microscopy, culture or PCR from GeneXpert test. An influenza case was defined as an individual with a positive PCR test for influenza. An influenza-tuberculosis coinfection case met criteria for both tuberculosis and influenza during the same admission. We implemented multivariable logistic and multinomial regression models to identify factors associated with (i) tuberculosis testing among enrolled patients; (ii) tuberculosis positivity among patients tested for tuberculosis; and (iii) tuberculosis single infection compared with influenza single infection or tuberculosis-influenza coinfection. Results: From February 2009 through December 2011, 13,603 patients were enrolled and influenza testing was conducted on 13,351 (98%) cases. Influenza virus was detected in 1157 (9%) patients. An influenza subtype was obtained from 1143 (99%) positive samples. Of these, 379 (33%) were influenza A(H1N1)pdm09, 387 (34%) were influenza A(H3N2) and 377 (33%) were influenza B. On multivariable analysis controlling for age and site, testing for tuberculosis among all patients with severe respiratory illness was associated with HIV infection (aOR 1.4, 95% CI 1.2-1.5), duration of symptoms ≥ 7 days (aOR 1.3, 95% CI 1.1-1.5), hospitalization > 7 days, (aOR 1.3, 95% CI 1.2-1.4), and receiving antibiotic (aOR 2.1, 95% CI 1.6-2.7) or tuberculosis treatment (aOR 1.3, 95% CI 1.2-1.5) during current admission. Among patients with available tuberculosis and influenza results, 15% (612/4219) tested positive for tuberculosis. Of the 4219 patients with available tuberculosis and influenza results, 3275 (78%) were negative for both pathogens, 571 (14%) were positive for tuberculosis alone, 332 (8%) were positive for influenza alone and 41 (1%) had influenza and tuberculosis coinfection. On multivariable analysis controlling for site, compared with tuberculosis-negative individuals, patients with tuberculosis were less likely to be coinfected with *Streptococcus pneumoniae* (aOR 0.5, 95% CI 0.3-0.8) and to receive oxygen therapy (aOR 0.7, 95% CI 0.5-0.8) and were more likely to be in the adult age groups (5-24 [aOR 2.0, 95% CI 1.3-3.2] or 25-44 years [aOR 1.7, 95 CI 1.2-3.0] compared with < 5 years), to be started on tuberculosis treatment (aOR 4.3, 95% CI 3.4-5.4) and to be admitted for ≥ 7 days (aOR 1.4, 95% CI 1.1-1.7). On multivariable analysis adjusting for age, patients coinfected with tuberculosis and influenza compared with patients with tuberculosis only were less likely to present with symptoms ≥ 7

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days (adjusted relative risk ratio (aRRR) 0.5, 95% CI 0.2-0.9) and were at increased risk of death (aRRR 3.1, 95% CI 1.1-8.9). On stratified multivariable analysis by duration of symptoms compared with those with tuberculosis only, individuals coinfected with both influenza and tuberculosis with symptoms for ≥ 7 days had an increased risk of death (aRRR 6.03, 95% CI 1.6-23.0) but not for those < 7 days (aRRR 0.6, 95% CI 0.1-4.9). The increased mortality was not seen in patients coinfected with tuberculosis and respiratory viruses other than influenza. Conclusions: Tuberculosis and influenza coinfection compared with tuberculosis single infection was associated with increased risk of death, particularly in individuals with symptoms ≥ 7 days. If confirmed in other studies and settings, this supports active provision of influenza vaccination and prioritisation for antivirals for patients hospitalized with tuberculosis.

P2-439

The epidemiology of severe influenza infection in England—the impact in the post-pandemic era

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Background: The UK Severe Influenza Surveillance System (USISS), established in 2010, is a Web-based scheme to collect surveillance data on hospitalised influenza cases from a sentinel network of NHS Acute hospital trusts in England. The system aims to monitor the impact of influenza on the population and describe the epidemiology of severe influenza in time, place and person. This abstract describes the impact and severity of influenza in the postpandemic era as determined by this scheme for the three influenza seasons from 2010 to 2013. Materials and Methods: The voluntary sentinel network consisted of up to 36 hospital trusts, recruited using stratified random sampling by hospital trust type, size and region. A case was defined as any person who was hospitalised and had laboratory-confirmed influenza A (H1 or H3) or B infection. Each participating sentinel hospital submitted a weekly aggregate report of all cases admitted the previous week, by age group and influenza type, at any level of care. Each hospital trust also submitted individual level data on any confirmed influenza cases admitted to intensive care units (ICUs)/high dependency units (HDUs) the previous week. Data are collected through a Web-based, secure platform. Data from week 40 2010 to week 13 2011 was compared with data from week 40 2011 to week 20 2012 and week 40 2012 to week 20 2013 for this analysis. Results: Twenty-three of 168 (13.7%) hospital trusts from across England participated during 2010/11, 36 of 170 (21.2%) in 2011/12 and 32 of 158 (20.3%) in 2012/13. The influenza seasons following the 2009 pandemic have been dominated by different subtypes. In 2010/11 the dominant subtype was influenza A/H1N1pdm09 (1262/1681 total hospitalised influenza cases, 75.1% of hospital admissions in 2010/11). The 2011/12 season was dominated by influenza A (473/543, 87.1%), specifically either influenza A not subtyped (283/543, 52.1%) or influenza A/H3N2 (190/543, 35.0%). The 2012/13 season was dominated by both influenza B (479/1341, 35.7%) and influenza A/H3N2 (364/1341, 27.1%). For all influenza types, the age groups most affected were 15-44 year olds in 2010-11 (739/1681, 44.0% of hospital admissions in 2010/11), 0-4 and 15-44 year olds in 2011/12 (157/543, 28.9% and 154/543, 28.4% respectively) and 2012/13 (326/1341, 24.3% and 392/1341, 29.2% respectively). In 2010/11, 14.1% (237/1,681) of hospitalised cases were admitted to ICUs/HDUs, 8.5% (46/543) in 2011/12 and 12.5% (167/1,341) in 2012/13. A greater proportion of H1N1pdm09 cases were admitted to ICUs/HDUs (16.3% in 2010/11, 95% CI: 14.3-18.4, 22.5% in 2012/13, 95% CI: 16.5-28.6) compared with influenza B (5.5%, 95% CI: 3.2-7.8, $P < .05$ in 2010/11 and 10.9%, 95% CI: 8.1-13.6, $P < .05$ in 2012/13), and H3N2 in 2012/13 (11.1%, 95% CI: 7.9-14.4, $P < .05$). Individual-level data were available for ICU/HDU admissions in 2011/12 and 2012/13; 65.2% of ICU/HDU cases reported an underlying risk factor in 2011/12 and 60.8% in 2012/13. The most common risk factors among the cases admitted to the ICU/HDU were chronic respiratory disease including asthma (18/46 cases in 2011/12 and 41/167 in 2012/13), chronic neurologic diseases (7/46 cases in 2011/12 and 17/167 in 2012/13) and chronic heart disease (5/46 cases in 2011/12 and 20/167 in 2012/13). Conclusions: An unexpected and large impact of influenza A/H1N1pdm09 was observed in 2010/11. However, the subsequent seasons also demonstrated significant impact and severity of influenza A/H3N2 and influenza B. As during the pandemic, chronic

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respiratory and neurologic diseases were the most frequently reported underlying clinical risk factors in cases of severe influenza.

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Homologous and heterologous antibody titer rises after influenza virus infections

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Background: Most influenza virus infections are associated with mild disease. One approach to estimate the occurrence of influenza virus infections in individuals is via repeated measurement of humoral antibody titers. The traditional criterion for infection during a period of influenza activity is a four-fold or greater rise in antibody titers across that period. We investigated the characteristics of antibody rises following confirmed influenza virus infections to assess the accuracy of this criterion and its sources of variation. Materials and Methods: We used data on participants in a Hong Kong community-based study who had influenza virus infection confirmed by RT-PCR, and had both baseline and convalescent antibody titers measured by hemagglutination inhibition and viral microneutralization assays against influenza A(H1N1), A(H3N2) and B viruses. Multivariate models were fitted in a Bayesian framework to characterize the distribution of changes in antibody titers following influenza virus infections. Results: In 76 subjects, homologous antibody titers rose by geometric means of 3.89 to 16.14 after infection for all three influenza virus types considered, with geometric standard deviations between 2.58 and 6.17 around the geometric means. Significant rises in antibody titers against A(H1N1)pdm09 were observed for seasonal A(H1N1) virus infections and vice versa. The most closely correlated titer rises were those for influenza A(H3N2) virus, followed by HI titers for A(H1N1)pdm09 and seasonal A(H1N1). Antibody titers for A(H1N1)pdm09 measured by viral microneutralization rose 3.1 (95% CrI: 1.0–9.5) times as much for those under 40 years of age relative to those older than 40 following infection by A(H1N1)pdm09 virus. Oseltamivir treatment was associated with lower rises in A(H1N1)pdm09 antibody titers by hemagglutination inhibition and viral microneutralization assays following A(H1N1)pdm09 virus infection. Conclusions: Rises in antibody titers following infection vary depending on age and the laboratory test used. The patterns in boosting of antibody titers following influenza virus infection could be taken into account to improve estimates of cumulative incidence of infection in seroepidemiologic studies.

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The incidence, aetiology and epidemiology of acute lower respiratory tract infection amongst children aged < 5 years in a high HIV prevalence setting, 2009-2012

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Background: Data on the epidemiology of acute lower respiratory tract infection (ALRI) and the contribution of influenza amongst HIV-infected and -uninfected children from Africa are limited. We aimed to describe the incidence, aetiology and factors associated with death among HIV-infected and -uninfected children aged < 5 years hospitalised with ALRI in South Africa from 2009-2012. Methods: Children aged < 5 years with ALRI were enrolled through active surveillance at 4 sites in 4 of the 9 South African provinces. Epidemiologic and clinical data were collected. Respiratory specimens were tested for the presence of influenza and 9 other respiratory viruses by multiplex real-time reverse-transcription polymerase chain reaction (PCR). Blood specimens were tested for *Streptococcus pneumoniae* by quantitative real-time PCR detecting the lytA gene. HIV testing was by ELISA for children aged > 18 months and PCR for those ≤ 18 months. Testing for tuberculosis by microscopy and/or culture and/or GeneXpert polymerase chain reaction was performed as part of routine care for some children. Cumulative annual incidence per 100,000 person-years was estimated at one site with available population denominators. Results: We enrolled 8722 children aged < 5 years with ALRI from 2009-2012; 64% (5602/8722) were aged < 1 year and 57% (5014/8721) were male. The overall HIV prevalence among tested children was 12% (700/5931). Among tested children, 26% (2217/8391) tested positive for respiratory syncytial virus (RSV), 7% (613/8392) for influenza and 4% (226/6441) for pneumococcus. Only 13% (1119/8722) were tested for tuberculosis and among these, 10% (107/1119) had laboratory-confirmed tuberculosis. Two percent (146/8633) of children with available information were started on tuberculosis treatment. The influenza detection rate was similar in HIV-infected (7%, 49/684) and HIV-uninfected (7%, 361/5136) children (odds ratio [OR] 1.0, 95% confidence interval [CI] 0.7-1.4). Pneumococcus was more commonly detected in HIV-infected children (7%, 41/623 HIV infected vs 3%, 47/1831 HIV uninfected; OR 2.0, 95% CI 1.4-2.9) and RSV was less common in HIV-infected children (13%, 88/684 HIV infected vs 27% 1408/5135 HIV uninfected; OR 0.4, 95% CI 0.3-05). The incidence of hospitalisation for ALRI was highest in children aged < 1 year (range 8283/100,000 population in 2010 to 10,258/100,000 in 2011). HIV-infected children aged <,5 years experienced up to 3 times greater incidence of ALRI than HIV-uninfected children (age-adjusted relative risk [aRR] 3.4, 95% CI 3.1-3.7 in 2009; aRR 2.5, 95% CI 2.2-2.8 in 2010; and aRR 1.3, 95% CI 1.2-1.5 in 2011). In multivariate analysis, compared with children without HIV infection who were hospitalised with ALRI, HIV-infected children were more likely to have respiratory symptoms for ≥ 2 days prior to admission (OR 1.4, 95% CI 1.1-1.7), require oxygen (OR

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1.1, 95% CI 1.1-1.8), be hospitalised for longer (OR 1.5, 95% CI 1.1-2.0 2-7 days; OR 3.5, 95% CI 2.6-4.8 > 7 days compared with < 2 days) and to die (OR 3.9, 95% CI 2.3-6.4). In contrast, they were less likely to be infected with RSV (OR 0.5, 95% CI 0.4-0.6). The case-fatality ratio was 8 times greater amongst HIV-infected (7%, 47/690) compared with HIV-uninfected (1%, 46/5204) children (*P* <.001). In multivariable analysis controlling for year and site, HIV infection (OR 4.2, 95% CI 2.5-7.0), pneumococcal coinfection (OR 2.6, 95% CI 1.1-6.1), receipt of mechanical ventilation (OR 8.7, 95% CI 3.4-22.2) and receipt of oxygen (OR 26.0, 95% CI 1.7-53.4) were independently associated with death; death was significantly less likely to occur in children testing RSV positive (OR 0.4, 95% CI 0.2-0.9)._Conclusions: Among children aged < 5 years in South Africa with ALRI, HIV infection was associated with substantially increased risk of death. RSV and influenza were the first and second most common viral aetiologies detected. HIV-infected children were less likely than HIV-uninfected children to test RSV positive, and mortality was lower in RSV-infected than -uninfected children. Influenza was not associated with HIV-infection status or mortality.

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The epidemiology of severe acute respiratory illness amongst hospitalized HIV-infected and -uninfected older children and adults aged ≥ 5 years—South Africa, 2009-2012

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Background: There are few published studies describing the epidemiology of severe acute respiratory illness (SARI) among older children and adults from high HIV-prevalence settings in Sub-Saharan Africa. We aimed to describe the incidence, aetiology and factors associated with death among HIV-infected and -uninfected individuals aged ≥ 5 years hospitalised with SARI in South Africa from 2009 through 2012. Methods: Individuals aged ≥ 5 years with SARI were enrolled through active surveillance at 4 sites in 4 of the 9 provinces of South Africa. Epidemiologic and clinical data were collected. Respiratory specimens were tested for influenza and 9 other respiratory viruses by multiplex real-time reverse-transcription polymerase chain reaction (PCR). Blood specimens were tested for *Streptococcus pneumoniae* by quantitative real-time PCR detecting the lytA gene. Testing for tuberculosis by microscopy and/or culture and/or GeneXpert PCR was performed as part of routine care. Cumulative annual incidence per 100,000 person-years was estimated at one site with available population denominators. Results: We enrolled 7191 individuals aged ≥ 5 years with SARI from 2009-2012. The HIV prevalence was 74% (4666/6339) among individuals with known HIV status. The annual incidence of SARI hospitalisation among individuals of all ages ranged from 388-619/100,000

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population during the study period and was highest in individuals aged 45-64 years (640-1285/100,000). HIV-infected individuals experienced a 13-19 times greater incidence of SARI than HIV-uninfected individuals (*P* < .001). Among individuals tested for respiratory pathogens, 9% (620/7065) tested positive for influenza and 9% (570/6632) for pneumococcus. Only 46% (3308/7191) of patients had specimens submitted for tuberculosis testing among whom 11% (372/3308) tested positive for tuberculosis. Ten percent (688/7171) of patients were started on tuberculosis treatment during the current admission. The influenza detection rate was lower in HIV-infected (8%, 350/4605) than HIV-uninfected (11%, 185/1649) individuals (odds ratio [OR] 0.7, 95% confidence interval [CI] 0.5-0.8). Pneumococcus was more commonly detected in HIV-infected individuals (10%, 475/4537 HIV infected vs 4%, 62/1614 HIV-uninfected; OR 2.9, 95% CI 2.2-3.8). Based on multivariable analysis, among patients hospitalized with SARI, compared with patients without HIV infection, HIV-infected patients were more likely to receive tuberculosis treatment on admission (adjusted odds ratio [aOR] 3.1, 95% CI 2.2-4.3) and have pneumococcal infection (aOR 2.3, 95% CI 1.7-3.1) and less likely to be infected with influenza (aOR 0.7, 95% CI 0.5-0.8). HIV-infected individuals were also more likely to have symptoms for > 2 days prior to admission (aOR 1.6, 95% CI 1.3-1.9), receive antibiotics on admission (aOR 2.5, 95% CI 1.7-3.7), be hospitalised for longer (2-7 days: aOR 1.5, 95% CI 1.2-2.0; > 7 days: aOR 2.9, 95% CI 2.3-3.7; compared with < 2 days) and die (aOR 1.7, 95% CI 1.3-2.3). The case-fatality ratio was 1.5 times greater among HIV-infected (8%, 351/4610) compared with HIV-uninfected (5%, 85/1653) individuals (*P* = .001). In multivariable analysis, independent risk indicators for death included HIV infection (OR 1.8, 95% CI 1.4-2.5), age group ≥ 25 years (25-44 years: OR 1.5, 95% CI 1.0-2.3; 45-64 years: OR 2.8, 95% CI 1.8-4.2; and ≥ 65 years: OR 4.3, 95% CI 2.5-7.2) compared with 5-24 years, starting tuberculosis treatment during admission (OR 1.8, 95% CI 1.4-2.4), having respiratory symptoms for ≥ 2 days (OR 1.5, 95% CI 1.1-2.1), admission to intensive care (OR 9.6, 95% CI 1.9-49.1) and receiving oxygen therapy (OR 2.6, 95% CI 2.1-3.2). Conclusions: We observed a high HIV prevalence (> 70%) among patients aged ≥ 5 years hospitalised with SARI. Influenza was identified in 9% of all hospitalisations. HIV-infected older children and adults hospitalised with SARI in South Africa were more likely to test positive for pneumococcus and to be started on tuberculosis treatment and less likely to test positive for influenza than HIV-uninfected individuals. The incidence of SARI hospitalisation and in-hospital mortality was elevated in HIV-infected compared with HIV-uninfected older children and adults. The odds of in-hospital mortality increased with increasing age. Improved access to highly active antiretroviral therapy for HIV-infected older children and adults may assist in reducing the high burden of hospitalisations and mortality.

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Global seasonal patterns in human influenza A (H5N1) incidence

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Background: Influenza A (H5N1) has a documented case-fatality proportion of 60% in humans and the potential to become a worldwide pandemic if mutations improve human-to-human transmissibility. Despite abundant research on seasonal patterns in annual epidemic influenza strains, it is unknown whether similar patterns exist in human influenza A (H5N1). Such knowledge would allow targeted use of resources to surveil, screen, and quarantine at-risk individuals. Thus, we set out to characterize global seasonal patterns of A (H5N1) in humans. Materials and Methods: We performed an exhaustive literature review of A (H5N1) infection in humans published in all languages from the first case report in December 1997 to April 2013. We reviewed multiple databases, including PubMed, Google Scholar, Scopus, and the World Health Organization (WHO) Global Alert and Response entries. We included all articles reporting data on either 1) a human case with confirmed A (H5N1) infection as defined by WHO criteria, or 2) a human case with suspected A (H5N1) infection amid a cluster of confirmed cases. Two independent reviewers (RBP and MM) assessed each article against a predefined set of inclusion criteria; a third reviewer (NK) resolved disagreements. We abstracted a

predefined set of variables from each study, including month, year, country, and mortality. We further classified cases by season (summer: June-August; fall: September-November; winter: December-February; spring: March-May) and by hemisphere (northern vs equatorial). We used Poisson regression to predict changes in case incidence as a function of season and hemisphere (northern vs equatorial). As a post-hoc analysis to investigate a possible contribution of climate to seasonal changes in case incidence, we performed similar regression analyses after grouping countries according to the Köppen-Geiger climate classification, which incorporates three dimensions of climate: main climate (equatorial, arid, warm temperate, snow, polar), precipitation, and temperature. The five climate groups we created were 1) Thailand, Viet Nam, Myanmar, Laos, Cambodia, Bangladesh, and Nigeria; 2) Indonesia; 3) China and Hong Kong; 4) Egypt, Iraq, Azerbaijan, Pakistan, and Djibouti; and 5) Turkey. Results: We identified 571 cases of human influenza A (H5N1) between December 1997 and April 2013. Cases occurred in 16 countries, primarily in Southeast Asia and the Middle East. Case incidence was higher in winter (n = 234; $P < .0001$), spring (n = 176; $P < .0001$), and fall (n = 83; $P = .03$) than in summer (n = 57). Seventy-five percent of all cases occurred in winter and spring combined. Additionally, there were significant interactions with hemisphere; the winter and spring peaks in case incidence were more dramatic in the northern hemisphere versus equatorial countries ($P = .0001$ and $P = .002$, respectively, for northern*winter and northern*spring interactions). The winter and spring peaks in case incidence replicated across all five climate groups, although climate showed several significant interactions with season. Conclusions: Influenza A (H5N1) incidence is nearly three times higher in winter and spring combined than in fall and summer combined; these patterns are robust across varied climatic groups. Our findings in humans mirror previous research on seropositivity patterns in avian vectors, which are in turn associated with migration patterns of wild birds and may contribute to the strong seasonal incidence pattern among human cases. Our results are also consistent with prior research showing that cold and dry conditions characterizing winter climates improve influenza virus transmission in animal models. In light of our findings, preferentially increasing surveillance and resource deployment during winter and spring months may be an effective strategy to curb influenza A (H5N1) mortality in resource-limited settings.

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Epidemiological characteristics of hospitalizations associated with circulating seasonal influenza strains: pooled analysis from the "Global Influenza Hospital Surveillance Network" (GIHSN)

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Background: The predominant circulating strains and level of severity of influenza epidemics vary between years and between countries. High-quality surveillance networks are relevant tools to put in place to control and have an overview of influenza epidemics. During the 2012-2013 influenza season, the GIHSN network comprised 21 hospitals in the world. With a broad regional representativeness and a platform of international public-private partnerships, it will lead to a better understanding and knowledge of influenza epidemiology. The GIHSN, with its expected sustainability over sequential influenza seasons and standardization of data collection, has for its main objective to evaluate the burden of severe influenza disease, defined as hospitalization related to community-acquired influenza or complications following influenza infection, and to quantify the distribution of different influenza strains among severe cases. Materials and Methods: Core documents were created to compile the essential elements of the influenza epidemiologic research project. All sites followed homogenously these documents in terms of the purpose of the study, design, target population, data and variables needed, to have uniformed data to allow for the pooled analysis. This multicentre prospective epidemiologic surveillance study in Spain (5 hospitals), France (5 hospitals), Turkey (7 hospitals), and Moscow (1 hospital) and St. Petersburg (3 hospitals) regions in the Russian Federation was conducted during the main influenza season. Hospitalized patients of all ages presenting with influenza like-illness (ILI) within 7 days between the onset of symptoms and admission were swabbed. Patients were first enrolled if they were hospitalized in the previous 48 hours, and presented an admission diagnosis possibly associated with an influenza infection. Positives for influenza were rea- time reverse transcription polymerase chain reaction (RT-PCR) positive for influenza A (H3N2), or A (H1N1)pdm09, or B. Patient characteristics between positives and negatives for influenza were compared using chi-square tests. A *P* value < .05 was considered statistically significant. Graphic representations were produced for pooled results of the distribution of respiratory viruses across the defined influenza season, influenza-associated hospitalizations across age groups, and the disease attributable to each strain of the influenza virus. All data management and analysis were performed with Stata version 12. Results: Of the 8078 patients screened, 4922 were eligible and tested, 1015 (28%) were positive for influenza, 297 (8.3%) were positive for other respiratory viruses, and 2270 (63%) were negative. Influenza A (H1N1)pdm09 was dominant (n = 555; 56%), followed by influenza B (n = 273; 27%) and influenza A (H3N2) (n = 172; 17%). Influenza B was most common in Spain and France in the persons older than age 65, compared with influenza A H1N1pdm09 that predominated in younger people in the Russian Federation and Turkey. The age group most affected

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by influenza this season were patients 18 to 50 years old. The flu season started globally mid-January with a peak extending from week 5 to week 8 and ending around week 16, 2013. Sociodemographic characteristics such as age, the number of chronic conditions, and smoking habits were significantly different between positives and negatives, with a *P* value of < .001. Conclusions: Influenza H1N1pdm09 was highly circulating this season in younger persons in Eastern Europe and Central Asia, and influenza B was present mostly in the elderly in northern and southwest Europe. There was peak at a later appearance for influenza B compared with influenza H1N1pdm09. More investigation is needed to further assess the risks of being hospitalized due to influenza. However, the data represented from this network provide robust comparisons between positives and negatives for influenza, and epidemiologic data provide a global overview of the influenza season 2012-2013. Increased sample size within this network will allow for more accurate and representative data. Nevertheless, this study has shown that a multicentre hospital-based study is feasible, and maintaining a homogenous practice across sites through strong coordination and support ensures the quality of pooling of data. Funding: This network activity is partly funded by Sanofi Pasteur.

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Influenza A(H1N1)pdm09 fatal cases in women of reproductive age: risk determinants and virological assessment

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Background: Pregnant women have a higher risk for severe disease and death as a consequence of influenza infection. We studied the risk factors and virologic features of influenza A(H1N1)pdm09(H1N1p) associated with fatal cases among women of reproductive age from Rio de Janeiro (April 2009-December 2010), a scenario that is still poorly explored in Brazil. Materials and Methods: The study population comprised 826 women of reproductive age (15-44 years) from which 41 progressed to death. Clinical specimens (oro-nasal swabs) as well as demographic/clinical /epidemiologic information were collected by the Epidemiological Surveillance Team/Rio de Janeiro, using a nationally standardized questionnaire. After RNA extraction, influenza detection was performed using real-time RT-PCR (CDC protocol). Polymorphisms at positions HA222 and NA275 were identified by pyrosequencing (Deyde VM, et al. *Antimicrob Agents Chemother*. 2010;54:1102). Descriptive, bivariate analyses (chi-square/Fisher exact test and *t* test for means) and multiple logistic regressions (considering variables of epidemiologic relevance/plausibility) were carried out and considered significant when *P* < .05. Results: Among the 826 studied women, the mean (± SD) age was 27.8 (± 7.7) years and 57.5% were pregnant. The mean per capita income was US$ 321.52 (± 254.73). Approximately 62.1% presented dyspnea and about a third of sample (30.2%) reported any comorbidity, such as cardio- or pneumopathy, renal disease, metabolic disorders or immunodeficiency. About 60.0% showed any abnormality as the X-ray result, most frequently an interstitial infiltrate (73.9%). Influenza A(H1N1)pdm09 infection was detected in 23 (60.5%) and 309 (44.1%) of fatal and nonfatal cases, respectively (*P* = .035). Infected pregnant women were about 3 times more likely to progress to death than those not infected (68.4% vs 43.2%; AOR = 3.2, 95% CI 1.1-9.7). Among flu cases, deaths were significantly associated with dyspnea (94.6% vs 62.1%; *P* = .002), the presence of any comorbidity (32.7% vs 31.3%; *P* = .043), metabolic disorders (5.5% vs 4.5%; *P* = .021), a lower per capita income (US$ 217.07 ± 112.96 vs 353.61 ± 260.55; *P* < .001) and a longer time from symptom onset and hospitalization (4.6 ± 3.0 vs 2.7 ± 3.0; *P* = .010). The last, a risk determinant for a fatal outcome in pregnant (4.0 ± 2.8 vs 2.5 ± 2.9 days, *P* = .011). Among infected nonpregnant women, the presence of any comorbidity was identified as an independent risk factor for death (AOR = 6.9; 1.9-28.8, *P* = .002). Polymorphisms at HA position 222(G/N) were significantly associated with progression to death in both categories (*P* < .001). Finally, mutation H275Y, associated with antiviral resistance, was not detected in our sample. Conclusions: These findings underline the fatal role of influenza A(H1N1)pdm09 infections among pregnancy. Routine vaccination and early usage of NAIs must be stressed, as it is critical to circumvent further complications and

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progression to death by influenza among this risk category. Also, our figures corroborate previous findings on the low frequency of NAI resistance and the association between D222G mutation and severe/fatal disease. Financial support: Carlos Chagas Filho Foundation for Research endowment for Rio de Janeiro (FAPERJ), process n. 111.802/2012; Science and Technology Department (DECIT), Ministry of Health and National Counsel of Technological and Scientific Development (CNPq), Brazil.

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Hospitalization fatality risk of influenza A(H1N1pdm09): a systematic review

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Background: During the 2009 influenza pandemic, uncertainty surrounding the seriousness of human infections with the H1N1pdm09 virus hindered the calibration of the public health response. One measure of seriousness is the hospitalization fatality risk (HFR), defined as the probability of mortality among cases of H1N1pdm09 that required hospitalization for medical reasons. The objective of our study was to review published data on the HFR of H1N1pdm09. Materials and Methods: We searched for relevant studies in PubMed, MEDLINE and EMBASE. Studies that reported population-based estimates of the HFR or sufficient data to retrieve a HFR in the first year of the pandemic were included. We excluded studies that reported estimates of the HFR in population subgroups such as pregnant women or those at higher risk of severe outcome if infected (eg, individuals with underlying chronic diseases). Results: We included 103 estimates of the HFR from 101 published studies, reporting a total of 82,133 hospitalized cases and 3743 deaths. Only 7% of the studies were published within the calendar year of 2009. In total, our analysis was based on reports from 43 countries or regions in every continent except Antarctica. We identified heterogeneity in published HFR estimates, with estimates of the risk of death ranging from 0% to 48% in individual studies. The point estimates of the HFR increased substantially with age, with estimates ranging from 0% to 5% in children compared with 14% to 27% in the elderly. Patients with underlying health conditions had an increased risk of death if hospitalized. Conclusions: There was some variability in published estimates of the HFR, but much less variability than we found in our review of published estimates of the case fatality risk (Wong J, et al. *Epidemiol* 2013). The HFR of H1N1pdm09 increased with age and in persons with underlying health conditions. Lower HFR in children could be due to reduced seriousness of infection or a lower threshold for admitting children. Early in the next pandemic, estimation of the HFR may provide a reasonable picture of seriousness of infection and thereby inform risk assessment of the severity of the pandemic strain.

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Latitude gradients and climate associations of influenza-like illness in Vietnam

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Background: Climatic factors have been shown, both from ecologic and experimental studies, to play a substantial role in driving the epidemiology of influenza-like-illness (ILI). Absolute humidity (AH) has

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been identified to play a particularly important role in temperate countries. Here we studied potential climatic drivers of ILI epidemiology in Vietnam (latitudinal range, 8° to 24°N), substantial climatic diversity, and a large population (90 million). Our study focused specifically on the seasonality (annual periodicity) of ILI. Materials and Methods: ILI notifications to the Ministry of Health were aggregated by month (from January 1993 to December 2011) and province. Estimated commune population sizes were derived from the General Statistical Office of Vietnam. Monthly (from January 1993 to December 2011) average min, mean and max temperatures, absolute and relative humidity, sunshine and rainfall in 68 climatic stations were obtained from the Vietnam Institute of Meteorology, Hydrology and Environment. Estimated global monthly values for the above climatic variables by 2.5° x 2.5° pixel were obtained from the NCEP/NCAR project. ILI time series for each province were detrended, centered, reduced and square-root transformed. Annual periodicities were quantified from the value of the Fourier spectrum at the period of 1 year. Climatic variables that separated into difference threshold cutoff (varying from the minimal to the maximal value of the variable) were entered in a regression tree analysis to identify those that best explain the observed ILI seasonality. A seasonally forced SEIR model was developed in which the seasonality of the forcing on the transmission can be controlled independently of its variance. Results: During the 18-year study period, 26,023,574 cases of ILI were reported. ILI reporting incidence for the whole country showed a larger peak from September to November, and a smaller one from February to April. The seasonality of ILI increases from south to north, parallel to the gradient observed for certain climatic variables. Regression-tree analysis showed a strong influence of temperature and humidity with the seasonality of AH explaining most of the variance in ILI seasonality. The relationship between seasonality of AH and seasonality of ILI epidemiology revealed a threshold effect that is also predicted by the SEIR model. This suggests that the nonlinearity of the transmission process is responsible for this threshold effect. Extrapolation of these results to a global climate surface of the annual periodicity of AH predicts the spatial distribution of seasonal versus nonseasonal ILI epidemiology that is in agreement with previously published results. Conclusions: Our analyses have demonstrated that the periodicity of AH in Vietnam may play a role in driving the seasonality of ILI. These results may be applicable to other countries.

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An outbreak with mixed infection of influenza A/H3N2 and pandemic H1N1 in Alappuzha district of the state of Kerala, India

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Background: In late June and July 2011, an upsurge in patients with influenza-like illness (ILI) was reported from peripheral hospitals in Alappuzha district of Kerala state. An investigation was carried out to determine the causative agents. Methods: Investigations were carried out in the outpatient departments of sentinel hospitals. Using the World Health Organization case definition for ILI, 204 patients with ILI were identified and their nasal/throat swabs were collected. Real-time RT-PCR–based diagnosis was performed to detect influenza type A and B, and their subtypes. Madin-Darby Canine Kidney cell line was used for virus isolation. Haemagglutinin (HA) and neuraminidase (NA) genes of isolated influenza viruses were sequenced and phylogenetic analysis was carried out. Results: Of 204 specimens tested, 102 (50%) were influenza A PCR positive. PCR subtyping results revealed that 96 (94.11%) influenza A positive samples were influenza A/H3N2 and six (5.89%) were A (H1N1) pdm09. All the influenza A negative samples were also negative for type B. Phylogenetic analysis of eight A/H3N2 isolates showed that seven of these were genetically related to A/Victoria/361/2011/H3N2, which was the 2012-2013 northern hemisphere vaccine component, and one isolate showed 99.04% identity with A/Perth/16/2009, which was the 2010-2012 northern hemisphere vaccine component. These isolates acquired amino acid substitutions K158N, N189K, and T212A as in A/Victoria/361/2011. Further, the isolates showed amino acid substitutions N145S, A198S and V223I and formed clade 3 of the Victoria group. Genetic characterization of four A (H1N1) pdm09 isolates indicated that the viruses predominantly clustered with A/California/07/2009 (2010-2013 northern hemisphere vaccine strain) in clade 7, which is globally the most widely circulating

clade. A (H1N1) pdm09 isolates were also screened for the H274Y mutation in the NA gene and all were sensitive to oseltamivir. Seven influenza A/H3N2 isolates were studied for the presence of substitutions at positions R118K, E119Q, D151E, R152K, R224K, E227D, E276D, R292K, N294S, and Δ244-247, and all isolates remained sensitive to oseltamivir. Conclusions: From this investigation, we conclude that influenza type A (H3N2) was the etiologic agent of the outbreak, and pandemic H1N1 virus co-circulated during the ILI outbreak in Alappuzha district. No substantial differences in the sequences were noticed in either A/H3N2 or A (H1N1) pdm09. Both viruses was found to be sensitive to oseltamivir.

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Risk factors for hospitalization with influenza-associated severe acute respiratory illness in 2012, South Africa

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Background: There are few data on the risk factors for severe influenza infection in high HIV prevalence settings such as South Africa. Data describing risk factors for severe disease will assist in allocation of resources such as vaccines and antiviral drugs. In addition, clinical risk factors will be of value to doctors treating severe acute respiratory illness (SARI). Influenza vaccination is available for HIV-infected patients, children, and older adults in the public health system in South Africa; however, coverage remains low. We aimed to describe the factors associated with severe influenza infection by comparing the characteristics of influenza-positive patients with outpatient influenza-like-illness (ILI) to influenza-positive patients hospitalized with SARI in South Africa. Methods: Patients with ILI and SARI were enrolled at two outpatient clinics and two hospitals in similar geographic areas as part of an active, prospective sentinel surveillance programme for respiratory viruses. Patients fitting clinical case definitions (standard World Health Organisation defined case definitions) were systematically enrolled. Respiratory specimens were tested by multiplex real-time reverse transcription polymerase chain reaction (PCR) assay for respiratory viruses: influenza, adenovirus, enterovirus, rhinovirus, parainfluenza 1-3, human metapneumovirus, and respiratory syncytial virus. HIV results were obtained from medical records, voluntary counselling and testing or anonymized testing as part of the surveillance programme. Children under 18 months of age were screened by HIV enzyme-linked immunosorbent assay and confirmed by PCR if positive. Variables considered as risk factors for influenza-related SARI included the following: HIV infection, any underlying chronic illness (other than HIV infection), age and coinfection with other respiratory viruses. Influenza-positive patients with ILI were compared with patients admitted to hospital with influenza-related SARI using logistic regression analysis. Results: During the 2012 influenza season from the beginning of June 2012 to the end of October 2012, 1045 patients with SARI and 914 patients with ILI were enrolled. Influenza testing results were available for 93% (1816/1959) of patients. The detection rate for influenza was 9% (90/953) in SARI patients and 23% (196/863) in ILI patients ($P < .001$). The HIV prevalence in patients with influenza-associated SARI and available data on HIV status was 48% (30/62) compared with 38% (56/146) in patients with influenza-associated ILI ($P = .2$). On univariate analysis, underlying illness (excluding HIV) (odds ratio [OR]: 4.2; 95% confidence interval (CI): 2.2-7.9), HIV infection (OR: 1.5; 95% CI: 0.9-2.7), rhinovirus coinfection (OR: 3.5; 95% CI: 1.3-9.6) and age (reference age 3-5 years: age ≤ 2 OR 4.1, 95% CI: 2.6-6.4; age 6-15 OR 0.4, 95% CI: 0.2-0.7; age 16-55 OR 2.4, 95% CI: 1.6-3.5; age ≥ 56 OR 7.2, 95% CI: 4.3-12.2) were associated with influenza-associated SARI. In the multivariable model, HIV infection (OR: 2.9; 95% CI: 1.2-7.1) and age remained associated with SARI (reference age 3-5 years: age ≤ 2 OR 6.7, 95% CI: 1.6-28.7; age ≥ 56 OR 8.4, 95% CI: 1.0-46.6). Conclusions: Among patients with respiratory influenza infection in South Africa, very young

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age, older age and HIV infection are risk factors for SARI compared with milder ILI. These data support calls for improvement in coverage of influenza vaccination in HIV-infected people, infants and the elderly.

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Hospital surveillance for severe acute respiratory infections (SARI) in nine countries in the WHO European Region—risk factors for a severe outcome

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Background: One of the gaps in influenza surveillance that was highlighted by the emergence of the influenza A(H1N1)pdm09 virus was the need for influenza surveillance systems that routinely monitor severe influenza to compare the severity of different influenza seasons, to monitor high-risk groups and to track viruses causing severe disease. Sentinel hospital surveillance for severe acute respiratory infection (SARI) has recently been established in countries in the WHO European region. Case-based SARI data are presented here with the objective to describe characteristics of SARI cases and to investigate the association between risk factors and a severe outcome in influenza-positive cases. Materials and Methods: All countries collecting case-based data on SARI patients between 2009 and 2012 were invited to participate in the study. For inclusion, as a minimum, data on age, sex, clinical symptoms, presence and specification of underlying conditions, and influenza (sub)type test results were required. Information on SARI case-definition(s) applied in the hospitals and sampling protocols for collecting respiratory specimens from patients were also collected. Descriptive analysis was performed to investigate the patient characteristics for not tested, negative and influenza-positive SARI cases. For countries that collected additional data on severity (ICU admission, death), we assessed the association between possible risk factors (ie, age, sex, underlying conditions, influenza subtype) and a severe outcome (ICU admission or death). Bivariate analysis was performed to identify factors associated with ICU admission or death. All variables associated with a severe outcome in the bivariate analyses at a significance level of $P < .15$ are included in a multivariate logistic regression analysis to identify factors independently associated with ICU admission or death. A factor is defined significant when $P < .05$.Results: Nine countries (Albania, Armenia, Belarus, Georgia, Kazakhstan, Kyrgyzstan, Romania, Russian Federation and Ukraine) fulfilled the inclusion criteria. Between 2009 and 2012, a total of 13,301 SARI cases were reported. The percentage of children aged 0-14 years ranged from 23.5% to 85.7% for the different countries. Data on ICU and death were available for five countries. Preliminary bivariate analysis indicated that the following factors were significantly associated with death vs nondeath: age > 15+ (OR = 7.01; 2.09-23.53), presence of underlying conditions (OR = 6.29; 2.15-18.37), being immune-compromised (OR = 3.00; 1.17-7.69), presence of liver conditions (OR = 4.59 (1.47-14.38) and influenza A(H1N1)pdm09 infection (OR = 16.43; 2.19-123.30) vs influenza A(H3N2). In the multivariate analysis using logistic regression, the preliminary results indicate that 1) influenza A(H1N1)pdm09 virus was significantly associated with death, and 2) immune-compromised status may also be an independent risk factor ($P = .05$). Conclusions: The establishment of sentinel SARI surveillance in hospitals is a

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first step toward monitoring influenza-related severe acute respiratory disease and is a promising system to obtain information on high-risk groups and to track the viruses that cause severe disease. Although the SARI data collection in the region is not identical (possible bias in age, sampling and health care use) and reports of ICU admission and death are available for part of the countries only, we have a better understanding of the role of influenza at the severe spectrum of the disease. Preliminary results indicate that influenza A(H1N1)pdm09 and being immune compromised are independent risk factors for death. Nine countries contributed data for this study, the majority of which are low-middle income countries with low vaccination uptake and antiviral use. Information on risk factors will help in targeting and prioritizing vulnerable populations for vaccination and antiviral treatment in these countries and may be useful for other countries in the European region.

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Rate of decline of antibody titres to influenza A/California/7/2009 (H1N1) by haemagglutination inhibition and virus neutralization assays in a cohort of seroconverting adults in Singapore

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Background: The rate of decline of antibody titres to influenza following infection can affect results from serosurveys, and population level decline in such titres may explain the pattern of repeat epidemics observed in pdmH1N1 influenza A. We describe changes in antibody titres in a cohort of adults who seroconverted during the initial wave of pdmH1N1 infections in Singapore. Materials and Methods: We followed up a cohort who seroconverted (≥ 4-fold increase in haemagglutination inhibition (HI) titres to A/California/7/2009 H1N1) during a seroincidence study in 2009 and obtained 2 additional blood samples in April 2010 and September 2010, which were assayed for antibodies to A/California/7/2009 H1N1 by both HI and virus microneutralization (MN) assays along with the sample from 2009 with the highest HI titres (between Aug and Oct 2009). We analysed pairwise mean fold change in titres and the proportion with HI titres ≥ 40 at the three time points; for MN, a cutoff point of ≥ 160 was used as this best correlated with a HI titre of 40 in our assays. We also fitted a multinomial probit regression model to titre distributions to explore if the rate of decline differed by demographic variables like age and gender. Results: After excluding 2 participants with a ≥ 4-fold increase in titres (on both HI and MN), we had 65 participants who contributed 3 samples each; 63 (97%) and 49 (75%) of these had a decrease in HI and MN titres, respectively, from the 2009 sample to the last sample in 2010. Titres by both assays decreased significantly, and in 6 individuals aged 55 and older, the decrease was significantly greater than in those aged below 55. The posterior means of the sampling period 2 effect were estimated as –3.42 (95% Credible interval [CrI], –4.33 to –2.61) and –1.99 (95% CrI, –2.72 to –1.30) by HI and MN assays, respectively; for period 3 these were –3.88 (95% CrI, –4.86 to –3.01) and –1.99 (95% CrI, –3.38 to –1.87), respectively. Older individuals had more rapid decline in measurable antibodies: mean –1.22 (95% CrI, –2.95 to 0.42) and –3.30 (95% CrI, –5.21 to –1.50) for sampling periods 2 and 3 in subjects aged ≥ 55 by HI assay; –2.80 (95% CrI, –4.30 to –1.33) and –2.56 (95% CrI, –4.03 to –1.12) by MN assay. Statistically discernible differences in gender over the sampling periods were observed by HI assays only: –1.43 (95% CrI, –2.40 to –0.52) and –1.35 (95% CrI, –2.33 to –0.40) for sampling periods 2 and 3, respectively, for female relative to males. While 71% and 72% had titres ≥ 40 and ≥ 160 by HI and MN in 2009, this had decreased to 14% and 37% by September 2010. Conclusions: After 1 year, only 14% individuals with serologically ascertained pdmH1N1 infection had the traditionally accepted seroprotective HI titres of ≥ 40. The drop in titres was more marked by HI than MN, with implications for serologic studies. The

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significance for reinfection is uncertain, but the rapid decline may explain the multiple infection waves observed. There was also evidence that titres decreased more rapidly in older individuals.

P2-453

Predictive value of telenursing complaints in influenza surveillance: a prospective cohort study in Sweden

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Background: Data source alternatives to mandated reporting by microbiologic laboratories and sentinel physician practices have been sought to improve the timely detection of influenza outbreaks. This study examines the use of data from calls to telenursing services as population-level predictors of influenza outbreaks and their progression. It employs data from the Swedish telenursing service Healthcare Direct/1177 and an electronic health data repository covering an entire county population. Specifically, the aim is to examine which grouping of telenursing chief complaints is most strongly correlated with influenza case rates and to assess the prospective epidemiologic performance of this grouping as predictors of influenza activity. Materials and Methods: Data from two influenza seasons (2007-08 and 2008-09) were collected in Östergötland county (population 427,000), Sweden, to determine which telenursing chief complaint grouping had the largest correlation with influenza case rates and offered the longest lead time. The best performing alerting threshold for this grouping was established using receiver operating characteristics curves. This influenza activity prediction (IAP) grouping and threshold were prospectively evaluated using data from three subsequent seasons (2009-10 to 2011-12). Correlations with influenza case rates were calculated and estimates of the area under curve (AUC) and positive predictive value (PPV) were computed as performance indicators. To denote the strength of correlations, limit values were applied as suggested by the Cohen Scale (small, medium and large effect sizes defined as 0.10, 0.30, and 0.50, respectively). The limits for interpreting the AUC were set to 0.90, 0.80, and 0.70, denoting very strong (outstanding), strong (excellent), and acceptable discriminatory performance, respectively. Results: The grouping of chief complaints with the largest correlation strength on a daily basis (r = 0.66; P < .001) and longest lead time (14 days) to influenza case rates in the retrospective data was fever (child, adult) and syncope. On a weekly basis, the strength of the correlation was larger (r = 0.91; P < .001), while the lead time remained at 2 weeks. The alerting threshold was determined to a moving average of 0.9 calls/day/100,000 above the baseline level. The strength of the correlation between telenursing call rates for the IAP grouping and influenza case rates was in the prospective evaluation, on a daily basis, slightly smaller than observed in the retrospective analysis but still large (r = 0.59; P < .001). The correlation strength was smaller during the first part of the period including the pandemic outbreak in 2009 (r = 0.56; P < .001) than in the second part of the period including only seasonal outbreaks (r = 0.64; P < .001). Similarly, the weekly correlation strength was smaller than observed from the retrospective data (r = 0.80; P < .001). Also here it was smaller during the first part of the evaluation period covering the pandemic outbreak (r = 0.76; P < .001) than in the later part including only seasonal outbreaks (r = 0.86; P < .001). The AUC for the 14-day predictions on a daily basis was 0.87 (PPV = 0.75) for the entire prospective evaluation period; the specificity was 0.88 and the sensitivity was 0.67. The performance was acceptable for the part of the evaluation period including the 2009 pandemic outbreak (AUC = 0.84; PPV = 0.58), while it was strong (AUC = 0.89; PPV = 0.93) for the remaining period including only seasonal outbreaks. On a weekly basis, the AUC was strong 0.81 (PPV = 0.90) for the entire prospective evaluation period; the specificity was 0.94 and the sensitivity was 0.68. Also on a weekly basis, the performance of predictions was acceptable for the

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pandemic outbreak (AUC = 0.78; PPV = 0.79) and strong for the seasonal outbreaks (AUC = 0.83; PPV = 1.00). Conclusions: The telenursing complaints fever and syncope showed in a prospective evaluation large strengths for correlations with influenza case rates and strong discriminatory performance in predicting seasonal influenza outbreaks. The telenursing surveillance method achieved poorer results during the 2009 pandemic outbreak when health behaviors did not follow anticipated patterns. We recommend use of telenursing data in surveillance of seasonal influenza.

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Age-specific differences in influenza virus type and subtype distribution in 12 European countries, 2012-2013

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Background: Limited surveillance data on hospitalised cases of laboratory-confirmed influenza suggest that the distribution of seasonal influenza virus types and subtypes differs between age groups. It is unclear if this also applies to influenza cases seeking primary care. The European Influenza Surveillance Network (EISN) is based on sentinel surveillance networks in each of the 29 participating countries. It routinely reports data on influenza-like illness (ILI) and acute respiratory infection (ARI) by age group, but these data are aggregate and thus not linked to any virologic information. Conversely, EISN laboratories do not routinely report patient age for the clinical sentinel specimens testing positive for influenza. The aim of this study was therefore to find out if the influenza virus type and subtype distribution differs between age groups and what implications this could have from the public health perspective. A more strategic objective was to explore if this data collection and analysis add value to our understanding of influenza epidemiology in Europe and should be considered as an additional element of routine surveillance to, for example, better target the vaccination campaigns. Methods: Toward the end of the 2012-2013 influenza season, all EISN laboratories were asked to submit a disaggregate subset of their data for this season to the European Centre for Disease Prevention and Control (ECDC). The variables were limited to reporting week, patient age, and influenza type and subtype. The age groups analysed were 0-4, 5-14, 15-64 and ≥ 65 years of age. The data were cleaned and variables unified between different countries. The main analysis was the comparison of the subtypes in different age groups, between countries and at European level. Results: Twelve of 29 EISN member states (accounting for 36% of the EU population), reported patient age, influenza virus type and subtype for 7872 positive specimens. France and Spain contributed 64% of all reported specimens. The positivity rate of the samples varied across countries, ranging from 19% to 72%.However, the distribution of positive samples by (sub)types remained similar across most countries. In cases 5-14 years of age, 75% tested positive for influenza B virus, whereas all other age groups had an even distribution of influenza A and B viruses in 2012-2013. Among the influenza A viruses subtyped, A(H1N1)pdm09 viruses dominated over A(H3N2) viruses in all cases up to 64 years of age (68% vs 32%), whereas in those aged 65 years and above, A(H3N2) viruses dominated (63% vs 37%). Conclusions: This study is the first cross-European study analysing the influenza type and subtype distribution across age groups. The study corroborates previous findings of differences in distribution of subtypes across age groups and warrants further analysis specifically between the two influenza B virus lineages. In the 2012-2013 influenza season, the B viruses were dominating in school-aged children, whereas all other age groups had an even distribution of influenza A and B viruses. This could be an indication of different transmission dynamics in different populations, possibly due to differences in age-specific population immunity based on previous infection or vaccination. These are likely explanations that should be further explored seroepidemiologically and by taking vaccination status into account. Obviously, also health-seeking behaviour may have caused a bias toward children being tested more often. Based on the results of the study, the ECDC will further explore the possibility of end-of-season collection of this type of disaggregate detection data, as this may support the targeting of the vaccination campaigns to specific age-groups.

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Molecular epidemiology and serological characterization of influenza virus infection in Nepal

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Background: A cross-sectional study in 2012, based at the National Influenza Center Nepal, was carried out with the objective to characterize the circulating influenza viruses in Nepal. Methods: A total of 1536 throat swab specimens, obtained from patients with influenza-like Illness (ILI) at National Influenza Surveillance Network (NISN) sentinel hospitals, were transported to the National Influenza Center, maintaining reverse cold chain, within 48 hours. Viral RNA was extracted using the QIAmp viral RNA kit. Polymerase chain reaction assay (PCR) was performed following CDC real-time PCR protocol for detection and characterization of the influenza virus. Randomly selected 10% of PCR-positive specimens were subjected to virus isolation in a Madin Darby Canine Kidney (MDCK) cell line and characterized by haemagglutination inhibition assay. Results: Of the 1536 throat swab specimens collected from ILI cases, influenza viruses were detected in 890 (57.9%) specimens. Influenza A infection was detected in 176/1536 (11.4%) cases, of which 174/1536 (11.3%) were influenza A (H1N1) pdm 09 and 2/1536 (0.1 %) were influenza A/H3 subtype. Influenza B was detected in 664/1536 (43.2%) cases. Influenza A (H1N1) pdm09 and influenza B coinfection was observed in 50/1536 (3.2%) cases. Influenza A (H1N1) pdm 09, A/H3 and B virus were antigenically similar to the novel influenza A/California/07/2009-like (H1N1) V type viruses, A/Victoria/361/2011 (H3N2) viruses, and B/Brisbane/60/2008 and B/Wisconsin/1/2010 viruses, respectively. Although sporadic cases of influenza were observed throughout the year, the peak was observed during July to November. The highest numbers of influenza A (H1N1) pdm09 and influenza B were found in September and in children (< 15 years of age). Conclusions: All types of influenza viruses are in circulation in Nepal, with the peak during July-November. Comparison of genetic patterns of influenza virus in consecutive years is necessary to link viral genetic changes and seasonal outbreak of influenza viruses in Nepal.

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Risk factors associated with hospitalized severe acute respiratory illness (SARI) in rural South Africa—2009-2012

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Background: Although severe acute respiratory illness (SARI) is the leading cause of death among children under five years of age worldwide, little is known about the epidemiology of SARI in parts of rural sub-Saharan Africa. In 2009, prospective hospital-based SARI surveillance began in South Africa to estimate disease burden in adults and children. Combining data from this surveillance system and the existing Agincourt health and demographic surveillance system (HDSS) in the same catchment area, we investigated risk factors for hospitalization for SARI in rural South Africa.

Materials and Methods: We conducted a case-control study. Cases were defined as hospitalized patients who lived in the Agincourt HDSS area and met SARI screening and enrollment criteria at Mapulaneng and Matikwana hospitals, Mpumalanga Province, 2009-2012. We matched SARI cases to their records in the HDSS using record linkage analysis algorithms. For each case, ten HDSS controls were randomly selected without replacement, matched by age group (< 5 years and ≥ 5 years) and village of residence. The HDSS database contains basic demographic variables and household characteristics, including socioeconomic status (SES) quintiles created from principal components analysis based on household assets in 2009 (households with the least assets are in the 1st quintile; those with the most in the 5th). To identify risk factors associated with hospitalized SARI, we conducted multivariable conditional logistic regression stratified by age group and adjusted by village of residence. Results: Of the 2215 hospitalized SARI patients at the two hospitals, we identified 258 (12%) who were registered in the HDSS. These patients did not differ significantly from those who were not found in the HDSS except for hospital site. Most HDSS-cases sought care at Matikwana Hospital (n = 209; 81%), whereas non-HDSS cases were evenly distributed between Mapulaneng Hospital (n = 938; 48%) and Matikwana Hospital (n = 958; 49%). Influenza virus was detected by polymerase chain reaction in 9.1% of all SARI patients (n = 202) and 9.3% of matched cases (n = 24). In bivariate analysis for age group < 5 years, male sex was significantly associated with hospitalization with SARI (OR = 1.5, 95% confidence interval [CI] = 1.0–2.1). Compared with those in the 1st SES quintile, participants in higher quintiles had higher odds of being a case (3rd quintile OR = 3.0, 95% CI = 1.1–8.4; 4th quintile OR = 2.7, 95% CI 0.9–7.5; 5th quintile OR = 3.3, 95% CI = 1.2–8.9), and being enrolled in a crèche or preschool was associated with a lower odds of being a case (OR = 0.5, 95% CI = 0.3–0.9). In multivariable analysis for this age group, high SES quintile (OR for 5th quintile = 4.3, 95% CI = 1.2–15.0) and crèche or preschool enrollment (OR = 0.3, 95% CI = 0.1–0.6) remained independently associated with being a case. In bivariate analysis for age group ≥ 5 years, male sex was associated with lower odds of being a case (OR = 0.5, 95% CI = 0.4–0.8), as was being a refugee from Mozambique post-1992 compared with not being a refugee (OR = 0.3, 95% CI = 0.1–0.7). Having 1–10 cattle at home compared with having no cattle was associated with increased odds of being a case (OR = 2.8, 95% CI = 1.3–5.7), as was belonging to the 4th SES quintile compared with the 1st SES quintile (OR = 4.7, 95% CI = 1.3–17.4). In multivariable analysis for this age group, only belonging to the 4th SES quintile remained significantly associated with being a case (OR = 4.5, 95% CI = 1.2–16.9). Conclusions: Risk factors for SARI hospitalization are similar for adults and children in rural South Africa. Possible explanations for the lower odds of SARI hospitalization observed among children in crèches and preschools might be higher vaccination coverage or access to hospitals. For both age groups, the association with higher SES may suggest better access to hospitals among those with more assets. Influenza is an important etiology of SARI in rural South Africa among all ages. To better guide public health recommendations to reduce the burden of SARI and influenza, future research should elucidate the associations observed with preschool enrollment and SES in this population.

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Influenza virus occurrence in asymptomatic and symptomatic patients during pandemic and postpandemic period in Sao Paulo, Brazil

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Background: Influenza A(H1N1)pdm09 had a major impact on global health, causing thousands of deaths in 2009. Facing the pandemic situation, the transmission control measures were not completely effective in preventing the spread of the disease. Knowledge about the infection aspects and the different profiles of clinical presentation is crucial for effectiveness of these measures during a pandemic. This study aimed to analyze the occurrence of influenza virus in asymptomatic and symptomatic individuals at a tertiary hospital of Sao Paulo city, Brazil, in the pandemic and postpandemic period. Materials and Methods: Nasal swab samples from children with respiratory symptoms, their caregivers asymptomatic at attendance, asymptomatic HIV-infected patients and asymptomatic health care workers were collected during the months of influenza circulation in 2009, 2010 and 2011. These samples were tested for influenza A and B by CDC real-time RT-PCR.

Results: Twenty-seven children (27%; 27/100) and 194 adults (64.7%; 194/300) were vaccinated against influenza in the year of sample collection. Of the tested samples, 21.3% (85/400) were influenza positive; 86% (73/85) were influenza A and 14% (12/85) were influenza B. The influenza rate in symptomatic children was 46% (46/100) and in asymptomatic adults was 13% (39/300). The chance of an unvaccinated child who was five years or older of having influenza was 4.8 times greater than an unvaccinated child under five years old. HIV-infected patients with influenza infection had an average CD4 count statistically lower (354.2 cells/mm^3) than noninfluenza patients (533.8 cells/mm^3). Of the caregivers who had contact with an influenza-positive child, 23.9% (11/46) were also infected with influenza, and the highest rate (34.8%; 8/23) occurred in 2009. Influenza A(H1N1)pdm09 detection progressively decreased and influenza B detection increased over the postpandemic years. Conclusions: Asymptomatic influenza detection was higher in caregivers of symptomatic children, followed by HIV-positive patients. Annual vaccination was associated with a lower frequency of virus infection, both in asymptomatic adults and in symptomatic children.

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The asymptomatic fraction of influenza virus infections: a systematic review

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Background: Influenza virus infections lead to a wide range of clinical manifestations, from severe pneumonia to subclinical or even asymptomatic disease. There has been substantial controversy over the proportion of influenza virus infections that are asymptomatic, referred to as the asymptomatic fraction (AF). Knowledge on the AF, variation in the AF in certain groups, and the potential infectiousness of asymptomatic cases are extremely important in designing public health control strategies such as contact tracing and quarantine, and in estimating the burden of disease. Materials and Methods: We conducted a systematic review to assess and summarize the AF of influenza virus infections. Laboratory-confirmed influenza infection is defined by an infection confirmed by RT-PCR or viral culture in respiratory specimens, or serologic evidence of recent infection indicated by a 4-fold or greater rise in antibody titer in paired sera. The AF is defined as the proportion of influenza-infected individuals who experienced no symptomatic illness. Results: After reviewing 407 titles and abstracts, we identified 39 articles, the majority (80%) of which were reported after the H1N1 pandemic in 2009. Studies were classified into 5 categories: cross-sectional studies of general population or high-risk groups (8), community-based transmission studies (15), case-control studies of outbreaks (10), randomized-control trials of interventions (4), and healthy volunteer challenge studies (2). In cross-sectional studies, sera from the general population or high-risk groups (eg, health care workers [HCW], schoolchildren, military) were collected to determine seroconversion, and the AF was estimated to be between 0%-93%. In community-based transmission studies, the AF was estimated to be between 8%-30%. There was also evidence that peak viral shedding was similar between symptomatic and asymptomatic infected individuals, although the duration of shedding was shorter for asymptomatic individuals. Cohort studies in other high-risk groups (eg, HCW and travelers) tended to give a much higher estimate of AF. For case-control studies of outbreaks in schools and ships, the AF was estimated to be between 1%-59%. Randomized controlled trials of pharmaceutical intervention showed evidence that, although the use of antivirals as prophylaxis would not prevent influenza infection as reflected by seroconversion, the AF in the group given antiviral prophylaxis was higher than in the placebo group. In volunteer challenge studies, the AF was approximately 30%. Conclusions: Approximately 8%-30% of influenza virus infection are estimated to be asymptomatic from community-based household transmission studies and volunteer challenge studies. Estimates of the AF are affected by the study designs, and the definitions of infection and symptomatic illness. Community-based household transmission studies and volunteer challenge studies may have one advantage over other study designs of being able to study the infectiousness of asymptomatic individuals.

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The study of heterosubtypic neutralising antibody responses against H5N1 influenza viruses in human subjects using a comparative serology approach

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Background: The continuous rapid genetic and antigenic evolution of H5 subtype influenza viruses has major implications for the sensitivity of serologic assays and can limit the efficacy of prepandemic human vaccines and the ability to undertake effective serosurveillance in susceptible populations. Materials and Methods: A panel of serum samples collected from the Italian population between 1992 and 2007 and previously found to be positive for antibodies against H5N1 as determined by serial radial hemolysis (SRH) were evaluated using pseudotype-based neutralisation assays (PPN) in combination with haemagglutination-inhibition (HI) assays using a clade 1 and a clade 2 H5N1 serologic antigen. Results: Sixty-eight human sera were evaluated for their ability to neutralize A/Vietnam/1194/04 H5 and A/chicken/Italy/13474/99 H7 pseudotypes. IC50 neutralizing antibody titres against the Group 1 A/Viet Nam/1194/04 H5 pseudotypes ranged from < 40 (cut-off) to 1:5120-10240. Seven of 68 (10.3%) sera were found to be negative against A/Vietnam/1194/04 pseudotypes. IC50 neutralizing antibody titres against the Group 2 A/chicken/Italy/13474/99 H7 pseudotype ranged from < 40 (cut-off) to 1:160-320. Forty-seven of 68 (69.1%) sera were found to be negative against A/chicken/Italy/13474/99 H7. Conclusions: From the results obtained it can be concluded that the pseudotype assay can efficiently measure cross-reactive antibody responses that are not detected by the HI assay. It is postulated that these responses are directed against epitopes on the HA2 stalk. All three serologic assays (PPN, SRH, HI) measure antibodies with different (functional) overlapping specificities, contributing to a comprehensive analysis of humoral immunity to influenza viruses.

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Comparison of two influenza seasons (2011-12 vs 2012-13) in Georgia

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Background: Influenza is one of the main causes of acute respiratory illness among humans worldwide. Routine influenza surveillance plays an important role in determining influenza seasonal activity, trends and the severity of each season. For this purpose, ILI and SARI surveillance has been conducted in Georgia since 2009. Based on these surveillance data in this study, we compared two influenza seasons, 2011-12 versus 2012-13. Materials and Methods: For the purpose of the study, the following information has been used: 2011-12 and 2012-13 influenza seasons ILI/SARI weekly reports from sentinel sites and virologic data (rRT-PCR was performed for influenza virus detection in combined nasal and pharyngeal swabs). Laboratory investigation was conducted at the National Influenza Center. Received data were analysed using SPSS. Results: During the 2011-2012 season, distribution of ILI cases by age group were as follows: 0-4 years – 36.9%; 5-14 y – 30.8%; 15-29 y – 9.9%; 30-64 y –10.7%; > 65 y – 11.4%. The ILI referral rate was highest on week 11. SARI admissions were highest during week 10. There were five laboratory-confirmed fatal cases. A total of 1070 specimens were tested, of which 246 were found positive: 34 – A/H1p; 188 – A/H3; 22 – B; there were 2 cases of coinfection with A/H1p and A/H3. All fatal cases were associated with A/H3. Virologic detection peak was reached in March 2012. Both influenza A subtypes, H1p and H3, circulated simultaneously; however, A/H3 was the dominant virus for season 2011-2012 in Georgia. During the 2012-2013 season, distribution of ILI cases by age group were as follows: 0-4 years – 33.6%; 5-14 y – 35.3%; 15-29 y – 13.5%; 30-64 y –14.3%; > 65 y – 3.7%. ILI and SARI referral rates were highest on week 7. Of 1331 collected samples, 332 were found positive for influenza A/H1p and

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106 for influenza B. Virologic detection peak was reached in mid-February 2013. Influenza A/H1p was the dominant virus in January and February, while influenza B mostly circulated in March and April during the 2012-2013 season. Twenty-nine laboratory-confirmed fatal cases due to A/H1p were reported during the 2012-2013 season. Age of the fatal cases ranged from 2 to 75 years; most deaths (23 cases; 79.3%) were in the 30-64 year age group. Conclusions: Influenza seasons 2011-2012 and 2012-2013 were caused by different influenza viruses: A/H1p and A/H3(mostly) for the previous season and A/H1p and influenza B virus for the last season. Presumably, the severity of the last season can be explained by the dominant circulation of the A/H1p influenza virus compared with the 2011-2012 season.

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Perinatal outcomes and children's health after prenatal exposure to influenza and influenza vaccination

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Background: In Norway, a cohort of pregnant women and their children, the Norwegian Influenza Pregnancy Cohort (NorFlu), was established during the influenza A H1N1pdm09 pandemic. The cohort comprises about 3200 mother and child pairs, 2600 with biological samples from mother (serum and peripheral blood cells) and cord blood. Information on immunization, health and diseases is available from questionnaire data and national health registries. These resources enable us to study perinatal outcomes and children's mental and cognitive development, as well as risk factors for severe influenza in pregnancy and the effect on children's immune response after prenatal exposure to influenza A H1N1pdm09 virus infection or influenza immunization. Materials and Methods: NorFlu questionnaires cover exposure to vaccination, influenza infection and antiviral medication as well as maternal and child development and health. Follow-up questionnaires at 6, 18 and 36 months have been collected so far, and long-term follow-up is planned. Cohort data are linked to national health registries for selected outcomes and exposures: the Medical Birth Registry of Norway on perinatal health, the Norwegian Patient Registry on childhood developmental disorders and maternal health outcomes, the Norwegian Immunisation Register for vaccination data and the Surveillance System for Infectious Diseases for data on confirmed maternal influenza infection. Clinical assessment of a sample of 3-year-old children (N = 600) focusing on developmental health and immune status will be performed starting October 2013. Information on prenatal exposure to immunization and infection will be established through immunologic analyses of maternal and cord blood at birth, maternal and child blood after 3-5 years, as well as questionnaire and register data. Outcomes will be studied according to four maternal exposure groups: 1) vaccinated and infected, 2) only vaccinated, 3) only infected, and 4) neither vaccinated nor infected. The possibility of using cellular immune responses as biomarkers for infection and vaccination status will be explored. Results: Preliminary results revealed that 15% of the women had influenza during pregnancy, and 6 of the mothers were hospitalized due to influenza (1.5%). According to self-reports, 74% were "pretty sick" or "very sick" and 85% were sick for more than three days (average of 11 days). In addition, 57% were vaccinated against influenza (Pandemrix) and 2% used influenza medication. Protective HI titers were found in 40% of the blood samples from the mother. When comparing vaccinated versus nonvaccinated, no differences in length of gestation and birth weight were found. Conclusions: Short and long-term consequences of exposure to influenza in utero clearly need to be clarified, as do the potential modifying effects of vaccination or antiviral treatment. The relative importance of antibody-mediated compared with cell-mediated immunity in the protection against influenza is unclear. Furthermore, the impact of the specific immunologic challenges in pregnancy on women's immunologic response to influenza infection is largely unknown. Providing unique data including maternal and fetal biological samples, NorFlu has the potential to contribute significantly to increasing our knowledge in all these areas.

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Estimates of immunity and infection in the Netherlands over the influenza A/2009 (H1N1) pandemic by analysis of cross-sectional protein microarray data

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Background: Yearly epidemics of influenza A differ greatly in size and in the age distribution of cases. This is true for influenza A pandemics caused by novel or reappearing subtypes as well, and can be traced to varying levels of preexisting immunity for the strain that is seeding an epidemic or pandemic. Although a fair amount is known about the infection attack rates during the A/H1N1 pandemic of 2009, quantitative estimates of the levels of preexisting immunity are largely lacking. Materials and Methods: We estimated patterns of immunity and infection by analysis of two cross-sectional population-based serologic surveys carried out before and after the influenza A/H1N1 pandemic of 2009. Samples were analysed using hemagglutination inhibition assay and a protein microarray test using hemagglutinin (HA1) of a range of human and avian viruses as antigen. Estimates of preexisting immunity and infection attack rates were obtained using mixed-model mixture analyses in which the mixing distributions identified susceptible, immune, and recently infected persons. Results: The results indicate that levels of preexisting immunity were low in children aged 5-9 years (3%) and very high in adults (80%). Conversely, infection attack rates were high in children 5-9 years (64%) and very low in adults. The microarray and hemagglutination inhibition data yielded comparable estimates of the infection attack rates, but only the microarray data enabled estimation of the age-specific levels of immunity, due to its higher sensitivity at low antibody concentrations. We validated the results by analysis in two- and higher-dimensional mixture models using a range of human and avian antigens. Conclusions: Levels of preexisting immunity were high and young children were the main driver of the recent H1N1 pandemic. Overall levels of heterosubtypic antibody cross-reactivity were high. Our results offer insight into the complex evolutionary dynamics of influenza A and have potential implications for vaccine design.

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Estimating the protective effect of hemagglutination-inhibition antibody against pandemic influenza A(H1N1) infections

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Background: Hemagglutination-inhibition (HI) antibody level has been used as the main correlate for protection against influenza infections for decades. Conventionally, an HI antibody titer of 1:40 is associated with a 50% reduction in susceptibility against influenza infections. Materials and Methods: We analyzed 8745 longitudinal serum samples collected from 1737 blood donors (5 samples per donor on average) in Hong Kong between June 2009 and Dec 2011 to estimate the association between HI antibody titer levels and susceptibility to pandemic influenza A/H1N1 (pdmH1N1) infections. Specifically, the presence of donors who were infected more than once during this period allowed estimation of the protective effect of not only the absolute HI antibody titer levels but also the

level of HI titer rises incurred from previous infections. We approximated the temporal distribution of pdmH1N1 infections by the product of the weekly number of influenza-like illnesses (ILIs) registered by ILI surveillance and the proportion of ILI specimens that were lab-confirmed to be pdmH1N1. We developed an age-stratified survival analysis model to estimate the infection attack rates of pdmH1N1 in 2009-2011 and the protective effect of pdmH1N1-specific HI antibodies. Results: Individuals who had an HI antibody titer of 1:40 after 2-, 4-, and 8-fold titer rises were 34%, 47% and 62% less susceptible, respectively, than those who were seronegative. Individuals who had an HI antibody titer of 1:20, 1:40, 1:80 and > 1:80 after a 4-fold titer rise were 27%, 47%, 66% and 82% less susceptible than those who were seronegative. The infection attack rates among ages 20-29, 30-39, and 40-59 were 34%, 21%, and 22% in the 1st wave (June 2009 - May 2010) and 25%, 21%, and 20% in the 2nd wave (June 2010 - Dec 2011). Conclusions: HI antibody titer level is a strong protection correlate for influenza infections. In addition, the level of HI titer rises previously incurred is also an important correlate, especially when the HI titer level has significantly dropped due to antibody waning.

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Influenza vaccines and influenza antiviral drugs in Africa: are they available and do guidelines for their use exist?

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Background: Influenza is preventable through vaccination. Persons in Africa may be at increased risk of influenza-related health complications because of untreated comorbidities and limited access to health care. The World Health Organization reports that only 2 of the 54 countries in Africa have access to influenza vaccine, yet anecdotal evidence points to the marketing of influenza vaccine more widely. Little is known about the availability and use of influenza vaccine and factors affecting decision-making regarding its use in Africa. Materials and Methods: We distributed a self-administered questionnaire to key influenza experts working in Africa. This cross-sectional questionnaire was available in hard copy and electronic format in English and French. Questionnaires were distributed in person or by email between January 2012 and January 2013. When survey responses from the same area did not match, the response from the representative of the Ministry of Health or equivalent was considered valid. If there was no clear valid response, we tried to determine a valid response through personal communication with an in-country influenza expert. Results: We contacted influenza experts working in 40 countries, of which 31 (78%) completed the survey. There were 2 or more respondents for each of 12 of the 31 respondent countries (39%). A representative from La Réunion also completed the survey. Of the 31 countries surveyed, 16 (52%) reported the availability of seasonal influenza vaccine: 6 in the private sector only (Democratic Republic of Congo, Senegal, Togo, Uganda, Zambia, and Zimbabwe) and 9 in both private and public sectors (Cameroon, Côte d'Ivoire, Egypt, Kenya, Madagascar, Mauritius, Morocco, Republic of Congo, and South Africa). La Réunion also reported the availability of vaccine in both private and public sectors. Six countries reported using the Southern Hemisphere vaccine and 4 the Northern Hemisphere; for 6, the vaccine composition was unknown. La Réunion also reported using the Southern Hemisphere vaccine. Vaccine coverage data were only available for 4/16 (25%) countries that reported the availability of seasonal influenza vaccine and ranged from <0.5% to 2% of the population. Among the 17 countries that reported having any influenza vaccine, 5 (Côte d'Ivoire, Egypt, Ghana, Mauritius, and Morocco) reported having a national policy: 5 target healthcare workers, 4 target young children, 4 target the elderly, 4 target persons with underlying medical conditions, 3 target pregnant women, and one targets pilgrims going to Hajj. Six (67%) of the 9 respondents for countries without influenza vaccine who felt national decision-makers would be willing to introduce the vaccine said the country would do so within the next 4 years. These same respondents ranked a demonstration of public health burden within the country and availability of international funding as most important in influencing decision-makers to introduce and promote the use of the influenza vaccine. Evidence that illness and death can be prevented by influenza vaccination and evidence of a cost benefit to the national health system were also considered important, but did not rank as high. Of the 31 respondent countries, 23 (74%) reported purchasing and/or receiving pandemic influenza vaccine in or after 2009, and 19 (83%) of these reported using these vaccines. Four countries (Kenya, Mali, Senegal and South Africa) and La Réunion reported conducting influenza vaccine research in the years 2002 and/or 2013. Finally, 19 (65%) of the 31 respondent countries reported the availability of antiviral drugs to treat influenza: 9 in both the public and private sector, 6 in the public sector only, and 4 in the private sector only. La Réunion also reported the availability of antiviral drugs in both the private and public sectors. Cameroon, Egypt, Ghana, and South Africa reported having national guidelines for the use of these antiviral drugs. Conclusion: Influenza vaccine was available in nearly two thirds of the African countries surveyed, but coverage estimates remain largely unknown. Describing the local burden of disease and identifying funding will be essential to encourage these countries to use influenza vaccine more widely.

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Rapid knowledge translation during the 2009 influenza pandemic

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Background: The emergence of a novel influenza A (H1N1) viral strain originating in swine resulted in intense research activities and publication of a large volume of literature during the 2009 pandemic. The mandate of the National Collaborating Centre for Infectious Diseases (NCCID) is to protect the health of Canadians by encouraging and facilitating the integration of evidence and emerging research on infectious diseases to inform public health programs and policy. To help public health practitioners manage this exploration of research information and close the gap in knowledge related to the public health pandemic response, NCCID launched a weekly publication in November 2009, the *Purple Paper*, to make findings from pH1N1 peer-reviewed research articles accessible to public health practitioners and policymakers in a timely manner. In addition to summarizing and contextualizing research findings for the Canadian situation, this rapid knowledge translation initiative maintained flexibility in its approach and content in order to address the evolving need information local public health information as the pandemic unfolded. This presentation will describe the methodology used to conduct rapid knowledge translation during the 2009 influenza pandemic and discuss how this approach may be adapted for other infectious disease-related public health emergencies. Materials and Methods**:** A National Center for Biotechnology Information (NCBI) daily citation email alert was set up using broad pH1N1-related search terms to be as inclusive as possible in the capture of relevant publications. At the end of each week, a PubMed search of pH1N1 citations was carried out to ensure no publications were missed. The filtering procedure consisted of first excluding non-peer-reviewed publications and giving priority to publications about the following public health topics: epidemiology of pH1N1, community mitigation strategies, infection prevention and control, vaccine and vaccine-related issues, and biological aspects of the pH1N1 virus that could affect the public health response to the pandemic. Although not a major focus, papers on clinical aspects of the disease were included on a case-by-case basis. The methodology and relevance of selected studies were scored based on preset criteria. Every issue of the newsletter was externally reviewed before dissemination. Results: Fourteen issues of the *Purple Paper* were published between November 2009 and March 2010. Of more than 1100 publications that were captured by the literature search over the same period of time, information from 91 relevant articles were synthesized for the *Purple Papers*. As the pandemic evolved, the type of information that appeared in the *Purple Papers* also evolved. A “pandemic response debrief mini-series” that gathered feedback from local public health practitioners about their jurisdiction’s pandemic response was introduced in the 9th issue in February 2010 to provide a conduit for information-sharing among practitioners across the country. This ad-hoc mechanism for communication identified gaps in knowledge and opportunities for training in the best practices for public health emergencies, while highlighting areas that warranted further investigation. Conclusion: The *Purple Paper* was the NCCID’s first attempt to extend its role in knowledge translation during a public health emergency. The NCCID remained fluid during the second wave of the 2009 influenza A (H1N1) pandemic in order to address the changing information needs on the frontlines of public health. In addition to synthesizing the much needed information from research literature, the *Purple Paper* provided an open channel of communication among frontline practitioners for sharing their pandemic response experiences. This rapid knowledge translation mechanism could be adapted for other infectious disease emergencies in public health.

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Communicating influenza antivirals' stockpiling strategies across Europe: a quantitative and qualitative study

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Background: The world is subject to seasonal influenza epidemics and pandemics. In recent years high profile occurrences—H5N1 ("bird flu") and H1N1 ("swine flu")—have attracted considerable attention among experts and lay audiences. Recent worries about outbreaks of a new H7N9 strain of bird flu in East Asia and its spread to other continents show the importance of understanding how influenza policy responses are shaped and developed as well as the extent to which these policies can be harmonized. In addition to preventive vaccination, many European governments have pandemic contingency plans that involve the use of influenza antivirals to combat the spread and mitigate the severity of the infection. Such antivirals include oseltamivir (Tamiflu) and zanamivir (Relenza). Yet, unlike the use of seasonal flu vaccines, the prescription and stockpiling of antivirals as a risk-reduction strategy remains largely understudied. Material and Methods: This paper presents the first results of a study of the pros and cons of European governments antiviral stockpiling strategies to combat influenza. The project was developed in 2012-2013 and concentrated on weighing the benefit and risks of existing approaches, how they are assessed by governments and what are their implications in terms of risk communication to patients and public trust. The research team conducted elite interviews of key regulatory actors in five European countries (France, Germany, The Netherlands, Sweden, and United Kingdom), as well as an opinion survey targeting a large representative sample (age, gender, ethnicity, employment, etc) of 5648 respondents from across Europe, including: France (n = 1061), Germany (n = 1008), The Netherlands (n = 1010), Spain (n = 504), Sweden (n = 1,051), and the United Kingdom (n = 1014). In order to make European transnational comparisons possible, the survey contained 16 closed and/or open questions that can also be directly compared to studies conducted in the United States by Löfstedt et al. (2012) and Chrakraborty and Löfstedt (2012). The draft questionnaire, initiated at Maastricht University, was developed together with cognitive psychologists at Dialogik, Stuttgart University, who received informal input from medical experts and the survey data from the country. Studies was collected and collated by Ipsos MORI, a UK polling agency. In addition, the European survey included an investigation of the public's views of flu outbreaks, and results of this specific line of investigation will be discussed in a separate article. Results: The paper reviews the range of antiviral stockpiling strategies adopted in the five European countries from the perspective of risk policy and risk communication; evaluates the possible impact of these strategies on public perception, behavior and compliance; presents an analysis of and suggest improvements to the risk communication of the different strategies, with a view to building rather than destroying trust among critical actors (industry, government and patients). Conclusions: Patients have a poor knowledge of institutional sources of information. They have a poor understanding of the benefits and risks of antiviral treatments. As a result, their trust in institutions is fragile and they are likely to stop treatment when a safety issue is raised. Therefore, the main conclusion is that much needed improvements should be based on taking risk communication on board as well as introducing "reasoned transparency" initiatives to build institutional trust and help patients make better risk decisions.

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Where backyard poultry producers seek help for sick poultry: implications for avian influenza prevention in Bangladesh

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Background: In Bangladesh, backyard poultry raisers lack awareness of avian influenza and infrequently follow government recommendations for prevention. Optimal communication through accessible channels can inform rural poultry raisers about avian influenza and its prevention. This study aimed to identify appropriate communication channels to reach backyard poultry raisers and to explore knowledge and perceptions of poultry healthcare providers at the village level. Methods: During June-August 2009 in two Bangladeshi districts, we conducted in-depth and short interviews: 70 with backyard poultry raisers and six with local veterinary medicine sellers in two villages, and five with government veterinary professionals at the sub-district and union levels (the lowest government administrative unit). Results: Most (86% [60/70]) raisers sought care for their backyard poultry at the local medicine sellers, 14% only used home remedies and none sought care from government veterinary professionals. Some local medicine sellers had shops in the village market where raisers went with poultry health problems and others visited rural households to provide poultry healthcare services. The majority (4/6) of the medicine sellers did not have formal training on veterinary medicine. Medicine sellers either did not know about avian influenza or considered avian influenza to be a disease common among commercial but not backyard poultry. The government veterinary professionals had degrees in veterinary medicine and elaborate knowledge about avian influenza and its prevention based on their training and/or experience with avian influenza prevention and control. The government professionals had their offices at the sub-district or union level and reported that they did not have the staffing to reach the backyard raisers at the village level and lacked adequate supply of medicines. Conclusion: The local medicine sellers play an important role in providing healthcare to backyard poultry in villages and are an accessible information source for the rural raisers. Integration of these local medicine sellers in the government's avian influenza control programs is a potentially useful approach to increase rural raisers' awareness about avian influenza that should be evaluated.

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The World Health Organization Battle against Respiratory Viruses (BRaVe) initiative: bridge science and policy to foster innovative therapies to tackle viral respiratory infections

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Background: Acute respiratory infections kill an estimated 3.9 million people annually and in developing countries comprise the leading cause of mortality in children younger than 5 years of age. In recent studies, investigators have demonstrated that an important share of respiratory infections is caused by viruses. Depending on the setting and population, up to at least half of respiratory infections are viral in origin. Preceding RVIs may hold the key to events predisposing the patient to secondary bacterial infections and may precipitate factors affecting other organ systems. In addition to the effect of common RVIs on annual infection rates, emerging respiratory viruses (eg, the SARS coronavirus, the novel coronavirus MERS-CoV, and, most recently, the avian influenza A [H7N9]) virus) represent threats to global health. The potential effect of RVIs on mortality and morbidity and in terms of healthcare systems remains underappreciated. Globally, RVIs are either treated inappropriately or never treated. Although progress has been made in reducing pneumonia mortality, current initiatives do not address the contribution of RVIs to the burden of disease and do not include steps to mitigate it. The notion that RVIs can be treated successfully is relatively new to the medical

community. There is a need to articulate new global approaches and foster an accelerated path to the discovery, development, and implementation of therapeutic and management strategies. Materials and Methods: The development of an integrated research framework and response initiative, designated the Battle against Respiratory Viruses (BRaVe), was conceived to address these needs with input from partner organizations and non-WHO experts and has become a cross-cutting effort within WHO. Two informal technical consultations were held by WHO, with support from the Fondation Mérieux and the Wellcome Trust, to identify key knowledge gaps and research needs and tools to address these gaps. More than 60 persons attended, representing academic investigators, the pharmaceutical industry, funders, clinicians, public health decision-makers, WHO staff, and others. This led to the development of a research agenda for BRaVe and a call to action to various stakeholders. The aims of the research agenda are to (1) identify specific research priorities for RVIs to address over the next 5 to 10 years; (2) provide a framework for resource allocation that addresses public health, operation, and social science-related needs in under-resourced countries; (3) facilitate interactions and collaboration among basic science and clinical investigators in both public and private sectors, as well as public health experts and representatives from funding organizations and the pharmaceutical industry; and (4) monitor the progress made in filling knowledge gaps to facilitate the development of new evidence-based policies. Results: Building on the current momentum, WHO proposes to create a global forum or "think-tank" to prioritize gaps in knowledge and needs and develop workable solutions. This think-tank would benefit from the input of a variety of stakeholders beyond the usual players and scientific silos and from the exchange of ideas from multiple sectors. As currently envisioned, this forum would be at least a 3-year initiative to address 3 key topics in parallel: new antiviral treatments for viral respiratory infections; innate and adaptive immune responses in the pathogenesis of disease; and optimization of clinical management, diagnostics, and public health strategies, especially in low-resource settings. Conclusion: WHO calls for a change in the paradigm for approaching viral respiratory diseases from pathogen-specific interventions to multi-stakeholder engagement to shape the future. The increased awareness and coordination among stakeholders will effectively link public health communities around a single major cross-cutting issue, and accelerate progress toward innovative solutions while addressing the major issue of respiratory viral infections.

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Herald 1918 pandemic waves: importance for contemporary pandemic response strategies

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Background: Over the last decade, epidemiologists, demographers, medical historians, and virologists have put a considerable amount of effort into studying the 1918 "Spanish" influenza pandemic, the most devastating pandemic in modern history, with a mortality estimated at 2% of the global population. A key question has been whether the unusual outbreaks of influenza-like illnesses between February and August in 1918 in many regions of the world could be considered the first manifestation of the pandemic—a "harbinger" of the lethal pandemic in autumn of that year. Here we review the evidence supporting the occurrence of mild herald 1918 pandemic waves and discuss the policy implications of our findings for pandemic preparedness. Methods and Findings: We reviewed clinical descriptions of influenza-like-illness outbreaks occurring in the spring and summer of 1918 in Europe and the United States; analyzed historic mortality time series data from national statistics, church and graveyard records from Europe, the Americas, and Asia; and incorporated evidence from recently uncovered virological data on the 1918 pandemic virus. Results: We found that unusual respiratory outbreaks in the spring and summer of 1918 were characterized by a brief episode of febrile illness and a high infection rate, with occasionally a more severe clinical picture that included severe respiratory distress, fulminating pneumonia, and death. In New York City, Copenhagen, and Mexico City, these outbreaks were relatively mild but characterized by the signature pandemic age pattern, with an increased number of deaths among young adults, which set the wave apart from

typical seasonal influenza. Senior populations in Europe and the United States were spared pandemic-related mortality; however, this was not the case in more remote settings in Latin America. Senior sparing may have been a result of childhood exposure to a 1918-like virus that protected those born before 1870, but only in geographical settings that were well connected at the time. A final piece of epidemiological evidence is provided by illness data from US and British populations indicating moderate to high clinical protection between successive pandemic waves. Specifically, the spring wave provided 35% to 94% protection against clinical illness during the fall wave (56%–89% protection against death), suggesting that the pathogens responsible for the spring and fall waves were similar enough to convey immunity. Finally, retrospective virological findings in autopsy specimens from US soldiers who died of pneumonia during May and August 1918 corroborate the notion that nearly identical influenza viruses circulated in the spring/summer and fall of 1918 in Europe and North America. Conclusions: Taken together, epidemiological, clinical, and virological evidence consistently support the occurrence of a relatively mild but highly transmissible pandemic herald wave during the spring/summer of 1918 characterized by an unusually high mortality among young adults that otherwise provided immunological protection during the lethal autumn wave. Based on historical evidence, we conclude that a prudent pandemic response is critical during the first year in which a novel pandemic virus is in circulation, even when early epidemiological data indicate low severity. The robust public health response to the 2009 pandemic is a case in point, in that the possibility of subsequent waves of increasing severity had to be taken into account in risk mitigation planning, even though this threat never fully materialized.

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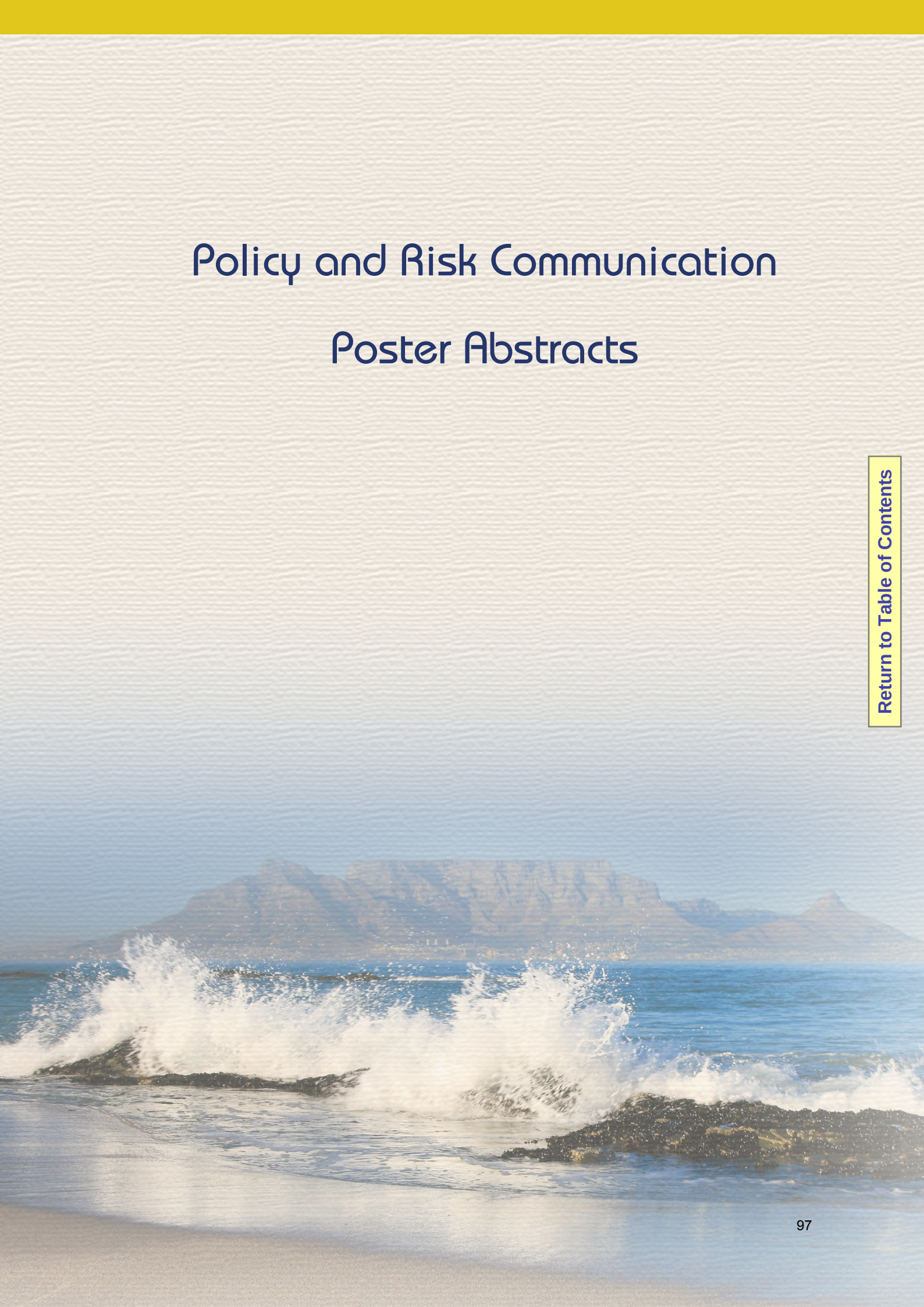
Poster Abstracts



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Review of influenza vaccine efficacy and effectiveness in China

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Background: Seasonal influenza vaccine use in China has several decades of history, but more use started in 1998. However, influenza vaccination coverage is still around 1%, which is far behind developed countries. Data on post-marketing influenza vaccine efficacy/effectiveness in China are incomplete, and this paucity of domestic data might influence policies and result in low vaccine coverage. It's vital to assess all such data in both Chinese and English to describe influenza vaccine efficacy/effectiveness in China to inform a national vaccination policy. Broad inclusion criteria were used to conduct a comprehensive review of studies related to influenza vaccine efficacy/effectiveness in China. Materials and Methods: We searched the CNKI (China National Knowledge Infrastructure) database for Chinese articles and Pubmed, Embase, Global Health, CINAHL, WHOLIS, Web of Science, LILACS for English articles before 2012 November. The main search strategy was "'influenza vaccine' AND 'efficacy OR effectiveness' AND 'China'." Eligibility criteria included original data, human subjects, related to currently used influenza vaccine, data from mainland China, single vaccination, and protection rate or other necessary data to calculate vaccine efficacy or effectiveness. We extracted data from final eligible articles on study period, influenza season, site, design, vaccine, participants, outcome, and protection rate. We analyzed influenza vaccine protection rate by vaccine type, study design, and populations. Results: We identified 4880 publications in total, discarded duplicates, and had 4771 publications. After a primary scan, 395 qualified for full text review, of which 56 articles (50 Chinese, 6 English) were eligible to have data extracted. Among them, 41 articles studied only seasonal influenza vaccine, 13 only 2009 pandemic vaccine, and 2 both. In the 43 seasonal influenza vaccine articles, there were 5 randomized control trials (RCT, all Chinese) in 4 discrete influenza seasons and 38 observational studies (4 English and 34 Chinese) in influenza seasons from 1996-2012, except 1998-1999. Only 2 articles (1 English and 1 Chinese) used laboratory-confirmed influenza as the outcome, and both were observational studies. The English language article reported effectiveness in 6- to 59-month-year-old children at 47% (95% CI, 35.30-55.89) in 2009-2010, and 56% (95% CI, 41.78-67.04) in 2010-2011. The Chinese article looked at all ages in 2009-2010, but found no significant protection (P = .319). Of 5 RCT articles, 2 articles reported efficacy in healthy children aged 6-35 months (48% against ILI) and 3- to 12-year-old children (69% against clinically diagnosed influenza (ICHPPC-2-defined) respectively. One hospital-based study looked at the efficacy in 3- to 14-year-old children (38% against common cold). Three articles reported efficacy in adults aged 20-60 years, 18-39 years, and 20-50 years (67%, 73%, and 73% against ILI, respectively). One article reported efficacy against ILI in adults aged 60 years and older at 69%. In other observational studies, 23 articles reported effectiveness against ILI in children (aged 6 months to 18 years), median at 55%; 6 articles reported effectiveness against ILI in 18- to 60-year-old adults, median at 65%; 25 articles reported effectiveness against ILI in adults over 60 years old, median at 49%. Five articles reported ineffective protection against ILI in HCWs. All 15 articles with 2009 pandemic influenza vaccine data were observational studies (2 English and 13 Chinese). Laboratory-confirmed influenza protection rates were reported in 3 articles in school-aged children at 57%, 87%, and 100%, respectively, and 1 article in 6- to 59-month-old children at 47%. Only 1 Chinese article looked at the effectiveness in pregnant women (40% against ILI). Conclusions: 41 of 43 seasonal influenza vaccine articles used clinical symptoms as an outcome. Only 2 used laboratory-confirmed influenza, one showed moderate protection for children, and the other one showed no protection for all ages combined. Influenza vaccine efficacy/effectiveness studies should have laboratory-confirmed influenza as an endpoint because ILI and other clinical symptoms are too difficult to measure reliably and also because they can be caused by agents other than influenza. To inform a national influenza policy in China, ongoing studies in different high-risk populations, especially pregnant women, children, HCW, and elderly using laboratory-confirmed influenza as the outcome are needed.

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Influenza-related knowledge, attitudes, and practices among pregnant women: a cross-sectional study in Suzhou, China

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Background: WHO recommends that pregnant women receive influenza vaccination. However, few pregnant women are vaccinated against influenza in China. The factors that may be responsible for this low vaccination rate include pregnant women's attitudes towards influenza vaccination, vaccine availability at antenatal clinics, the cost of the vaccine, advice offered pregnant women by their physicians. We surveyed pregnant women regarding their knowledge, attitudes, and practices (KAP) regarding influence in order to gain evidence for use in interventions designed to increase the rate of influenza vaccination in pregnant women. Materials and Methods: We used a multi-stage cluster sampling method to recruit pregnant women at different stages of pregnancy for this cross-sectional study in Suzhou, Jiangsu Province, in 2012. The questions were designed to determine their knowledge of influenza effects and symptoms, transmission routes and prevention methods; influenza vaccine protection and its effects in adults and in the fetus, vaccine cost, doctors' advice and the other factors that might influence the decision to get a vaccination; where and how respondents wished to get information about influenza and its vaccine. For quality control, we trained the interviewers and supervised the implementation of the survey. We did a pilot study and used repeated but restructured questions to test the questionnaire's reliability. We analyzed the data using SAS 9.2 software. Results: A total of 1673 pregnant women, in all stages of pregnancy and from both urban and rural settings were recruited and completed the questionnaire. The average age of respondents was 26.5 ± 4.2 years. The detailed study results are as follows. *Influenza illness KAPs:* 1017 of 1673 (60%) respondents knew of the seriousness of influenza, but 836 (50%) were not aware of its routes of transmission. Additionally, 1153 women (69%) thought that contracting influenza during pregnancy would be harmful to the baby, but they did not know how it would harm the baby. Only 30% of respondents knew that pregnant women were at increased risk for severe illness and death during the influenza A (H1N1) pandemic in 2009. *Influenza vaccination KAP:* Compared with women with limited/no (illiterate) education, those with a high level of education were more likely to know that an influenza vaccine was available for pregnant women ($P < .05$). Additionally, 239 (14.3%) respondents responded correctly regarding the time of year when they should be vaccinated against influenza. We found that 654 (39.1%) women knew the correct vaccination frequency, but only 115 (6.9%) knew they be vaccination before becoming pregnant. Interestingly, only 9.9% of respondents believed that pregnancy increases the risk of acquiring an influenza infection. Respondents reported that they would be more likely to allow vaccination if they were not pregnant ($P < .05$), with.91.5% reporting they would refuse influenza vaccination because they felt it might harm the unborn baby. *Assessment of possible interventions:* Among pregnant women who were inclined to receive an influenza vaccine, the main reasons for acceptance were high vaccine efficacy for themselves (67.3%), professional medical recommendations (69.0%) and information regarding safety and high efficacy for their unborn children (69.7%). Conclusion: We identified several important gaps in influenza and vaccine-related KAPs among pregnant women in China. Acceptance of influenza vaccination during pregnancy and knowledge of vaccine-related risks for pregnant women and their unborn children were low. We recommend more education for pregnant women regarding personal health behaviors for preventing influenza infection, as well as the benefits of influenza vaccine for both pregnant women and their children. Education of health care workers is an essential component of increasing acceptance of vaccination among pregnant women because of their influence in vaccine-related decision-making. More basic influenza-related information should be provided through mass media to make it easily accessible by the general public.

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Health literacy of influenza prevention in Taiwan—a scale development

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Background: In 1990, the concept of health literacy was created in the United States to examine people's ability to utilize medical recourses and comprehend doctor's advice regarding chronic diseases. The WHO, US HHS, and IOM defined health literacy as "The degree to which individuals have the capacity to obtain, process and understand basic health information and service needed to make appropriate health decisions." In the field of infectious diseases, health literacy studies are rare, with the exception of studies on HIV carriers. Taiwan has an influenza vaccination program that began in 1998, and began to stockpile influenza antivirals since the emergence of avian influenza virus A (H5N1) in 2003. During the H1N1 novel influenza pandemic in 2009, Taiwan launched the H1N1 influenza vaccination program on November 1, hoping to create herd immunity. However; inoculation decreased following the death of a 7- year-old boy 1 month after being inoculated with the H1N1 influenza vaccine. This study tries to implement the concepts and elements of health literacy into influenza prevention that led to the development of this scale. We used a qualitative method to develop our scale which would serve our specific purpose since there is no existing scale that would cater to our study. We took a bottom-up approach in the development of the scale for health literacy in influenza prevention. Thus, with the qualitative method and bottom-up approach, the scale we developed can become a tool to assess the public's ability to understand and interpret influenza prevention messages. Materials and Methods: We reviewed literature and held study groups in order to establish the knowhow and the definition of health literacy's role in influenza prevention. In addition to making decisions on the context, pattern, and question types, we conducted 4 focus groups consisting of experts, mass media, and 2 opinion leader groups. The purpose of the focus groups was to check the applicability of the scale used to develop the questions. Researchers first developed the draft scale, allowed the 4 focus group symposiums to discuss the preliminary scale, then researchers classified and analyzed the scale through transcribed texts of the focus group sessions. Lastly, the scale was reviewed by 6 experts, and revised to be the final scale. Results: In our study, we set up a structure for health literacy in influenza prevention, which included the operative definition "the degree to which people have the capacity to obtain, understand and apply basic health information and utilize services, with subjects both over the age of 18 and have completed at least junior high school education". The scale framework is set up based on the 3 levels of prevention and with the5 preventative points of health protection, symptom conscientious, diagnosis, treatment, and self-care in mind. We want to test one's ability to obtain, understand, apply and use basic health information and services. Because this scale was developed to test ability, every question has a correct answer, thus can be scored easily. Additionally, due to time constraints, the question difficulty was low and there were no open ended questions. The final scale developed, through the analysis of the data collected from focus groups, scale analysis, and behavior survey, was finalized to be formed from 7 categories and 22 questions. Conclusion: Through our focus group symposiums we found that gaps exist between influenza prevention information provided by the government and the amount received and understood by the public. This study identified a potential area to apply the concept of health literacy, with special focus on infectious disease prevention. Although this study was developed initially to test health literacy in regards to influenza prevention, it needs to be further assessed and tested for the usability in other applications.

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The use of focus group interviews to evaluate China's risk communication on H7N9 avian influenza in 2013

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Background: The first human cases of the novel avian influenza A H7N9 virus were identified in February and March 2013 in China. As of May 27, 2013, the accumulated nationwide number of H7N9 human cases reached 130, including 37 dead and 76 patients who had recovered (China National Health and Family Planning Commission). In order to assess the effects of the risk communication work conducted by Chinese health authorities, we conducted nine focus group interviews with residents recruited from communities in Beijing, Zhejiang and Gansu provinces. These three study locations represent areas with low case numbers, high case numbers and no identified cases, respectively. Materials and Methods: Three focus group interviews of 10 participants each were conducted in all three study locations. A well trained moderator from the Chinese Center for Health Education ran the focus groups with the same pre-drafted and tested moderator guide. The interviews were recorded into audio files; additionally notes were taken on site by two note takers. Results: In general, each of the 90 focus group participants were satisfied with the communication work carried out by the Chinese health authorities during the H7N9 outbreak. Compared with the risk communication during the 2003 SARS outbreak, the participants expressed their great appreciation for the more transparent, open and timely communication in this H7N9 outbreak. The participants' questions of highest concern were: How to prevent this disease? What is the source of this disease? What are the routes of transmission? What medicines are effective to cure this disease? The top five most trusted information channels were China CDC, National Health and Family Planning Commission, the local health authorities, national level media and the WHO. Although some of the participants stated that they prefer to get information from Weibo (the Chinese version of Twitter), they were quick to add that they only trust credentialed Weibo accounts for their H7N9 information. The information that the focus group participants found the most useful included: public health recommendations on prevention, updated epidemiological data particularly as related to the patients' occupations and the geographic distribution of the cases, treatment effectiveness and the official statement regarding lack of evidence on human to human transmission of H7N9. Some of the participants stated that they used these indicators to judge their susceptibility and the severity of the outbreak. An overwhelming majority of the participants felt less concerned about this outbreak because they were able to get the official daily epidemiological data updates from TV, newspaper and websites. All of the participants could recall at least one of the public health recommendations. The majority of them indicated they have followed the recommendations such as frequently washing their hands, opening the windows to air the room, staying away from crowded public places and avoiding contact with live poultry. Although the bulk of the participants indicated that they received information from health authorities affirming that thoroughly cooked poultry products are safe to eat, many still admit that they have stopped consuming poultry products or sharply decreased their consumption amount since the media coverage reporting the H7N9 outbreak on April 1, 2013. A few of the participants expressed that they still have concerns that have not yet been addressed, such as if keeping pet birds at home is safe. Conclusion: China effectively utilized risk communications principles during the H7N9 avian influenza outbreak by releasing timely information through multiple trustworthy channels. This information has met the needs of the public and guided their behavior. Although at this point the source of this novel avian influenza outbreak remains a mystery, the Chinese public is highly satisfied with the health authority's communication work. They trust the government has been transparent about the epidemic data and has effectively handled the outbreak of this disease.

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Increasing influenza vaccination in health care workers: which policies work in Europe?

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Background: Health care facility–associated infections are a major threat to patient safety and health outcomes. Seasonal influenza vaccination coverage rates (VCRs) among health care workers (HCWs) remain low in Europe, despite strong recommendations. The objective of this study was to determine the essential elements of European influenza vaccination policies that could help to increase VCRs among HCWs. Methods: Influenza vaccination policies (2008-2012) of 15 European countries were assessed. Data on existing policies were collected via European National Vaccine Industry Groups and from individual country experts. Analysis of the 15 national immunization policies focused on four main categories: 1) management of vaccination programs; 2) role and responsibilities of HCWs in vaccination programs; 3) role of information/communication campaigns; and 4) ease of access to vaccines. Spearman correlation coefficients (rho) of single or sets of two policy elements with HCWs' VCRs were calculated. Results: The results of this study identified several policy elements that might help to increase influenza vaccination uptake among HCWs. In eleven countries, teaching about influenza vaccination is included in the curriculum in medical schools. Countries with this policy generally showed higher influenza VCRs among HCWs than countries that did not include influenza vaccination education in medical schools (rho = 0.464; *P* = .082). This was the only stand-alone policy with a *P* value < 0.1. Despite a small sample size, correlations of sets of two policy elements were calculated, which assessed interaction between policy elements. Not all combinations showed a significant impact on vaccination behavior. Policy elements with the most influence on HCW VCRs included combinations with annual influenza vaccination awareness campaigns via radio/TV (rho = 0.535; *P* = .04), campaigns via press/media conference (rho = 0.540; *P* = .038), or with campaigns via flyers/folders in medical waiting rooms (rho = 0.524; *P* = .045). In addition, various combinations of health care policy elements that led to increases in VCRs (*P* < 0.1) included influenza vaccination recommendations specifically targeting HCWs, dedicated objectives for HCWs to achieve in vaccinating patients against influenza, annual monitoring of VCRs, the reimbursement of HCW vaccination costs and media activities such as radio, flyers/folders in medical waiting rooms, and press/media conferences. Conclusions: Several elements of influenza vaccination policies could be combined in a multifaceted approach at the national level to improve HCW influenza VCRs to reduce the risk of health care–associated infection both for HCWs and their vulnerable patients. These include strong national vaccination recommendations, influenza education in medical schools, and information on annual influenza vaccination via the media.

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Intentions to perform nonpharmaceutical protective behaviors during influenza outbreaks: a cross-sectional study of a representative sample of the Swedish adult population

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Background: Failure to monitor the beliefs and attitudes of the public has been identified as a weakness in European preparedness strategies to mitigate infectious disease outbreaks. We set out to examine whether intentions to improve personal hygiene and increase social distancing during influenza outbreaks can be explained by trust in official information, perceptions of threat and the ability to cope as examined by protection motivation theory (PMT), self-reported assessments of health, and sociodemographic factors. Materials and Methods: The study used a cross-sectional design to analyze associations between intended protective behaviors and items in a theoretical model of explanatory factors. A random sample of 1,011 persons ranging between 20 and 90 years of age was drawn from the Swedish national population register. A telephone survey was carried out during the first quarter of 2012. Individuals agreeing to participate were asked to complete a paper-based survey, prior to the call, querying for sociodemographic data and data elements from the SF-8 self-reported health questionnaire. The remaining data were collected in the interview. Behavioral intentions were assessed by items asking whether the respondent would try to carry out a protective behavior in accordance with the PMT. The protective behaviors were restricted to two categories: increased personal hygiene (use of disinfectants and other hand wash products; frequent washing of hands when having touched common objects, such as door knobs) and social distancing (staying home from work or school; avoiding use of public transportation). For each endpoint, uni- and multivariate logistic regression analyses were applied using the items in the model as explanatory variables. These included trust in official information, variables corresponding to PMT items (threat appraisal items and the coping appraisal items), variables representing the SF-8 summary items, and sociodemographic characteristics (age, gender, educational level, living with partner, living with child, and employment). Results: Two-hundred and fifty-four persons in the total population sample (n = 1011) could not be reached by a telephone call. Of the 757 persons reached, 443 provided a complete response, leading to a 59% response rate to the telephone survey and a 44% participation rate with regard to the total sample. Every second respondent (50.0%) reported high trust in official information about influenza. Regarding social distancing measures, 9% of the respondents scored strong agreement with the stated intention to stay home when not ill during seasonal influenza outbreaks, and 11% scored strong agreement with this intent during pandemic outbreaks. Twenty-three percent of the respondents intended to avoid use of public transportation during a seasonal outbreak, while 29% of the respondents scored strong agreement with this intention during a pandemic outbreak. Regarding measures related to personal hygiene, 77% of the respondents scored strong agreement with the stated intention to use hand wash products during seasonal outbreaks, while 85% of the respondents reported this intention for pandemic outbreaks. Regarding the intention to frequently wash hands, 46% reported strong agreement in association to seasonal influenza outbreaks and 60% in association to pandemics. Multivariate models revealing coping appraisal as the dominant explanatory factor for behavior showed strong discriminatory performance for staying home while not ill (seasonal outbreaks AUC 0.85 (95% CI 0.82;0.89), pandemic outbreaks AUC 0.82 (95% CI 0.77;0.85)) and acceptable performance with regard to avoiding public transportation (AUC 0.78 (0.74;0.82), AUC 0.77 (0.72;0.82)), using hand wash products (AUC 0.70 (0.65;0.75), AUC 0.76 (0.71;0.80)), and frequently washing hands (AUC 0.71 (0.66;0.76), AUC 0.75 (0.71;0.80)). Conclusions: We observed a relatively high trust in official recommendations and a higher prevalence of intentions to improve personal hygiene than to increase social distancing in association to influenza

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outbreaks. Coping appraisal was the dominant factor found to explain the observed behavioral intentions. Threat-affect played a relatively small role in lay decision-making, despite uncertainties associated to narcolepsy cases surrounding a recent mass vaccination campaign. Developing interventions that support the general population's efforts to perform self-protective behaviors during outbreaks, and longitudinal studies of such behaviors across several influenza seasons are also warranted in European countries.

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Banning the slaughter of poultry in live bird markets: crisis or chance to change?

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Background: Live-bird markets (LBMs) can provide unavoidable opportunities for contact between humans and animals. Many consumers accustomed to buying fresh poultry and selecting the chickens they like the most, especially in Taiwan, Hong Kong, and China. However, LBMs have been considered the most important origin of novel avian influenza viruses (AIVs) since the first highly pathogenic avian influenza A/H5N1 outbreak occurred in Hong Kong in 1997. Therefore, closure of LBMs is the key to successful influenza control. On April 25, 2013, one day after the first human H7N9 case was identified, the government of Taiwan announced a ban on slaughtering live poultry. This study was designed to (1) compare differences between support and opposition to this policy among LBM workers, and (2) find the best channel for informing LBM workers about their risks of infection. Materials and Methods: A face-to-face survey of 34 LBMs was conducted from May 10 to May 16, 2013. The questionnaire covered (1) occupational history; (2) knowledge of disease, risk perception, and attitudes; and (3) viewpoints on the H7N9 epidemic in China and the banning of poultry slaughter. Results: We administered 80 questionnaires and had an 80% response rate. The mean age of respondents was 53.4 ± 10.91 years. Additionally, 53.7% of the respondents were female, 48.8% had a primary education or lower, and most (95%, 76/80) had been working as LBMs for more than 10 years. Traditional media, such as TV or newspapers, were their main sources of information (93.8%, 75/80); only 17.5% used such novel media as the internet or a mobile phone. Most of the respondents (92.5%, 74/80) did not trust the government as a source of information, and only 25% believed what professionals said. Surprisingly, 85.7% (54/63) of respondents did not think this ban was necessary to prevent and control AI. A significant proportion of those opposed to this measure considered refusing to follow the new policy (92.6% [50/54], P = .0018), believing that electronic slaughtering kept them safe (92.7% [51/55], P < .001) and protected consumers (98.1% [52/53], P < .001). Moreover, 93.6% of them thought the AIV H7N9 outbreak would not occur in Taiwan. Additionally, 80% of respondents said that their revenue has decreased more than 50% and that they were not willing to follow the new policy (P = .006). The government offer of a subsidy of US $3000 dollars did not motivate them to use electronic slaughtering techniques (P = .08), although those who trusted the government indicated they would cooperate with the government policy (P = .02). We also obtained important qualitative information for further improvement. Although most LBM workers felt uncomfortable about banning poultry slaughter in LBMs, some of them did follow government policies because of trust. Therefore, winning trust from those who are affected is the key to implementing policies successfully. This ban has caused some LBM workers to become unemployed, which has created family problems. However, traditional media can play an important role in explaining the influenza risk factors and providing public health education. Unfortunately, hearing TV reports in Taiwan about the growing number of H7N9 cases and deaths in China every day only made Taiwanese listeners become afraid of losing business in the LBM and led them to refuse to follow government policies. In fact, the LBM workers thought the government should pay more attention to surveillance and control in poultry farms rather than focusing on end point surveillance in LBMs. Conclusions: We would like to make the following recommendations: (1) the government should fully understand LBM workers' concerns and be willing to address them during policy planning; (2) media must fulfill their social responsibility to

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provide the best public education possible regarding to the current scientific findings for novel H7N9 viruses and their modes of transmission; and (3) policies should be continuously evaluated for timely improvement. Future research directions should involve cross-country/area comparisons among Taiwan, Vietnam, Hong Kong, and China.

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Influenza awareness and vaccine advocacy in the Asia-Pacific region: the role of the Asia-Pacific Alliance for the Control of Influenza (APACI)

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Awareness of the burden of influenza is increasing in the Asia-Pacific region; however, there is no consensus on the best way to prevent and treat the disease and ensure policies for the use of seasonal influenza vaccines, specific treatments, and effective communication strategies are in place. The APACI was established in 2002, with a mission to reduce the burden of disease within the Asia-Pacific region. The changing influenza landscape following the 2009 pandemic required APACI to move from an informal structure to a company limited by guarantee and registered in Hong Kong as a not-for-profit organization in April 2011. It has a vision to be a lead organization on influenza education in the Asia-Pacific region, with a set of objectives which complement those of the World Health Organization's (WHO) Global Influenza Programme. One of the goals of APACI is to facilitate the development of an influenza foundation (or similar group) in every Asia-Pacific country, and over time, for each of these foundations to be interlinked, utilizing a comprehensive communication and education program. Influenza foundations have been established in India (IFI), Thailand (IFT) and Indonesia (IIF), while linkages with similar organizations or activities exist with Australia, China, Hong Kong, Korea, New Zealand, and the Philippines. Each of these nine countries has a representative that forms the APACI membership. In 2012, APACI, in partnership with the IFT and Thailand Department of Disease Control, held the First Asia-Pacific Influenza Summit meeting, followed by the First Asia-Pacific Forum on the Antiviral Treatment of Influenza in Bangkok, Thailand, to encourage regional discussion on influenza control. Additional APACI education initiatives have involved the development of a website (www.apaci.asia), an online regional newsletter "Influenza" (formerly "Asian Focus"), a regular media bulletin, translated influenza resources (available online), and collaborative research projects. APACI will continue to foster the development of new alliances and support existing alliances to promote influenza awareness in the region.

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SCHOLARSHIP International Research Scholarship

Informing future policy for influenza vaccination programs in sub-Saharan Africa: forging a path for decision-making.

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Background: New Global Burden of Disease estimates attribute nearly 2% of all-cause mortality during the first five years of life to influenza. Of influenza deaths, 99% are estimated to occur in low

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and middle income countries. Effective influenza vaccines have been available for decades, but their use in sub-Saharan Africa has been limited; only South Africa includes influenza vaccine in its National Immunization Program. The objective of this study was to assess the process that would be necessary for decision-making for programmatic introduction of influenza vaccine in countries in sub-Saharan Africa and to identify potential solutions to the specific challenges such countries would face during the decision-making process. Materials and Methods: We conducted surveys of national and international vaccine policy makers and influenza and influenza vaccine thought leaders for this study. We also interviewed a South African policy maker to understand the specific process that was used to evaluate influenza vaccines in South Africa and to understand how such deliberations could inform vaccine policy decision-making in other sub-Saharan African countries. Surveys and interviews gathered data from respondents regarding the key minimal information that would be needed in order to consider introduction of an influenza vaccination program. Strategies and processes at the global level which may assist in accelerating decision-making and/or policy implementation were also queried. We synthesized and analyzed these data to inform a strategy for influenza vaccine policy consideration in the sub-Saharan African context. Results: In order to implement influenza vaccination programs in their countries, policy-makers in sub-Saharan Africa stated that they required regional or sub-regional data on each of the following: influenza disease burden, vaccine effectiveness and safety, the potential programmatic impact on cold chain capacity, and program sustainability. In South Africa, the National Department of Health has launched an influenza vaccination campaign in the public health sector, targeting groups at higher risk for severe disease. South Africa, largely temperate, has an influenza season falling in the southern hemisphere winter. Therefore, vaccines are recommended to be administered sufficiently early before the season. The decision-making process used in South Africa emphasizes the importance of adequate influenza surveillance data to support policy decisions. Tropical Africa may experience less distinct influenza seasonality. This, along with often-limited or non-existent influenza surveillance, provides a challenge to informing optimal timing of immunization. Lack of surveillance data has also left questions regarding risk groups and disease burden largely unanswered. Many diverse international organizations (governmental, non-governmental, and academic) are contributing data which might inform sub-Saharan influenza vaccination strategies. UN agencies can provide technical assistance for annual influenza vaccine delivery and immunization. Sub-Saharan African countries have limited expertise with data on influenza epidemiology and with influenza vaccines. Such regional capacity must be strengthened if countries are to make the best evidence-based decisions about influenza vaccine use and to maintain a sense of program ownership. A regionalization of efforts would be the most realistic and feasible approach for future influenza vaccine policy consideration as it would leverage individual country strengths and capacities. The pathway for influenza vaccine introduction in sub-Saharan Africa could be as follows: strengthening laboratory capacity and influenza surveillance; conduct of research studies, such as burden studies, in a few key countries; improved communication of data; development of policy strategies; building vaccine production capacity; identification of financing and development of political will; and reliance on well-informed technical advisory committees. Additional concerns regarding influenza vaccine implementation included capacity to administer vaccine outside of existing routine immunization systems, program sustainability, and the importance of local and regional program control. Conclusion: Influenza historically has been perceived as a mild disease that is uncommon in sub-Saharan Africa. Public health authorities have generally not considered influenza vaccine a high priority. Occasionally, local epidemics or global pandemics have drawn policy-makers' attention. Successful introduction of sustained influenza vaccination into the region will require adherence to a clear plan that includes strong evidence-based decision-making processes, evidence of operational feasibility, and assurances of sustainability.

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Focal adhesion kinase regulates efficient influenza A virus entry and RNA replication

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Introduction: Virion internalization is dependent on host-cell signaling cascades induced by viral attachment to extracellular receptors that mediate viral entry. Phosphatidylinositol 3-kinase (PI3K) is activated by various stimuli through multiple pathways, including receptor tyrosine kinases. Integrin-mediated PI3K signaling is dependent on focal adhesion kinase (FAK), a cytoplasmic tyrosine kinase that is a major component of focal adhesion complexes associated with the actin cytoskeleton. Activation of PI3K by influenza A virus (IAV) attachment is required for efficient IAV entry and infection. FAK activation leads to Y397 phosphorylation, which serves as a binding site for Src kinases PLCγ and PI3K that in turn mediate maximal FAK activation through Y576/577 phosphorylation. Active FAK phosphorylates paxillin-Y118 to affect actin reorganization. The role of FAK in virus entry was reported for Kaposi sarcoma herpes virus (KSHV) and herpes simplex virus 1 (HSV-1) and the antiviral response to other viruses but not for influenza virus. Therefore, we investigated the role of FAK during influenza virus infection. Materials and Methods: Viruses and cells: egg-grown PR8 (A/Puerto Rico/8/34), X31 (A/Aichi/2/1968-X31), CA (A/CA/04/2009), CA_{mad}, (mouse-adapted CA), WSN (A/WSN/1933), and NC (A/New Caledonia/20/1999) virus stocks and culture supernatants were stored at −80°C until used. Yields were measured as $TCID_{50}$ in MDCK cells. MDCK and A549 cells were grown in MEM and F12K media, respectively. Normal bronchial epithelial (NHBE) cells were grown in transwell inserts and cultured in air-liquid interface (ALI) media. A549-FAK-WT and A549-FAK-KD cells were generated by stable overexpression of wild-type or kinase-dead FAK, respectively. Confocal microscopy: cells were pretreated with FAK or PI3K inhibitors for 2 hours and infected with influenza virus at MOI 5 in the presence or absence of inhibitor. Cells were fixed in 4% paraformaldehyde, permeabilized with 0.1% Triton-X 100, and blocked with 5% horse serum in PBS. Primary and AlexaFluor-conjugated secondary antibodies were diluted in blocking buffer. SDS-PAGE and Western blotting: A549 whole-cell extracts were harvested in RIPA buffer. Cell extract proteins were separated by SDS-PAGE. For Western blotting, proteins were transferred to nitrocellulose membranes, probed with specific primary antibodies and HRP-labeled secondary antibodies. ELISA: Levels of FAK-pY397 and AKT-pS473 in mock-infected or influenza virus infected were detected by ELISA (Invitrogen) according to the manufacturer's protocol. Quantitative RT-PCR of influenza RNA species: total RNA was isolated from infected A549 cells and cDNA complementary to the 3 viral RNA species were synthesized using NP-specific primers with unrelated nucleotide tags for cDNA synthesis. First-strand synthesis system for RT-PCR kit was used according to manufacturer's instructions. Human GAPDH was used to normalize viral gene levels. Results: We observed that viral attachment to sialic acid was required for efficient PI3K-dependent FAK-Y397 phosphorylation; this correlated with Y118 phosphorylation of the FAK effector protein paxillin. FAK inhibitor treatment resulted in a dose-dependent reduction of viral titers, which was also confirmed in inhibitor-treated NHBE cells. Additionally, A549 cells overexpressing a kinase-dead FAK mutant produced lower titers compared with cells overexpressing wild-type FAK (FAK-WT). Virus infectivity and NP expression at 24 hpi were also reduced in FAK inhibitor-treated cells. We observed minimal localization of virus with early endosomes in the presence of FAK inhibitor that also resulted in virion accumulation at the periphery of cells. Neither FAK-pY397 inhibition nor FAK-KD overexpression affected cortical actin or microtubules but did affect the actin meshwork and stress fibers. Finally, although inhibition of FAK-pY397 had little effect on RNA synthesis between 0.5 and 8 hpi, it significantly reduced influenza vRNA, cRNA, and mRNA synthesis between 8 and 20 hpi. Conclusions: This study highlights novel regulatory roles of FAK in both influenza virus entry and RNA replication. Our data suggest that FAK links receptor-mediated PI3K signaling to influenza virus-induced actin reorganization and endosomal sorting. Independent of its role on entry, FAK also regulates influenza RNA synthesis at later times of infection. Importantly, this suggests that inhibiting FAK activation could be a novel antiviral approach to influenza virus infection, one that is also being investigated in the field of cancer therapy.

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Airborne transmission in ferrets is associated with a change in glycan receptor specificity of an α2,3 sialic acid-specific 2009 pandemic influenza A virus

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Background: Influenza A viruses of animal origin pose a pandemic threat to humans when they are able to replicate efficiently and transmit between humans by respiratory droplets. Infection by influenza viruses is mediated via binding of the viral hemagglutinin (HA) glycoprotein to terminally attached α2,3 or α2,6-linked sialic acid (SA)-containing complex glycans on airway epithelial cells. Receptor binding specificity is an important determinant of host-range restriction and transmission of influenza viruses. Avian influenza viruses generally bind to glycan receptors terminating in α2,3-linked SA, whereas human-adapted viruses predominantly bind to glycan receptors terminating in α2,6-linked SA. In the human respiratory tract, the epithelium of the trachea primarily expresses α2,6 SA, whereas the lung contains both α2,3 and α2,6 SA. Ferrets, a well-established animal model for influenza, have SA distribution similar to humans, whereas mice predominantly express α2,3 SA and little α2,6 SA. The HA of human influenza viruses preferentially binds to glycans composed of **α**2,6-linked SA terminally attached to long oligosaccharide branches comprising at least 3 sugars (“long” **α**2,6 SA) that are predominantly distributed on the apical surfaces of the human and ferret respiratory tract. Materials and Methods: We studied the 2009 pandemic H1N1 (pH1N1) virus that predominantly binds α2,6 SA, replicates well in the upper and lower respiratory tract of ferrets, and is highly transmissible in humans and ferrets. To determine the importance of receptor specificity in tissue tropism and transmissibility, we generated distinct pH1N1 viruses with amino acid changes in the HA to alter receptor-binding preference. One virus contained two mutations designed to increase binding to **α**2,6 SA (**α**2,6 pH1N1) and the second virus had four mutations designed to switch binding preference from **α**2,6 to **α**2,3 SA (**α**2,3 pH1N1). The replication of the viruses was compared in mouse and ferret models, and respiratory droplet transmission was evaluated in the ferret model. Results: Despite the predominance of **α**2,3 SA in mice, all three viruses replicated to high titers, achieving a peak on day 1 or 3 postinfection, with declining titers on day 7. No significant difference was observed among the **α**2,3 pH1N1, wild-type pH1N1, and **α**2,6 pH1N1 viruses. The median mouse lethal doses for all three viruses were similar. Replication of all viruses was also similar in the respiratory tract of ferrets, and the viruses infected the same cell types in the ferret lung. Typically α2,3 influenza A viruses show inefficient airborne transmission in ferrets. Surprisingly, both the α2,3 pH1N1 and α2,6 pH1N1 viruses transmitted equally well by respiratory droplet in ferrets. Airborne transmission of the α2,3 pH1N1 virus was associated with reversion of an engineered mutation at position 222 in the receptor binding site of the HA, whereas the other three engineered changes remained unaltered. This reversion occurred in the upper and lower respiratory tract of ferrets but not in lung homogenates of infected mice. Biochemically, the mutation at residue 222 in the α2,3 pH1N1 virus led to a substantial increase in its binding to long α2,6 SA, without altering its binding to α2,3 SA. Conclusions: A change in receptor preference from **α**2,6 to **α**2,3 SA does not alter the virulence of the pH1N1 virus in mice or replication in the upper and lower respiratory tract of mice or ferrets. Our results demonstrated that influenza viruses can adapt within a single passage in ferrets to acquire the property of respiratory droplet transmission. These findings provide biologic evidence that association with long α2,6 SA is one of the important determinants of respiratory droplet transmission in ferrets.

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The cell-surface mucin MUC1 is a potential receptor for influenza A virus infection

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Background: Influenza A virus (IAV) is a highly contagious zoonotic pathogen. IAV predominantly infect target epithelia through sialic acid (Sia)-dependent binding, although Sia-independent infection does occur. However, Sia are only small carbohydrate moieties and do not fully explain the entire narrative of viral binding and entry to a host cell. Importantly, a definitive receptor for influenza virus infection has yet to be described. The lipid envelope of the influenza virion contains 2 virus-encoded glycoproteins, hemagglutinin (HA) and neuraminidase (NA), that protrude from the virion surface. The influenza HA is involved in attachment of the virus to terminal Sia of glycoproteins on the cell surface to initiate entry. Receptor engagement by HA triggers uptake of the virus by endocytosis. However, how the signal of viral binding is transmitted across the plasma membrane to initiate the formation of endosomes at the binding site is not understood. Cell surface (cs-) mucins consist of a large, extracellular, O-glycosylated polypeptide backbone that extends 200-500nm above the apical surface. Cs-mucin glycosylated branches terminate in Sia and the entire extracellular glycoprotein structure maintains a dominant presence at the apical surface of epithelial cells. Additionally, the cs-mucins contain a transmembrane domain enabling extracellular domain shedding and a short cytoplasmic tail capable of triggering epithelial signalling cascades in response to microbial binding. Imparting a capacity to carry cargo into the cell, the cs-mucin extracellular domain is constantly internalized by clathrin-mediated endocytosis for recycling. We hypothesized that cs-mucins mediate IAV internalisation into host cells and that upon binding, cytoplasmic tail signalling induces endosome formation. Materials and Methods: Human lung-derived type II epithelial cells (A549) were infected with the laboratory IAV strain A/Puerto Rico/8/34 (PR8) and were monitored for evidence of cs-mucin and PR8 co-localization using confocal microscopy. Alteration to cs-mucin expression levels during the course of infection was determined using flow cytometry and confocal methods. siRNA technologies, were also used to diminish the capacity of A549 cells to produce the cs-mucin MUC1 and the rate of viral entry into cells determined. To confirm the potential for MUC1 to bind and aid internalization with the virions, PR8 was co-incubated with recombinant MUC1 and the mixture added to siRNA treated cells. The ability for the MUC1-bound PR8 to infect was then re-examined. Results: Colocalization of PR8 and MUC1 occurred at the surface of infected cells as visualized by confocal microscopy, but neither MUC13 nor MUC16 co-localized with the virus. All cells infected with PR8 also expressed MUC1 but the reverse was not true. In addition, cells increased cs-mucin expression during the course of infection, with MUC16 showing the most marked increase over time. Interestingly, heightened MUC16 expression occurred most prominently in cells neighbouring infected cells. We utilized siRNA technology to temporarily knock down MUC1 expression by A549 cells and examined the effects on viral invasion and subsequent replication kinetics. Flow cytometry showed cells diminished in their ability to express MUC1 were not infected by the virus, and cell supernatants assayed for the number of plaque forming units/mL revealed slower replication kinetics than for untreated cells. MUC1-bound PR8 mixtures were able to rescue the ability to infect siRNA treated cells. Conclusions: These findings provide the first definition of a likely candidate for a specific sialic acid-expressing cell-surface receptor that mediates endocytic entry of IAV into the epithelium of the host. We are now rigorously exploring the mechanisms of viral attachment to cs-mucin MUC1 and its subsequent entry into the cell. We are also further exploring the influence of MUC13 and MUC16 expression by lung epithelia during infection. This research will open up novel avenues for research into the contribution of these receptors as important effector molecules in the dynamic mucosal barrier to infection. This work and J McAuley's attendance at the conference is supported by funding from NHMRC Program Grant 567122.

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Properties and prevalence of neuraminidases of human H3N2 influenza viruses that bind sialic acid via their catalytic site

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Background: The haemagglutinin (HA) glycoproteins of human H3N2 influenza viruses isolated since 2005 exhibit poor binding to sialic acid–containing receptor analogues and these viruses are unable to agglutinate turkey erythrocytes. Possibly as a way to compensate for this poor HA binding, the neuraminidase (NA) genes of many of these H3N2 viruses have acquired mutations that result in viruses being able to bind to cells via the catalytic site of this glycoprotein. This is distinct from the HB haemagglutination site previously reported to be present in the neuraminidases of many avian viruses. This binding via the neuraminidase has complicated the interpretation of antigenic characterisation of viruses as determined by haemagglutination-inhibition assays, as it can lead to artificially low titres. Materials and Methods: Viruses were obtained from the WHO Collaborating Centre for Reference and Research on Influenza, National Institute for Medical Research, Mill Hill, London, UK. The growth characteristics of the various influenza viruses were determined by plaque assays. NA binding was determined by haemadsorption assays and the kinetic parameters were determined with baculovirus expressed NA using MUNANA as substrate. Results: Viruses that bind via their NA were found to be more likely to be selected for during propagation in MDCK cells as opposed to MDCK-SIAT cells that have greater levels of receptors. They carried mutations that resulted in amino acid substitutions at either of two positions around the catalytic site: residues 151 or 148 (N2 numbering). These 2 groups of mutants had distinct enzymatic properties determined using NA expressed using a baculovirus system. Mutant NA proteins with substitutions at residue 151 mutants had greatly reduced enzymatic activity. Virus preparations with this amino acid substitution always also contained a significant proportion of wild type virus possibly because it is needed as a helper virus in cell culture. The 148 mutation, which results in the loss of a conserved carbohydrate attachment site, gave NA that had very similar kinetic properties to wild type. In culture these viruses could be propagated in the absence of wild-type virus. Therefore although the 151 mutant viruses may be debilitated in their growth properties the 148 mutant viruses seem to be as viable as wild type and are able to propagate in a wider range of cells types. Conclusions: These observations illustrate another way for influenza virus to alter its receptor binding properties and therefore potentially its tropism. It also reinforces the concept that viruses can readily mutate, not only in eggs but also upon passaging in tissue culture cell cultures.

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Host-specific differences in membrane fusion activity of H1N1 influenza A viruses

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Background: The repeated introduction of animal influenza A viruses into human population highlights the necessity to understand which viral properties contribute to interspecies transmission. Viral HA-mediated recognition of sialic acid receptors in a new host species is well known to be essential for the transmission. Less is known about the potential role of the membrane fusion activity of the HA. To address this question, we compared fusogenic properties of H1N1 Eurasian avian-like swine influenza viruses isolated in 1970s-1980s in Europe with properties of putative avian precursors of these viruses. Pandemic and seasonal human influenza viruses were also tested for a comparison. Materials and Methods: The HA-mediated membrane fusion activity of the viruses was characterized using high-sensitivity hemolysis assay. To study pH dependency of viral cell entry, we determined inhibition of viral infection in MDCK cells by lysosomotropic agent ammonium chloride, which

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interferes with the acidification of low pH cellular compartments. Stability of the viruses at low pH was studied by incubating them at a pH between 5.0 and 7.0 for 15 min and titrating remaining infectivity in MDCK cells. HA sequences were determined by reverse transcription and PCR amplification of viral RNA followed by Sanger didesoxy terminator sequencing. The effects of amino acid substitutions on the HA membrane fusion activity was studied using the pCAGGS-HA plasmid containing the HA gene of A/duck/Bavaria/1/77 (H1N1) virus modified by site-directed mutagenesis. Fusion activity of modified HAs was tested by expessing them in HeLa cells and subsequent detection of polykaryon formation at low pH. Results: We found that the pH optimum of the HA-mediated membrane fusion of H1N1 swine influenza viruses was 0.2 to 0.5 pH units higher than the pH optimum of avian and human viruses. Swine viruses were less sensitive to neutralization by ammonium chloride than avian and human viruses (IC50%: 1.5- 2.5 mM of NH4Cl for swine viruses, 0.6-1.0 mM NH4Cl for avian and human viruses). By contrast, swine viruses were more sensitive than avian viruses to inactivation in low pH buffers. To identify amino acids responsible for the observed differences between Eurasian avian-like swine viruses and their putative avian precursors, we compared viral HA sequences and found 8 host-specific amino acid differences. We introduced the swine-virus-like amino acids into the avian HA (A/duck/Bavaria/1/77) and compared membrane fusion activity of the mutants. Two of the mutations tested, T393S and S457F, were found to raise the pH of HA-mediated fusion of the avian HA by 0.2 pH units. Conclusion: Our studies revealed that HAs of avian and human H1N1 viruses have lower pH optimum of membrane fusion activity and are more stable at low pH than closely related swine viruses. Alteration of these properties occurred soon after the introduction of an avian virus into pigs in Europe, suggesting that fusion-related characteristics of the HA can represent a host-range factor restricting interspecies transmission of influenza viruses.

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A statistical strategy to identify recombinant viral ribonucleoprotein of avian, human, and swine influenza A viruses with elevated polymerase activity

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Background: The segmented genome of influenza virus makes it possible for reassortment of gene segments when co-infection of different virus strains occurs in the same host. Apart from the 1918 pandemic H1N1 virus, where the origin is controversial, the subsequent 3 influenza pandemic strains contain polymerase subunits reassorted from strains of various origins. Both the 1957 H2N2 and 1968 H3N2 pandemic viruses contain PB2 and PA of human origin reassorted with PB1 of avian origin. The pandemic H1N1/2009 strain contains polymerase genes originated from the North American triple-reassortant swine lineage, of which the PB2 and PA genes are of avian origin and the PB1 gene is of human origin. It is possible that, in addition to acquiring a novel HA or/and NA surface protein, a virus with polymerase subunits of different origins may have an increased pandemic potential. Thus it is necessary to develop a more systematic strategy to analyze chimeric polymerase complexes with genes from multiple sources. Here, we applied statistical methods to analyze the polymerase activities of reassorted vRNPs originated from 3 strains of different origins. In particular, we aimed at testing the feasibility of using this strategy to determine whether temperature, the origin of single subunit of vRNP and/or interactions between subunits of vRNP affect the polymerase activity. Materials and Methods: In this study, we reconstituted chimeric vRNPs with 3 different viral strains isolated from avian, human and swine hosts. We wanted to identify the effect of the origin of single vRNP subunit or interactions between different subunit on polymerase activity. Eighty-one chimeric vRNPs were reconstituted in 293T cells at different temperatures. Polymerase activity was determined by luciferase reporter assay, and statistical strategy was applied to identify the single vRNP subunit effect or the interactions between different subunits. Results: It was found that PB2, PB1, NP, PB2-PB1 interaction, PB2-PA interaction and PB1-NP interaction had significant effect on polymerase activity at 37^{o}C, and several single subunits and interactions were identified to lead to elevation of polymerase activity. Furthermore, we applied a 27-run fractional factorial design to obtain a subset of 27 chimeric vRNPs from the 81

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combinations of vRNPs. A 27-run (3^4-1) fractional factorial design was applied as a model to select a subset of 27 chimeric vRNPs out of the 81 combinations. With this fractional factorial design, a total of 24 different subsets of data could be selected. It was found that the average rank on the significance of single subunit effect and 2-factor interactions of the 24 subsets of fractional factorial designs is similar to the rank in the 81-combination full factorial design. This suggests that, by running an experiment with a fractional factorial design, it is possible to capture the major factors or interaction effects that would be observed from the results generated from the full factorial design. Conclusion: This approach can be used to identify the major single subunits or interactions that affect the polymerase activity. In addition, the use of fractional factorial design can obtain reliable results without the need to experimentally reproduce all possible vRNP combinations.

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Virology and Viral Receptors

Poster Abstracts

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Studies on the receptor-binding properties of influenza viruses H1N1 to sialic acid

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Background: In April 2009, a new influenza A H1N1 circulated among the population worldwide, generating the first pandemic of the 21st century. At the time of the emergence of the influenza A(H1N1)pdm09 in early 2009, two other influenza A viruses had been circulating in humans, the H3N2 viruses, AH3, and the seasonal H1N1 viruses, AH1. In the following years it has become apparent that the AH1 viruses were displaced but the H3N2 viruses were not. The extinction of one subtype by another during a pandemic was previously suggested to be due to short-lived transcending cross-subtype immunity. A hypothesis recently made for the extinction of the AH1 virus after A(H1N1)pdm09 appearance is the generation of neutralizing antibodies against conserved regions of HA or NA. However, it is interesting to note that in coinfections in ferrets aimed at generating potential reassortants between influenza A subtypes, only pure A(H1N1)pdm viruses were recovered, suggesting that the new virus could outcompete the old ones even in naive animals. In influenza AH1 virus, amino acids at position 190 and 225 of HA affect replication and transmission. Both the new pdmH1 and the seasonal AH1 viruses that circulated until 2008 have 190D and 225D, but other amino acid differences between the two viruses may confer differences in sialic acid binding. The aim was to evaluate the hypothesis of whether the displacement of the AH1 virus might be associated with a lower affinity for the sialic acid receptor in the human respiratory tract and to describe the replacement phenomenon in an in vitro system. Materials and Methods: To assess differences in growth kinetics, HAE cells were individually infected with three viruses: (H1N1)pdm09, AH1and AH3 at an MOI = 0.0001. Viruses were harvested from the apical surface every 12 h post infection and viral titres were obtained by plaque assay in MDCK cells. In addition, viruses were evaluated in competition assays in HAE cells: (H1N1)pdm09 vs AH1; (H1N1)pdm09 vs AH3 and AH1 vs AH3 at a defined ratio of 50:50. At 0, 12, 24, 48 and 72 h post infection, viruses were collected from the apical surface and plaqued on MDCK cells to assay for viral titres. In addition, to determine the virus ratio in each mix, collected viruses in each time point were plaqued in MDCK cells and isolated by picking 30 visible plaques. From each grown plaque an RNA extraction was performed and a fragment of the hemagglutinin gene of (H1N1)pdm09, AH1 and AH3 was amplify by RT-PCR. Results: From the single HAE cells infection, it was observed that at 12 h post infection the(H1N1)pdm09 and the AH3 viruses replicated with similar titres while the AH1 didn't show viral growth. Although at 24 h post infection the viral growth for the three viruses was similar, further at 72 h the AH1 virus showed important decrease in viral titre (2.83 x 10^5PFU/mL) compared with (H1N1)pdm09 (3.33 x 10^7PFU/mL) and AH3 (1.82E x 10^7PFU/mL). In competition assays, at 12 and 24 h post infection, the two combinations containing the AH1 showed slightly higher titres than the mix containing the (H1N1)pdm09/AH3. However, at 48 and 72 h, the combination (H1N1)pdm09/AH3 showed increased viral titres than the two combinations containing the seasonal AH1 virus. From the RT-PCR analysis, at 24 h post infection, the 70% of analysed plaques from the mix (H1N1)pdm09/AH1 amplified for the AH1 and the remaining 30% amplified HA for both viruses. In contrast, in the mix AH1/AH3, 46% of the plaques were positive for AH1 and 40% plaques were positive for both viruses. Conclusions**:** Individual infections with (H1N1)pdm09 and AH3 viruses showed high titres and similar kinetics; however, the AH1 replicates at lower titres. During simultaneous infections, virus combinations containing the AH1 showed lower titres than (H1N1)pdm09 vs AH3. These results suggest that AH1 viruses appear to have a more stringent preference for α-2,6 SA than the newly emerged pdmH1. Also, competition for receptors may contribute to the subtype replacement during a pandemic.

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Viral scission factors: M2-mediated influenza virus budding

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Background: Although many of the interactions required for influenza virus assembly have been determined, the molecular machinery needed to complete the budding process has only recently been elucidated. We recently determined that the influenza virus M2 protein contains an amphipathic helix (AH) that is capable of altering membrane curvature and causing membrane scission. These studies uncovered a novel and essential role for the M2 protein in mediating virus budding; however, there remain many unanswered questions about the M2 protein and the process of influenza virus budding. Materials and Methods: M2-mediated budding was investigated in vitro using giant unilamellar vesicles (GUVs) formed by electroformation and visualized by confocal microscopy. Full-length M2 protein was purified following baculovirus expression and incorporated into GUVs. Alternatively, peptides corresponding to the wild-type M2 AH, or incorporating M2 AH mutations, were chemically synthesised and added to preformed GUVs. Single GUV imaging was used to calculate the biophysical properties of M2-mediated budding. Peptides were synthesised using the amino acid sequences of the A/Udorn/72 and A/California/09 strains of influenza virus. The effects of additional viral proteins on M2-mediated budding were determined using SUPER-templates and giant plasma membrane vesicles (GPMVs) generated from transfected HEK-293T cells. M2 mutations were introduced into the influenza virus genome using reverse genetics and the replication of the recombinant viruses assessed. Results: Investigation into the role of the M2 protein showed that the protein possesses a well-conserved amphipathic helix that is capable of altering membrane curvature in a cholesterol-dependent manner. In GUVs containing reduced levels of cholesterol, such as would be found at the neck of a budding virion, we saw that M2 caused positive membrane curvature and scission, a process that is dependent on the M2 amphipathic helix. By imaging single M2 AH peptide-treated GUVs in real time, we were able to calculate the kinetics and biophysical properties of M2-mediated budding. Using reverse genetics to recover influenza virus containing a mutated amphipathic helix, we saw that the M2 amphipathic helix is necessary for membrane scission and for the release of budding virions. Further results using SUPER-templates and GPMVs showed that additional viral proteins are capable of modifying membrane curvature and function to define and initiate the budding event, whereas the M2 protein mediates membrane scission and completes the budding process. Conclusions: Our results showed that the M2 protein is capable of altering membrane curvature in a cholesterol-dependent manner, serving an essential role during influenza virus budding by mediating membrane scission. The activity of the M2 protein is modified by expression of additional influenza virus proteins that define the site of assembly and enable the process of viral budding to proceed with maximal efficiency.

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The binding avidity of human H3N2 viruses to α2,6-sialic acid linked receptors has decreased during the evolution of the HA gene in viruses isolated from 2000 onward

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Background: Since their emergence in 1968, influenza A (H3N2) viruses have undergone considerable genetic and antigenic evolution, and this has been found to be associated with changes in receptor-binding properties. The H3N2 viruses circulating from 1968 to 1972 had a similar pattern of binding to avian strains. However, H3N2 viruses isolated after 1975 were no longer able to bind to

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sialic acid α2-3Gal specific gangliosides. H3N2 viruses isolated since the early 1990s lost the ability to agglutinate chicken RBCs. More recently, most H3N2 viruses circulating from 2002 onward can no longer be efficiently isolated from and replicate in embryonated eggs. In addition, the majority of H3N2 viruses isolated from 2005 were unable to agglutinate turkey RBCs via the haemagglutinin (HA), and had poor growth properties in Madin-Darby canine kidney (MDCK) cell cultures. The isolation rates were increased by using MDCK-SIAT1 (SIAT) cells, which had been made to stably overexpress the α2,6-linked sialic acid receptors, compared with MDCK cells. However, little is known about the molecular basis for the altered receptor-binding specificity and affinity as a result of the evolution of H3N2 viruses from 2000 onward. In this study, the binding specificity and affinity of representative human H3N2 viruses from 2000 were measured and the relationship with amino acid changes in H3 HA are discussed. Materials and Methods: Viruses used in this study were from stocks held in our laboratory, and recombinant viruses were generated by reverse genetics. Virus binding to receptor analogues was measured using surface biolayer interferometry (BLI). For crystallographic studies, HA was expressed in insect cells and purified by affinity chromatography. HA crystals diffracted to 1.85 Å and the structure was solved using molecular replacement. Results: Sequence analyses of HAs from viruses isolated between 2001 and 2010 showed that a series of substitutions around receptor-binding site had occurred: W222R and G225D around 2001; Q156H, S189N, V226I and S227P from 2003; S193F and D225N from 2004; and K158N and N189K from 2009. The avidity of H3N2 viruses for the human receptor analogue, α2,6-sialyllactosamine, as measured by surface BLI, decreased overtime with a 4-fold reduction between 1968 and 2001 and then a further estimated 200-fold reduction in binding over the period of 2001-2004. By 2010, viruses failed to bind to human receptor analogue under standard assay conditions, but some very limited binding could be detected at increased virus concentrations. The binding affinity of RG/Finland/486/2004, which had the substitution D225N on HA, was significantly decreased and the virus lost the ability to bind to turkey RBCs. Serial passaging of viruses with the D225N substitution in MDCK cells or eggs gave rise to substitutions around the receptor-binding site, and these viruses had significantly increased binding to receptor analogues and could agglutinate turkey RBCs. The crystal structures of HA-receptor analogue complexes formed with HAs from viruses isolated in 2004 and 2005 have been determined and compared with the H3HA 1968 structure. The structural study confirmed that substitutions in the receptor-binding site, particularly at residues 222, 225 or 226, altered the interactions between the HA and the sialic acid receptor. Conclusions: Following the evolution of the HA gene, the binding avidity of human H3N2 viruses to α2,6-sialic acid linked receptors has decreased since 2000, which caused difficulties in virus propagation in vitro as well as in antigenic analysis. Amino acid substitutions at HA1 position 225 correlate with recent changes in affinity for human receptor and is responsible for the loss of the ability to bind to turkey RBCs.

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Tropism of human and influenza virus infection in the human respiratory tract using ex vivo tissues

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Background: Influenza is an infection primarily of the respiratory tract, and infection ranges from the upper regions (nasopharynx) and conducting airways to the lung parenchyma. This tropism has an impact on a determination of disease severity and transmission, with infection of the alveolar epithelial cells leading to cell death and respiratory distress but infection of the upper airways enhancing the possibility of transmission from human to human. Previous investigations using lectin binding data proposed that avian viruses with a α2-3 binding preference would infect the lower respiratory tract but human viruses with a α2-6 binding preference would infect the upper respiratory tract, leading to enhanced transmission. We have previously utilized fresh tissue samples from the nasopharynx, bronchus and lung and infected these tissues with avian and human viruses in order to study infection

and innate tissue responses. Over a 4-year period we collected and analyzed the infection of a wide range of influenza viruses, including the recent H7N9 strains from China, in order to re-evaluate whether this tropism paradigm required revisiting. Materials and Methods: Ex vivo culture of human bronchus and lungs was performed as previously published using 3 human H1N1 isolates, 2 H1N1pdm isolates, 8 H3N2 isolates, 2 H5N1 isolates, 5 H9N2 isolates, 5 H1N1 swine isolates, 2 H1N2 swine isolates, 1 H5N8 avian isolate, 3 H7N7 isolates and the 2 recent H7N9 isolates from mainland China that have caused recent cases of human disease. 27 of these isolates had glycan array performed in order to determine binding preference to α2-6 or α2-3 sialylated N and O-glycans. The tissues were obtained from patients undergoing surgical biopsy or resection for pathological diagnosis, and consent had been given for the use of these tissues for influenza research. The tissues were infected with influenza virus with a titer of 10^6 50% tissue culture infectious doses ($TCID_{50}$)/mL, a titer similar to that used previously for 1 h at 37°C. The bronchial mucosa was placed on a surgical sponge with its apical epithelial surface upwards in a 12 well plate, while the lung parenchyma was placed into the 12 well plate directly with 1.5 mL of culture medium. The increasing virus titers along the time course provided evidence of productive virus replication. Bronchial and lung tissues were also subjected to mass spectrometric analysis in order to determine glycans present in these tissues that might be potential receptors for different influenza virus strains. Results: A total of 113 bronchial and 185 lung samples were infected between 2008 and 2012 and 3 bronchial and lung samples were infected in 2013. Apart from 2 swine viruses (A/Swine/Hong Kong/4167/1999 and A/Swine/Hong Kong/915/2004), all viruses tested showed replication in the lung, with an efficiency of replicating ranging from 25%-100%. Sialidase treatment abolished infection in the H1N1pdm and H5N1 infected tissues. In the bronchial explants, apart from A/Swine/Hong Kong/NS29/2009 all viruses showed replication ranging from 21-100%. In particular we found that the recent H7N9 strains had a very efficient degree of replication in the lung and bronchus with extensive infection in ciliated and non-ciliated cells in the bronchus and type 2 pneumocytes in the lung. There was also limited H5N1 replication in bronchial epithelium. Mass spectrometry and lectin histochemistry showed that both α2-6 and α2-3 sialylated glycans were present in the bronchial tissues. Conclusions: When a large number of viruses and samples was studied it appears that the distinction of bronchus versus lung tropism for influenza may be less evident as previously published and viral or host factors apart from haemagglutinin affinity for α2-6 or α2-3 sialic acid.

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Analysis of influenza C virus possessing substitutions in its CM2 transmembrane region

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Background: CM2, the second membrane protein of influenza C virus, consists of 115 amino acids and is encoded by the RNA segment 6 (M gene) of the virus. Although the biochemical characteristics and properties as an ion channel of CM2 have been exclusively examined so far, the role(s) of CM2 in virus replication remained to be clarified. Recent studies using virus-like particles (VLPs) lacking CM2, and recombinant viruses and VLPs possessing mutations at the CM2 posttranslational modification sites have indicated that CM2 is involved in uncoating and packaging processes. However, the specific roles of CM2 extracellular, transmembrane (TM) and cytoplasmic domains in virus replication still remain to be determined. In the present study, to study the effect(s) of CM2 TM domain on virus replication, we generated a recombinant influenza C virus (rCHC), in which the CM2 TM domain is substituted to the TM domain of Haemagglutinin-Esterase-Fusion (HEF) glycoprotein of the influenza C virus, and analysed the virus growth, virus morphology, and virus proteins synthesized in virus-infected cells and incorporated into virions. Materials and Methods: Reverse genetics: The mutant M gene cDNA, in which the 69 nucleotides (positions 872 to 940) of C/Ann Arbor/1/50 M gene was replaced with the 78 nucleotides (positions 1900 to 1977) of C/Ann Arbor/1/50 HEF gene, was PCR-amplified and cloned into the Pol I vector, generating pPolI/M-CHC. To generate a recombinant virus,

rCHC, the pPolI/M-CHC was transfected into 293T cells together with the other Pol I plasmids and virus-protein expressing plasmids. Analysis of virus-infected cells: HMV-II cells infected with recombinants were pulse-labeled and chased, and then immunoprecipitated with antibodies against virus proteins. For membrane flotation analysis, the infected cells were fractionated through sucrose equilibrium ultracentrifugation and then subjected to immunoprecipitation. To detect cell-surface virus proteins, cells were biotinylated and precipitated with streptavidin-agarose, followed by Western blotting. Analysis of viruses: The virions grown in HMV-II cells and eggs were purified by ultracentrifugation through 30% sucrose, and then subjected to Western blotting or electron microscopy. Results: 1) The rCHC virus grew less efficiently than did the recombinant wild-type (rWT) virus. 2) The mutant CM2 protein synthesized in the rCHC-infected cells was matured slightly slower than was the authentic CM2, but the amount of CM2 expressed on the rCHC-infected cells was comparable to that on the rWT-infected cells. 3) Pulse-chase experiment of virus-infected cells showed that the affinity of M1 to the plasma membrane in rCHC-infected cells was lower than that in rWT-infected cells, although there was no apparent difference in the affinity of HEF between the two cell populations. 4) The ratio of M1 to HEF in the rCHC virions was lower than that in the rWT virions, whereas the ratio of CM2 to HEF in the rCHC virions was higher than that in the rWT virions. 5) The rCHC virions mainly exhibited pleomorphic short-rod forms, whereas most of the rWT virions were long filamentous particles. Conclusions: The reduced M1 affinity to the plasma membrane of the rCHC-infected cells correlated with the reduced amount of M1 in the rCHC virions. Thus, the HEF TM domain inserted into CM2 appeared to affect the ratio of M1 and CM2 in the virions, leading to the differences in the morphology and replication of the viruses. This finding is reminiscent of our previous observation that the influenza C virus morphology was affected by M1 affinity to the membrane.

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Characterization of influenza A viruses with different amino acid residues at PB2-627

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Background: Viral ribonucleoprotein (vRNP) of influenza A virus is responsible for transcription and replication of the viral genome. Residue 627 of one of its subunit, PB2, has been shown to have significant effect on host specificity, polymerase activity and viral replication. The majority of avian influenza viruses carry a glutamic acid (E) at PB2-627, while most of the human influenza viruses identified before emergence of 2009 H1N1 pandemic carry a lysine (K). The amino acid change E627K in PB2 is believed to favor avian virus adapting in mammalian hosts. Although glutamic acid and lysine are commonly found at PB2-627 in nature, some other amino acids are also possible at this residue. For example, some avian viruses possess valine and some human viruses possess arginine at this residue. However, the effects of these less frequent amino acids at PB2-627 are not fully understood. Furthermore, the possibility of emergence of virus with other amino acids at PB2-627 cannot be excluded. This prompted us to generate and characterize the recombinant viruses with a random amino acid mutation at this position. Materials and Methods: Degenerated primers were designed to generate random amino acid mutations at residue 627 of the PB2 gene in A/PR/8/1934 (PR8). Recombinant PR8 viruses with random mutations at this position were generated by reverse genetics techniques in 293T cells. The recombinant viruses generated were inoculated in the mammalian system (MDCK cells) or the avian system (10-day-old embryonated chicken eggs). The viruses harvested were plaque purified and the amino acids at PB2-627 were identified by DNA sequencing. The polymerase activities of vRNPs with different amino acids at PB2-627 were compared and the growth kinetics of the recombinant viruses were also determined in mammalian and avian cells. The virulence of the recombinant viruses were compared in balb/c mice. Results: Sequencing result of the recombinant viruses revealed that amino acids at PB2-627 are not restricted to those isolated from nature. It was observed that the recombinant viruses derived from the avian system behaved differently from those derived from the mammalian system in polymerase activity assay and *in vitro* growth kinetics assay. The recombinant viruses derived in the avian system generally had lower polymerase activity and slower growth rate than the wild-type virus in mammalian

cells. On the other hand, some recombinant viruses derived in the mammalian system had low polymerase activity and slow growth in avian cells. Although all the recombinant viruses with different amino acids at PB2-627 replicated in mammalian cells, the virulence of the recombinant viruses in mice vary. It was found that recombinant viruses with isoleucine at PB2-627 led to more significant weight loss and higher mortality in mice than the recombinant viruses with the avian marker at PB2-627, albeit lower than wild-type virus. Conclusions: This study demonstrated that the amino acid identity at PB2-627 is not exclusive to glutamic acid and lysine. Introducing other unnatural amino acid mutations at this position can also lead to viable viruses in mammalian and avian cells. Some of these novel amino acid mutations may also lead to highly virulent virus in mammals. Although the mechanism leading to the role of PB2-627 is yet to be elucidated, more understanding on the effect of this residue on the viral polymerase can surely contribute to the evaluation of the pandemic potential of any novel virus that may emerge in the future.

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Influenza B viruses in swine: virus tropism in swine respiratory organ explant cultures

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Background: Swine has been considered an animal reservoir of pandemic influenza A virus (IAV), for example, the 2009 H1N1 pandemic virus, swine is acting as a “mixing vessel” for the reassortment of swine, human and avian IAVs. Certain influenza B virus (IBV) strains were also found to be readily infecting piglets as early as in 1969. However, tissue tropism of IBV in swine is understudied, at least in 2000s, mainly due to the misconception that IBV causes milder disease than IAV. IBV has in fact circulated in many parts of the world causing regular seasonal epidemics in humans with mortality rates sometimes higher than that in IAV seasons. Here, our research group hypothesizes that swine could be a neglected host of IBV, apart from human and seal, due to the previous infectivity of IBV in this animal, as well as the fact that swine has close contact with human and possesses a similar sialic acid (influenza virus receptor) distribution profile as the human respiratory tract. We aim to examine the characteristics of IBV tissue tropism using swine tracheal and lung explant models, and risk assess swine susceptibility to a panel of IBV strains from both Yamagata and Victoria lineages of different years. Materials and Methods: The tracheal and lung explants were prepared from fresh swine respiratory organs from approximately 6-month-old pigs, and cultured with maximal similarity to the *in vivo* conditions. A panel of IBV strains, from both Yamagata and Victoria lineages and from different years, were used to infect the tissue explants at 37°C or 39°C according to the original physiological temperature of the tissue. The virus replication efficiencies were evaluated through viral titration and immunohistochemistry of the collected supernatant and formalin-fixed tissue explants respectively at 1, 24, 48 and 72 h postinfection. Seasonal IAVs (H1N1 - A/OK/447/08 and H3N2 - A/OK/370/05) were used as controls. Results: Most of the tested IBVs showed productive replication in the swine lung explants. Swine tracheal explants, on the other hand, supported the replication of limited IBV strains. Most of these IBVs belong to the Victoria lineage, which spread across the years from 2005 to 2011. IBVs that could replicate in swine lung explants reached their maxima at 48 hpi or sometimes later. This is comparatively slower than the replication rates of seasonal IAVs (H1N1 & H3N2) used in the study, which usually showed significant increase at 24 hpi with still increasing virus yields at 48 hpi in some cases. However, the overall increase in titres between the IBVs and seasonal IAVs were similar. In swine tracheal explants, both IBVs and seasonal IAVs showed limited replications with similar trends of having maxima being reached at 24 hpi. Conclusions: The successful replication of IBVs in swine explants cultures indicates the possible susceptibility of swine to IBV and provides the essential basis for further investigation on the likelihood for swine to be an animal reservoir of the virus, as well as the threat it may pose to humans. Continuous studies on the replication kinetics of a greater number of IBVs in swine explant cultures across a wider range of years, countries and lineages will probably be our future target.

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The human AXL receptor tyrosine kinase is a novel bifunctional receptor for influenza A virus

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Virus binding to a cell surface receptor and subsequent entry into host cells are critical steps determining viral species specificity and organ tropism. However, there are few therapeutics that can target virus entry. Influenza virus A (IAV) is known to use sialic acid-containing molecules as a primary receptor. In addition, it has also been demonstrated to use C-type lectin receptors (CLRs), such as DC-SIGN, L-SIGN, SIGN-R1, MMR, and MGL, molecules as alternative receptors. These molecules may expand the tissue specificity of IAV infection. By using an RNAi (interfering RNA) library screening to search for cellular factors required for influenza virus infection and replication, we have identified a cell surface molecule AXL as a cellular factor required for virus infection. AXL is a member of the TAM (Tyro 3, AXL, Mer) protein family, and is a receptor tyrosine kinase (RTK), which is a single-pass type 1 transmembrane protein. We found that IAV replication was suppressed by knockdown of AXL in A549 cells and enhanced by overexpression of AXL in 293 cells, indicating a positive role for AXL in IAV infection. Furthermore, treatment with a polyclonal antibody against AXL during virus binding stage (first 20 minutes), but not at the later stage, completely blocked virus infection, even in the presence of sialic acid receptors. Under the same condition, the sialidase treatment completely blocked IAV infection despite the presence of AXL. The IAV binding to the cell surface was decreased by knockdown of AXL using specific shRNAs targeting to AXL. The IAV infection induced transient AXL phosphorylation, and inhibition of AXL kinase activity by a specific inhibitor leads to impaired IAV uptake into cells. Taken together, our findings indicated that AXL serves as a virus receptor in conjunction with sialic acid, for IAV at least in some cell types. We further used co-immunoprecipitation assay to show that AXL binds with the viral envelope proteins neuraminidase (NA), matrix protein 2 (M2) and, to a lesser extent, hemagglutinin (HA). Paradoxically, over-expression of AXL reduced the extracellular virus titer by trapping the newly generated viral particles on the cell surface. This property is reminiscent of the sialic acid receptor. In conclusion, AXL is a novel dual-function receptor for IAV in some cell types. It offers a new target for antiviral therapy.

P1-175

Receptor binding studies of 2009 H1N1 pandemic emergence

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Introduction: 2009 H1N1 pandemic viruses arose from a reassortment of a triple reassortant classical swine virus with an avian-like Eurasian swine virus. This reassortment gave rise to a novel combination of haemagglutinin (HA) and neuraminidase (NA), with the HA coming from classical swine and the NA from Eurasian swine. The HA and NA are likely to be major factors in determining the transmissibility, and therefore pandemic potential, of these viruses. Triple reassortant classical swine viruses had arisen previously from a reassortment of a classical swine H1N1 virus with a seasonal human H3N2 and an avian H1N1, forming an H3N2 virus, which then reassorted back with a classical swine H1N1 forming H1N1, H1N2, and H3N1 viruses. These triple reassortant viruses led to a number of sporadic zoonoses in the years leading up to the 2009 pandemic; however, these viruses were not found to spread from human to human. These studies aim to determine the factors necessary in the HA and NA for the emergence of the 2009 pandemic. The HA in the early pandemic viruses harbour a number of conserved substitutions when compared with the H1 HA of classical swine viruses. A number of these residues are located in positions that have been previously shown

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to affect HA receptor binding. Materials and Methods: Biophysical receptor analogue binding assays were used to determine virus receptor binding characteristics. These assays are based on an Octet RED system (ForteBio). The assays measure the binding of virus to sialoglycopolymers bearing trisaccharide receptor analogues 3-Sialyl-N-acetyllactosamine (3-SLN) or 6-Sialyl-N-acetyllactosamine (6-SLN). Assays measure virus binding to a range of different biosensor sugar loadings to a constant concentration of virus (normalized by solid-phase NP ELISA). The extrapolated saturation point of binding is then plotted against relative sugar loading, allowing a measure of relative virus avidity. Modified binding assays have also been carried out to measure the relative balance of HA and NA activity. Results: A number of common substitutions around the HA receptor binding site were identified after sequence alignments of classical swine and early pandemic strains. These substitutions were introduced into viruses both of a triple reassortant classical swine and early pandemic lineage by reverse genetics. These viruses were then used in receptor analogue binding assays. Both triple reassortant and pandemic H1N1 viruses were found to bind to α2,6 linked sialic acid strongly with no detectable binding to α2,3 linked sialic acid. There was little difference in the binding of these two separate lineages. A number of substitutions were found to alter the relative α2,6 linked sialic acid avidity. Assays were carried out to measure the relative HA/NA balance of pandemic and triple reassortant H1N1 viruses to ascertain the importance of the novel combination of HA and NA seen in the 2009 pandemic. Conclusion: These studies allow better understanding of the factors underlying HA receptor binding as well as HA/NA balance, which allowed the emergence of the 2009 H1N1 pandemic.

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Hemagglutinin receptor specificity and structural analyses of aerosol transmissible H5N1 viruses

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Background: 5N1 is a highly pathogenic virus for poultry and endemic in several countries; although human infections are sporadic, they are accompanied with a high mortality. It is well documented that avian viruses primarily recognize α2,3 sialic acid receptors (avian type) and human viruses recognize α2,6 receptors (human type). Moreover, recent studies suggest that binding to α2,6 sialosides is a perquisite for aerosol transmission in ferret models and is a risk factor for transmission in humans. To date H5N1 viruses have not acquired human-type receptor specificity, raising interest in what it would take for this to happen. Recently 3 reports showed that H5N1 viruses with HA mutations that shift receptor specificity from avian type to human type are able to achieve aerosol transmission in ferrets (LM Chen, et al. *Virology.* 2012;422:105; S Herfst, et al. *Science.* 2012;336:1534;M Imai, et al. *Nature.* 2012;486:420). In 2 cases, receptor specificity was determined using assays that assess a binary specificity for human (α2,6) or avian (α2,3) type specificity (S Herfst, et al. *Science.* 2012;336:1534;M Imai, et al. *Nature.* 2012;486:420), while the third was determined by glycan array analyses that assess differential binding to sialic acid containing glycans that occur on the human airway. Here we employ glycan array analysis to assess the fine receptor specificities of the 2 other transmissible H5N1 viruses, including all of the intermediate mutants in the receptor domain and stalk region that were found to be required for transmission (S Herfst, et al. *Science.* 2012;336:1534;M Imai, et al. *Nature.* 2012;486:420). Materials and Methods: We used the A/Vietnam/1203/04 (VN1203) H5 mutants in the Cal/04 background as inactivated whole viruses and created recombinant soluble

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trimeric HA proteins for VN1203 and A/Indonsia/5/05 (INDO5). We applied these viruses and hemagglutinins to a sialoside glycan array created to asses influenza a viruses receptor binding specificity containing 58 glycan structures (CM Nycholat, et al. *Angew Chem Int Ed Engl.* 2012;51:4860). To determine the effect of the stalk mutations on the stability of the viruses; we incubated these at 50◦C with over time and analyzed their ability to agglutinate turkey erythrocytes and growth in MDCK cells. Finally, we crystallized the VN1203 hemagglutinin with or without the stalk mutations. Results: We found that both virus and recombinant hemagglutinins containing Q226L G228S or N224K Q226L show a shift in receptor binding from α2,3 to α2,6 sialosides. The loss of the carbohydrate at position 158 on the head of the hemagglutinin strengthens this effect. The stalk mutations, however, did not affect receptor binding. Importantly the 2 mutant H5 HAs exhibit a slightly different receptor binding profile. For example the VN1203 seems to prefer linear structures, whereas INDO5 prefers N-linked glycans. Furthermore, for all mutants we observed residual α2,3 sialoside specificity, which is not seen for fully human-adapted viruses. We were able to show that the stalk mutation in INDO05 indeed introduces extra stability; however, we did not observe a structural determinant for this effect, whereas the crystals structures obtained for VN1203 with or without the T318I mutation where nearly identical. Conclusions**:** Acquisition of α2,6 sialoside binding for H5N1 viruses to become aerosol transmissible in mammals can be achieved by a different sets of amino acid substitutions. Both viruses analyzed in this study showed predominant binding to α2,6 sialosides, but exhibited different fine specificities. Although the specificities of the 3 transmissible viruses overlap (LM Chen, et al. *Virology.* 2012;422:105; S Herfst, et al. *Science.* 2012;336:1534;M Imai, et al. *Nature.* 2012;486:420), it is not clear if binding to a single type of glycan or multiple glycans is required for effective transmission in ferrets. Expanded glycan array libraries comprising glycans present on mammalian airway cells are needed to systematically evaluate receptor determinants required by influenza for aerosol transmission.

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The effect of cholesterol on cytokine expression in human lung epithelial cell and macrophage infected with 2009 pandemic influenza A virus

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Background: Nowadays obesity is considered as a chronic disease. Pandemic H1N1 (pH1N1) influenza virus had caused worldwide panic in 2009, and many statistics suggested that obesity may be one of the risk factors associated with higher mortality. Most of the deaths were caused by cytokine storm, and the effect of a high-concentration cholesterol environment on influenza virus infection is still unknown. Therefore, in this study we intend to explore the role of cholesterol in 2009 pH1N1 infection in human lung epithelial cell and macrophage. Materials and Methods: The pH1N1 virus strain used was isolated in the Virology Lab of the National Taiwan University Hospital in 2009 and propagated in MDCK cell in MEM containing 2 μg/ml TPCK-Trypsin. A549 and U937 cells were cultured in DMEM and RPMI-1640 medium, respectively. Cholesterol was dissolved in absolute ethanol before passing through a 0.22 μm filter. In immunofluorescence assay for sialic acid expression, cells were fixed with 10% formalin and stained with MAA-Avidin, Anti-biotin Cy3, and SNA-FITC. In addition, anti-F4/80 antibody was used to confirm that the U937 was induced to macrophage-like cell. In a cell toxicity assay, MTT was used to observe cell viability in A549 and MDCK, trypan blue exclusion assay used for U937. The mRNA expression of cytokines was measured by real-time qPCR using SYBR green system. Results: Virus growth curve showed no significant difference between growth in culture medium containing 0.04 mM and 0.08 mM cholesterol in MDCK. Cell toxicity assay showed that over 70% of MDCK and A549 survived, and no significant change was observed in U937 after treating with high concentration of cholesterol. The monoclonal antibody against F4/80, a macrophage-specific marker, was used to confirm that U937 was induced to macrophage-like cell. In immunofluorescence, we found that the expression of alpha 2,3- and alpha 2,6-linked sialic acid in MDCK and A549 was higher at high concentration of cholesterol. Higher expression of HA was observed, and significantly higher mRNA expression of IFN-beta, TNF-alpha,

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and IL-6 in MDCK cells infected with pH1N1 at high concentration of cholesterol. The expression of cytokines in A549 and macrophage-like cells also had dose-dependent results. We also observed that the expression of cytokines was suppressed when treating with lovastatin, a drug that inhibits cholesterol synthesis. Conclusion: In this study we demonstrated more expression of sialic acid in cells at high concentration of cholesterol and observed a significantly higher expression of cytokines and HA in MDCK infected with pH1N1. For A549 and U937 macrophage-like cell we also obtained some significant results.

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Assembly restrictions on the evolution of the influenza neuraminidase transmembrane domain and its impact on viral propagation

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Background: The signal anchor sequence of influenza neuraminidase functions both as a targeting sequence to the endoplasmic reticulum and as a transmembrane domain (TMD) that dictates the topology and trafficking of the protein. As such, the TMD must maintain its hydrophobicity and appropriate length to perform these functions that ultimately enable neuraminidase to contribute to viral spreading. Materials and Methods: The contribution of the neuraminidase TMD to the production of functional neuraminidase was evaluated in a transfection-based system. These results were combined with a bioinformatic approach that assessed the hydrophobicity and residue positioning of the neuraminidase TMDs over time, subtype, species (swine and human) and region (North American and Eurasian). This analysis was complimented with an experimental system (GALLEX) in *E. coli* that can monitor TMD associations, which enabled the mechanism of the neuraminidase TMD assembly to be determined and explained how the TMD contributes to the maturation of neuraminidase. Based on these results, genetic rescue viruses were generated with specific neuraminidase TMDs and the resulting effect on viral propagation was assessed in MDCK cells. Results: The TMD from subtype 1 neuraminidase (N1) is required for the production of functional N1. This is because the TMD is necessary to stabilize the stalk domain by tethering it to the membrane in a tetrameric conformation that matches the oligomeric state of the enzymatic head domain. The tetrameric assembly of the N1 TMDs were found to be dictated by key polar residues on the polar faces of what appears to be an amphipathic helix. This likely explains why the human subtype 1 neuraminidase TMDs have become less and less hydrophobic since 1918. Most intriguingly, the TMDs from the recent pandemic H1N1 strain of swine origin are the least hydrophobic to date, and this property could be used to track the Eurasian swine decent of the neuraminidase gene segment, suggesting that the TMD may in fact be a previously overlooked determinant. To test this possibility, several specific N1 TMD chimera viruses were generated. In various independent generations of the specific TMDs that we analyzed, additional mutational selection occurred only in the neuraminidase TMD that either increased or decreased the TMD hydrophobicity in a predictable manner that had an effect on viral propagation. Conclusions: The N1 TMD contributes to the assembly process by tethering the stalk to the membrane in a tetrameric conformation that is based on the polar characteristics in its amphipathic helix. The counter evolution of the N1 TMDs against the very feature that defines them, their hydrophobicity, and the ability to track the Eurasian origin of the N1 segment in pandemic H1N1 strain of swine origin highly suggests this is an overlooked determinant. Supporting this notion is the observation in our lab that only specific and characteristically predictable N1 TMD chimera viruses can be produced by recombinant genetics, further exemplifying the selective pressure on this potential key determinant.

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The increased pH threshold of the influenza virus HA fusion activation affects virus infectivity and efficiency of live and inactivated influenza vaccines

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Background: Influenza viruses enter the host cell via receptor-mediated endocytosis and deliver the viral genome to the cytoplasm by means of the fusion of viral and endosomal membranes. The threshold pH at which the hemagglutinin (HA) of influenza virus undergoes irreversible conformational change that is necessary for fusion is strain specific. Whereas for human influenza viruses, this modification takes place at a pH range of 5.1-5.4, avian viruses undergo conformational change at a pH of 5.6-6.0. The long term cultivation of human influenza viruses in chicken embryos or mammalian continuous cell lines often leads to HA mutations increasing the pH value of the HA conformational change, making HA less resistant to low pH and heating. We investigated the impact of HA stability on the virus infectivity, immunogenicity of live influenza candidate vaccines, and HA antigen content in the inactivated influenza vaccine preparations. Materials and Methods: Human influenza viruses of different subtypes and avian highly pathogenic viruses were used in the study. The pH threshold of the HA activation was measured in the hemolysis assay with human erythrocytes. Virus stability was estimated toward a low pH and an elevated temperature. Virus infectivity in mouse upper respiratory tract was determined by mouse ID_{50} titration. Viral uptake by the mouse respiratory epithelial cells was estimated using immunohistochemical staining. Virus immunogenicity was evaluated in ferrets after intranasal immunization. The total protein content was measured by Bradford assay. The quantity of immunocompetent HA antigen in inactivated purified virus preparations was determined by a single radial immunodiffusion assay. The mutation (K58I) was introduced by site directed mutagenesis. Results: We found that the H3N2 egg-derived viruses A/Brisbane/10/07, IVR147 and A/Uruguay/716/07 recommended by the WHO for vaccine production already showed decreased virus stability to an acidic pH and elevated temperature when compared to antigenically similar primary isolates. This phenotype was caused by HA1 (A138S, L194P) and HA_2 (D160N) substitutions increasing the pH threshold (by 0.2-0.6 pH units) of the HA conformational change. The subsequent propagation of egg-derived H1N1 and H3N2 seed viruses in MDCK and Vero cells induced HA_2 N50K (H1N1) and D160E (H3N2) mutations thereby improving virus growth, but further decreasing the virus stability. We showed that these mutations dramatically decreased the immunogenicity of live influenza vaccine candidates in ferrets. In addition, we demonstrated, that viruses with the low pH threshold of HA fusion have certain advantage during production of inactivated vaccine batches yielding the higher HA antigen content. Moreover, since all studied highly pathogenic avian influenza viruses are characterized by an elevated pH range of the HA conformational change, we assumed that low efficiency of H5N1 vaccines might be also explained by the unstable nature of their HAs. To prove this, we implemented a single amino acid substitution K58I, known to decrease the pH of HA fusion, into the HA_2 subunit of the A/Vietnam/1203/04 (H5N1) virus containing attenuated cleavage site. Resulted mutant virus acquired increased stability toward a low pH and an elevated temperature, displayed 30 times higher infectivity in the mouse upper respiratory tract, as determined by ID_{50} titration, and provided a better viral uptake by the mouse respiratory epithelial cells, as demonstrated by immunohistochemical staining. Conclusions: The threshold pH of the influenza virus HA fusion activation is an important host range factor and has a great impact on the efficiency of live and inactivated influenza vaccines by reducing virus stability, immunogenicity, and the HA antigen content.

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Hemagglutinin receptor binding preferences of seasonal and pandemic H1N1 influenza A viruses in Taiwan

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Background: Influenza virus infection is initiated by virus attachment to cell-surface sialoside receptor via influenza hemagglutinin (HA). The carbohydrate binding specificities have been found to be different among avian and human influenza A viruses and may affect the tissue tropism and transmission. Numerous reports have demonstrated that amino acids of the receptor binding domain of HA protein contribute to its relative binding affinities for α2-3 or α2-6 linked receptors. In April 2009, the first outbreak of pandemic influenza H1N1 in Mexico spread rapidly across the world via human-to-human transmission. Our sequence analysis revealed numerous amino acid variations in receptor binding sites between pandemic and seasonal H1N1 viruses. Materials and Methods: To investigate whether pandemic H1N1 viruses display different receptor binding specificity from seasonal H1N1, we compared the carbohydrate binding specificity of seasonal H1N1 influenza and the 2009 pandemic H1N1 strains that was isolated from Taiwan during 2000 to 2010 by using a high-throughput carbohydrate solution array. Results: Seasonal H1N1 accepted not only α2,6 sialylated glycans but also α2,3 sialylated glycans. However, some of the 2009 pandemic H1N1 viruses were predominantly bound to α2,3 sialylated glycans, and the viruses isolated in 2010 had changed the binding profiles from 2009, revealing the virus adaptation in the human population. These results indicated a distinct carbohydrate binding repertoire between seasonal H1N1 and the 2009 pandemic H1N1 viruses. Conclusions: This study provided not only systematic analysis of receptor binding specificities for influenza A clinical isolates, but also useful information to monitor the antigenic shift of influenza viruses. These findings may help us understand influenza virus infection and transmission.

P1-181

Genetic variants of influenza A (H1N1) pdm09 virus circulating in Kenya, 2010 to 2011

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Background**:** Since the emergence followed by rapid global spread to cause the 2009 pandemic, influenza A (H1N1)pdm09 viruses remained predominant among human influenza viruses that circulated in Kenya during the 2010-2011 season. In this study we sought to determine genetic variation amongst influenza A (H1N1) pdm09 viruses isolated in Kenya during the postpandemic period with reference to prototype influenza A/California/7/2009 strain and others obtained elsewhere during the same period. Materials and Methods: A total of 30 influenza A (H1N1) pdm09 isolates obtained from nasopharyngeal specimens (NP) and received from USAMRU-K's sentinel surveillance network covering the whole of Kenya were analyzed. Virus HA1 subunit of hemagglutinin gene segment was amplified by RT-PCR and nucleotide sequences determined followed by conversion of the sequences to amino acid code before performing phylogenetic analyses. Results: The study showed all Kenyan isolates had 6 parallel amino acid substitutions when compared with the prototype A/California/07/2009 vaccine reference strain. These included P83S, D97N, S185T, S203T, I321V, and E374K. Additionally, there were further mutations that occurred stochastically in individual isolates. Most of these changes, however, did not affect virus antigenic sites; except the P83S amino acid substitution at site Cb and a few other changes at sites Ca1, Sa, and Sb in individual isolates.

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None of the amino acid changes in the Kenyan isolates resulted in either gain or loss of N-glycosylation motif in the viruses. Phylogenetic analyses showed all Kenyan isolates clustered closely but separately from the vaccine strain. Conclusions: This study show that influenza A (H1N1) pdm09 viruses which circulated in Kenya during the 2010-2011 season had genetic changes in the HA1 of hemagglutinin gene segment not seen in the seasons A/California/07/2009 WHO vaccine strain recommendation. Most of these changes, however, did not affect the virus antigenic and glycosylation sites and therefore did not significantly alter the virus antigenic properties. This findings are in agreement with those from similar studies carried elsewhere and support the decision by WHO to retain A/California/07/2009 as the vaccine strain component for influenza A (H1N1)pdm09 viruses for the 2011-2012 season.

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Solving the complexities of influenza A virus infection of macrophages: macrophage galactose-type lectin can mediate virus attachment and entry

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Background: Infectious entry of influenza A virus (IAV) into macrophages (MΦ) represents an early event in recognition of the virus by the innate immune system. There is evidence to suggest that MΦ contribute to innate defence by limiting the release of newly synthesised IAV particles from infected cells through abortive replication, a “dead end” route of infection. However, MΦ have been reported to support productive infection and release of viral particles following exposure to some highly pathogenic IAV. In addition, seasonal IAV strains show marked differences in their ability to infect murine MΦ, including airway MΦ. Therefore, MΦ have been implicated in playing a major role in IAV disease pathogenesis. In this study, we performed experiments to improve our understanding of the complexity of early interactions between MΦ and IAV. Previously, we reported that the MΦ mannose receptor (MMR) can play a role in IAV infection. We now provide direct evidence that the MΦ galactose-like ligand (MGL) can serve as a cell surface attachment receptor for IAV, and importantly, facilitate virus entry into cells. Materials and Methods: Lec1 Chinese hamster ovary (CHO) epithelial cells express cell surface sialic acid (the primary IAV attachment receptor) yet are largely resistant to IAV infection. We made stable Lec1 cell lines expressing murine (MGL)-1 to directly assess the ability of MGL to serve as an IAV receptor by restoring permissivity to infection. We investigated the ability of MGL to serve as an entry receptor for IAV by constructing mutants lacking a portion of the MGL cytoplasmic domain containing the internalization motif. Moreover, we used RAW264.7 MΦ (expressing only MGL) and primary MΦ isolated from the peritoneal cavity of C57BL/6 mice (expressing MMR and MGL) or $MMR^{-/-}$ mice (expressing only MGL) to confirm a role for MGL in the enhancement of IAV infection of murine MΦ. Results: Expression of murine MGL-1 by Lec1 cells restored permissivity to IAV infection in the presence or absence of cell surface sialic acid, and infection was blocked in the presence of a multivalent inhibitor of MGL (asialofetuin). These data indicate that MGL can act as a primary IAV receptor or as a coreceptor with sialic acid. MGL expressed on Lec1 cells promoted rapid internalization of IAV, and this was impaired by mutation of the internalization motif, indicating that MGL can play a role in IAV entry into target cells. Up-regulation of MGL on mouse RAW264.7 MΦ correlated with increased sensitivity to IAV infection, and this could be blocked by asialofetuin. Furthermore, asialofetuin also blocked IAV infection of MΦ from $MMR^{-/-}$ mice, indicating that expression of MGL, in the absence of MMR, is sufficient to facilitate the infectious entry of IAV into MΦ. Conclusions: Together, these studies identify MGL as a likely cell surface receptor for IAV on MΦ. Mutations in the internalization motif of MGL indicate that this molecule may serve as an “entry receptor” rather than acting purely as an “attachment” receptor for IAV. This novel information regarding MΦ receptor-mediated recognition of IAV may provide some insight as to why particular IAV strains circumvent uptake and destruction by MΦ.

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Pandemic influenza mortality in developing countries is dramatically reduced by targeting interventions to those with endemic diseases

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Background: Low-income countries suffer the highest rates of morbidity and mortality during influenza pandemics. This is illustrated by data from the 1918/1919 pandemic suggesting much higher mortality rates in India (> 4%) compared with Europe and the United States (< 1%). Even during the mild H1N1 pandemic of 2009, 59% of the estimated 284,500 deaths occurred in low-income countries in Africa and Southeast Asia. We have previously shown that the illness attack rate in a developing country is ≈ 50% higher than that in developed countries. In the present study, we show that underlying predisposing conditions usually absent in developed countries cause an increase in case fatality rate. These include chronic respiratory diseases, tuberculosis, malaria, dengue fever, malnutrition, and HIV and helminthic infections. Using mathematical models for the similarly sized towns of Madang, Papua New Guinea (PNG), and Albany, Australia, we compared the impact of a moderate influenza pandemic in a developing vs developed country setting. Materials and Methods: Models for both towns are individual based and spatially explicit and use the latest census data. Each household is explicitly represented and populated with occupants categorized in appropriate age categories. The dynamics of influenza spread were modelled using (1) a transmission probability function, with parameters obtained from a literature survey, and (2) daily population mobility and resulting contact that was estimated using field data. The prevalence and risks of increased mortality due to coinfection with predisposing diseases (ie, chronic respiratory diseases, tuberculosis, malaria, dengue fever, and HIV and helminthic infections) were sourced from published literature from resource-limited tropical countries, or if not available, conservative estimates were applied. Results: We used a pandemic of moderate severity consistent with that which occurred in 1957, having a basic reproduction rate of 1.5 and case fatality rate of 0.25%. In an unmitigated pandemic with these baseline characteristics, the PNG model predicted an attack rate of 46%, a value almost 50% higher than that predicted by the Australian model. The increased attack rate resulted mostly from larger family sizes and greater person-to-person contact in PNG. In addition, due to the high prevalence of predisposing diseases, the case fatality rate was 0.39% in the PNG setting, an approximately 60% increase compared with that in Australia. Overall mortality in the PNG model was predicted to be increased by a factor of 2.2 when compared with the Australian setting (80 of 100,000 in Australia vs 179 of 100,000 in PNG). School closure and other social distancing interventions were found to be less effective in reducing the attack rate in PNG than in an Australian setting, due to lower school attendance rates and greater contact rates in PNG. Reducing the number of preventable and curable underlying comorbidities in PNG to near elimination levels (eg, helminths, malaria, and dengue, for which good progress has been made over the past decade) led to a decrease in case fatality rate, to 0.3% and thus an overall reduction in mortality of approximately 25%. Antiviral treatment and vaccination-based interventions targeted at comorbidity risk groups having predisposing diseases were found to be more effective at reducing the excess mortality rate due to influenza infection compared with generalised social distancing interventions. Conclusions: The results showed that developing countries suffer from higher mortality rates compared with industrialised countries during influenza pandemics for 2 reasons: an increased attack rate due to greater person-to-person contact and an increased case fatality rate due to coinfection with predisposing diseases. Long-term reduction of the number of malaria and helminth infections through control programs and the direct targeting of risk groups with known or easily diagnosable predisposing conditions (eg, HIV infection, tuberculosis, and malnutrition) with vaccine and/or antiviral-based interventions may significantly reduce pandemic mortality in developing countries. Our current model is limited by the unavailability of detailed data on the interaction of endemic diseases and influenza. Better data are urgently needed to improve our understanding of pandemic influenza spread in developing countries and to refine models used to quantify the effectiveness and cost effectiveness of interventions.

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The social resolution of influenza transmission in a human population

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Background: Changes in risk of infection for many human pathogens can be attributed to a combination of changes in behaviour and changes in our immune system. For respiratory viral infections, it has been proposed that self-reported social mixing patterns can be used to explain the behavioural component of infection, and theoretical studies based on these data are used routinely to assess public health policy. Although there is some statistical evidence from age-aggregated cross-sectional serologic data that such models capture infection risk, it is not conclusive. Further, it is not known what resolution of age-structured model—both in terms of number and size of age groups—reproduces observed attack rates best. Methods: We constructed a flexible model framework with which to compare alternate hypotheses, under the assumption that both age and contact behaviours are known. The population was divided into arbitrarily finely resolved age and contact classes, the latter based on self-reported social contacts. Our formulation generalised a number of previously published models: depending on the number of age groups and contact classes used, the framework produced a simple mass-action model, an age-structured formulation, or a model dependent only on the distribution of number of contacts. We compared predictions from the different models with self-reported behaviour data and serologically confirmed infection rate taken from a 2009 Hong Kong study, in which paired sera were used to identify which of the 762 participants had been infected (defined as a four-fold or more rise in neutralisation titre between baseline and follow-up visit). Results: We found strong evidence that an individual's risk of infection is strongly influenced by the average social mixing behaviour of their age group, rather than by their personal reported contacts. Further, we found that finely resolved age classes are required to reproduce the observed distribution of infection. The best performing model also assumed that transmission is based on reported physical contacts rather than on all reported contacts. This model successfully reproduced the observed drop in risk of infection after childhood and the small peak that occurs in individuals of parental age. Conclusions: Our results demonstrated that self-reported contacts have useful epidemiologic value, in that the average behaviour of a population can be used to predict individual infection patterns. Further, we have identified the likely social resolution of influenza transmission during the 2009 Hong Kong pandemic. Although different countries have different social and demographic structures, if key age transitions—specifically, the postchildhood drop in risky contacts and subsequent parental rise—are fundamentally important to epidemic dynamics, it should be possible to profile different countries' social structure and use these aggregate population data to tailor predictions about infection attack rates. Such information would be relatively straightforward to collect, and could prove valuable in the future when targeting interventions during an outbreak.

O-830

Origins of the basic reproduction number, R_0, and its value in influenza research

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Background: The basic reproductive number, R_0, was first introduced in the 19th century to examine population growth dynamics. After success in the early 20th century looking at the transmission

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dynamic of diseases such as malaria, it has, in later years, gained wider use in infectious disease epidemiology, notably foot and mouth disease and human influenza. From its early origins, in simple population growth dynamics, R_0 has gained prominence in the more complex world of infectious diseases such as influenza and has taken up the position of centre stage in pandemic planning and predicting the use of mitigation procedures. Much has been made of the potential power of R_0 as a useful tool, using values derived from historical pandemics, notably 1918, as a measure of its worth. Method: A literature review was carried out to explore the historical origins and parameters by which the concept of R_0 was derived. Complexity theory was used as a starting point to examine the plausibility of being able to derive R_0 and use this value to predict the course of an epidemic and the value of specific measures in its mitigation. Results: Unlike population growth dynamics, many factors come into play in defining the course of an epidemic of an infectious disease such as influenza: population density and mixing, case ascertainment in data capture, viral virulence, transmissibility, etc, all of which confound the predictive value of R_0 derived from early cases and hence its usefulness in examining the impact of preventative measures. Conclusion: The role of R_0 is well established in its field of origin in human population growth dynamics and has had some success in quantifying epidemic activity in simple infectious diseases, in closed communities. Because of the nature and unpredictability of influenza, more work needs to be carried out to understand the role of R_0 in directing the use of influenza mitigation measures. New emerging data from administrative health care systems provide a real opportunity to help with this understanding.

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The First Few Hundred—case and contact series investigations—experiences from the United Kingdom

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Background: The First Few Hundred (FF100) has been defined as the comprehensive epidemiological and virological investigation of the initial cases of a new strain of pandemic influenza virus and their close contacts. The approach was first developed following the World Health Organization's *Global Surveillance During an Influenza Pandemic (2009)* guidance and aims to contribute to the early assessment of the key characteristics of a pandemic or other new respiratory virus to inform prevention and control measures. This system has been designed to be easily applied to other novel pathogens, particularly those passed by the respiratory route. An FF100 investigation was carried out during the 2009 influenza pandemic in the United Kingdom (UK), which involved detailed follow-up of the initial laboratory confirmed pandemic cases and their close contacts. The investigation was instrumental in providing data used for real-time modelling predictions for this novel influenza virus and informing policy decisions. Following the pandemic, comprehensive assessments of the FF100 approach were carried out and recommendations for future improvements were made. This abstract serves to illustrate the developments that have taken place in the postpandemic era.
Materials and Methods: In order to implement the recommendations of the various postpandemic assessments, the following steps were taken:

- Creation of a FF100 Steering group
- Revision of the epidemiological investigation protocols
- Revision of the data requirements, including the role of the FF100 approach for other emerging respiratory infections
- Review and revision of the web-based informatics tool
- Review of biological sampling aproaches

These activities were carried out in consultation with numerous stakeholders including local health protection teams, modellers, statisticians, clinicians, virologists and public health experts. Results: A key development was the need for an individual pandemic case register. The case register serves to produce a national line list of probable and confirmed cases and is designed to count cases and to gather key data to rapidly understand the emerging clinical and epidemiological characteristics of a novel respiratory pathogen in time, place and person and provide initial case-severity estimates. The case register combines epidemiological data collected by local teams with laboratory data from the

reference and regional laboratories. In the event of a pandemic, the case register has been designed to also function as a sampling frame for the enhanced FF100 follow up investigation. This permits a sample of the first laboratory confirmed cases and their household contacts to be investigated in depth in the early stages of any pandemic. The data requirements of the FF100 investigation have been refined to enable efficient data entry. Web-based, integrated informatics tools have been developed to facilitate both the case register and FF100 system. The case register informatics system automates the integration, de-duplication and matching of laboratory and epidemiological data on a scheduled basis, enhancing with verified personal identifiable information. Confirmed cases are then selected from the case register to enter the FF100 enhanced investigation in a separate data collection system. Due to the emergence of Middle Eastern Respiratory Syndrome Coronavirus (MERS-CoV) and the first confirmed case detected in September 2012 in the UK, the FF100 informatics tool has been specifically developed to facilitate the collection of enhanced epidemiological information from any further cases of MERS-Co-V and contacts in the UK. Conclusions: Since the 2009 influenza pandemic, considerable progress has been made to implement recommendations and lessons learned for the early scientific investigations of a pandemic in the UK. Key developments include the implementation of a national case register that has the flexibility to be used in any emerging infection situation and also the revision of the data collection requirements for FF100 investigations. Preagreed protocols and operational guidance have been developed along with informatics tools. These protocols and informatics tools will be further field tested. It is hoped that this work will lead to a process which will ensure optimal early assessments of a pandemic.

O-832

Immunogenicity, effectiveness and population impact of influenza vaccine in England during seasonal and pandemic periods

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Background: In England influenza vaccine is recommended for those with chronic illness, healthcare workers and those aged 65 years or above. During the 2009 pandemic these recommendations were extended to include children and pregnant women, as well as all adults who wished to be vaccinated, but uptake was low and pandemic vaccine was not widely available until after the peak of circulation. During the 2010/11 wave of H1N1pdm2009 circulation vaccine was available prior to circulation. Vaccine immunogenicity and efficacy can be measured during trials but vaccine effectiveness and population impact needs to be measured in real-world settings. Materials and Methods: We conducted a household-level community cohort study (The MRC/Wellcome Flu Watch study) over the influenza seasons of five sequential years (2006-2011). Randomly selected households were followed up from autumn to spring with weekly reporting of respiratory illness. Participants were asked to submit nasal swabs on day 2 of any respiratory illness for RT-PCR identification of influenza. Baseline sera were examined for antibodies to circulating strains of influenza using haemagluttinin inhibition assays. We compared immunogenicity, effectiveness and population impact of influenza vaccine against influenza A during seasonal influenza periods (2006/7, 2007/8 and 2008/9), the 2009/10 pandemic autumn/winter wave and the 2010/11 pandemic wave. We calculated: the proportion vaccinated prior to recruitment and following recruitment; the proportion of those vaccinated prior to recruitment who had protective antibody titres of ≥ 32 to the dominant strain of influenza circulating; the age adjusted odds ratio of having protective titres in vaccinees compared to the unvaccinated (using logistic regression); vaccine efficacy (VE) against influenza A (1-age–adjusted incidence rate ratio)*100 using poisson regression comparing those vaccinated prior to recruitment and those who

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remained unvaccinated; and the “Population Prevented Fraction” (PPF - the additional percentage of cases that would have occurred in the hypothetical scenario of no one in the population receiving vaccine) using STATA punaf commands on poisson regression models of age adjusted effect of vaccine. PPF measures were weighted to the age and region structure of the population of England. Results: Amongst the 2051 participants followed up during periods of seasonal influenza, vaccine coverage was 26% (19% prior to baseline 6% after baseline). 66% of vaccinees had protective antibody titres (age adjusted OR 10.00, 95% CI 7.93-12.61, P < .001). VE was 61% (95% CI % 15%-82%, P = .017). The PPF was 19% (95% CI 9%-29%). Amongst the 3398 participants followed up over the 2009/10 pandemic wave vaccine coverage was 19% (4% prior to baseline and 15% after baseline). 54% of vaccinees had protective antibody titres (age adjusted OR 9.99, 95% CI 5.62-17.69). No PCR confirmed cases occurred in the participants vaccinated before baseline (VE 100% 95% not calculable). The PPF was 4% (-7%-15%). Amongst the 879 participants followed up over the 2010/11 pandemic wave vaccine coverage was 37% (21% prior to baseline and 16% after baseline). 77% of vaccinees had protective antibody titres (age adjusted OR 21.51, 95% CI 12.38-37.35). VE was 50% (-195%-150%). The PPF was 18% (-14%-62%). Conclusions: Influenza vaccine effectiveness against seasonal influenza was around 60% and at usual vaccine coverage levels prevents around 19% of cases that would otherwise have occurred. The analysis confirms that despite similar levels of vaccine immunogenicity and effectiveness during periods of pandemic influenza circulation the population impact of vaccine during the 2009/10 period of circulation was greatly diminished by late availability. This illustrates the need for accelerated vaccine production and/or universal vaccines to allow more effective population protection during a pandemic.

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Influenza A virus coinfection kinetics: gaining insight into pathogenesis through mathematical models

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Background: Influenza A virus infected individuals often become coinfected with a bacterial pathogen, which results in a significant increase in morbidity and mortality. A better understanding of how these pathogens interact with each other and with the host is of key importance. Materials and Methods: We pair quantitative data from infected mice with kinetic modeling and analyses to quantitatively describe how influenza A virus and *Streptococcus pneumoniae* influence each other, and how their virulence factors contribute to infection kinetics. Results: Our model and analyses suggest that infection with PR8 reduces the ability of alveolar macrophages (AMs) to phagocytose pneumococci, which leads to the observed outgrowth of bacteria; and that the subsequent pneumococcal infection enhances viral release from infected cells, which leads to the observed short rebound of viral titers. Subsequent data have confirmed our prediction that AM-mediated clearance of pneumococci is impaired, which may be due to a depletion of these cells during influenza infection. Conclusions: Together, these results provide new insights into the mechanisms of influenza coinfection and a platform for examining the processes involved in the interactions of pathogens during a dual infection.

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Potential population-level actions of CTL-inducing vaccines to mitigate pandemic influenza

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Background: Influenza vaccines that could protect against more than one season's viruses, and preempt emergence of pandemic strains, would be highly desirable. Observations in human populations support animal evidence for the importance of memory CD8+ T cell (CTL) responses to reduce disease severity and ongoing transmission of influenza, prompting development of vaccines containing highly conserved HLA class I restricted epitopes. We incorporated emerging insights from animal models regarding mechanisms of CTL-inducing vaccine protection into population-level epidemiologic models simulating emergence of a novel influenza strain. Optimal vaccine implementation strategies were considered within this framework, in the context of alternative assumptions regarding naturally acquired and vaccine-induced immune protection. Outcomes of interest included mitigation success and likely emergence of antigenically novel variants under immune selection pressure. Materials and Methods: We employed a susceptible-exposed-infectious-recovered (SEIR) model to perform scenario analyses of CTL-inducing vaccine impact. Vaccine was assumed to reduce infectiousness relative to naive hosts, by a factor between 25% and 75%. The population was divided into four strata, representing vaccinated or unvaccinated naive hosts (assumed mostly children) and vaccinated or unvaccinated influenza "experienced" hosts with some degree of prior immunity against the novel strain (assumed mostly adults). Mixing was either homogeneous or weakly assortative, with increased contact rates arising from the naive class. Four scenarios were considered: (A) a "virgin soil" outbreak in a population completely unprotected in the absence of vaccine; (B) the existence of partial CTL-mediated cross-protection from prior exposure was incorporated, with vaccine action saturating at a fixed efficacy threshold or in scenario (C) "boosting" prior immunity above natural levels; scenario (D) included the presence of neutralizing cross-protective antibodies in a proportion of hosts in addition to the assumptions of scenario (C). For each case, virus transmission characteristics were parameterized to ensure a moderate rate of spread (R_{eff} = 2) at pandemic onset, prior to vaccine implementation. Results: The impact of vaccine administered to a truly naive population (scenario A) was entirely predictable, with 50% coverage halving attack rates for the highest (75%) efficacy assumption. When prior immunity existed, even high levels of vaccine uptake did little to mitigate the pandemic (scenario B) unless the vaccine was capable of boosting protection in experienced adults (scenario C), in which case the impact of scenario A were recovered. Scenario D revealed a complex interplay between prior immunity and mixing assumptions. In the presence of cross-reactive sterilizing antibody, initial growth rates in the naive population were required to be higher to achieve the same "baseline" R_{eff} as in scenarios A-C, fuelling a rapid early epidemic. Moreover, vaccine administered to hosts protected by antibody was effectively "wasted," driving a requirement for higher population-level vaccine coverage to constrain epidemic growth. Prioritising vaccine administration to children was an efficient use of finite resources in all scenarios except A and was particularly critical in scenario D given the superior protection afforded by cross-reactive antibody compared with cellular immune responses. For the moderate pandemic scenarios considered here, and in the presence of prior immunity, a modest reduction in the probability of emergence of antigenically novel variants was observed with increasing population vaccine coverage (this reduction may be greater if vaccine further reduces within-host selection pressure). An exploration of more severe scenarios ($R_{eff} \geq 3.5$) revealed a tendency for vaccination to exacerbate antigenic drift, particularly if distributed over several months, resulting in prolongation of the epidemic. Conclusions: This analysis highlights present uncertainties in predicting the impact of widespread administration of CTL-inducing vaccines against a pandemic virus. These uncertainties derive from limited understanding of existing constraints of cellular immune responses on the virus population. Results of models that incorporate preexisting constraints indicate that CTL-inducing vaccines are likely to be most efficient when preferentially distributed to naive hosts. The impact of

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such vaccines is predicted to be greatest for more antigenically novel viruses such as H5N1 or H7N9, against which there is little or no preexisting humoral immunity in the population at baseline.

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A stepwise probability model for evaluating the bias and precision of influenza vaccine effectiveness estimates from observational studies

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Background: Because influenza vaccination is now widely recommended, randomized clinical trials are no longer ethical in many populations, and observational studies remain the only option for assessing influenza vaccine effectiveness (VE). The recently introduced test-negative design, in which patients with influenza-like illness (ILI) who seek medical care and test positive (negative) for influenza infection are considered cases (controls), is now the most popular study design to estimate influenza VE, due to its convenience. We developed a stepwise probability model for evaluating influenza VE estimates from observational studies and use it to compare estimates from test-negative studies with those resulting from case-control studies in which controls are selected from the entire community. Materials and Methods: Our model consists of five steps: vaccination, infection (with influenza virus or a different respiratory pathogen), developing an ILI, seeking care for ILI and undergoing a laboratory test for influenza infection, and the results of the test. The model's parameters are the conditional probabilities of the outcomes in each step given the outcomes of the previous steps. We used the model to obtain mathematical expressions, stated in terms of its parameters, for the true VE and for the bias of VE estimates (difference between estimated and true VE) from test-negative and case-control studies. These expressions were used to (1) derive conditions under which each estimate was unbiased and (2) conduct numerical comparisons between the estimates, using parameter values estimated from results of randomized clinical trials. When the bias of VE estimates from influenza studies is assessed, it is important to specify whether one is estimating VE against influenza illness or VE against medically attended influenza because the true VE depends on this distinction. We assume that the study design was not affected by the outcome of interest. Results: When VE against influenza illness is estimated, estimates from test-negative studies are unbiased under the following assumptions: (1) The test for influenza infection has perfect sensitivity and specificity, (2) the probability of a noninfluenza ILI is the same in vaccines and nonvaccines, and (3) the ratio of the probabilities of seeking care for ILI, comparing vaccines and nonvaccines, is the same for patients with an influenza or a noninfluenza ILI. When VE against medically attended influenza is estimated, the test-negative study-based estimate is unbiased under assumptions (1), (2), and assumption (4) that a person with a noninfluenza ILI has the same probability of seeking care regardless of vaccination status. The case-control study-based estimate is always biased, unless the vaccine is ineffective. In our numerical computations, we observed that when the VE against influenza illness was estimated, the absolute biases of estimates from the test-negative studies were usually smaller than those of the corresponding estimates from the case-control studies. On the other hand, when the VE against medically attended influenza was estimated, the estimates from case-control studies usually had a smaller absolute bias than the corresponding estimates from test-negative studies. When the VE against influenza illness was estimated, the biases of the estimates from the test-negative and case-control studies ranged from −12 to 18 and from −12 to 42 percentage points, respectively. On the other hand, when the VE against medically attended influenza was estimated, these biases ranged from −26 to 12 and from −1 to 1 percentage points, respectively. As expected, the absolute biases of both estimates increased as the ratio of the probabilities of seeking medical care for ILI between vaccinated and unvaccinated individuals moved away from 1. Conclusions: Our model facilitates the evaluation of the bias of influenza VE estimates from observational studies. We argue that it is important to specify the outcome (influenza illness or medically attended influenza) against which the vaccine is supposed to protect. In our model, test-negative studies produced less biased estimates when the outcome of interest was influenza illness,

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whereas estimates from case-control studies had smaller biases when the effectiveness of a vaccine in protecting against medically attended influenza was estimated.

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Reassessing antiviral distribution strategies for future pandemic events based on new conceptualizations of impact and controllability

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Background: Following the emergence of influenza A(H1N1)pdm in 2009, national governments have been evaluating the strengths and weaknesses of their pandemic plans. In Australia, pre-2009 plans focused primarily on responding to a severe pandemic with a 1918-like signature of high severity and high clinical attack rate (CAR). The generally mild nature of the 2009 pandemic posed challenges for targeted case-based intervention strategies designed to limit transmission, such as wide-scale antiviral deployment. Here we evaluate alternative strategies for the use of antiviral agents to minimize both individual- and population-level impact of infection and transmission. We conducted our analyses under a framework built on the operationally relevant concepts of pandemic impact and pandemic controllability. Materials and Methods: The mathematical model employed allows for a suite of factors that potentially affect program effectiveness, including (1) variations in the underlying transmissibility and severity of the epidemic; (2) the expected relationship between severity and "visibility" of the epidemic, in which there is an increased tendency for those with mild or moderate disease to seek medical intervention in epidemics of greater severity; and (3) logistical and capacity constraints on the community delivery of antivirals. We consider alternative uses for antiviral agents, dynamically distributed during the evolving epidemic: treatment of severe cases only, treatment of all "diagnosed" cases, or case treatment with provision of antiviral prophylaxis to a proportion of contacts. We further consider the provision of continuous pre-exposure prophylaxis to health care workers (HCWs) and explore the likely consequences of refocusing efforts toward those at greatest risk of severe outcomes following initial "whole of population" mitigation attempts. We assessed the utility of the alternative intervention strategies through measures including the final CAR of the epidemic and the early growth rate of the epidemic. We used a Latin-hypercube-sampling framework for our analyses, simulating many thousands of epidemics with varying biologic and epidemiologic parameters and assessed our findings across a range of prior assumed pandemic impact scenarios. Results: Consistent with previous modelling studies, treatment of only the severely ill rarely impacted population epidemic characteristics, with the exception of very high-severity, low-transmissibility epidemics. Extending treatment to mild and moderate cases in the community led to measurable reductions in the CAR for less severe epidemics of low intrinsic transmissibility. Additional use of antivirals for timely and continuing postexposure prophylaxis of contacts had the ability to further improve population-level outcomes under several pandemic impact assumptions. HCW pre-exposure prophylaxis was highly beneficial across all pandemic impact scenarios. Although a reduction in final CAR was only very rarely achieved for high-transmissibility scenarios, substantial benefits in terms of a reduction in case load were often achieved over the first month of an intensively applied community-based antiviral delivery strategy due to slowing of disease spread. Conversely, recognition of the resource-intensive nature of such an activity necessitates identification of indicators that mitigation attempts are unlikely to succeed, prompting a refocusing of the response toward a risk-targeted approach. In simulations in which the decision was made to refocus efforts, those strategies that delivered any measure of community-based drug distribution over and above treatment of the severely ill conferred additional benefits to the population. Such findings were particularly clear in models capturing an explicit "at-risk" subpopulation for which the risk of hospitalisation and death was enhanced. Conclusions: Using a model of antiviral distribution that accounts for the real-world complexities of case identification and distributional capacity, we explored the effectiveness of alternative use case strategies across a range of assumed pandemic impact scenarios. Our findings suggest that there is a clear rationale for maintaining an antiviral stockpile of sufficient size to allow for treatment of both hospitalised and mild/moderate cases because substantial additional benefits can be gained from logistically feasible community-based distribution strategies, without compromising

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management of the severely ill. Although such intensive case-targeted approaches may only be maintained over a period of several weeks, substantial easing of pressure on acute medical services in this time frame is anticipated.

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A preliminary report of the preparedness and response to avian influenza A(H7N9) infections, Taiwan, 2013

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Background: Outbreak of human infections with avian influenza A(H7N9) was reported by China in March 2013. This is the first time that humans infected by avian influenza A (H7N9) virus and cause fatalities. Human infections by this virus continue to be reported in China. Because of frequent cross-strait exchanges, human H7N9 infections in China are a menace to Taiwan society. This article described Taiwan's initial responses to A(H7N9) infections from 31 March to mid-April. Materials and Methods: The responses are collected and sorted from everyday work logs of the Central Epidemic Command Center for H7N9 influenza. Results: The Infectious Diseases Advisory Committee meeting convened by Department of Health on April 3 and recommended to enact the "Pandemic Influenza Preparedness Plan", using 4 major strategies and 5 defense lines in response. Listed the H7N9 influenza as Category V Notifiable Infectious Disease; organized the Central Epidemic Command Center for H7N9 influenza; and collaborating and managing the resources to ensure effective response with relevant departments. The preliminary responses are outbreak surveillance, border quarantine, medical system assembly, risk communication and response, antiviral medicine stockpile, and vaccine preparation. To response timely, continue updating the newest outbreak information in domestic and international societies. Conclusions: Although the possibility of human to human transmission is still low, we should remain vigilant in face of the A(H7N9) threats.

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Improvements made in pandemic preparedness in 32 countries between 2008 and 2012

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Background: The US Centers for Disease Control and Prevention (CDC) provides donor funding and technical assistance to support low and middle-income countries build and enhance their capacity for influenza surveillance and pandemic preparedness. Our objective was to measure and describe changes in pandemic preparedness among countries supported through bilateral cooperative agreements, and explore their related causes, using evaluations conducted between 2008 and 2012. Materials and Methods: In 2008, 2010, and 2012, 32 countries assessed and scored their pandemic preparedness across 12 capabilities, using a standardized tool developed by CDC. Median scores were calculated by country and preparedness capability and compared across years using the Wilcoxon rank-sum test. Additional methods for analyzing the data will be explored. Results: The following results are preliminary. All countries improved their pandemic preparedness between 2008 and 2012 and 23 of 32 showed statistically significant gains ($P < .05$). Increases in median scores were observed for all 12 capabilities between 2008 and 2012: country planning ($P < .01$), research ($P < .01$), communications ($P < .01$), epidemiology ($P < .05$), laboratory ($P < .01$), routine influenza surveillance ($P < .01$), national respiratory disease surveillance ($P < .01$), outbreak response ($P < .01$), resources for containment ($P < .01$), community-based interventions ($P < .01$), infection control ($P < .01$), and health sector response ($P < .01$). The biggest improvements in pandemic preparedness were made between 2008 and 2010 whereas between 2010 and 2012 four capabilities improved, 2

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remained the same and 6 decreased, including the capability of resources for containment that decreased by 17% (P < .01). Conclusions: The following conclusions are preliminary. Countries supported by CDC have made significant improvements in influenza pandemic preparedness between 2008 and 2012. Donor funding and technical assistance provided to countries is likely to have contributed to these improvements, though information on other sources of funding and support, for example, from country governments, is unavailable to study. In addition, improvements may have been precipitated by the global circulation of Influenza A (H1N1)pdm09 virus. Further research is necessary to determine the sustainability of pandemic preparedness.

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4Flu—an individual-based simulation tool to study the effects of vaccination on seasonal influenza in Germany

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Background: Influenza vaccines contain influenza A and B antigens and are adjusted annually to match the antigenic characteristics of circulating viruses. Before 1985, influenza B viruses only belonged to the B/Yamagata (B/yam) lineage, but since then, viruses of the antigenically distinct B/Victoria (B/vic) lineage have emerged. Since 2001, both B lineages have cocirculated with the influenza A subtypes A(H3N2) and A(H1N1). Current trivalent influenza vaccines (TIV) contain antigens of both A subtypes, yet only one B lineage, resulting in frequent vaccine mismatches. Since 2012, the WHO has recommended vaccine strains from both B lineages, paving the way for quadrivalent influenza vaccines (QIV) containing both B lineages. The aim of this study is to model the concomitant transmission of four influenza strains in Germany and to compare the effects of vaccination with TIV and QIV on infection incidence. Materials and Methods: We developed an individual-based stochastic simulation model for the simultaneous transmission of four influenza strains (A[H1N1], A[H3N2], B/vic, and B/yam) in a population that corresponds to one-thousandth of the German population, structured by age and risk status. The age distribution matched the German demography throughout the 50 years of simulation time. Individuals are connected in a dynamically evolving contact network based on the German POLYMOD age-mixing matrix. The transmission probability per contact is calculated by relating the largest eigenvalue of the contact network to the basic reproduction number R_0 and then multiplying by a seasonal forcing factor. Furthermore, infections are introduced at a low rate from outside. Special care has been taken to incorporate a realistic immunity model allowing for maternal protection, boosting of existing immunity, gradual loss of immunity, and cross-immunizing events between the B lineages and between drift variants. New drift variants are introduced on average after 3.5 (A[H3N2]) or 7 years (other variants). Only part of the existing immunity is active against newly introduced variants (simulated by a sudden loss of immunity for part of the population). Vaccinations are performed annually in autumn; coverage depends on the age of vaccinees, their risk status, and previous vaccination status. Vaccine efficacy depends on age; appearance of new drift variants may lead to a vaccine mismatch (ie, reduced vaccine efficacy). The first 10 years of simulation time are used to initialize the contact network, the next 20 years to initialize the immunologic and epidemiologic state of the population (applying the reported TIV coverage), and during the final 20 years, individuals either receive TIV or QIV, using a mirrored simulation approach, which allows for matched comparisons. Results: After calibration to observed incidence data, our simulations led to a median annual infection incidence of 10.6% in young adults. Compared with no vaccination, TIV reduced the annual incidence from 15,049,000 to 8,943,000 infections; QIV further reduced it by 4.3% or 11.2% of influenza B infections. As in any simulation study, our results strongly depend on the parameter values used. If the average duration of

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naturally acquired immunity is varied from 4 to 12 years (baseline, 6 years), the annual incidence decreases from 11,267,000 to 6,735,000 (TIV scenario), yet the percentage of infections additionally prevented by QIV remains constant at about 4.3%. If we selectively double the assumed duration of naturally acquired influenza B immunity to 12 years, influenza B infection incidence decreases, yet QIV still annually prevents 284,000 more infections than TIV. If R_0 is varied from 1.2 to 2.0 (baseline, 1.575), the annual incidence grows from 3,354,000 to 12,973,000 (TIV scenario), yet the annual number of infections additionally prevented by QIV remains nearly constant at 395,000 infections for $R_0 \geq 1.5$. Conclusions: 4Flu describes the spread of influenza viruses under different vaccination strategies in a dynamically evolving contact network, reflecting German demography. Our simulation results indicate that vaccination with TIV substantially reduces the number of influenza infections compared with no vaccination. Based on our model, QIV would further reduce influenza infection incidence by 4.3%.

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Defining a spatial model of disease transmission: the effect of resolution and contact on patterns of peak incidence

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Background: The emergence of moderate or severe pandemic strains of human influenza will challenge the capacity of key health care resources such as intensive care units. To anticipate the magnitude and timing of the peak incidence and to accurately assess the value of interventions designed to reduce peak incidence, we need a clear understanding of the underlying epidemic process across the spatial scale of interest. *We hypothesized that the representation of the population and the way its members travel over the geographic scale within which resources can be allocated may significantly affect the accuracy of model-based predictions.* Methods: In this work, we fixed the scale of interest (size of the whole region) and focussed on the resolution of the representation. Resolution refers to the number and size of the spatial units in the representation: higher resolutions have a larger number of small units (districts). Our aim was to determine the impact of resolution and mobility on key epidemic outcomes, such as peak incidence. We used a spatially explicit stochastic metapopulation model to determine the effect of the spatial resolution of the population density and the population mobility on the key epidemic outcomes. We simulated a pathogen representative of influenza with SIR natural history. The recovery time is on average 2.6 days and R_0 is 1.8. Mobility (mixing of the population) was determined by an offset power law kernel. We used both generated and real regional population data with urban and rural areas; the real data were obtained from LandScan data; we chose Guangzhou, Rio de Janeiro, Delhi, and New York. We simulated three mobility scenarios by varying the power in the kernel (and fixing the offset) and examined the impact on the epidemic outcomes of decreasing the resolution of the regional representation. Finally, we used Latin hypercube sampling (LHS) to investigate a range of kernel parameters in these populations to determine what mobility levels caused the epidemic to vary with resolution. Results: Cumulative attack rate (CAR) was the same at all resolutions and mobilities tested, but the epidemic trajectories (amplitude and timing of the peak incidence) were different. Clear thresholds for the resolution of the representation existed, such that resolutions below this threshold overestimated peak incidence. This effect was most obvious at lower mobility levels, when a high-resolution model resulted in increased epidemic duration and low peak incidence compared with a population with higher mobility, where the increased level of contact between districts facilitated the quick spread of the epidemic. These patterns were found in both the theoretical population and in our four chosen regions. LHS analysis confirmed that the effect of resolution is most important in populations that are less mobile. Conclusions: We showed that spatial models defined at the wrong resolution may result in the wrong peak incidence and epidemic properties such as speed of spread and duration. If accurate predictions can be made about the epidemic over a region of a certain scale, it greatly enhances the ability of public health officials to effectively limit the impact of the epidemic. On the other hand, if the resolution is incorrect (too low), not only is peak incidence overestimated but also

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the predicted time of the peak is much earlier, leading to the possibility that carefully timed control effects for the epidemic will fail.

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Pandemic preparedness in the WHO European region: why we must continue

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Background: Since the detection of the first human case of avian influenza A(H5N1) in 1997, the outbreak of SARS in 2003 and the launching of the revised International Health Regulations (2005) there has been a global focus on preparing countries to mitigate future outbreaks of infectious disease in particular influenza pandemics. The WHO Regional Office for Europe (Regional Office) has been working closely with Member States, the European Centre for Disease Prevention and Control, the US Centers for Disease Control and Prevention, and other international partners over the past decade to strengthen capacities to detect and respond to emerging pathogens. The WHO European Region is diverse with 53 member states ranging from high to low and middle-income countries. There are significant differences in the resources and technical capacities available to develop and improve pandemic preparedness. The 2009 pandemic showed that there are still gaps to be addressed if member states are to be prepared to face the next, possibly more severe, pandemic. In 2013, the world is reminded about the importance of well-developed preparedness plans with the emergence of avian influenza A(H7N9) in China and the Middle East respiratory syndrome—coronavirus (MERS-CoV). These events highlight the need for targeted interventions in the WHO European region in areas such as laboratory capacity, risk assessment, treatment of cases of severe acute respiratory infections and to have operational pandemic vaccine deployment plans which are all fundamental pillars in pandemic preparedness. Materials and Methods: Since 2003, the regional office has carried out more than 80 missions and 25 meetings and workshops covering all member states to improve capacities to detect and respond to emerging pathogens and thus improve pandemic preparedness in the region including: support the development of National pandemic preparedness plans, in-country national pandemic preparedness assessments, laboratory strengthening, simulation exercises, training in rapid response and detection, epidemiology and clinical management. Results: In the last decade, substantial achievements have been made such as: by 2009 national pandemic preparedness plans were published on national web sites by 38 countries; introduction of sentinel surveillance for influenza-like illness and hospital based severe acute respiratory infections in 9 eastern European countries; 8 member states developed plans for vaccine deployment to receive vaccine donation; the number of countries with the WHO recognized National Influenza Centres increased from 34 in 2003 to 41 in 2013. However, there are still gaps to be filled, for example only 4 countries have published revised pandemic plans following the 2009 pandemic, all situated in Western European Member States. Furthermore, 3 months after the emergence of A(H7N9) and MERS-CoV, approximately only 50% of member states have established the laboratory capacity to confirm cases of human infection. Finally, rapid sharing of information among clinicians for the management of severe cases is not well-established in the Region. Conclusions: The ongoing outbreaks of avian influenza A(H7N9) and MERS- are reminders that maintaining the capacities to detect and respond to emerging pathogens is as important as ever and that there is still a global need to improve pandemic preparedness. Laboratory capacities, surveillance systems and pandemic plans have substantially improved in WHO European region in recent years with the 2009 pandemic as an important real life exercise. But there are still important gaps to be filled: More generic preparedness plans covering all emerging respiratory pathogens, better integration of pandemic preparedness with implementation of the IHR and a more tailored approach to meet the divergent needs of countries in the Region is the way forward for the regional office. By applying a needs-oriented approach and focusing on the areas that are lacking behind, the regional office is striving to reduce the inequalities in pandemic preparedness in the region, in line with the WHO European policy for health—Health 2020.

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Cost effectiveness of quadrivalent influenza vaccination in England and Wales

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Background: The current seasonal influenza vaccines are trivalent, containing strains from both influenza A subtypes (H1N1 and H3N2) and from one of the two currently circulating influenza B lineages (Victoria or Yamagata). The aim of this study was to estimate the clinical impact and cost effectiveness of including both B lineages each year in a quadrivalent vaccine. Materials and Methods: The population of England and Wales was compartmentalised by immune and infection status (susceptible, exposed, infectious, recovered, and vaccinated), and a previously published age-stratified "SEIRS" dynamic transmission model (Pitman, R et al. *Vaccine*. 2013;31:927-942) was adapted for quadrivalent vaccination to model the temporal trend of influenza virus infection incidence. A conservative estimate of the duration of vaccine-induced immunity of 1 year was used over an analytical time horizon of 20 years. The dynamic nature of the model accounted for indirect vaccine protection (herd immunity). No cross-protection between influenza subtypes and lineages was assumed. Previously published estimates of the clinical burden (annual number of general practitioner [GP] consultations, hospitalisations, and deaths) attributable to influenza were used to estimate the recent age-stratified probabilities of each of these outcomes following influenza virus infection. The model then simulated the disease burden of influenza, for each vaccination scenario, in terms of clinical, quality of life, and economic burden. The costs and outcomes of quadrivalent vaccination were compared with those of trivalent vaccination, assuming price parity with trivalent equivalent. Results were subject to extreme value, univariate, and probabilistic sensitivity analyses. In the base case, all of the quadrivalent and trivalent vaccines administered in children and adolescents were assumed to be live attenuated. Alternative scenarios explored the implications for cost effectiveness of varying the live-attenuated proportion for quadrivalent vaccines at a fixed level of uptake. Results: Given the variability of influenza, ranges of clinical burden are presented to encompass the best and worst possible cases, as estimated by the extreme value analysis based on currently available data. The range of infections averted by the use of quadrivalent vaccination, relative to trivalent vaccination, was estimated to be 11,000 to 5,000,000 infections in an average year. This reduction resulted in an average annual decrease of between 800 and 500,000 GP consultations, between 40 and 33,000 hospitalisations, and between 10 and 15,000 deaths. The inclusion of both influenza B lineages in the seasonal vaccine is expected to save costs. In the probabilistic sensitivity analysis, quadrivalent vaccination was estimated to save a cumulative discounted sum, over 20 years, of between 630,000 and 1,550,000 QALYs and between £660m and £2300m in costs. In all sensitivity analyses, quadrivalent vaccination saved costs. In the alternative scenarios, exclusive use of quadrivalent live-attenuated vaccine to immunise all child and adolescent vaccinees, at 50% uptake, was cost effective relative to scenarios in which any quadrivalent inactivated vaccines were used in these age groups. Conclusions: The use of quadrivalent seasonal influenza vaccines, at price parity with their trivalent equivalent vaccines, appears to be highly cost effective and most likely cost saving. The use of quadrivalent live-attenuated vaccine in children and adolescents appears to be cost effective relative to the use of quadrivalent inactivated vaccine. This study was sponsored by AstraZeneca.

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Coverage of high risk groups for influenza vaccination in South Africa, 2011-2013 influenza seasons

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Background: Influenza is a major public health threat throughout the world, causing substantial morbidity and mortality each year. In South Africa, it is estimated that 5000 to 10,000 deaths and 70,000 to 100,000 hospitalizations are due to influenza each year. To mitigate the effect of 2009 influenza A(H1N1) pandemic, the South African Department of Health initiated the first national influenza vaccination campaign in 2010. Subsequently annual vaccination campaigns were held, targeting individuals at increased risk for severe complications from influenza, which included young children, the elderly, women who are pregnant or postpartum (within 2 weeks after delivery), and persons of any age with underlying medical conditions (such as heart disease, lung disease, and HIV infection) . The vaccines were available at public health facilities nation-wide. This review aims to describe the influenza vaccination coverage among the targeted high-risk groups. Materials and Methods: Trivalent inactivated influenza vaccine was centrally procured at the national level and distributed to provincial depots for further distribution to public health facilities. Provinces had a 6-8 week campaign period to administer the vaccines to the high-risk groups. All vaccinations were recorded daily on tally sheets and were sent to the district level for collation and analysis. High-risk group populations were accessed from the South African National Department of Health, Statistics South Africa (Stats SA), the Actuarial Society of South Africa AIDS Demographic Model 2008lite and the South African Health Review 2011. Results: The number of vaccines annually procured was 924,469, 845,350, and 800,000 in 2011, 2012 and 2013, respectively. The number that was administered was 833,085 (90.1%), 706,374 (83.6%) and 711,910 (89.0%) in 2011, 2012 and 2013, respectively. The reasons some vaccine was not administered were due to inadequate planning in some districts and subdistricts, eg, there were inadequate staff, transport, supplies, and data management. Influenza vaccinations in South Africa falls under the responsibility of the Communicable Disease Control programme whereas other vaccinations fall under the Expanded Programme on Immunisation. Therefore coordination of all the relevant role-players posed challenges. Late delivery of vaccines to facilities and inadequate social mobilisation were also major challenges. In South Africa in 2011, it is estimated that 20.5 million individuals of a total population of 51.7 million fall into at least 1 of the high-risk groups (children 6 months to < 5 years = 5.1 million, pregnant women = 0.8 million, adults > 65 years = 2.5 million, individuals with specific high-risk underlying conditions including HIV = 11.6 million). Vaccine coverage among the high-risk groups for 2011, 2012, and 2013 were estimated as follows: < 5 year olds—4.4%, 3.2% and 3.3% respectively, pregnant women—13.8%, 14.1% and 12.3% respectively, > 65 year olds—2.0%, 2.2% and 2.1% respectively and > 5 years of age with chronic medical conditions, including HIV—5.3%, 5.0% and 5.4% respectively. Conclusions: Coverage estimates for the high risk groups in the public health sector were relatively stable over the last 3 years. Our results indicate that not all the vaccines were utilised despite the target population (≈ 20.5 million) being much higher than the available vaccines. This raises concern as the country is in the process of expanding its influenza vaccination programme. Of particular interest is that coverage in the high risk group of pregnant women is higher than in other target groups. Seasonal influenza vaccine coverage should be closely monitored in coming years to identify specific barriers to vaccine uptake as this would be important to inform strategies to improve influenza vaccine coverage.

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Using simulation to evaluate enhanced pandemic influenza surveillance strategies

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Background: The collection of enhanced epidemiologic data in the early stages of an influenza pandemic can help to estimate its likely impact and inform decision making about the appropriate scale of response. Strategies for enhanced surveillance include monitoring household members and nonhousehold contacts of detected cases for illness. This approach was used in the United Kingdom during the 2009 H1N1 influenza pandemic to collect enhanced data on the "first few hundred" confirmed cases. The key characteristic of interest is the reproduction number R_0, which provides information on how quickly a pathogen is likely to spread through a population. Enhanced surveillance can expedite the accurate estimation of R_0 by increasing the rate of case detection. Estimates of R_0 can help inform decision making about the optimal use of antiviral stockpiles and the suitability and timing of social distancing measures such as school closure. In addition, there is growing awareness that estimation techniques must be sensitive to biases introduced by disease visibility and the surveillance and testing strategies that are used. For example, previous work has demonstrated that transmissibility estimates based on early detected cases can be too high because higher transmission events are likely to be overrepresented early in an epidemic outbreak. It is therefore desirable to explore the effects of reporting and testing biases in surveillance strategies so that, when possible, they can be accounted for when estimating disease parameters. An effective surveillance plan must balance the need to collect sufficient data to enable accurate estimation of disease parameters against the sustainability of obtaining quality data alongside other response activities. Here we describe a simulation model for evaluating alternative enhanced surveillance strategies to develop an evidence basis to inform optimal contact tracing efforts. The model further allows consideration of when enhanced data collection may be stopped, based on the precision of the estimated epidemic parameters. Materials and Methods: We used computational models to simulate both a disease outbreak and a surveillance strategy. The output of the disease model provides a "true" picture of all cases occurring in the outbreak, whereas the output of the surveillance model provides the subset of cases that we anticipate will be detected using that particular strategy and hence which are available for the purposes of parameter estimation. We simulated disease spread using a susceptible-exposed-infectious-removed (SEIR) model in a population with demographic, household and contact network structure. Demographic and household structure are taken from recent Australian census data, and contact network structure is based upon contact data collected in the European POLYMOD study. Different outbreak scenarios can be simulated by modifying disease parameters, including the duration of exposed and infectious periods, transmissibility, and clinical severity (which may influence infected cases' propensity to seek medical attention and hence alter the likelihood of detecting cases). Different surveillance strategies can be simulated by varying the number of contacts who are monitored over and above immediate household members, the duration for which they are monitored, and the number of cases collected before enhanced data collection is halted. Results: Initial simulations revealed the extent to which the effectiveness of any given surveillance strategy will depend on the transmissibility and severity of the disease. For example, even when transmissibility is low, if a disease is relatively mild (and visibility is consequently low), the outbreak may have been underway for some time before the first case is detected. The subsequent rapid growth in cases has the potential to exhaust data collection capacity almost as rapidly as would be expected in a more transmissible and severe (and hence more visible) scenario. Conclusions: Simulation models provide useful frameworks for the evaluation of pandemic influenza surveillance strategies, facilitating the development of evidence-based data collection protocols driven by key information needs.

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Estimates of age-specific influenza-related mortality and hospitalizations in the United States from 1997 to 2009

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Background: Granular estimates of the burden of influenza can inform vaccine policy and guide prevention and control efforts. However, direct assessment of the burden of influenza is confounded by the fact that influenza cases are rarely confirmed using laboratory tests and that clinical manifestations are nonspecific and can vary from symptoms of mild common cold to severe pneumonia. Indirect statistical modeling techniques have been used by the Centers for Disease Control and Prevention (CDC) in the United States to assess the total burden of influenza. In the present study, we adapted these statistical modeling methods to generate updated granular estimates of the number of influenza-attributable deaths and hospitalizations in the United States, stratified by age and risk status for 12 influenza seasons, while controlling for the concurrent circulation of respiratory syncytial virus (RSV). Materials and Methods: Using data extracted from the Nationwide Inpatient Sample (NIS) and US National Vital Statistics System throughout 12 influenza seasons (July 1997 to April 2009), we generated time series for respiratory deaths (any mention), defined as all deaths with a "J" code listed anywhere among the recorded ICD-10 codes, for age groups < 5, 5-17, 18-49, 50-64, and ≥ 65 years. Similarly, using data extracted from the NIS, weekly time series for influenza-related hospitalizations were generated for the same age groups and risk statuses (according to comorbidities). A multiple linear regression model was applied to each age stratum to statistically associate respiratory deaths to influenza A or B, while controlling for RSV. The model incorporated US CDC weekly and seasonal virological surveillance data to track the epidemiologic patterns and seasonal variations of RSV and influenza incidence by type and subtype. Population census data were used for denomination. Results: For the 12-year period included in the study, across all ages, the model attributed a seasonal average of 19,100 respiratory deaths in the entire US population to influenza (using a broad definition of respiratory mortality that included any mention of respiratory disease, cough, abnormalities of breathing, fever, or viral infections not otherwise specified); 28,169 cardiorespiratory deaths (any mention); and 10,682 pneumonia and influenza (P&I) deaths (any mention). On average, influenza A accounted for 71% of influenza-related deaths, whereas influenza B accounted for 29% of influenza-related deaths across all ages. Influenza A-related mortality exceeded that related to B in all except three seasons (2000-2001, 2002-2003, and 2008-2009, for which influenza B-associated mortality accounted for 95%, 78%, and 84% of all influenza-attributable deaths). Regardless of influenza type, the rates of influenza-attributable deaths increased with age, with an estimated annual average of 86% of all influenza deaths (16,504) observed in the ≥ 65 years age group. By comparison, individuals aged < 18 years accounted for only 183 influenza deaths (1%). During the study period, the model attributed to influenza a seasonal average of 297,548 respiratory hospitalizations (using the broad definition [any mention]), 350,212 cardiorespiratory hospitalizations (any mention), and 151,224 P&I hospitalizations (any mention). Influenza-attributable hospitalizations increased with age, with individuals aged ≥ 65 years accounting for 53% of all influenza-attributable respiratory hospitalizations compared with 12% in individuals aged < 18 years. Rates of both influenza-attributable deaths and hospitalizations were 8- to 9-fold higher in the high-risk group than in the low-risk group (influenza-attributable all-age respiratory deaths per 100,000 persons: 18.9 vs 2.3; influenza-attributable hospitalizations per 100,000 persons: 179.7 vs 20.3). Conclusions: These data suggest a substantial burden of influenza-related mortality and hospitalizations in the US during the last decade. The mortality and hospitalization estimates are consistent with previous results and provide an up-to-date assessment of the possible burden of influenza in the United States. These estimates are critical to guide decision making and public health communication. Funding: GlaxoSmithKline Biologicals SA; ClinicalTrials.gov: NCT01599390.

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The global mortality impact of the 2009 influenza pandemic: report from the GLaMOR project

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Background: Assessing the 2009 influenza pandemic mortality impact is essential for optimizing public health responses to future pandemics. WHO reported 18,631 laboratory-confirmed pandemic deaths, but the total pandemic mortality burden was likely substantially higher. We set out to estimate the 2009 pandemic mortality burden through statistical modeling of mortality data from multiple countries. Material and Methods: We obtained weekly virology and national age-specific underlying cause-of-death time series for 2005-2009 for 20 countries and administrative zones covering ≈ 35% of the world population. In a first stage, we applied a multivariate linear regression model to measure pandemic respiratory mortality in each collaborating country. In stage 2, we used 10 country indicators in a multiple imputation model to project the mortality burden in all world countries. We used a variety of external data sources and sensitivity analyses to validate our findings. Results: Between 138,000 and 189,000 (range, 123,000 to 203,000) influenza A/H1N1 pandemic respiratory deaths occurred globally during April-December 2009. The majority of these deaths (62%-85%) occurred in persons aged < 65 years. The mortality from all causes of death was likely twice as large but could not be measured with precision. We found a striking regional heterogeneity, with up to 20-fold higher mortality in the Americas than Europe. For comparison, the model attributed 210,000 (range, 148,000-249,000) respiratory deaths to influenza in an average recent season, with only 19% in persons aged < 65 years. Study limitations include a limited economic and regional diversity among stage 1 countries and an inability to study mortality in subsequent pandemic waves (2010-2012). Conclusions: Our conservative global pandemic respiratory mortality estimate is ≈ 10-fold higher than the WHO laboratory-confirmed mortality count. Although the pandemic mortality burden estimate was similar in magnitude to that of seasonal influenza, there was a marked shift toward mortality among the young so that many more life-years were lost. The burden varied greatly among countries; some carried virtually no mortality burden, whereas others suffered a “worst-in-a-decade” influenza event. A collaborative network to rapidly collect and analyze mortality and hospitalization data in global settings is needed to quickly establish the severity of future pandemics and improve the public health response. Work should be attributed to Netherlands Institute for Health Services Research (NIVEL), Utrecht, The Netherlands. Support: This study was supported by the WHO on a contract with NIVEL (APW 20043611 and 200617995). LS acknowledges funding support from the RAPIDD program of the Science and Technology Directorate, Department of Homeland Security, and the Fogarty International Center, National Institutes of Health. MDVK acknowledges salary support from the Medical Research Centre and the Bill and Melinda Gates Foundation. Acknowledgements: The authors thank the following members of the WHO Task Force for their advice and support: I

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Model estimates of age-specific influenza-related outpatient visits in the United Kingdom in recent years

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Background: Accurate assessment of the overall burden of influenza is difficult; physicians usually rely on clinical observation for a diagnosis, which is rarely confirmed with laboratory testing. Furthermore, other viruses can cause “influenza-like” syndromes (eg, respiratory syncytial virus [RSV], human metapneumovirus). Virus-guided regression modeling techniques allow estimation of influenza burden while controlling for the potential influence of other circulating respiratory pathogens. Using this method, Pitman et al (*J Infect*. 2007;54:530-538) estimated the number of influenza-associated general practitioner (GP) consultations for 2002-2003 in England and Wales. We used similar methods to update the burden of outpatient visits related to influenza types by age groups in England and Wales between 1996 and 2008. Methods: Virology data from the Health Protection Agency national surveillance system were used to generate weekly time series of numbers of specimens positive for influenza A or B or RSV. Using data extracted from the General Practice Research Database (GPRD), we generated weekly time series for multiple influenza-related health outcomes. These included all respiratory disease broadly defined (all READ H codes plus cough, otitis media, and viral infections not otherwise specified), influenza-like illness, pneumonia, and influenza for age groups < 5, 5-17, 18-49, 50-64, and ≥ 65 years. We applied a similar multiple linear regression model to each age stratum to statistically associate GP office visits with influenza A or B while controlling for RSV. The final model was optimized by the exclusion of summer months, trimean (the mean of the previous, current, and following observations) smoothing of the time series, consideration of two periods for the pathogen time series (seasons until 2000-2001 and later), and lagging of the RSV viral series by 2 weeks for GP office visits for patients aged > 18 years. Weekly denominators were generated from the GPRD data. Results: For the 13-year study period, the average number of seasonal influenza-attributable GP office visits using the broad definition of respiratory disease was 857,996. More than one-third of these visits (322,117 [37.5%]) occurred in children aged < 18 years. The relative proportion of GP office visits related to influenza A and influenza B was 76% and 24%, respectively. For most respiratory outcomes, the rates of GP office visits related to influenza A was relatively constant across all age groups and higher than those related to influenza B, except for OM for which no burden of influenza A or B was observed in persons aged ≥ 65 years. The highest influenza B burden was observed in children aged 5-17 years (996 visits/100,000 persons, comparable to influenza A only in this age group) and decreased with age, reaching 76 visits/100,000 persons in the ≥ 65 years age group. The highest influenza A burden was observed in children aged < 5 years (2436 visits/100,000 persons). Conclusions: In this study, we generated critical burden parameter estimates needed to accurately model the economic impact of influenza. Detailed and updated estimates of the total burden of influenza, including how it affects various age groups and groups at risk for influenza complications, are essential to optimize decisions

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Assessing changes in vaccination programmes of seasonal influenza: an evidence synthesis and modelling study

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Background: Influenza vaccine policies that maximise health benefit through efficient use of limited resources need to be identified. In most countries, influenza vaccination programmes have targeted individuals over the age of 65 years and those in high-risk groups, in line with WHO recommendations. Although an increasing wealth of longitudinal surveillance data sets are available, integrating the multiplicity of data sources to inform the construction of realistic models to test alternative strategies remains challenging. Here we synthesize information from surveillance data sets in England and Wales to evaluate the impact of changes in the seasonal influenza vaccination programme. Materials and Methods: Using a contemporary evidence-synthesis statistical approach, we optimised use of virological, clinical, epidemiologic, and behavioural data to develop an age- and risk group-stratified transmission model that reproduces the timing and magnitude of the strain-specific behaviour of influenza over 14 seasons in England and Wales, having accounted for the vaccination uptake over this period. We estimated the reduction in cases and deaths achieved by the historical programme compared with no vaccination and the reduction had different policies been in place over the period. Results: We found that the current vaccination programme has led to an average reduction of 0.4 infections per dose of vaccine and 2 deaths per 1000 doses. Targeting transmitters by extending the current programme to 5- to 16-year-old children would increase the efficiency of the total programme, resulting in an overall reduction of 0.68 (0.52, 0.81) infections per dose and 2.1 (1.4, 3.6) deaths per 1000 doses. In comparison, choosing the next group most at risk (50- to 64-year-old adults) would only prevent 0.43 (0.35, 0.52) infections per dose and 2 (1.2, 3.3) deaths per 1000 doses. Conclusions: This study proposes a framework to integrate influenza surveillance data into transmission models. Application to data from England and Wales confirms the role of children as key infection spreaders for seasonal influenza. The most efficient use of vaccine to reduce overall influenza morbidity and mortality is thus to target children.

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Would targeted interventions change health outcomes in an influenza A (H7N9) pandemic?

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Background: Mathematical models can be used to predict the spread of influenza and the effectiveness of alternative health policies in mitigating influenza pandemics. Models range in complexity from simple models representing homogenous populations to highly detailed representations including ages and comorbidities. We set out to create a mathematical model of the

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spread of pandemic influenza to determine the impact that targeting interventions to individuals at higher risk of influenza-related mortality due to age or comorbidities has on health outcomes. Materials and Methods: We used MatLab (MathWorks, Natick, Massachusetts) to create a stochastic susceptible-exposed-infected-recovered (SEIR) disease transmission model with output parameters reflecting a severe influenza pandemic. We divided the infected population into three subcategories representing asymptomatic individuals, symptomatic circulating individuals, and symptomatic noncirculating individuals. Asymptomatic individuals were not at risk of influenza-related mortality, and symptomatic noncirculating individuals have reduced interaction with the rest of the population. We used differential equations to represent age groups and comorbid subpopulations with unique hospitalization rates and mortality rates. We then adjusted the size of each subpopulation to represent the demographic breakdown of New York City. We based our transmission matrix on contact patterns between age groups to estimate age-specific infection patterns. We performed sensitivity analysis on all input variables and calibrated the model so that the attack rate and case-fatality proportion were similar to those seen in 1918. After performing a base-case analysis with no vaccination, we compared two vaccine distribution policies: (1) vaccinating 20% of the population in a nontargeted fashion and (2) vaccinating 20% of the population with the highest risk of influenza-related mortality based on age and comorbidities. We tested our vaccine distribution policies in two vaccine preparedness scenarios: (a) vaccine distributed four months after the start of the pandemic and (b) vaccine distributed nine months after the start of the pandemic (as occurred in the 2009 pandemic). We used infections and deaths averted to determine the effectiveness of each health policy. Results: In our base case with no vaccination, the attack rate was 32.1% (2,621,400 infected individuals) with a case-fatality proportion of 2.49% (65,402 influenza-related mortalities). If we assume that our vaccine will be ready for distribution nine months after the beginning of the pandemic, both nontargeted and targeted vaccination policies resulted in nearly identical numbers of infections (2.62 million in our city of 8.2 million individuals) and deaths (65,402) as our base case. If vaccines were distributed four months after the start of the pandemic, nontargeted vaccination would avert 7500 infections and 199 deaths and targeted vaccination would avert 5000 infections and 251 deaths. We performed threshold analyses; for targeted vaccination to avert 5% more deaths than untargeted vaccination, it would have to be implemented 51 days after the start of the pandemic. Conclusions: We compared health outcomes of targeted versus nontargeted vaccination during a severe influenza pandemic. We found that the timing of vaccination determines the importance of targeting the intervention to groups at higher risk of mortality secondary to their age or comorbidities. When vaccination occurs four months or nine months after the start of the pandemic (similar to the 2009 pandemic), much of the population has already achieved natural immunity through infection and recovery, making targeting high-risk groups less effective and limiting the effectiveness of the intervention itself. If vaccine production technology improved so that distribution could occur sooner, a significant proportion of the population would still be susceptible, and targeting high-risk groups could significantly reduce the number of influenza-related deaths. We note that our model does not target individuals more likely to transmit disease (such as children); additional research should be conducted to determine the value of prioritizing such groups for vaccination. Nevertheless, our results show that modeling high-risk subpopulations separately may only be important in making policy decisions when extremely rapid mass vaccination is possible, technology that is currently unavailable.

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Estimated proportion of antivirals given to influenza cases in the UK during the second and third waves of the 2009 H1N1 pandemic—results from the Flu Watch Study

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Background: Antivirals are a major component of pandemic preparedness plans. In order for antivirals to make a population-level impact during a pandemic, they need to be correctly targeted at people with influenza. We used community level estimates of PCR-confirmed influenza illness and antiviral use from the Flu Watch study to estimate the national proportion of PCR confirmable influenza cases who received antiviral treatment. Materials and Methods: Flu Watch is a community level, household-based cohort study in the United Kingdom. This analysis reports antiviral use during the second and third waves of the UK pandemic (autumn/winter 2009/10 and winter 2010/11 respectively). Participants filled in weekly prospective illness surveys asking about any respiratory symptoms and any medicines (prescribed or over-the-counter) taken for those symptoms. They were also asked to self-administer nasal swabs on day 2 of any respiratory illness for RT-PCR identification of influenza. We measured the proportion of PCR confirmed influenza A cases who were treated with antivirals and estimated the population-level rates of PCR-confirmed influenza cases who were treated with antivirals. We applied these rates to census data to estimate the number of national antiviral courses used on people with influenza. We then divided this estimate by external data on the number of national courses dispensed (Mytton O, et al. *Euro surveill.* 2012;17(14):15) to estimate the proportion of dispensed courses taken by people with PCR confirmable influenza. Results: Only 2% (0%-6%) of 144 PCR-confirmed influenza cases received antivirals during their illnesses. After applying Flu Watch rates of antiviral-treated PCR-confirmed influenza to the national population, we estimate that only 5% (2%-16%) of the 1.2 million courses dispensed nationally were used by people with PCR-confirmable influenza. Conclusions: Despite the wide availability of antivirals during the second and third waves of the pandemic, only a small proportion of those with PCR confirmed influenza received antivirals minimizing the potential population-level impact. In addition, even given the fact that our study will have missed PCR-confirmable cases our analysis suggests that a high proportion of antivirals that were used are likely to have been dispensed to people without influenza. In the United Kingdom, most of the antiviral courses in the second wave of the pandemic were prescribed by pandemic telephone and website services. Difficulties in ensuring the correct people receive antivirals may be exacerbated when patients are assessed over the phone or internet. In order to maximize the population-level impact of antivirals, we need better point of care tests to distinguish influenza illnesses from other respiratory illnesses. Given the difficulties in ensuring good uptake and distribution to those with influenza it may be more effective to target resources on those with the greatest risk of complications (ie, those with chronic disease who are more at risk of serious outcomes).

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Anydemic, a new kind of real-time stochastic model for decision-making: feasibility study

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Background: During an epidemic or pandemic situations, decision-makers use models to compare or to adapt strategies, particularly small world models. However, there is a need for designing stochastic models allowing many more options such as a multiagents-based population model (in a ratio of 1/100 for instance), several ways of life and status, various kinds of asymmetric relations between agents, several simultaneous outbreaks, and heterogeneous exposure to epidemic infectious agents. Such a model must be able to take into account geographic data and to compare various strategies combining several interventions. Ideally, to be used during a health crisis, this model must input real-time epidemiologic data and use software running fast on a personal computer or on mobile technologies. This work aims to 1) design a stochastic model based on a multiagent systems (MAS) paradigm, which allows simulation at different levels (individual and global) and has all the advantages listed previously, and 2) evaluate a prototype version called Anydemic 1.0, simulating a seasonal influenza outbreak in one country (France). Methods: *Mathematical approach:* All calculations are based on a stochastic pretopologic approach, mixing stochastic methods and a multiagents frame described with a pretopology modeling. Each parameter is defined either by a Poisson distribution or by a triangular distribution (minima-maxima interval, mode). *Software:* Data collected in text format are transformed into frames, using TOS (Talend Open studio); Anydemic 1.0 pretopologic computations used AnyLogic 6 professional software. *Population simulation:* A population mock-up is designed in a ratio of 1/100 (660,000 people), taking into account gender, age, housing (house, building, elderly home), size of family, occupation, activity (medical doctor, nurse, physiotherapist, other health care worker, teacher, other activity), local daily transfer mode, interregional transfer, place of residency, place of work, vaccination status, health status (safe, chronic disease, pregnancy). *Day simulation:* Each day has 4 time units: night + morning at home, transfer to work, a day of work, transfer back home. Weekend days and holiday periods are taken into account. *Transport simulation:* Each morning (working days), each active agent (except during sickness leave) goes to work with a conveyance. If using public transport, he is supposed to be in touch with other people. Each end of day, he comes back home using the same conveyance. *Influenza simulation:* Influenza infection is divided into the following phases: safe, noncontagious incubation, contagious incubation, onset of symptoms, symptomatic noncontagious, recovered and protected (immunized) or dead. Each infected person may get medical contact(s), sickness leave(s) and hospitalization. *Other simultaneous outbreak simulation:* Several seasonal infectious agents are taken into account: influenza A(H1N1), A(H3N2), B, RSV. *Transmission simulation:* During each time unit, each agent gets a computed number of contacts depending on importance of household, activity and conveyance type. Among these contacts are a variable proportion of contagious persons (contagious incubation, onset of symptoms). Protection measures (vaccine, hand washing, protective masks, post contact, “preemptive” or curative use of neuraminidase) are characterized by 2 parameters: usage percentage, effectiveness. *Data sources:* INSEE 2009 census (www.recensement.insee.fr) and INSEE databases (www.insee.fr), Ministry of Education (www.education.gouv.fr), Ecosanté (www.ecosante.fr), medical references (http://www.ncbi.nlm.nih.gov/pmc/). *Inputs:* Parameters frames: inhabitants, educated people, health care professionals and teachers, elderly home, household, cohabitation, children by family type, number of index cases, consultation rate, sickness leave or school absenteeism rates, hospitalization rate, duration of each phase of the disease, transmission rate, protection measures (rate of use, effectiveness), transportation mode, extraregional transportation. *Calculation:* Outputs are recomputed at the end of each time unit (4 times per day). *Outputs:* Attack rate by age group, gender, occupational status (health care workers), housing, vaccination status and region, medical consumption, hospitalizations, death rate by age group and vaccination status, school absenteeism and sickness leaves. *Validation process:* Influenza attack rate by age group and region and sickness

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leaves in France from 2008 to 2013; data provided by the GROG national network (www.grog.org). Results: Observations of epidemic weeks in the past were shown by the simulation results.

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Assessing outbreak containment success in case of accidental laboratory escape of potential pandemic pathogens

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Background: The recent work on modified H5N1 has stirred an intense debate on the risk associated with the accidental release from biosafety laboratories of potential pandemic pathogens. Here we assess the risk that the accidental escape of a novel transmissible influenza strain would not be contained in the local community. Materials and Methods: We make use of a stochastic spatially explicit individual-based model of influenza transmission. This allows the infection transmission chain to be analyzed at the level of each single individual, and all of the microscopic details of the progression of the epidemic in the population can be accessed for each stochastic realization of the escape event. The escape events are identically initialized in a BSL facility in a European country by assuming one initial infected worker. Once the initial conditions for the outbreak are set, the model generates stochastic ensemble estimates of the unfolding of the epidemic. After the pathogen escape event has been detected, we consider the following set of containment interventions: (1) isolation of the laboratory, (2) laboratory workers' household quarantine, (3) contact tracing of cases and subsequent household quarantine of identified secondary cases, and (4) preventive school and workplace closure, on a spatial basis, at the very beginning of the epidemic, and reactive during the entire epidemic. Results: We found that the containment depends on the timely implementation of nonpharmaceutical interventions and contact tracing, and it may be effective (> 90% probability per event) only for pathogens with moderate transmissibility (reproductive number no larger than R_0 = 1.5). Containment could be feasible even for larger values of R_0 only in cases of very timely interventions—both in recognizing the accidental release and during contact tracing—and high probability of detecting secondary cases in the same household, school, or workplace as a newly identified case. Qualitatively, results do not vary much by considering different seeding locations. However, containment probabilities are affected by several factors, including population density and demographic structure, with probability of giving rise to a global event that is 3 to 5 times lower in rural areas. Conclusions: Our results suggest that public health authorities should be prepared to put in place a set of social distancing interventions (eg, contact tracing and closure of schools and workplaces on a geographical basis), with efficacy in containing the outbreak that is not guaranteed. In addition, our findings strongly suggest the location of new biosafety laboratories worldwide should be carefully chosen, for instance, with priority given to rural areas, and when this is not feasible, by taking into account density and structure of the population in urban areas.

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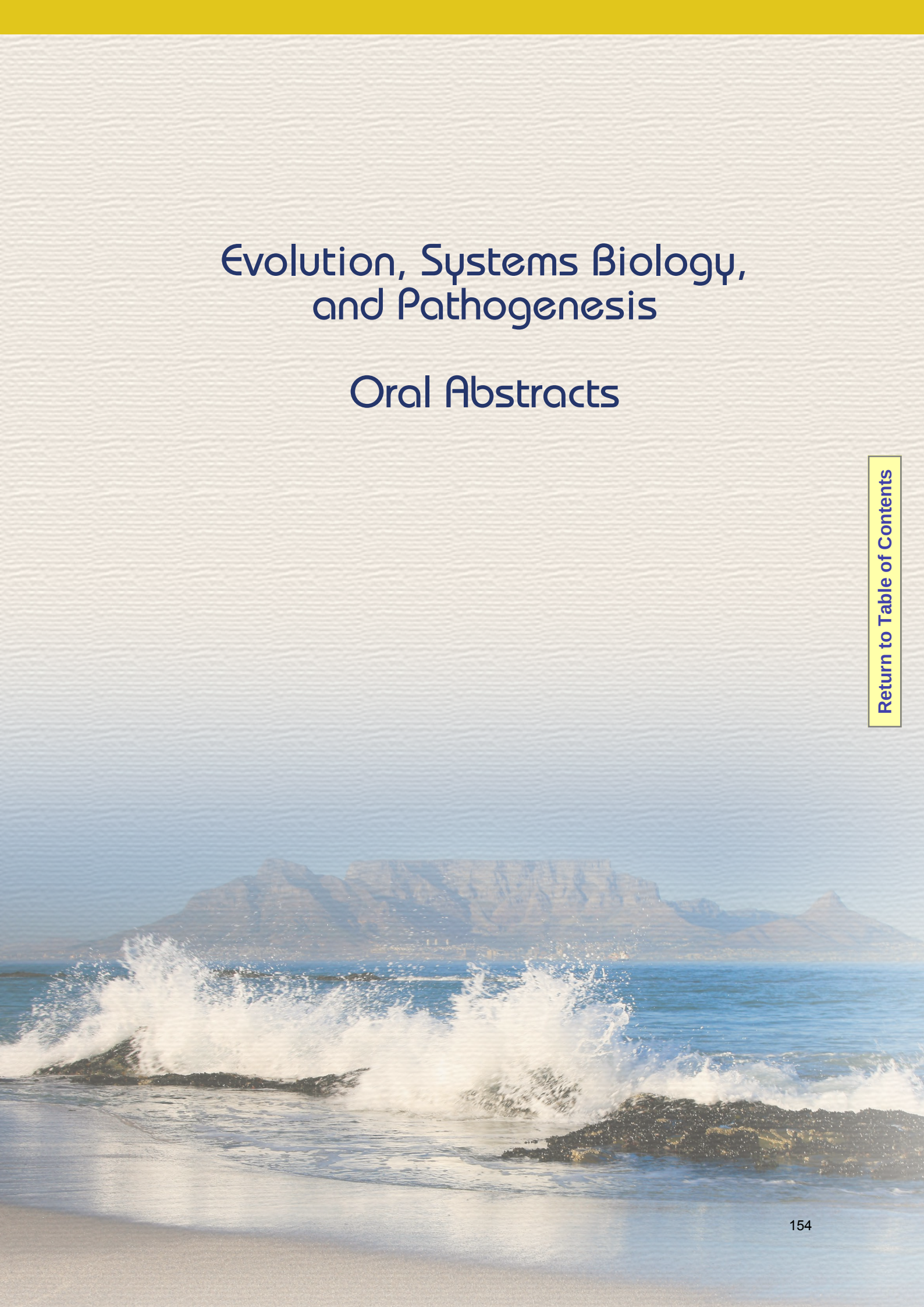

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Selection on hemagglutinin imposes a genetic bottleneck during mammalian transmission of H5N1 influenza viruses

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Background: Avian H5N1 influenza viruses sporadically infect humans with a lethality rate approaching 60% among confirmed cases, but to date have not acquired the capacity for sustained human-to-human transmission. The rapid replication and high mutation rates of influenza viruses can generate high levels of within-host genetic diversity, but little is known about the impact of this diversity on the evolution of H5N1 viruses during replication within hosts and transmission between hosts. Methods: We used deep sequencing to characterize genetic diversity of H5N1 reassortant viruses that were found to transmit between ferrets via respiratory droplets in a previous study (Imai M, et al. *Nature.* 2012;486:7403). In that study, transmission of H5 viruses occurred in a total of 6 ferret pairs. We extracted viral RNA from archived nasal washes collected from these animals at multiple time points and amplified HA, NA and M gene segments using RT-PCR. Amplicons from each sample were pooled, randomly fragmented and barcoded for multiplex sequencing on the Illumina MiSeq in 300-cycle runs. Sequences were analyzed using CLC Genomics Workbench, R and LinkGE, a custom Perl script that detects and enumerates combinations of physically linked single nucleotide polymorphisms in paired-end sequence data. Results: Deep sequencing of HA genes confirmed that mutations observed in association with mammalian transmission (as identified by Imai et al) were present in ≈ 100% of replicating viruses. However, we detected multiple additional mutations that rapidly increased in frequency from ≤ 5% to ≥ 20% of viruses during 3-5 days' replication in inoculated ferrets. Overall HA nucleotide diversity also increased markedly during this timeframe. HA nucleotide diversity was then dramatically reduced in contact ferrets upon respiratory droplet transmission; infection in these animals was established by only 1-2 distinct HA gene segments. Minor HA variants present in as little as 5.9% of viruses within the source animal became dominant in ferrets infected via respiratory droplets. In contrast, nucleotide diversity in NA and M gene segments changed little during replication or transmission. Statistical analyses confirmed that transmission was associated with a significant reduction in nucleotide diversity of HA, but not of NA or M. Conclusions: Mammalian respiratory droplet transmission of H5 reassortant viruses is associated with a significant genetic bottleneck caused by selection focused on HA. Minor HA sequence variants present in as little as 5.9% of viruses in an infected source animal can become dominant in contact animals after droplet transmission. Our results therefore suggest that selection could favor establishment of infection in mammals by viruses bearing "favorable" HA genes, even when such viruses are in the minority in the source population. Furthermore, surveillance efforts based on consensus sequencing may fail to detect the early emergence of genetic markers associated with virulence or transmissibility of H5N1 viruses in mammals.

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A systems biology approach reveals novel host response genes that differentially regulate influenza virus pathogenicity

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Background: Influenza viruses can cause severe disease in humans, and while the mechanisms are incompletely understood, host response dysregulation is thought to contribute. To uncover novel host factors that regulate influenza virus pathogenicity, we used a systems biology approach to computationally identify transcriptional network "hub-like" genes (i.e., genes exhibiting highly connected expression behavior) in lungs of influenza virus-infected mice. Hub-like gene expression behavior implies a regulatory influence over the transcriptional response and a potential role in controlling virus pathogenicity. The contribution of two hub-like genes to influenza virus pathogenesis was tested by using knockout mouse models. Methods: Groups of wild-type female C57BL/6 mice were mock-infected or infected with 10^3, 10^4, 10^5, or 10^6 plaque-forming units (PFU) of influenza A/California/04/2009 (H1N1; "CA04") or 10^2, 10^3, or 10^4 PFU of influenza A/Vietnam/1203/2004 (H5N1; "VN1203"), and lung tissues were harvested at 1, 2, 4, and 7 days postinfection for global transcriptional analysis by microarray. The weighted gene coexpression network analysis (WGCNA) algorithm was applied to a compendium of differentially expressed genes from all infections, dosages, and time points to identify gene coexpression modules and individual genes exhibiting putative hub-like regulatory behavior over these modules. The role of two highly ranked hub-like genes (i.e., *Ripk3* and *Saa1*) in influenza virus pathogenesis was assessed by comparing virus-induced disease in wild-type mice and Ripk3 or Saa1/Saa2 knockout (KO) mice after infection with a sublethal dosage of CA04. Results: Ripk3 KO mice exhibited less severe disease, significantly reduced weight loss, and faster recovery compared with wild-type mice, implying that Ripk3 activity is detrimental for the outcome of infection. In contrast, Saa1/Saa2 double KO mice exhibited more severe disease, increased weight loss, and reduced survival, suggesting a protective role. Conclusions: These results illuminate novel host genes that differentially regulate influenza virus pathogenicity and lay the foundation for future studies aimed at clarifying the mechanisms of influenza virus-induced disease and the discovery of new treatment strategies. This project was funded in whole or in part with federal funds from the United States National Institutes of Allergy and Infectious Diseases, National Institutes of Health and the Department of Health and Human Services under contract HHSN272200800060C.

O-836

The importin-α7 gene is a determinant of influenza virus cell tropism in the murine lung

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Background: Influenza A viruses are a continuous threat to humans due to their ability to cross species barriers and adapt to new hosts. Since viral transcription and replication takes place in the nucleus of the host cell, the viral polymerase complex needs to adapt to the mammalian nuclear import machinery upon interspecies transmission. It has been shown that differential use of importin-α isoforms governs host adaptation of influenza viruses. While growth of highly pathogenic avian influenza viruses depends on importin-α3, growth of mammalian viruses depends on importin-α7

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expression. Materials and Methods: To analyze the role that importin-α7 plays in susceptibility to mammalian influenza virus infection in mice, we monitored survival and weight loss in infected animals. We investigated virus tropism in the murine lung by histological analysis, magnetic resonance tomography, confocal microscopy, and transmission electron microscopy. The cellular immune response was analyzed by multicolor flow cytometry and the cytokine response by using an antibody array for 22 different cytokines and chemokines. Additionally, we measured virus growth and clearance by murine primary macrophages. Results: In this study, we performed a comparative analysis of the viral replication efficiency and cell tropism in the lungs of wild-type (WT) and importin-α7 knockout ($\alpha7^{-/-}$) mice using a recombinant H1N1 influenza virus that carries a GFP reporter gene in the NS gene segment. We observed that upon GFP virus infection, 90% of the WT mice succumbed to infection within 8 days, while $\alpha7^{-/-}$ mice lost less weight and 50% of the infected animals survived an otherwise 100% lethal infection. Analysis of viral kinetics in infected lungs by immunohistochemistry, ex vivo magnetic resonance tomography, confocal laser scanning microscopy, and correlative transmission electron microscopy revealed extensive infiltration and destruction especially of the alveolar epithelium in WT mice. In contrast, lung tissues of $\alpha7^{-/-}$ mice were mostly intact and bronchial epithelium was preferentially infected, suggesting that importin-α7 is required for virus replication in the alveolar epithelium. Further, higher levels of proinflammatory cytokines and chemokines (MCP-1, IL-12, IL-6, and IL-10) were detected in WT compared with $\alpha7^{-/-}$ mice. Moreover, virus RNA-positive macrophages were detected in the lungs of infected WT but not $\alpha7^{-/-}$ mice using immunohistochemical staining. However, no significant differences were observed concerning the biologic activity (virus replication and virus clearance) of primary macrophages isolated from WT or $\alpha7^{-/-}$ mice. In general, cellular immune responses measured by flow cytometry did not significantly differ between WT and $\alpha7^{-/-}$ mice. Conclusions: In summary, these observations support the view that the importin-α7 gene mediates virus tropism in the mammalian respiratory tract without affecting the cellular immune response in general.

O-837

Antigenic and receptor-binding properties of recent human H3N2 viruses

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Background: Human H3N2 influenza viruses have undergone antigenic drift for 45 years since the pandemic of 1968. Most of the amino acid changes that are selected by neutralizing antibodies occur in loops surrounding the receptor binding site, so it is not surprising that mutations that alter antigenic properties can also alter receptor binding affinity and specificity. We have investigated the immunodominant antigenic sites from 2006 to 2012 as seen by antibodies in human serum after immunization with the trivalent inactivated influenza vaccine, compared with changes in the glycan structures bound by H3N2 viruses. Materials and Methods: Viruses that were isolated in mammalian cells and never passaged in eggs were obtained from Wadsworth Center, Baylor College of Medicine, St. Jude Children's Research Hospital and the Children's Hospital of Oklahoma. Viruses were grown in MDCK cells and purified from 1-2 liters of medium. The purified viruses were labeled with Alexa488 and screened on the Consortium for Functional Glycomics Glycan Array at Emory University. Plasma or serum was obtained from healthy subjects who had received inactivated trivalent influenza vaccine and from subjects who had suffered an influenza-like illness. The sera were analyzed for antibodies against native virus and by hemagglutination-inhibition tests, using a range of H3N2 vaccine viruses as well as recombinant wild-type Wisconsin-like HA and mutants derived from it. Escape mutants were selected in MDCK cells using human sera. Results: Human H3N2 influenza viruses, isolated from 1968 to 2012, that had been isolated and passaged in mammalian cells, showed different binding specificities on the Glycan Array; the earliest human H3N2 viruses preferentially bound short, branched sialylated glycans while recent viruses bind better to long polylactosamine chains terminating in sialic acid. Some viruses bind glycans with α2-3 linked sialic acid; for 2010 and 2012 viruses this binding, and also virus entry into MDCK cells, was inhibited by oseltamivir, due to binding of α2-3 sialylated glycans by neuraminidase Gulati, et al. *PLoS One*. In press; e66325). The 2013 circulating strains had a mixed population of wild-type neuraminidase and the D151 mutant genes,

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and they bind α2-3 as well as α2-6 sialylated glycans unless oseltamivir is added to block the α2-3 binding, so it is possible that antineuraminidase antibodies will contribute to further antigenic drift of the H3N2 viruses. At the same time, we have tracked the antibodies induced by the H3N2 component of the seasonal vaccine. We reported that antigenic site B of H3 HA was immunodominant in subjects vaccinated in 2006-7 or 2008-9 (Popova, et al. *PLoS One*. 2012; e41895). We have now extended these studies to subjects who received the 2010-11 vaccine (H3N2 component A/Perth16/2009) or 2012-13 (A/Victoria/361/2011). In Perth, site B was also dominant, and we used sera from Perth-vaccinated subjects to select escape mutants of the Perth virus. Sera from two different subjects selected the same escape mutant, with changes in site B of H156Q and G186V, and the other sera tested also showed reduced binding to this escape mutant. Both of these mutations are present in the next vaccine strain, Victoria, so it appeared that these escape mutants had predicted antigenic drift. However, only a handful of the cell-isolated strains from 2012-13 in the GISAID database have mutations at H156 or G186; these site B mutations that were antigenically distinct were specific to the egg-isolated virus. Antibodies in sera of people who became infected in 2012-13, regardless of vaccine status, showed higher reactivity with local circulating strains than to the vaccine virus. Conclusions: Viruses with quite different binding specificity and avidity are equally fit to infect and transmit in the human population. The year-to-year variation in receptor binding specificity is a consequence of amino acid sequence changes driven by antigenic drift, and the limited number of sequence changes between antigenically distinct epidemic variants is due to dominance of one or two antigenic sites.

O-838 SCHOLARSHIP Promising Investigator Scholarship

The obese host as a driver of influenza virus evolution

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Background: Obesity has been associated with increased severity of influenza infection. Previous studies in animal models and epidemiologic evidence from humans have demonstrated that nutritional status can affect virulence of RNA viruses including influenza. These changes may occur due to altered immune responses or increased inflammatory state, both of which are present in the obese state. The global epidemic of obesity combined with increased disease severity in obese subjects led us to question whether obesity could influence influenza virus evolution. Materials and Methods: To determine if the obese lung microenvironment could differentially affect viral adaptation, A/California/04/2009 virus was passaged five times in either wild-type, lean, genetically obese (*ob/ob*) or diet-induced obese (DIO) C57Bl/6 male mice. Lean and DIO mice were then infected with parental and passaged viruses and monitored for morbidity and mortality. In addition, viruses were grown in 10-day-old embryonated chicken eggs and used to reinfect wild-type mice as well as used in standard replication assays on MDCK cells. Results: Interestingly, virus passaged in obese mice induced greater morbidity, pathology and mortality in both lean and DIO animals compared with lean-passaged virus. In addition, obese-passaged virus had increased replication kinetics and yielded larger plaque sizes compared with lean-passaged controls. Studies are under way to define the molecular determinants associated with the enhanced pathogenicity; however, the obese virus retained increased pathogenicity following egg passage, suggesting genetic changes to the virus itself. Conclusions: These studies suggest that the expanding obese population may not only be more susceptible to disease, but may also differentially regulate viral evolution toward more virulent viral strains. Further studies are also investigating the mechanism involved in increased pathogenicity and suggest that viral evolution in the obese host may be influenced as early as the primary infection, with obese mice shedding virus days longer than lean controls.

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O-839

Increased expression of β6 integrin is associated with enhanced inflammation and lung damage during influenza infection

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Background: Integrins are key modulators of cellular function that can also influence the innate immune response. The tightly regulated β6 integrin (Itgb6) is unique in that it is only expressed in epithelial cells during development and injury. Several studies have demonstrated a critical role for Itgb6 in controlling acute lung injury and the development of fibrosis during microbial infections. Because we found an increased expression of Itgb6 in the influenza virus–infected mice, we investigated the role of Itgb6 in pathogenesis. Materials and Methods: To determine the role of Itgb6 during influenza infection, C57Bl/6 or Itgb6$^{-/-}$ KO mice were intranasally inoculated with 10^4 TCID50 A/California/04/09 (H1N1) and monitored for morbidity and mortality. At different days post-infection, bronchoalveolar lavage (BAL) and tissues were collected via histopathologic methods by H&E staining and flow cytometry to monitor viral titers and cytokine/chemokine levels. Cytokine/chemokine profiles and surfactant protein D levels were also assayed from the BAL using a Milliplex kit and ELISA. Immune cell populations in lungs and BAL were monitored by flow cytometry (using markers for CD11c, CD11b, MHCII, and Ly6C) and by immunohistochemistry. Several different methods of assessing lung function were also conducted, including determining wet/dry lung weight to monitor for edema, permeability by Evans blue dye injection, and fibrosis/lung damage by hydroxyproline assay. Results: Itgb6$^{-/-}$ KO mice survived longer (MLD_{50} $10^{5.3}$) during infection as compared with wild-type C57Bl/6 mice (MLD_{50} $10^{2.5}$). Enhanced survival was accompanied by improved lung function as measured by edema, permeability, and lung damage/fibrosis. Itgb6$^{-/-}$ KO mice also had less inflammation with significantly lower levels of pro-inflammatory cytokines in the BAL and fewer neutrophils in the lungs. Interestingly, the number of lung macrophages was also increased. These changes were independent of differences in viral titers. We hypothesized that the enhanced protection in the Itgb6$^{-/-}$ KO mice may have been due to higher levels of surfactant protein D, which is known to be protective during influenza infection. Importantly, enhanced protection is not limited to influenza infection; Itgb6$^{-/-}$ KO mice were also protected during a lethal bacterial challenge and even during a secondary bacterial infection following influenza virus infection. Conclusions: Our results suggest that the β6 integrin plays a previously unrecognized role in influenza pathogenesis, regulating inflammation and the resulting lung damage by modulating communication between the infected epithelium and the innate immune response. Studies are underway to determine whether modulation of Itgb6 could be used as a therapy during severe influenza infection.

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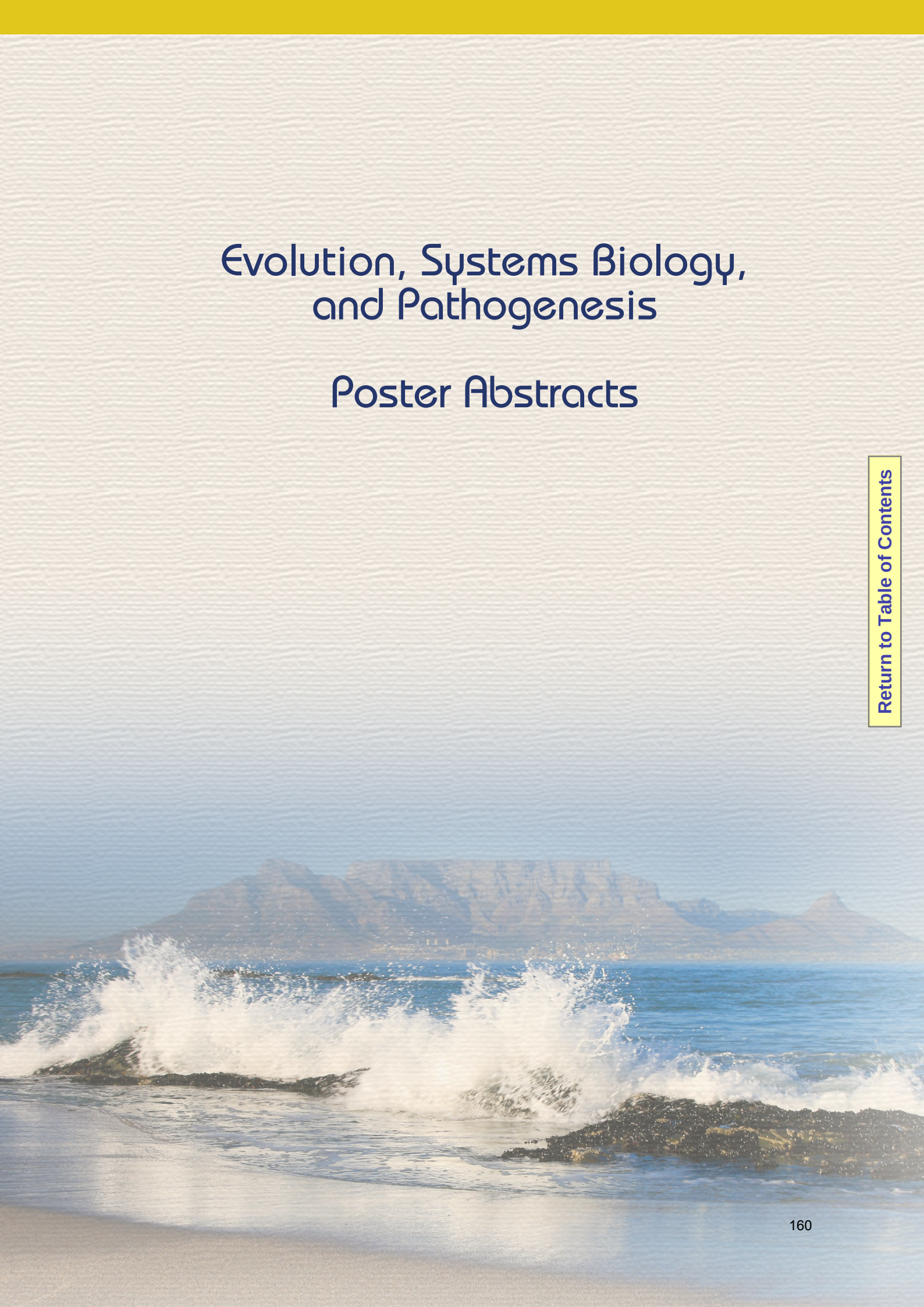

P1-210

Systems biology of respiratory virus infection: are host-response networks more complicated than social networks?

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Background: As high-throughput technologies allow ever broader and deeper views into the intricacies of biological systems, it is clear that the ability to work with numbers, networks, and vast amounts of data will be critical to deciphering biological complexity and to advancing new therapies to combat virus infection and disease. Materials: We are using systems biology and computational approaches to understand and model integrated views of host-response networks, virus-host interactions, viral evasion of host defenses, and viral pathogenesis. Much of our work is focused on highly pathogenic respiratory viruses, including pandemic and H5N1 avian influenza virus and SARS coronavirus. Innovative experimental systems, such as the Collaborative Cross mouse genetics platform, and ongoing advances in metabolomics, lipidomics, and next-generation sequencing, are expanding our systems-level views to encompass host genetic variation, metabolic pathways, epigenetics, and the uncharted territory of noncoding RNA transcripts. Results: We have observed changes in the expression of diverse classes of short and long noncoding RNAs in response to virus infection, suggesting that a detailed knowledge of noncoding RNA regulation and function will be necessary for a full understanding of viral pathogenesis. Moreover, we are becoming increasingly aware of the critical roles of host genetics and system dynamics in determining infection outcome. Conclusions: The combination of data integration, scale, and system complexity often appears overwhelming. However, computational biology is not alone in having to deal with abundant data and complex networks: Facebook keeps track of more than 900 million users and more than 125 billion friendships. Is computational biology up to a similar, or greater, challenge? Perhaps there are lessons to be learned or help to be gained from the world of information organization and access, computing, and social networking.

P1-211

Contribution of neuraminidase to the reassortment efficiency of wild-type with cold-adapted influenza viruses

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Background: Live attenuated influenza vaccine (LAIV) consists of reassortant viruses, with hemagglutinin (HA) and neuraminidase (NA) gene segments inherited from circulating wild-type (WT) parental viruses, and the 6 internal protein-encoding gene segments from cold-adapted attenuated master donor viruses (genome composition 6:2). Successful and quick generation of vaccine reassortants for LAIV by classical reassortment technique depends considerably on a variety of factors, including the phenotypical properties of the WT parental strain. Recently, many strains identified by the WHO as those likely to resemble the strains in circulation during the next epidemic season have been found to be highly sensitive to nonspecific serum inhibitors. The standard protocol for developing LAIV reassortants includes treating anti–master donor virus (anti-MDV) serum with receptor-destroying enzyme to remove nonspecific inhibitors. However, properly treated serum may still contain residual levels of heat-stable inhibitors. The presence of residual nonspecific inhibitors in immune anti-MDV serum may falsify the antibody pattern and complicate the development of reassortant vaccine strains based on inhibitor-sensitive WT epidemic viruses. In this study, we describe the obstacles in developing LAIV vaccine strains depending on the inhibitor-sensitivity phenotypic peculiarities of WT viruses used for reassortment. Materials and Methods: A total of 44 influenza A and B viruses were chosen for this study. Viruses were propagated in 10- to 11-day-old

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embryonated chicken eggs at 32°C-33°C. MDV and WT viruses were coinfected into embryonated chicken eggs by classical reassortment technique. Genomic composition analysis was performed using a 3130xl Genetic Analyzer according to the manufacturer's instructions. Hemagglutination inhibition (HAI) assay was performed using standard techniques at room temperature with 1.0% suspension of chicken or human 0(I) Rh^+ red blood cells. To analyze the sensitivity of influenza viruses to nonspecific serum inhibitors, normal guinea pig serum was used. Serum was heat inactivated for 10 min at 80°C to eliminate temperature-sensitive inhibitors, and used for HAI assays with 4 hemagglutinating units of each virus. A virus was considered to be resistant to nonspecific serum inhibitors if the HAI titer was ≤ 40, and designated as sensitive to nonspecific serum inhibitors if the HAI titer was ≥ 80. Results: The obstacles in developing LAIV vaccine strains depending on the phenotypic peculiarities of WT viruses used for reassortment were described. Eight hundred forty-nine reassortants of influenza A and B WT viruses with MDVs obtained using routine egg-based reassortment techniques were analyzed. Genomic composition analysis of these reassortants revealed that over 80% of reassortants based on inhibitor-resistant WT viruses inherited WT NA, compared with 26% of reassortants based on inhibitor-sensitive WT viruses. In addition, the highest percentage of 6:2 vaccine genotype reassortants was achieved when WT parental viruses were resistant to nonspecific serum inhibitors. It was also demonstrated that NA may play a role in influenza virus sensitivity to nonspecific serum inhibitors. Replacing NA of inhibitor-sensitive WT virus with the NA of inhibitor-resistant MDV significantly decreased the sensitivity of the resulting reassortant virus to serum heat-stable inhibitors. Conclusions: Analysis of reassortants based on influenza viruses circulating in different years revealed that clones with a 6:2 vaccine genotype were isolated much more frequently when using WT parental viruses resistant to nonspecific serum inhibitors. Our data suggest that NA may play a role in the sensitivity of influenza viruses to nonspecific serum inhibitors and affects reassortment efficiency. Thus, viral sensitivity to inhibitors seems to depend on a combination of unique properties of HA and NA. Clearly, LAIV vaccine development would benefit from a better understanding of the genetic features and mechanisms of influenza virus serum resistance. This work was partially supported by PATH.

P1-212

The timing and magnitude of the pulmonary macrophage response influences the outcome of virulent influenza infection

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Background: The innate immune system provides immediate and non-specific protection against invading pathogens. During virulent influenza infection, innate immune cells are recruited to the lung and secrete pro-inflammatory cytokines to control viral replication. However, excessive and unregulated innate immune responses have been demonstrated to contribute to the manifestation of more severe disease and death. Toll-like receptor 2 (TLR-2) is a cell surface receptor expressed on many cell types, including neutrophils, macrophages, and dendritic cells. The intranasal administration of the TLR-2 agonist dipalmitoyl-*S*-glyceryl cysteine (Pam2Cys) to mice results in the recruitment of innate immune cells and increases pro-inflammatory cytokine levels in the pulmonary tract. We have shown that these modifications to the pulmonary environment induced by Pam2Cys treatment significantly reduce the impact of virulent H1N1 (PR8) influenza virus infection. In this study we determined the ways in which recruitment of inflammatory mediators affects the outcome of virulent influenza infection. Materials and Methods: Pam2Cys is insoluble in aqueous solution and was therefore conjugated to polyethylene glycol (PEG) to increase its solubility for intranasal administration. C57BL/6 mice were treated with either saline or Pam2Cys 3 days prior to virulent influenza infection. The pulmonary viral loads, cellular infiltrate, and pro-inflammatory cytokine levels were then examined throughout the course of infection. Results: Following viral challenge, mice treated with saline developed influenza-induced symptoms that included hunched posture, ruffled fur, weight loss, and dehydration that necessitated euthanasia 7-8 days following infection. Unexpectedly, the pulmonary viral titers were reduced at this time point, indicating that viral burden is not a correlate of influenza-associated mortality. Four days postinfection, dramatic increases in the numbers of

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interstitial macrophages (up to 5-fold higher) and to some extent alveolar macrophages in the lung coincided with the onset of disease. At the same time, MCP-1 levels reached a maximum in mice treated with saline on days 3 and 7 postinfection. IFN-γ levels were also found to be elevated following infection. In contrast, mice treated with PEG-Pam2Cys did not experience weight loss or symptoms associated with PR8 infection, and the virus was ultimately cleared from the lungs by 8 days postinfection. As early as day 1 following infection, the lung viral burden in these mice was reduced, suggesting that Pam2Cys treatment has an immediate effect on initial viral titers. During the first 3 days of infection, mice treated with Pam2Cys had elevated numbers of neutrophils, alveolar macrophages, interstitial macrophages, and $CD11c^+$ dendritic cells—effects associated with Pam2Cys treatment. By day 4 postinfection, total cell numbers and individual cell populations declined and remained stable until viral resolution. These mice did not experience the surge in macrophage cell numbers observed in animals treated with saline late in infection. Immediately following the viral challenge, IL-1β induction was significantly higher in PEG-Pam2Cys–treated mice than in saline-treated mice. Levels of IL-6, IFN-γ, IL-12p70, IL-10, and TNF-α did not differ between PEG-Pam2Cys– and saline-treated mice. Conclusions: In this study we found that inflammatory cytokines, including IL-6 and TNF-α, were not related to influenza-associated disease or to survival, as has been reported in other studies. We did, however, find that the early localization of innate immune cells reduced the impact of virulent influenza infection, whereas the significantly larger and late recruitment of innate immune cells marked the onset of influenza-associated disease. It seems to us that prophylactic and/or therapeutic drug strategies based on PEG-Pam2Cys could be a useful means for combating virulent influenza infection.

P1-213

Effects of modifying the genetic robustness on the evolutionary capacity of influenza viruses

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Background: Genetic robustness is described as the capacity for a biological system to maintain the phenotype despite genetic modifications that could occur during replication. Mutational robustness is modulated by the codon usage, as synonymous codons differ in their propensity to mutate nonsynonymously and nonconservatively. With their error-prone polymerase, RNA viruses have elevated mutation rates and exist as a swarm of closely related sequences called quasispecies. At each replication cycle, many of the newly synthesised genomes differ from the consensus sequence, with possible deleterious effects on the viral population fitness. Viral populations can thus explore and occupy various regions of the genotypic sequence space, and their dynamics within this space greatly depends on the mutational robustness. Most published studies on viral robustness compared preexisting viruses, those being either “natural” strains or viruses engineered for other purposes. We chose a different approach for influenza viruses and decided to compare viruses specifically designed for their differences in robustness. The theoretical robustness (measured by the codon robustness indexes described by Dr Marco Archetti, University of Oxford, UK) of an H1N1pdm09 influenza virus was thus modified by rationally changing the codon usage within the PA gene, and the consequences on virus diversity and evolvability were studied. Materials and Methods: Based on the potential effect of a point mutation, the six possible codons for coding leucine and serine were classified into three groups (“stop,” for which only one point mutation is required to introduce a stop codon; “high,” with a high likelihood to change the amino acid for one with different properties; and “less,” with a high likelihood to be silent or to maintain similar physicochemical properties). For both amino acids, the codons within the PA gene were all replaced by one of these three groups. Theoretical calculations showed that the three modified and the wild-type viruses should indeed differ in their robustness. Using a reverse genetic system, the four viruses were rescued. Their evolutionary capacity was evaluated by passaging each virus in mutagenic conditions and in different cell types. Results: Using MDCK cells, the evolutionary capacity of the four viruses was first compared after serial passages at low m.o.i. under weak mutagenic pressures. The consequences on their ability to resist an increase in the mutagenic pressure were then investigated by passaging them once more but in stronger

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mutagenic conditions. In parallel, the four viruses were adapted to two cell lines derived from the respiratory tract of human and pig to test the potential effect of cell type on their evolutionary capacity. The viral fitness and genetic heterogeneity were assessed to evaluate the effect of differences in robustness. Viral fitness was determined by growth capacity and ratios of noninfectious/infectious particles, while the viral population architecture (mutation frequencies, identification of sngle nucleotide polymorphisms) was analysed by next-generation sequencing using an Illumina GAII methodology developed to cover the whole genome of influenza viruses. Conclusions: Beside modifications in the polymerase fidelity of viruses, reshaping a population's evolutionary capacity by manipulating the genetic robustness was shown to be a useful tool to attenuate viral pathogenicity. Until now, this approach was mainly limited to nonsegmented single-stranded positive-sense RNA viruses (poliovirus, plant viruses). We are presenting here the initial results about the role of robustness in the pathogenesis of a negative-sense RNA virus that replicates in the nucleus, ie, influenza virus. This study is only the first step in our broader interests to decipher the mechanisms by which mutational robustness could be used to develop new anti-influenza strategies.

P1-214

The evolutionary mutability of influenza A(H3N2) and B viruses circulating in Russia during 2007-2013

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Background: Influenza A and B viruses are most relevant among infection agents, as they cause severe respiratory infections in humans. While influenza A viruses comprise a large group of different subtypes, influenza B viruses are divided into the Victoria and Yamagata lineages. Molecular genetic characteristics of influenza A and B viruses, circulating in 2007-2013 in the Russian Federation, were studied. Phylogenetic analysis and genetic markers of susceptibilities of influenza A and B viruses to antiviral drugs were performed. Materials and Methods: PCR-positive samples were inoculated on 70%-90% confluent MDCK cell lines in flat-sided tubes after pretreatment with TPCK trypsin to facilitate virus entry. Isolates were sequenced by using the Sanger sequencing method (automatic analyzer AB 3130). The three gene segments (HA, NA, protein M) were phylogenetically analyzed on the basis of their nucleotide/protein sequences. Results: The Russian influenza virus strains were clustered on the basic of sequencing results and were divided in different genetic groups. During the 2007-2008 epidemic season, influenza H3N2 strains fell into the A/Brisbane/10/2007 genetic clade and all of them carried the signature genetic markers Q173K in HA and V215I in NA. In the 2010-2011 season, the isolates circulating in the east remained within the A/Perth/16/2009-like clade and had the substitutions I260M, P162S, R261Q in HA and L338F, N342D, N402D in NA. All strains, isolated in the 2011-2012 epidemic season belonged to the A/Victoria/208 genetic clade and were divided in two genetic groups: A/Stockholm/18/2011 (N145S, N144D (resulting in the loss of a glycosylation site), V223I, D487N in HA) and A/Hong Kong/3969/2011 (N145S, T48I, S45N (resulting in the gain of a glycosylation site), N278K, N189K и A198S in HA). In the 2012-2013 epidemic season, viruses fell into the A/Victoria/361 genetic clade and carried A198S, V223I, N312S, N278K, T212A substitutions in HA. The influenza B strains were divided into the Victoria and Yamagata lineages and carried substitutions K48R, K56D, V252M, Y40H, K129N (2007-2008 season), A146S, N202S, K88R, S229G (2010-2011), K253R, N202S (2011-2012). Conclusions: The Russian strains were closely related to the reference H3N2 and B viruses that WHO recommended for vaccines during 2007-2013 and were sensitive to neuraminidase inhibitors (oseltamivir and zanamivir) and were resistant to antiviral drugs rimantadine and amantadine. Also all H3N2 strains showed resistance to amantadine due to S31N substitution in the M2 ion-channel protein. The influenza B viruses had sensitiveness to oseltamivir and zanamivir (no substitutions E119V, R292K, H274Y, and R152K in NA).

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Reassortant viral lineages of influenza A

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Background: Influenza A evolves either by small mutations within a single gene, or by the reassortment of one or more genes between virions. Although many long-term trends in the mutational evolution of individual genes have been described, robust evaluation of patterns of reported reassortment is currently limited. Methods: We conducted a systematic literature review using the following search terms "influenza and (reassortment or reassortant)," to identify reported reassortant influenza A viral lineages. Duplicate claims were removed by repeatedly applying a filtering algorithm based on genetic sequence homology, to obtain 463 unique first-reported isolates (FRIs) out of a possible 14,666 publicly available full genomes with isolation dates up to and including 2010. Results: There was substantial variation in crude rates of reported reassortants by host type, geographic region and year of isolation. After adjusting for correlations between these three sample characteristics using a logistic regression model, only swine and years 1996-2002 remained as significant indicators of excess risk for identifying reassorted viruses. We then considered the phylogeny of 4 genes currently considered as primary threats to human health (H5, H3, N1, and N2). We constructed phylogenetic trees from the FRI set and compared them with trees describing random subsets of the same size drawn from all publicly available full genome isolates (random subsets of full genomes, RSFG). For some host-gene combinations, such as avian-H5, patterns in the FRI trees were similar to those in the RSFG trees. However, for other combinations such as swine-H3, swine-H1, and avian-H3; there were increased levels of diversity in the FRI trees and strong disparities in the representation of different host types. Conclusions: Despite publication bias in the reporting of reassortants, our results suggest that certain combinations of subtype and host may be more prone to successful reassortment. The phylogenetic patterns of FRIs, compared with the same-sized random subsets of all available full genomes, indicated a clear underrepresentation of samples from hosts identified as being high risk for the isolation of reassorted viruses, and those that have historically been involved in pandemic emergence. Looking forward, important host-subtype combinations could be more accurately described by systematic prospective studies of influenza in geographically diverse nonhuman populations.

P1-216

Substitution of hemagglutinin cleavage site contributes to the pathogenicity but not transmission of Egyptian HPAI H5N1 in chickens

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Background: The first cases of highly pathogenic avian influenza (HPAI) H5N1 were reported in Egypt in 2006. The pathogen has been declared endemic in the country and continues to evolve, although virus-associated mortality in infected chicken flocks has decreased over time; the reason for this drop in mortality remains unknown. Here, we investigated the role of a single amino acid substitution (R325G) in the hemagglutinin (HA) cleavage site on virus pathogenicity and transmission in chickens. Materials and Methods: Recombinant A/chicken/Egypt/1/2006, A/turkey/Egypt/7/2007, and their HA^{R325G} mutated counterparts were generated by reverse genetics. To compare the pathogenicity of these recombinant viruses in vivo in an avian host, we calculated their CLD50s in 6-week-old white Leghorn chickens. Results: All recombinant viruses replicated efficiently in Madin-Darby canine kidney cells and chicken eggs. The HA^{R325G} substitution significantly reduced virulence without altering the

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transmission efficiency of either HPAI H5N1 virus in chickens. Interestingly, birds in contact with the HA^{R325G} viruses did not get sick, whereas all their counterparts in contact with the parental strains succumbed to infection. Conclusions: The HA^{R325G} substitution increasingly identified in Egyptian H5N1 viruses since 2009 could therefore explain in part the lower mortality observed in poultry in the field. Reduced mortality with unaltered transmission provides further hurdles to control of H5N1 in Egypt.

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Surveillance and evolution of postpandemic influenza B viruses circulating in Argentina

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Background: Influenza is a public health problem that causes high morbidity and mortality especially in risk groups. Currently, two lineages of influenza B, denominated B/Victoria/2/87 (Victoria lineage) and B/Yamagata/16/88 (Yamagata lineage), co-circulate in the human population. Surveillance of influenza B viruses circulating in Argentina is an essential tool that enables early detection of new viruses and facilitates the decision of the annual formulation of the influenza vaccine. Materials and Methods: Influenza A or B positive clinical samples are routinely submitted to the National Influenza Centre through the National Influenza and Respiratory Viruses Laboratory Network for further characterization. A selection of positive influenza B clinical samples was inoculated in MDCK cells for viral isolation. Isolated strains (110 influenza B in 2010 and 57 influenza B in 2012) were characterized by haemagglutination inhibition (HI) using the WHO influenza reagent kit provided annually by the Centers for Disease Control and Prevention (Atlanta, Georgia, United States). In addition, in 2012, a RT-PCR real-time technique (kindly provided by Dr Olav Hungnes) was implemented and 678 clinical samples were tested for influenza B lineage differentiation; 57 of them were also tested by HI. For genomic analysis, 27 strains isolated in the 2012 season were selected. The sequences obtained were edited using the BioEdit program; the MEGA 4 and MEGA 5 programs were used to construct phylogenetic trees, with the neighbor-joining distance method and a bootstrap of 1000. Results: Between 2010 and 2012 a total of 314 influenza B viruses were isolated in MDCK cells: 185 (59%) in 2010 and 129 (41%) in 2012. Regarding the antigenic characteristics of 2010 isolates, the majority of them were closely related to the vaccine virus B/Brisbane/60/2008 belonging to B/Victoria lineage. The very low activity of influenza B viruses in 2011 did not allow obtaining isolates and therefore antigenic and genomic data. In 2012, a co-circulation of both lineages, Victoria and Yamagata, was detected. Most of the strains that belonged to the Victoria lineage (85%) were closely related to the B/Brisbane/60/2008 vaccine strain; meanwhile, influenza B isolates belonging to Yamagata lineage were antigenically closely related to the B/Wisconsin/1/10 variant. By RT-PCR real-time technique for differentiation of lineages, 678 original samples were characterized; 561 specimens (82.7%) were found to be of the Victoria lineage while 117 (17.3%) were of the Yamagata lineage. Fifty-seven samples studied by RT-PCR were also retested by HI assays and 100% of the results were consistent by the two techniques. The phylogenetic tree based on the influenza B Victoria lineage HA1 portion showed that 23 Argentinian viruses isolated in the 2012 season fell into clade 1A. In the phylogenetic tree for influenza B Yamagata lineage, three strains from 2012 fell into clade 2 and only one fell into clade 3. Conclusions: Viral isolation and monitoring of the antigenic and genetic evolution of the influenza B circulating strains are essential for early detection of new variants to ensure similarity with the corresponding vaccine strain. In light of the increase in the proportion of B/Yamagata/16/88 lineage viruses over the year 2012, a B/Yamagata/16/88 lineage virus was recommended for the 2013 southern hemisphere season trivalent vaccine. Because two antigenically distinct influenza B lineages have been circulating, the use of the quadrivalent vaccines recently approved by the FDA to be used in the United States in the 2013-2014 season may ensure a better correlation between the circulating influenza B viruses and the vaccine component in Argentina.

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Genome-wide analysis of reassortment and genetic drift of human influenza A (H3N2) viruses circulating between 1968 and 2011

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Background: Influenza A (H3N2) virus became widespread in humans during the 1968 H3N2 virus pandemic and has been a major cause of influenza epidemics ever since. The short-term evolution is shaped by reassortment and mutation. Antigenic drift results in frequent required updates of the vaccine. Using a dataset that spans the entire period of circulation of human influenza A (H3N2) viruses, it was shown that the influenza A (H3N2) virus evolution could be mapped into thirteen antigenic clusters. Materials and Methods: Here, 286 full influenza A (H3N2) virus genomes were analyzed to investigate intrasubtypic reassortment during 43 years of influenza A (H3N2) virus evolution. Further, selection pressures and nucleotide and amino acid substitution rates of all proteins, including the recently discovered PB1-N40, PA-X, PA-N155, and PA-N182 proteins, were studied. Results: Numerous reassortment events, scattered over the entire period of virus circulation, were found, most prominently in viruses circulating between 1991 and 1998. Persistent and nonpersistent reassortment events were detected, but no clear correlation with antigenic drift was found. Rates of nucleotide and amino acid substitution were most pronounced for hemagglutinin, neuraminidase, and PB1-F2 proteins. Selection pressures were highest in hemagglutinin, neuraminidase, matrix 1 and nonstructural protein 1. Conclusions: Reassortment occurs frequently and throughout almost the entire period of influenza A (H3N2) virus circulation, especially between 1991 and 1998. During this period, different antigenic lineages co-circulated and it appears that the viruses used various ways to increase virus diversity, presumably to improve fitness and/or to evade the host immune responses. This study of genotype in relation to the antigenic phenotype throughout the entire evolution of influenza A (H3N2) virus may ultimately lead to a better understanding of the evolution of influenza A (H3N2) virus.

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Evolution of hemagglutinin genes of influenza viruses isolated in Mongolia

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Background: Analysis of gene composition of influenza viruses isolated in different countries, especially hemagglutinin (HA) genes, has substantial importance for the real nature of influenza virus evolution. Here, we present an analysis of 21 H1, 22 H3 and 25 B nucleotide sequences from the representative strains influenza viruses isolated in Mongolia over the past 40 years. Materials and Methods: The released sequence data of the old influenza virus strains isolated before 2007 in Mongolia and the reference strains have been taken from GenBank. The influenza virus strains since 2007 have been isolated on MDCK cell lines by inoculating them with nasopharyngeal samples collected from ILI patients attending the sentinel surveillance sites of the National Influenza Center, Mongolia. The RNA of the cell culture–isolated influenza viruses has been isolated using QIAGEN purification kits according to the manufacturer's instructions. RT-PCR amplification was performed using the QIAGEN OneStep RT PCR kit on a Bioneer PCR system (Bioneer, Korea). The HA

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sequences were performed with a BigDye Terminator v3.1 cycle sequencing kit on an ABI 3130 Genetic Analyzer (Applied Biosystems, United States). The maximum likelihood trees of HA genes have been constructed using a PAUP test with MEGA4 and Geneious packages. Results: A phylogenetic analysis of the obtained results was performed in comparison to the published data on HA A(H1) (MI Nelson, et al. 2008), A(H3) (CA Russel, et al. 2008) and B (J Shen, et al. 2009). HA of the Mongolian strains had changes of 2-28 nucleotides, indicating on the continued genetic drifts of viruses with no novel gene flow events. Conclusion: In general the HA evolution of influenza virus strains isolated in Mongolia follows the worldwide evolution of influenza viruses with some time delay, which is shortening in the recent decade. The authors express their sincere thanks Mrs Noami Komadina, PhD, Head, Genetics Unit, WHO Collaborating Centre for Research and Collaboration on Influenza, Melbourne, Australia, for her valuable support in bioinformatics analysis of HA genes of influenza viruses isolated in Mongolia.

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Pathological study of ARDS complicated by influenza virus infection

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Background: Almost all fatal cases with highly pathogenic avian H5N1 influenza virus (H5N1) infection in humans and some fatal cases with 2009 pandemic influenza virus (A[H1N1]pdm09) infection were complicated by acute respiratory distressed syndrome (ARDS) to death. ARDS caused by influenza virus infection is generally classified as severe ARDS with P/F ratio< 100 mg, and the patients die due to respiratory failure rather than multiple organ failure early after the onset of the disease. We examined the lung pathology of fatal cases of severe ARDS to reveal the pathogenesis of ARDS complicated by influenza virus infection. Methods: Formalin-fixed paraffin-embedded lung tissues were analyzed with several methods as follows: immunohistochemistry for type A influenza NP antigen, proinflammatory cytokines and chemokines, in situ hybridization for influenza viral genomic RNA and mRNA, double immunofluorescence staining with cell marker proteins, and real time quantitative reverse transcription polymerase chain reaction methods for viral RNA and cytokine/chemokine mRNA. Results: Lung tissues showed a spectrum of histopathological changes of diffuse alveolar damages (DAD). The protein-rich edema fluid in the alveolar air spaces formed hyaline membranes in exudative phase. Myeloperoxidase (MPO)-positive and/or CD68 (clone KP-1)-positive neutrophils and monocytes/macrophages infiltrated prominently in the alveolar septa and alveolar spaces. Viral antigen-positive cells were mainly alveolar epithelial cells and monocytes/macrophages. Influenza virus mRNA was detected in epithelial cells using in situ hybridization presenting viral replication. For the H5N1-infected lung tissues, the expression levels of TNF-α, IL-6, IL-8, RANTES, and IP-10 correlated with H5N1 RNA copy numbers detected in the same lung region. Double immunofluorescence staining revealed that TNF-α, IL-6, IL-8, and IP-10 were expressed in epithelial cells and/or monocytes/macrophages. In particular, IL-6 was also expressed in endothelial cells. Conclusions: H5N1 and some A(H1N1)pdm09 infected alveolar epithelial cells and injured them directly. Marked accumulation of neutrophils and monocyte/macrophages suggested neutrophil-mediated lung injury. The expression levels of cytokines and chemokines, correlated with H5N1 copy number in the same lung sections suggested local uncontrolled inflammatory responses induced by H5N1 infection.

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Impact of the hemagglutinin protein acid stability on virulence and host range of H5N1 influenza viruses

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Background: Highly pathogenic avian influenza H5N1 viruses are currently endemic in poultry in Egypt and Indonesia. The H5N1 influenza virus continues to cause infections in humans and has killed 60% of patients diagnosed with the infection. Understanding of the molecular properties that allow the virus to adapt and transmit in mammals is critical for risk assessment of the pandemic potential of the H5N1 virus as it evolves. The hemagglutinin (HA) protein mediates binding and entry of the influenza virus into the host cells and plays an important role as a pathogenicity determinant. Following virus uptake into the endosomes, the HA protein is triggered by low pH to undergo irreversible structural changes that mediate membrane fusion. Human transmissible influenza viruses are thought to have more stable HA proteins compared with avian influenza viruses. Here, we investigated how a decrease in HA activation pH (an increase in acid stability) influenced the virulence and transmissibility of highly pathogenic H5N1 influenza virus in mammalian hosts. Materials and Methods: We generated isogenic A/Vietnam/1203/2004 (V1203) or A/chicken/Vietnam/C58/2004 (C58) H5N1 influenza viruses containing either the wild-type HA protein or an HA protein with a K58I acid-stabilizing mutation in the HA2 subunit. We then characterized the biochemical properties of the wild-type and HA2-K58I viruses and their replication and transmissibility in vitro in mammalian cell lines and in vivo in ducks, mice, and ferrets. Results: The K58I mutation further stabilized the C58 and V1203 HA proteins and decreased their activation pH from pH 5.9-6.0 to pH 5.4-5.5 without altering their expression, cleavage, or α(2,3)-receptor binding specificity. Compared with wild-type virus, the C58- and V1203-K58I viruses had similar replication kinetics in MDCK and normal human bronchial epithelial cells but were attenuated in human lung A549 cells, which were found to have a higher endosomal pH than MDCK cells. The K58I mutation resulted in a loss of growth, virulence, and transmission in ducks but increased growth and virulence in DBA2 mice compared with the wild-type C58 virus. Similar to the wild-type V1203 virus, the K58I-V1203 virus did not transmit to naive contact cage-mate ferrets. Interestingly, the K58I mutation resulted in a 100-fold increase in virus load in ferrets' nasal cavities early during infection in contrast to this mutation being attenuating in ducks. Conclusion: Overall, a single, acid-stabilizing mutation was found to enhance the growth of an H5N1 influenza virus in the mammalian upper respiratory tract. However, this mutation was insufficient to enable contact transmission in ferrets of H5N1 influenza viruses with avian receptor binding specificity. Additionally, our data suggest that mammalian species support a broader range of activation pH (at least pH 5.4-6.0) compared with a narrow range supported by ducks (pH 5.6-5.9). Thus, viruses with low activation pH might not be virulent in avian species but could infect and cause disease in mammals. Identification of HA protein activation pH as a determinant of host range will help in risk assessment of the pandemic potential of circulating H5N1 influenza viruses.

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Mass spectrometric analysis of the RIG-I interactome during influenza B virus infection reveals a potential role of RIG-I in stress granules

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Background: Influenza virus infection is detected by the cellular retinoic acid inducible gene I (RIG-I) receptor, which is a pattern recognition receptor (PRR). PRRs, like RIG-I-like receptors (RLRs), toll-

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like receptors (TLRs), and Nod-like receptors (NLRs), are essential in innate immunity. RIG-I-like receptors recognize cytoplasmic viral RNA and induce the production of type I interferons (IFNs) and proinflammatory cytokines via a cascade involving the downstream adaptor protein MAVS as well as the transcription factors IRF3 and NF-κB. Given RIG-I's central role in antiviral immunity, it is not surprising that many viruses have evolved sophisticated mechanisms to avoid its activation. We and others have previously identified the NS1 protein of influenza virus as an inhibitor of RIG-I activation. NS1 is a multifunctional protein that inhibits the activation of IRF3 and PKR, a protein kinase involved in antiviral activity. Furthermore, NS1 inhibits the induction of the interferon β promoter, probably by interaction with RIG-I, but so far the precise mechanism and factors involved are not known. To gain a better understanding of the role of RIG-I during influenza virus infection, we employed stable isotopic labeling of amino acids in cell culture (SILAC), a mass spectrometric technique that allows relative protein quantification, to identify novel RIG-I interaction partners. Materials and Methods: HEK 293T cells were grown in SILAC DMEM with three different isotope variants (heavy, medium and light) of arginine and lysine. After full incorporation of the isotopes, cells were transfected with STREP-RIG-I for 24 h and infected either with influenza B wild-type virus or a mutant virus expressing a truncated NS1 protein incapable of inhibiting the IFN response. The combined cell lysates were affinity purified with STREP tactin that specifically binds STREP-tagged proteins. The proteins were separated by 1D-SDS-PAGE and subjected to in-gel digestion with trypsin. The resulting peptide mixtures were analyzed on an LTQ-Orbitrap equipped with a Nano-LC (ReproSil-Pur C C18-A column, 75-µm internal diameter, 95-min elution gradient from 2% to 40% of solvent B [80% acetonitrile, 0.1% formic acid]). The five most intense peptide ions were fragmented by CID (collision-induced dissociation). Data were acquired using Xcalibur software. The MS/MS data were searched using the SEQUEST algorithm in Proteome Discoverer 1.4 against the *Homo sapiens* (NCBI) database (33,286 entries). Results: Among the proteins identified by MS in RIG-I's interactome, we found an enrichment of proteins involved in the stress granule response, like the ribosomal subunit 40S, transcription initiation factors, and ras GTPase-activating protein-binding protein (G3BP) a stress granule marker. These results indicate that RIG-I plays a role in the stress granule response, which is in line with recent findings showing that RIG-I colocalizes with the stress granules during infection with influenza A virus lacking the NS1 gene. Furthermore this work identified novel proteins with potential roles in the stress granule response. Conclusions: Stress granules are dense aggregations of translationally silence mRNPs with proteins in the cytosol that are formed when the cell is under stressful conditions like virus infections. Since stress granules control the mRNA translation, many viruses manipulate this stress response toward their own benefit. Here we show that RIG-I is present in stress granules, suggesting the hypothesis that stress granules may play an important role in the antiviral response.

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The evolutionary dynamics of lowly pathogenic avian influenza viruses in domestic ducks

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Background: Waterfowl are regarded as the major reservoirs of influenza A virus, which maintain various subtypes of lowly pathogenic avian influenza viruses (LPAI). Economic development over the past two decades has caused a great increase in the population of domestic ducks, and therefore reservoir hosts, in China. Domestic ducks have shorter life spans than wild birds and are often kept in denser populations. These agricultural developments may have altered the ecology and evolutionary dynamics of influenza viruses in China. Of all 16 subtypes of LPAI viruses, H3, H4 and H6 were the most frequently isolated from ducks in both North America and Eurasia. Potential changes in the ecology and evolution of the influenza viruses of ducks in China were investigated by considering these 3 major subtypes. Materials and Methods: Based on surveillance undertaken in Hong Kong (1976-1981) and southern China (2000-2006), isolates of H3 and H4 subtypes were selected from each positive sampling occasion for complete genome sequencing. Complete genomes of 185 H3 duck isolates and 149 H4 duck isolates were sequenced and analyzed with previously published H6

sequences from southern China (2000-2006), incorporated into the study dataset. A maximum likelihood (ML) phylogeny was constructed for each gene, and topologic confidence was assessed using various branch tests. Evolutionary rates of the viral lineages were estimated using relaxed molecular clock models and the Bayesian MCMC method implemented in the BEAST program. Selective pressure was measured by calculating the ratio of nonsynonymous (dN) to synonymous substitutions (dS) per site through the Datamonkey Web server. Results: HA phylogenies revealed that viruses from domestic ducks in southern China diverged from the contemporary virus "gene pool," and developed into local lineages, which persisted for some years. H3, H4 and H6 LPAI viruses in domestic ducks showed different level of divergence, with H6 the most divergent. Evolutionary rates of HA genes of recent (1990 onward) virus lineages in domestic ducks were substantially higher than those from early years (ie, 1970-80s) or from gene pool viruses. However, the dN/dS of all lineages remained at similar levels, indicating that they were not subjected to significantly different selective pressure. Multiple reassortments, based on the NA and internal genes of the HA lineages, were observed in the domestic duck viruses. The NA and internal genes of recent viruses from domestic ducks also showed higher evolutionary rates than viruses of the older or gene pool lineages. Conclusions: With the increase in the size of the domestic duck population in China, H3, H4 and H6 viruses from domestic ducks have shown some divergence from the influenza virus gene pool and exhibited higher evolutionary rates. These elevated rates might be due to the increased number of virus replication cycles in the much larger and denser domestic duck populations. LPAI viruses in domestic ducks also showed extensive reassortments to maintain a diversity of viruses, thereby creating an influenza ecology similar to viruses from wild birds. However, the greater separation of domestic ducks from wild birds by the agricultural system may also be responsible for virus lineages appearing. Due to their prevalence, and/or possible adaptation to the agricultural environment, these domestic duck lineage viruses might have a greater chance to spread to other domestic birds at farms or markets.

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A novel reassortant H2N3 virus isolated in China

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Background: The H2 influenza virus has been absent from the human population since 1968, but it still has been isolated from birds and swine. The first reported H2N3 avian influenza virus was isolated from migratory ducks in Japan. In 2006, H2N3 was detected in a swine herd in Missouri (United States), and phylogenetic analysis showed that the virus strains isolated from the diseased swine were avian/swine virus reassortant. However, there was no report of H2N3 influenza virus in China until now. Materials and Methods: An annual live poultry surveillance program was carried out in China; 40 environmental samples were collected monthly by each provincial influenza surveillance network lab. All collected samples were detected by real-time RT-PCR targeted for the influenza A matrix gene by network labs. The PCR-positive samples were sent to the Chinese National Influenza Center for further confirmation or virus isolation. Interestingly, one virus identified as H2N3 was isolated from a swab of a duck cage. Phylogenetic trees were constructed by the maximum likelihood method using MEGA (v. 5.05) after whole genomic sequences of the virus were obtained. Results: Phylogenetic analysis showed that the HA gene of A/environment/Guangdong/2/09 (H2N3) belonged to a Eurasian lineage. The NA gene possessed the highest homology with A/duck/Hokkaido/167/2007 (H5N3). The NP, PB1 and PB2 genes showed a close relationship with H4N6 strains isolated from wild ducks in Korea. The M gene showed the highest gene similarity with A/pintail/Aomori /1130/2008(H1N3). NS and PA had the highest homology with those of the H10N6 and H3N8 subtypes, respectively. The amino acid composition of the receptor-binding site of the HA gene was QGG, indicating that this virus was avian in origin. The drug resistance site analysis showed that this virus was still sensitive to amantadine and oseltamivir (Tamiflu). Conclusions: A novel avian-origin reassortant H2N3 influenza virus was isolated. To our knowledge, this is the first report in China. Our findings indicated that genetic reassortment occurred in different subtypes of influenza viruses, and

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suggested the importance of continuous surveillance of avian influenza virus in understanding the etiology and evolution of avian influenza virus.

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Single mutations in PB2 and NP mediate enhanced pathogenicity of 2009 pandemic H1N1 influenza A viruses in mice

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Background: Determinants of 2009 H1N1 influenza A virus pathogenicity are distinct from those known from studies with H5N1 or H7N7 highly pathogenic avian influenza A viruses (HPAIV). In order to determine novel molecular hallmarks of 2009 pandemic H1N1 pathogenicity, we compared the virulence of two clinical isolates (designated as HH05 and HH15) representing early and late pandemic phase strains in mice. We found that HH15 is more virulent ($logMLD_{50}$: 3.5 pfu) than HH05 ($logMLD_{50}$: > 5 pfu) in C57BL/6J mice. HH05 differs from HH15 by 12 amino acid substitutions in the PB2, PA, NP, HA, NA, and NS1 genes. Materials and Methods: In order to address the molecular basis responsible for enhanced 2009 pandemic H1N1 virulence in mice, we generated several recombinant influenza viruses by reverse genetics. Results: We identified single mutations in the viral nucleoprotein (NP) and in hemagglutinin (HA) that mediate enhanced pathogenicity in mice. Interestingly, these HH15-specific positions in HA, NA, NP, and NS1 were less prevalent in the early pandemic periods while their prevalence increased during the late stages of the pandemic. Conclusions: Taken together, our findings reveal novel determinants of 2009 pandemic H1N1 pathogenicity distinct from those described for HPAIV isolated from humans. Furthermore, high prevalence of these positions in currently circulating strains suggests that these sites were replaced during later phases of the pandemic, reflecting their selective advantage in the human population.

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Understanding a weakness of the 2013 H7N9 virus: a large-scale analysis of the NS segment from H9N2 viruses

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Background: The influenza A H7N9 virus from the 2013 outbreak in China is a serious pandemic candidate whose virulence and potential further evolution needs to be monitored closely. The segments coding for the internal genes were derived through reassortment from H9N2 viruses. As its NS segment codes for a truncated NS1 protein that lacks a putative PDZ binding motif, this could probably limit the H7N9 virus from interacting with host proteins to downregulate the innate immune response. In other strains, gain of a functional PDZ binding motif enhanced viral replication by up to 4-fold. It is therefore important to understand the distribution of this variant among its ancestral H9N2 viruses and examine the frequency of these viruses that have acquired full-length NS1 protein with a putative PDZ binding motif either through mutation or reassortment. Materials and Methods: In this study, we used Tachyon, a fast sequence search tool, to screen for reassortants in more than 1300 H9N2 viruses and verified some of these reassortants with GiRaF, a program that utilizes Bayesian coalescent inference and graph theory to predict reassortment in influenza viruses. Results: We found that there have been ongoing reassortment events and mutations that resulted in H9N2 viruses with NS segment coding for full-length NS1 protein with putative functional PDZ binding motif in the H9N2

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subtype in East Asia in recent years. H9N2 viruses with truncated NS1 protein with a nonfunctional PDZ binding motif are largely confined to China, whereas full-length NS1 protein with functional PDZ binding motif is distributed throughout the world. Conclusions: Based on these findings, it seems possible that the 2013 H7N9 virus could acquire a full-length NS1 protein with functional PDZ binding motif through either genetic mutations or reassortment within or outside China in the future. However, it remains to be established how gain of a functional PDZ binding motif would affect severity of this virus.

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Postpandemic genetic analysis of pandemic A(H1N1) 2009 influenza viruses in Ghana

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Background: Influenza pandemics are unpredictable, as illustrated by the recent detection of avian influenza A H7N9 infections among human patients in China. Therefore, there is a need for continued virologic surveillance. Under the national influenza sentinel surveillance system, A(H1N1) pdm09 virus was first detected in Ghana in August 2009. Since then, cases were recorded in all 10 regions of the country. Detection of A(H1N1) pdm09 virus outnumbered seasonal A(H1N1) virus by January 2010, the last date old seasonal A(H1N1) was identified in Ghana. Genetic analysis was deemed essential to understand the evolution of A(H1N1) pdm09 virus in Ghana. The haemagglutinin (HA) and neuraminidase (NA) genes of A(H1N1) pdm09 Ghanaian isolates were therefore sequenced and analyzed by comparison with isolates from other countries. Materials and Methods: We selected 35 representative A(H1N1) pdm09 virus isolates obtained during the onset, peak and postpandemic period from patients with influenza. The HA and NA genes were amplified from clinical material after first passage in cell culture and sequenced using a one-step reverse transcription polymerase chain reaction with primers previously described by the US Centers for Disease Control and Prevention. Sequences were assembled and edited using Lasergene Seqman software (DNASTAR). Consensus sequences were aligned with Bioedit (Ibis Biosciences) and other software for amino acid differentiation for molecular characterization of known pathogenic and drug resistance markers. Sequences from the HA region were compared with HA sequences of the original strain (A/California/07/2009). A Basic Local Alignment Search Tool (ncbi.nlm.nih.gov) was employed, and homologous sequences retrieved and used for further phylogenetic analysis using MEGA v5.1 software. Results: Sequence analyses of the HA genome showed that all 35 Ghanaian isolates possessed the P83S change located in the antigenic site E and also the S203T mutation specific to A(H1N1) pdm09 clade 7 viruses. The I321V variation was also present in all the isolates, and this change has been observed in certain European strains. The E374K change was associated with isolates from only 2009 and was not present in isolates from 2010 through 2012 in Ghana. All 35 isolates analyzed lacked the H275Y change in the NA gene, which is associated with drug resistance to oseltamivir. Conclusions: Following the 2009 pandemic, the dominant A(H1N1) pdm09 lineage in Ghana most resembles members belonging to clade 7. These viruses were not significantly different from the original strain and were susceptible to the antiviral drug oseltamivir. The D222G mutation associated with disease severity was not found among Ghanaian isolates. This collection of data highlights the importance of ongoing viral surveillance in Ghana, documenting viral changes and adaptations that lead to improved global reporting and informed health prevention and intervention strategies for influenza and could be applied to other respiratory viruses.

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Phylogenetic analysis of A(H3N2) influenza viruses circulating in Ukraine during years 2008-2012

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Background: Phylogenetic analysis of circulating influenza strains is essential for influenza surveillance. Influenza virus A(H3N2) was the predominant subtype in Ukraine in 2008-2009 and 2011-2012, but the small number of this strain was isolated in 2010-2011. The purpose of this study was to monitor the genetic drift of A(H3N2) influenza viruses, isolated in Ukraine in 2008-2012, by sequencing the genes coding surface glycoproteins of representative strains. Materials and Methods: Throat swabs from patients of sentinel sites in different regions of Ukraine were collected. The specimens were tested for influenza by real-time polymerase chain reaction (RT-PCR), and viruses were isolated in MDCK and MDCK-SIAT cell culture from PCR-positive samples. Phylogenetic analyses were performed using MEGA5 software and were aligned using the ClustalW algorithm. Phylogenetic trees were generated by the neighbour-joining method. The best-fit nucleotide substitution model (Hasegawa-Kishino-Yano 85 plus Gamma [HKY85+G]) was found using maximum likelihood estimates. A bootstrap technique with 1000 replications was used to test statistical validity of received data. Results: Phylogenetic analyses showed that A(H3N2) isolates of 2008-2009 were located in dominated cluster A/Brisbane/24/2008 with G50E, D122N, K140I and I223V substitutions in hemagglutinin. Only two A(H3N2) viruses were isolated in 2011-2012. An isolate of A/Ukraine/175/2011 was A/Pert/10/2010-like with D53N, Y94H, I230V and E280A substitutions in hemagglutinin. The Ukrainian strain had additional substitution K92E. Strain A/Khmelnitsky/1340/2010 was similar to the viruses of 2008-2009 and differed from them only by substitutions E401K in HA and I58T, T434N in NA. The strains that were isolated in 2011-2012 influenza season had an origin on the A/Victoria/208/2009 phylogenetic cluster. Ukrainian isolates were located in 5 different phylogenetic groups with several amino acid substitutions (some in glycosylation sites). Neuraminidase sequence analysis of these viruses showed similar results. Ukrainian viruses were genetically related to strains from all continents. It shows multiple influenza viruses enter the country during the epidemic season. Tested isolates didn't show mutations of resistance to oseltamivir (Tamiflu). Conclusion: The genetic drift of influenza A(H3N2) viruses in the Ukraine had the same direction as in Europe during the monitoring period.

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Sequencing artifacts in the type A influenza database and attempts to correct them

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Background: Type A influenza virus causes a wide range of disease in both man and animals, and considerable efforts have been made to study and sequence large numbers of isolates. Currently there are over 236,000 gene sequences representing over 50,000 strains in publicly available databases. However, the quality of the sequences submitted to these public databases are determined by the contributor and many sequence errors are present in the databases, which can affect sequence analysis and require significant curation of data by individual researchers to get usable data. As part of a high school class project, bioinformatics analysis was performed on all six internal gene segments of influenza A virus. Material and Methods: Sequences were selected in the Influenza Research Database Web site that were longer than the generally accepted lengths of the

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individual segments, with the hypothesis that these sequences would have an error in the sequence. Because of the greater variability in sequence length of the hemagglutinin and neuraminidase genes, only the six internal genes were examined. Multiple sequence alignments were performed for each segment, and viral sequences were divided into those with additional sequence upstream or downstream of the conserved noncoding ends of the gene segment and those with additional sequence internally. Specific attention was placed on sequences with additional nucleotides upstream or downstream of the highly conserved noncoding ends of the viral segments. The virus sequences were evaluated to try to determine the probable type of error associated with each sequence. Results: A total of 1081 sequences were identified that met this criteria when the database was queried in March 2012, which represents a 0.82% rate of potential errors. Three types of errors were commonly observed: noninfluenza primer sequence was not removed from the sequence; Taq polymerase added an adenine at the end of the PCR product; and the PCR product was cloned and plasmid sequence was included in the sequence. Internal insertions of nucleotides were also commonly observed, but in many cases it was unclear if the sequence was correct or actually contained an error, and therefore these sequences were not evaluated further. Students contacted some of the sequence submitters, alerting them that a sequence had been identified as likely having an error and if known what type of error it probably was. Submitters were requested to review the sequence and, if they agreed that the sequence was incorrect, that they revise the sequences in the public databases. A total of 215 sequences, or 22.8% of the suspect sequences, were corrected in the public databases in the past year in part because of the student project. Examination of the sequence database in 2013 showed an additional 138 sequences with possible errors were added to GenBank in the past year. A new class of students once again contacted submitters and requested they review and potentially correct the sequences, with at least partial success in getting sequences corrected. Conclusions: The integrity of the public sequence databases is largely dependent on the scientists who submit the sequence information. Some review by GenBank and other databases provides some identification of errors, primarily removing plasmid sequence from submissions, but because of the complexity and variability of sequences submitted, the error identification programs are limited. The identification of 138 new problematic sequences in the past year highlights that the error detection system is not working as well as intended. Additional awareness of the need for data integrity of sequences submitted to public databases is needed to fully reap the benefits of the huge amount of data available for analysis.

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Pathogenesis of influenza virus-induced alveolar damage

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Background: Influenza A virus (IAV) typically causes an acute infection of the upper respiratory tract. However, in some cases, IAV can cause an infection of the lower respiratory tract resulting in viral pneumonia, which can then give rise to acute respiratory syndrome (ARDS). Clinically, ARDS is characterized by severe respiratory insufficiency and a high case fatality rate. The severity of this disease is reflected in the lung lesions of infected patients. During the acute phase of ARDS, patients display diffuse alveolar damage characterized by the accumulation of fluid and leukocytes in the alveoli. Central to the development of these pulmonary lesions is damage to the epithelial-endothelial barrier. Destruction of this barrier results in fluid leakage from interstitium, fibrin deposition, and pulmonary hemorrhaging. Currently, much of our understanding of endothelial-epithelial damage during ARDS is derived from studies of bacterial sepsis, where the endothelium is the first site of damage. However, there are limited models available to describe how IAV, a pathogen that first infects the pulmonary epithelium, damages the endothelial-epithelial barrier. Here, we seek to summarize and analyze the available literature regarding the interactions between IAV and epithelial cells, macrophages, neutrophils, and the pulmonary endothelium in order to provide a new paradigm for disease pathogenesis. Materials and Methods: Relevant articles were identified by searching PubMed and Google Scholar using the search terms “epithelial cell,” “influenza,” “macrophages,” “neutrophils,” “endothelial cells,” “ARDS,” and/or “cytokine storm.” Relevant articles published post-1980 that were found in these searches, cited by articles found in these searches, or present in the

author's own files were reviewed. Results: The pulmonary epithelium constitutes approximately 90% of the epithelial-endothelial barrier in the alveolus while also being the primary target of IAV. Accordingly, damage to the epithelium plays a major role in IAV-induced ARDS. Very early during infection, IAV can directly target sodium channels and tight junctions on the pulmonary epithelium and induce alveolar fluid accumulation. Later, once IAV infection of the epithelium is widespread, IAV can also induce epithelial apoptosis and necrosis, further exacerbating lung injury. Epithelial cells can also produce various chemokines in response to IAV, including the neutrophil-recruiting chemokine CXCL8. Neutrophils subsequently adhere to the endothelium and extravasate from the blood to the alveolar wall and lumen. This egress of cells can damage the epithelial-endothelial barrier and further exacerbate fluid accumulation in the alveolus. Upon entering the alveolus, neutrophils produce toxic granules and neutrophil extracellular traps that damage both the alveolar epithelium and endothelium. Chemokines produced by epithelial cells, neutrophils, and to a lesser extent, alveolar macrophages can also recruit monocytes to the lung. Under the influence of the local milieu, monocytes can differentiate into macrophages. During the acute phase of IAV, macrophages may damage the epithelial-endothelial barrier in the lung by the production of a wide array of cytokines and toxic molecules. There is currently contradicting evidence as to whether macrophages cause apoptosis or repair of the pulmonary epithelium during IAV infection. In reality, it is likely that macrophages are able to fulfill both roles: causing epithelial apoptosis early during infection and then serving to repair the epithelium as the infection abates. Finally, although there is some evidence to suggest that IAV can infect, and damage, the pulmonary endothelium, perhaps of greater importance is the ability of the endothelium to act as a driving force behind the "cytokine storm" seen in severe IAV infections. Conclusions: Damage to the pulmonary epithelial-endothelial barrier during IAV infection is a complex and multifactorial event that involves not only epithelial and endothelial cells but also the residential and infiltrating leukocytes. This is in contrast to models of ARDS derived from for example septic shock, in which there is a more prominent role for damage to the endothelium than to the epithelium. This model of disease development would suggest that therapies directed at protecting the epithelial layer and accelerating re-epithelialization may be important in preventing severe pulmonary disease from IAV.

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Association of HA-222 polymorphisms in influenza A(H1N1)pdm09 viruses and fatal outcomes among women of reproductive age

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Background: Mutations in the influenza A(H1N1)pdm09 HA1-222 residue, with a substitution of aspartic acid to glycine, have been associated with severe disease and death. Because pregnant women are at a particularly high risk for further clinical complications by influenza infection, we investigated the role of HA-222 polymorphisms in disease severity among women of reproductive age infected by influenza A(H1N1)pdm09, presenting with ILI, SARI and fatal outcomes. Materials and Methods: The study population comprised 88 women of reproductive age (15-44 years; 54 pregnant and 34 nonpregnant) from Rio de Janeiro (southeast) and Rio Grande do Sul (south of Brazil), recruited from April 2009 to February 2013 in the scope of the Influenza Surveillance Program. The sample comprised ILI (n = 26), SARI (n = 28) and fatal (n = 36) cases, according to WHO case definitions. Clinical specimens (oro-nasopharyngeal swabs or aspirate) and demographic, clinical and epidemiologic information were collected by the epidemiologic surveillance teams, using a nationally standardized questionnaire. After nucleic acid extraction (QIAmp Viral mini-kit; QIAGEN), influenza detection was performed using real-time RT-PCR (CDC protocol). Polymorphisms at positions HA222 and NA275 were identified by pyrosequencing (Deyde VM, et al. *Antimicrob Agents Chemother*. 2010;54: 1102). Descriptive, bivariate analyses (chi-square/Fisher exact test and t test for means) and

multiple logistic regressions (considering variables of epidemiologic relevance/plausibility) were carried out and considered significant when P < .05. Results: Of 88 investigated women, 66 (75.0%) were infected by a wild-type virus (D222). Polymorphisms at HA-222 residue (n = 22) were identified as follows: D/N/G (n = 10); D/G (n = 7); D/N (n = 2); N/G (n = 2); and G (n = 1). Among ILI and SARI cases, D222 was found in 95.8% and 92.9%, respectively. In contrast, polymorphisms were identified in about 60.0% of fatal cases (*P* < .001). Pregnant women were not more severely affected than nonpregnant women (*P* = .117). Compared with the wild type, infections by HA-222 mutant viruses showed significantly higher frequencies of SARI (90.5% vs 62.5%; *P* = .012); fatal outcome (86.4% vs 26.6%; *P* < .001) and dyspnea (90.5% vs 62.5%; P = .012) and a lower frequency of ILI (9.5% vs 27.5%; *P* = .012). In addition, the presence of polymorphisms was significantly associated with duration of symptoms (6.1 ± 3.3 days vs 3.1 ± 2.4 days; *P* < .001). After multiple logistic regressions (final model, chi-square = 28.4; *P* < .001), HA-222 polymorphisms were independently associated with a fatal outcome (Adj OR = 27.6; 95% CI 5.7-133). Conclusions: These findings corroborate previous reports in the literature on the association between influenza A(H1N1)pdm09 HA-222 mutations and progression to death. The impact of HA-222 polymorphisms on clinical severity of disease is similar among pregnant and nonpregnant women. Financial support: Carlos Chagas Filho Foundation for Research endowment for Rio de Janeiro (FAPERJ), process n. 111.802/2012; Science and Technology Department (DECIT), Ministry of Health and National Counsel of Technological and Scientific Development (CNPq), Brazil.

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Genetic requirement for hemagglutinin glycosylation and its implications for the influenza A H1N1 evolution

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Background: Influenza A virus has evolved and thrived in human populations. Since the 1918 influenza A pandemic, human H1N1 viruses had acquired additional N-linked glycosylation (NLG) sites within the globular head region of hemagglutinin (HA) until the NLG-free HA head pattern of the 1918 H1N1 virus was renewed with the swine-derived 2009 pandemic H1N1 virus. Moreover, the HA of the 2009 H1N1 virus appeared to be antigenically related to that of the 1918 H1N1 virus. Hence it is possible that descendants of the 2009 H1N1 virus might recapitulate the acquisition of HA head glycoslylation sites through their evolutionary drift as a means to evade preexisting immunity. Materials and Methods: Here we evaluate the evolution signature of glycosylations found in the globular head region of H1 HAs using HA NLG mutant viruses rescued by reverse genetics, to determine their impact in virulence and transmission of H1N1 viruses. Results: We identified a polymorphism at HA residue 147 associated with the acquisition of glycosylation at residues 144 and 172. By in vitro and in vivo analyses using mutant viruses, we also found that the polymorphism at HA residue 147 compensated for the loss of replication, virulence and transmissibility associated with the presence of the N-linked glycans. Conclusion: Our findings suggest that the polymorphism in H1 HA at position 147 modulates viral fitness by buffering the constraints caused by N-linked glycans, providing insight into the evolution dynamics of influenza viruses with implications in vaccine immunogenicity.

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The molecular behavior of influenza A(H1N1pdm09) HA gene in Brazil four years after the pandemic event

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Background: The pandemic virus influenza A (H1N1pdm09) was first detected in May 2009 in Brazil, later presenting an extensive spread throughout the Brazilian population. Previous evolutionary studies clustered the early H1N1pdm09 groups in seven clades, based on specific mutations at the viral genome. Due to the evolutionary strategies of influenza viruses, it is necessary to constantly follow up on the new viral variants. The aim of this study was to reconstruct the phylogenetic profile of the H1N1pdm09 in Brazil during the two past epidemic seasons (2012 and 2013). Materials and Methods: We performed the Sanger sequencing protocol available from the CDC Influenza Division to a portion of the HA1 domain (1 to 886 nucleotides). A dataset with 352 HA sequences was constructed, including 82 sequences obtained from H1N1pdm09-positive Brazilian samples and other representative sequences from GISAID from 2009 to 2013. The sequences were aligned using ClustalW and the best-fitting nucleotide substitution model (HKY + Gamma distribution among sites) was estimated by means of a model test tool (both integrated within the MEGA 5.1 software). The maximum likelihood (ML) tree was constructed at the PhyML Web page (http://www.atgc-montpellier.fr/) and it was observed with FigTree v1.4.0 software. Results: The ML trees showed that Brazilian samples were grouped into five subgroups with specific signatures, all of them presenting the mutation S220T against vaccine strain A/California/7/2009. This mutation refers to clade 7 previously described in the literature, which has already been observed as a greatly diverse clade. Two subgroups were observed co-circulating during the months from March to July 2012, and the subgroup with the signature N277T seems to be specific to samples from South America. In 2013 three subgroups were observed, two co-circulated during March-April and the other in May with the signatures K60R, R222K, I251V and I333M. Conclusions: The HA diversification of the H1N1pdm09 virus is evident due to the co-circulating subgroups found in 2012 and 2013. Despite that the HA segment best represents the influenza evolution, care is needed when interpreting genetic data specifically related to a single segment. Complementary antigenic analyses are necessary to better assess the importance of these subgroups and the signatures described here.

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Evolutionary dynamics of influenza virus A(H3N2) during 1999-2012 in the southern, southeastern and northeastern Brazilian regions

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Background: Influenza infection is the most frequent cause of acute respiratory illness requiring medical intervention affecting individuals in all age groups. Of the distinct influenza viruses affecting humans, influenza type A, subtype H3N2 has been dominant in most seasonal influenza epidemics since 1968. Analysis suggests that the evolutionary dynamics of influenza H3N2 is modeled by a complex interaction between the high mutation rate of the virus, gene reassortments and selection exerted by the immune system. In Brazil, knowledge of influenza virus epidemiology and evolution is still incipient. Thus, our objective is to reconstruct the profile of evolution and geographic spread of influenza H3N2 in Brazil during 1999-2012 and to understand the role of Brazil in the dynamics of global virus spread. Materials and Methods: For this study, we used a total of 205 Brazilian sequences of the HA1 portion of the hemagglutinin gene (HA) collected between 1999 to 2012, as well as sequences from other South American countries (n = 58), Australia (n = 96), Hong Kong (n =

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72), United Kingdom (n = 58) and New York (n = 70) obtained from the GenBank and GISAID databases. Phylogenetic reconstructions were performed by the maximum likelihood and Bayesian methods using PhyML and MrBayes programs, respectively. Temporal structure of the phylogenetic tree was investigates with Path-O-Gen v1.3, whereas geographic structure was analyzed using the maximum association index (AI), parsimony score (PS) and monophyletic clade size (MC) statistics available from the BaTS program. Evolutionary and phylogeographic parameters were jointly estimated with the BEAST v1.7.5 program using the coalescence model "Bayesian skyline plot," a relaxed molecular clock and a reversible discrete model of geographic dispersion. Results: The phylogenetic tree of influenza A(H3N2) sequences showed a strong temporal structure, with a clear increase of genetic divergence over time. The mean rate of evolution of the HA1 portion of HA gene was estimated at 4.7 x 10^{-3} substitutions/site/year. The AI and PS statistics showed that despite the great intermix between influenza sequences from different countries and from different Brazilian regions, the distribution of viral sequences in the phylogenetic tree was not completely random. The MC statistic test was significant for all traits excepting New York and United Kingdom. The reconstruction of the worldwide dissemination dynamic of influenza A(H3N2) allowed us to verify that Brazil was tightly connected to other South American countries and to New York, while viral exchanges with other regions were very limited. Phylogeographic reconstructions also showed that there are no Brazilian samples in the trunk of the phylogenetic tree. Inside Brazil, the major flux seems to happen from southeastern to southern and from southern to northeastern Brazilian regions. Conclusions: This analysis indicates that Brazil has a secondary role in the emergence and dissemination of new influenza A(H3N2) lineages at a global scale. These results suggest that the influenza A(H3N2) viruses mainly came into Brazil via South and North American countries to the southeastern region, going then to the southern and finally to northeastern Brazilian regions. Further analysis associating evolutionary and seasonality data is important to clarify the dynamics of influenza in Brazil.

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Deciphering the virulence and transmissibility through sequence and structure of avian H1N1 influenza A viruses in mice and ferrets prior to mammalian adaptation

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Background: Among all subtypes of influenza A viruses (IAV) that are found in wild aquatic birds, only H1, H2 and H3 subtypes have caused epidemics and pandemics in human population to date. During reported human history, there have been three pandemics caused by H1N1 viruses in 1918, 1977 and 2009, either through the direct adaptation of the virus to mammals or due to the acquisition of several segments from avian H1N1 viruses. We previously showed that purely avian H1N1 viruses of North America have the potential to cause lethality in a DBA/2J mouse model, as well as increased morbidities and transmissibility in ferrets. Therefore, it is important to understand the dynamics of avian H1N1 viruses at the genomic level to address the questions regarding their potential virulence and transmissibility in mammals prior to mammalian adaptation. In this study, we analyzed the whole genome sequences of North American avian H1N1 influenza A viruses in an effort to identify the association between the genotype and pathogenicity of the viruses. Materials and Methods: Based on the genome sequence of 31 North American avian H1N1 influenza A viruses, we investigated the evolutionary relationships between these viruses and a selection of publicly available sequences from North American and Eurasian strains of human, swine, avian and 2009 pandemic viruses. We then examined the relationship between single residue variations in the proteome of 31 H1N1 influenza A viruses and the pathogenicity scores previously identified in DBA/2J mice. The strains with similar amino acid identity were grouped and the pathogenicity scores of each group were compared by ANOVA. Using available structures we examined the statistically significant single residue variations to assess potential structural changes that might influence protein function. Results: As expected, the 31 sequenced avian H1N1 strains are closely related to other publicly available North American avian

H1N1 viruses across all gene segments. However, their relationship to other H1N1 strains is more varied between segments. In terms of the physicochemical properties (eg, instability index, aliphatic index and grand average of hydropathy [GRAVY]), our analyses demonstrated that pathogenicity of avian H1N1 influenza A viruses are related to NEP protein in terms of aliphatic index and GRAVY (P = .0072 and .0119, respectively), NP protein in terms of GRAVY only (P = .025), and NS1 and PA proteins in terms of instability index (P = .0385 and .0382, respectively). We identified a total of 84 nonsynonymous single nucleotide polymorphisms (SNPs) in the proteome of avian H1N1 viruses that were statistically associated with pathogenicity ($P \leq .05$). We have examined the statistically significant SNPs in the effector domain of NS1 to understand the structural and functional importance of each substitution. Overall, 26% of the statistically significant residues are present at the effector domain and PI3K binding interface, indicating a selective pressure. “Pathogenic residues” appear to create a tighter and more favorable binding surface between the PI3K inhibitory domain and the NS1 protein. The two pathogenic variants in PA appear to impact the flexibility of the hairpin influencing the stability of the PA-PB1 interaction. We also observed variations in NP that increase the physical size of residues in positions along the cross helix in low pathogenic strains that might increase flexibility of the helix and decrease stability between the NP protein and RNA. Conclusions: Analyses to date indicate that amino acid differences in the PA, NP, NS1 and NEP proteins are associated with the pathogenicity of avian H1N1 viruses. The unique patterns in the genomes of North American avian H1N1 viruses might help us decipher the genetic codes for the virulence and transmissibility of influenza A viruses in mammals prior to adaptation. We are currently analyzing the interactions of other residues within and between the proteins in the proteome of avian H1N1 viruses. Furthermore, the future identification of these pathogenicity-associated amino acids in closely related wild aquatic birds has the potential to improve surveillance measures by identifying strains that might easily become adapted to mammalian hosts.

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Where are the highly pathogenic chicken H5N2 viruses in Taiwan: implication from problems of using animal influenza vaccine

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Background: Consecutive passage of low pathogenic avian influenza (LPAI) H5/H7 avian influenza viruses could result in their acquisition of pathogenicity and became HPAI in terrestrial poultry. Especially, those LPAI H5 and H7 viruses usually gain this ability after interspecies transmission from aquatic birds to terrestrial poultry after a series of continuous transmission chains in the field. In addition, the historical H5N2 episodes, such as occurred in Pennsylvania, 1983-84, in Mexico, 1994-95, and in Italy, 1997-98, had been directly linked to bird migration. The chicken H5N2 viruses have existed for about ten years in Taiwan since the first outbreak occurred between late 2003 and early 2004. However, the origin of those viruses is still an enigma. The chicken/Taiwan/A703-1/2008 (H5N2) virus in the 2008 outbreak presented two different intravenous pathogenicity indices: 0.68 (LPAI) in 8-week-old special pathogen-free (SPF) chickens and 1.86 (HPAI) in 6-week-old SPF chickens. Phylogenetic analysis of the limited surface gene sequences of these two Taiwanese chicken H5N2 viruses demonstrated that they were Mexico/95-like viruses. The interesting question is why and how these Mexico/95-like viruses introduced into chicken farms were repeatedly waiting to be addressed. The aims of this study were to investigate the origins and mechanisms of evolutionary behaviors of both chicken and duck H5N2 viruses isolated in Taiwan. Materials and Methods: The virologic and serologic surveillance for avian influenza was carried on in a live bird market during the two time periods (2005 to 2006 and 2008 to 2013). Both chicken and duck swabs were sampled biweekly (during seasonal months) or monthly obtained from healthy avians. We also collected

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chicken blood samples from December 2008 to April 2012. In total, 29 duck H5N2 viruses, one duck H5N6 virus, and one duck H5N8 virus were isolated during the study period, and additional several H5N2 viruses from late 2012 and early 2013 were genetically characterized with the previous Taiwanese chicken H5N2 viruses with other avian influenza viruses from neighboring countries and areas. The results of genetic and serologic examinations between chicken and duck H5N2 viruses by the maximum likelihood method, amino acid comparisons and hemagglutination inhibition assay (HI) assay, and their antigenic relationships with H5N1 viruses isolated in Hong Kong and China, were analyzed. Results: After fully sequencing and phylogenetic analyzing, the results showed that the HA and NA genes of Taiwanese chicken H5N2 viruses clustered together with those of the Mexican H5N2 strains isolated from the outbreaks during 1994-1995.This finding implies that the Taiwanese chicken H5N2 viruses did not derive directly from the Taiwanese duck H5 viruses. In fact, Taiwanese duck H5 HA genes comprised two sublineages: (1) H5N2 viruses isolated in 2009 grouped with the 2005 Korean H5N2 viruses from aquatic birds, and (2) the rest formed a specific and unique branch that was not close to any other H5 HA genes from the rest of the world. In addition, the internal genes of these Taiwanese chicken H5N2 viruses may come from Taiwanese chicken H6N1 viruses, but not from the aquatic birds in Taiwan or other regions. The serologic results of the continuous chicken serums collected from January 2009 to April 2012 also supported the seroprevalence rate of 7.80% (79/1013; HI titer ≥ 40) against CK/Hidalgo/28159-232/1994 (H5N2). Intriguingly, we also detected seropositive (90/1013; 8.88%) against Taiwanese duck DK/Taiwan /DV192/2006 (H5N2) virus. Conclusions: The unique gene constellations and phylogenetic results implied that Taiwanese chicken H5N2 viruses did not come from the aquatic birds, the natural reservoirs of AIVs. Thus, they probably came from unnatural sources. The serology data also revealed that both the Mexican-like H5 viruses would have been present in Taiwanese chicken and duck H5 viruses for a period of time, possibly driving the H5N2 virus being endemic. This study also raised awareness of the biosecurity and restrictions of using avian vaccine and also the urgent need for systemic and long-term surveillance of avian influenza virus.

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Antigenic cross-reactivity between Eurasian low pathogenic H5 and highly pathogenic H5N1 avian influenza viruses

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Background: Highly pathogenic avian influenza (HPAI) is an extremely contagious and systemic disease of poultry leading to high mortality. Especially, Asian-lineage H5N1 HPAI has been circulating in the avian population since 1996 and comprises at least 10 groups, or clades, of antigenically and genetically distinct strains. Clade 2.3.2 variants are now considered the dominant type in East Asia. H5N1 HPAI viruses have evolved considerably over this time, but no closely related low pathogenic precursor strains of the H5 subtype have been isolated. Antigenic differences between low pathogenic avian influenza (LPAI) H5 and HPAI H5 are not fully understood yet. In this study, we investigated the cross-reactivities between anti-LPAI H5 antisera and HPAI viruses. Materials and Methods: We used the 10 LPAI H5 viruses isolated from wild birds in South Korea from 2005 to 2011. These viruses were propagated and titrated using embryonated chicken eggs. For genetic characterization, phylogenetic analysis was performed with the MEGA 5 program using the maximum likelihood analyses. For preparation of chicken antisera against 10 LPAI H5 viruses, three chickens per each virus were immunized with 10^6EID_{50}/dose of inactivated virus combined with commercial oil adjuvant. Cross-reactivities of 10 anti-LPAI H5 antisera were tested using hemagglutination inhibition (HI) and microneutralization (MN) assay against 10 LPAI H5 and 4 HPAI H5 antigens, including A/Vietnam/1194/2004 (Viet/04), A/Indonesia/5/2005 (Indo/05), A/chicken/Korea/ES/2003 (ES/03) and A/mandarin duck/Korea/K10-483/2010 (Kr/10), belonging to clades 1, 2.1, 2.5 and 2.3.2, respectively. Results: Phylogenetic analysis showed that all HA genes of LPAI H5 belonged to Eurasian avian lineage and were similar to the viruses isolated in the Asian countries, such as Mongolia, China, Korea, and Japan, which share the East Asian–Australasian migratory bird flyway. The nucleotide

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homology between the HA genes of LPAI and HPAI H5 ranges from 87.7% to 90.7%, and at the amino acid level ranges from 90.1% to 92.8%. In particular, the HA gene of Kr/10 (clade 2.3.2) had the lowest homology with LPAI H5 viruses. In the HI and MN assay, 10 antisera reacted with 10 LPAI H5 antigens and partially reacted with ES/03 (clade 2.5) and Viet/04 (clade 1) HPAI antigens. However, Indo/05 (clade 2.1) and Kr/10 (clade 2.3.2) HPAI antigens were not reactive with anti-LPAI H5 antisera. Conclusions: Recent evidence has demonstrated that the presence of LPAI viruses may play an important role in host ecology and transmission of HPAI, providing enough immunologic memory to resist reinfection with HPAI. The results of genetic analysis indicated that all of the LPAI H5 viruses were phylogenetically distinct from HPAI H5 viruses. The HA genes of Kr/10 and Indo/05 had lower nucleotide homology with those of LPAI H5 viruses than other HPAI viruses. In the antigenic comparison, our study showed that LPAI H5 antisera exhibited different patterns of cross-reactivity with HPAI H5N1 variants. Particularly, the LPAI H5 antisera were cross-reactive to ES/03 and Viet/04. However, Kr/10, one of the currently circulating HPAI viruses in East Asia belonging to clade 2.3.2 and Indo/05, had low genetic and antigenic similarity with LPAI H5 viruses. Based on our results, some HPAI antigens were neutralized with anti-LPAI H5 antisera, but clade 2.1 and 2.3.2 were not. Further studies are required to analyze the relatedness of widespread of these clades in wild birds and low antigenic similarity with LPAI H5 viruses.

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Universal genomic amplification facilitates influenza B virus genomic sequencing, advanced diagnostics, and reverse genetics rescue

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Background: Influenza B virus (IBV) infections account for a significant part of the annual influenza epidemics. On average, IBVs account for more than 20% of the annual influenza infections, with up to ≈ 60% in some years. IBV is a segmented negative sense RNA virus and its genome consists of 8 segments of viral RNA. Although IBV genomic sequence information is critical for basic research, diagnostics, and vaccines to combat the IBV, there are a limited number of IBV sequences in public databases, especially complete genome sequence information. Materials and Methods: IBV RNA was purified using a ZR-96 Viral RNA Kit (Zymo Research, Irvine, CA). Universal primers were designed by aligning all of the publicly available sequences using MUSCLE. Primers were designed based on the consensus sequences of termini with consideration of natural variations. Amplicons were sequenced using next-generation sequencing platforms using procedures and protocols from manufacturers. New reverse genetics plasmids for rapid ligase-independent cloning of the IBV genome were developed by adding 22 bp stuffer fragments between the RNA polymerase I promoter and terminator. These modifications create a cloning strategy that can be accomplished using short regions (15 to 19 nucleotides) of identity between the plasmid and inserts and commercially available enzymes (eg, In-Fusion HD Cloning System, Clontech Laboratories). Recombinant IBVs were generated by cotransfection of 8 reverse genetics plasmids carrying the cDNA of each gene segment into 293T/MDCK co-cultured monolayer. Results: To develop universal primer sets to amplify any IBV genome in a single reaction vessel, we initially designed tailed primers that were complementary or identical to the universally conserved 9 and 10 nucleotides (Uni9 and Uni10) at the extremities of all IBV genomic RNA segments. Primers with various tails were tested and the one with best sensitivity and specificity was the Uni9/Uni10 primer set that comprises the 3 primers Uni9/FluB1, Uni10/FluB1, and Uni10/FluB2. Using the optimized RT-PCR cycling conditions, this primer set amplified the full genome of various influenza B viruses, albeit with low. Thus, to increase the sensitivity and better control the ratio of each segment in the genomic RT-PCR, primers were modified based on multiple sequence alignments. Using optimized RT-PCR cycling parameters, we obtained robust genomic amplification with very high sensitivity (limit of detection = 10 TCID50/RT-PCR reaction). In collaboration with various laboratories throughout the world, we applied this genomic amplification technology to amplify the genomes of more than 1000 contemporary IBVs. The genomic amplification products were successfully sequenced with different sequencing technologies, including Sanger sequencing and a variety of NGS platforms, such as Illumina HiSeq2000 and MiSeq, Life Technologies Ion Torrent sequencing, and Roche 454 pyrosequencing. This procedure has been

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used to amplify the complete genomes of more than 1000 IBVs to date. To rapidly generation recombinant IBVs from our genomic amplicons, we also developed a recombination-based cloning strategy that enables integration into reverse genetics plasmids without consideration of the sequence of the virus. The genomic amplicons of a lab-adapted B/Russia/69-like virus and a recent isolate B/Brisbane/60-10/2010 virus were cloned into the recombination-based plasmids. Sequence verified plasmids were cotransfected into co-cultured 293T/MDCK cells, and the IBVs were successfully rescued and characterized by comparison with their WT counterparts. Conclusions: We developed a universal single well/tube technology to amplify the complete genome of any past, present or future IBV, irrespective of strain genotype and/or specimen origin. DNA copies of more than 1000 IBVs were generated using this genomic amplification technology and were successfully sequenced using Sanger dideoxy sequencing or multiple next-generation sequencing platform. Additionally, specialized terminal sequences incorporated into the DNA products during the genomic amplification process and modified reverse genetics plasmids enable rapid simultaneous cloning of the entire genome into reverse genetics plasmids for recombinant virus engineering and/or vaccine seed stock creation.

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A new genotype resulting from the reassortment of clade 1.1 and 2.3.2.1 at the origin of the human and animal H5N1 virus cases in Cambodia in 2013

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Background: In Cambodia since 2004, A(H5N1) virus has been detected in 39 poultry outbreaks and 32 patients (27 deaths) to May 1, 2013. Until 2012, all A(H5N1) strains isolated in Cambodia belonged to clades 1 and 1.1, of which six distinct lineages were identified. Lineage 1 viruses shared the same ancestor with viruses from Thailand, while all the lineages detected since 2005 were closely related to viruses detected in Southern Vietnam. Materials and Methods: Samples were obtained between January and March 2013. The samples comprised 10 confirmed human cases, 6 poultry outbreak investigations and samples from environmental surveillance of live poultry markets. All samples included in this study tested positive for A(H5N1) virus by real-time RT-PCR. Full genome sequences were generated from 31 strains amplified after inoculation into MDCK cells, embryonated eggs, or directly from the original specimen. Phylogenetic analyses were performed using the maximum likelihood method on all the genes. Antigenic analyses were also performed using reference antisera. Results: The phylogenetic analysis of 8 genes revealed that the 31 A(H5N1) strains isolated and sequenced in Cambodia in 2013 resulted from reassortment between clade 1.1 and clade 2.3.2.1 viruses. While the HA and NA genes belong to the clade 1.1, genotype Z, the 6 internal genes are closely related to the viruses of the clade 2.3.2.1 (A/Hubei/1/2011-like). Antigenic analysis demonstrated a significant antigenic drift. Conclusions: In this study we report the emergence of a new genotype of clade 1.1 A(H5N1) virus in Cambodia associated with a significant antigenic drift. The link between the emergence of this new genotype and the increase in human cases observed in 2013 is yet to be determined.

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The 2012/2013 influenza season in Portugal: an antigenic and genetic characterisation of circulating viruses

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Background: The continuous monitoring of the antigenic and genetic properties of circulating influenza viruses is essential to detect any changes that may justify the selection of different vaccine candidates or changes in antiviral recommendations. During the 2012/2013 season, the influenza activity in Portugal was characterized by the co-circulation of influenza B/Yamagata and A(H1)pdm09 viruses with sporadic detections of influenza A(H3) and B/Victoria viruses. This study reports the antigenic and genetic characterisation of influenza viruses isolated in Portugal during the 2012/2013 influenza season. Materials and Methods: During the 2012/2013 influenza season, nasopharyngeal swabs were obtained through the National Influenza Surveillance Programme (ILI cases from primary health care–based surveillance, n = 1266) and from the Portuguese Laboratory Network for Influenza Diagnosis (hospital-based surveillance, n = 165). The National Influenza Reference Laboratory has characterised antigenically by hemagglutination-inhibition assays 388 influenza strains after isolation on MDCK-Siat1 cell culture (233 B/Yamagata, 138 AH1pdm09, 10 B/Victoria and 7 AH3 viruses). Ninety influenza viruses were selected from the beginning, middle and end of the season for HA1 genetic characterisation (43 B/Yamagata, 33 AH1pdm09, 8 AH3 and 6 B/Victoria). Results: In this season, the 145 isolated influenza A viruses showed good reactivity with antisera raised against vaccine viral strains (A/California/7/2009 and A/Victoria/361/2011). Genetically, most pandemic viruses (28 of 33) were from clade 6 (A/St. Petersburg/27/2011) and 5 from clade 7 (A/St. Petersburg/100/2011). Seven seasonal AH3 viruses clustered in group 3C (A/Victoria/361/2011) but one virus in group 5 (A/Alabama/05/2010). Isolated viruses from the B/Victoria lineage antigenically and genetically were similar to B/Brisbane/60/2008 (genetic clade 1A). The majority of influenza B/Yamagata viruses (which predominated in this season) showed higher reactivity with B/Massachusetts/02/2012 antiserum than with antiserum raised against the vaccine strain B/Wisconsin/1/2010. Sequence analysis showed that isolated B/Yamagata viruses were distributed between clade 2 (B/Estonia/55669/2011) and clade 3 (B/Wisconsin/1/2010). Most B/Yamagata viruses from clade 3 presented 6 amino acid substitutions (only one of these substitutions was located in an antigenic site of the HA: S202N) while all B/Yamagata from clade 2 revealed 7 amino acid substitutions (including 3 in antigenic sites: I150S, Y165N and S202N). Conclusions: Influenza A viruses, although genetically grouped in different genetic clades, all remained antigenically similar to the vaccine strains. This season, influenza B/Yamagata viruses have copredominated with influenza A(H1)pdm09; however, they showed a greater antigenic and genetic variability. Most of these viruses have diverged antigenically from the vaccine strain and genetically belong now to clade 2 (B/Estonia/55669/2011 and B/Massachusetts/02/2012). Compared with the vaccine strain, viruses from clade 2 presented, at least, 3 amino acid substitutions located at antigenic sites of B hemagglutinin. The few B/Victoria viruses detected in circulation remain similar to the former vaccine strain B/Brisbane/60/2008. This antigenic and genetic scenario was very similar to the one observed at the European level. All the different genetic clades of influenza viruses detected in circulation were uniformly distributed throughout the 2012/2013 season.

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Forming vaccine seed viruses—the interplay of viral genes in classical reassortment

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Background: To ensure that a sufficient quantity of seasonal split vaccine is produced each year, circulating isolates are reassorted with egg-adapted high growth strains. For example, H3N2 vaccine seeds are produced by coinfection of eggs with the circulating H3N2 isolate and the high growth A/Puerto Rico/8/34 (H1N1) strain (PR8). A reassortant virus is then selected as the vaccine seed, based on the presence of the seasonal haemagglutinin (HA) and neuraminidase (NA) genes and the high growth properties of the egg adapted strain. Retrospective analysis of vaccine reassortants indicate a high preponderance (50%) of viruses containing the seasonal PB1 gene, yet the mechanisms driving this gene selection and its impact on vaccine yields are not fully understood. Materials and Methods: In this study, we used reassortants of A/Udorn/307/72 (H3N2) virus (Udorn), a model seasonal H3N2 virus and the high-growth PR8 virus to investigate the selection of dominant gene constellations. Classical reassorting between PR8 and Udorn was performed to confirm the preferential selection of particular viruses in our model. Reverse genetics-derived viruses were grown in multiple eggs and the allantoic fluid analysed for infectious virus (plaque formation), HA content (haemagglutination assay), and particle number (by vRNA analysis). The ability of the polymerase complexes encoded by each virus to produce vRNA and viral mRNA in infected cells was determined by quantitative RT-PCR. Viral protein levels in infected cells, allantoic fluid, and split vaccine preparations were analysed by western blot and relative protein content quantified by densitometry. Additionally, competitive 10- and 9-plasmid transfections were used to identify the coselection of genes that could explain the preferential incorporation of the seasonal PB1. Chimeric PB1 plasmids were then produced, using site directed mutagenesis, to identify the regions directing the preferential incorporation observed. Results: The process of classical reassortment between PR8 and Udorn confirmed a high rate of inclusion of the seasonal PB1 gene with the seasonal HA and NA genes. Despite viruses containing the seasonal PB1 being selected much more often than those with other seasonal virus internal genes, its presence resulted in lower yields of virus and HA compared with the corresponding seed virus with the PR8 PB1. However, the 8-fold fewer virions produced was somewhat compensated for by a 4-fold higher HA per virion. Increased HA protein could not be explained by a difference in the replication or transcriptional activity of the polymerase complexes, and hence, we hypothesized that the PB1 selectively alters the translation of viral mRNA to result in higher HA densities. A higher HA/nucleoprotein (NP) ratio was also identified in past split vaccine preparations when the seasonal PB1 was present, also indicative of a higher HA density in these vaccine viruses. Together, these data suggest a nongrowth dependent mechanism for the selection of viruses containing the seasonal PB1. Competitive 9- and 10-plasmid transfections were then undertaken to investigate alternative mechanisms driving the seasonal PB1 inclusion. These competitive transfections showed a preference for the inclusion of the seasonal PB1 with the seasonal NA and not HA, which could not be accounted for by growth differences. Analysis of chimeric PB1 genes determined that this observed cosegregation of the seasonal NA and PB1 was not directed through the previously identified packaging sequences but through a novel interaction of the internal coding sequence of the seasonal PB1 gene. Conclusion: These studies demonstrate that the inclusion of the seasonal PB1 gene in candidate vaccine seed strains resulted in virus with more HA per virion but a poorer yield of HA overall. This less fit virus was selected on the basis of strong co-segregation of the seasonal PB1 with seasonal NA, directed through a novel interaction of the internal coding sequence of the PB1 gene and the seasonal NA. This study may be important for vaccine production and also allows an understanding of gene constellation restrictions during antigenic shift.

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Amino acid sequence analyses of Taiwanese avian influenza H5N2 viruses with those from neighboring countries

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Background: In 2003, the outbreaks of low pathogenic avian influenza (LPAI) H5N2 virus spread all over Taiwan. In 2008, similar outbreaks occurred by the same subtype, but the pathogenicity was closer to moderate pathogenic (MPAI). In 2012, several outbreaks of high pathogenic avian influenza (HPAI) H5N2 occurred in poultry with wide geographic distributions, including Chunghwa, Tainan, Kaohsiung and Penghu Island. Although there has been no human case reported, at least three poultry workers were identified to be infected with H5N2 through seroepidemiologic study by the Centers for Disease Control in Taiwan. Therefore, the specific aims of this study were: (1) to conduct risk assessment for all the Taiwan chicken H5N2 viruses, (2) to compare molecular changes of Taiwan duck H5N2 isolated in different years, and (3) to investigate the relationship between Taiwan duck H5N2 and those H5N2 isolated from waterfowls of the neighboring countries. Methods: We performed sequence alignment analysis, based on Taiwan duck H5N2 viruses isolated in our laboratory (one in 2005, six in 2009 and two in 2013), and compared them with H5N2 viruses isolated from chickens in Taiwan and different avian species in other countries, available from the NCBI and GISAID gene banks. In addition, viral sequences of Taiwanese H6N1 viruses were also quantitatively and qualitatively analyzed with H6N1 and H5N2 viruses isolated in Asia to fully understand the dynamic changes of both H5N2 and H6N1 viruses. Results: First, the HA cleavage sites of Taiwan chicken LPAI H5N2 viruses in 2003 and 2008 were REKR and RKKR, respectively, yet such sites of chicken HPAI H5N2 viruses in 2012 diverged into 3 types (REKR, RRKR and RKKR). Second, risk assessment of mammalian adaptation amino acids of these viruses found that these two chicken H5N2 viruses from both years had glutamine at position 627 and asparagine at 701 in polymerase PB2 protein. In addition, no N224K and Q226L comutations appeared in the HA receptor binding site of these two Taiwanese H5N2 as well as all Japan and Mexico H5N2 viruses, regardless of their high or low pathogenicity, indicating that they may not have acquired mammalian host adaptation and affinity to α2-6 receptor. Third, comparing Taiwan duck H5N2 isolated in different years and those from neighboring countries showed three patterns, including (1) high amino acid (AA) sequence identities in PA and M1 proteins among all these Asian duck/aquatic birds H5N2 viruses, (2) only high AA sequence identities in NA, PB1, PB2, NP, M2, NS1 and NS2 proteins between Taiwan 2005-2006 duck H5N2 viruses and Japan/Korea waterfowl H5N2 viruses, and (3) many unique mutations in HA protein among Taiwan 2005-06 duck H5N2 viruses, Taiwan 2009 duck H5N2 viruses, and those in Japan/Korea waterfowl H5N2 viruses. For example, Val 51 Met changes occurred in NA protein among Taiwanese duck H5N2 viruses in 2005-2006 compared with those in 2009. Conclusions: Taiwan's early duck H5N2 viruses isolated in 2005-2006 did have a close evolutionary relationship with the H5N2 viruses from the reservoir waterfowls in Japan and Korea, implying the importance of migrating birds in these two countries with impact on Taiwan domestic duck viruses. On the other hand, longer evolution of chicken H5N2 viruses in Taiwan without successful eradication has facilitated endemicity of these viruses over the years. Such endemicity over a long period of time may lead to an increase in the diversity of these H5N2 viruses with better adaptation and greater pathogenicity.

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Multiyear persistence of two pandemic A/H1N1 influenza virus lineages within West Africa

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Background: Despite frequent strong population bottlenecks following seasonal influenza epidemics, influenza A viruses are able to persist in humans through continual global migration, which repeatedly reseeds viral diversity at local scales. However, the role of African countries in this global dynamic remains poorly understood. Through the increased genetic sequencing of African influenza viruses following the emergence of novel swine-origin pandemic H1N1 viruses in 2009, we here identify the unique evolutionary dynamics of pandemic H1N1 viruses in Africa during 2009-2013, including multiyear persistence of viral lineages within Africa. Materials and Methods: Full-length HA and NA sequences of 699 pandemic A/H1N1 influenza viruses collected globally during 2009-2013 were downloaded from GISAID and included in this phylogenetic analysis. This dataset includes 100 viruses that were randomly sampled globally for each year during 2009-2012, as well all available viruses sampled globally during 2013 and all available viruses sampled during 2009-2013 from Africa for which full-length HA and NA sequences were available (n = 177, collected from 15 African countries: Burkina Faso, Cameroon, Cote d'Ivoire, Egypt, Ethiopia, Ghana, Kenya, Madagascar, Morocco, Niger, Nigeria, Senegal, South Africa, Tanzania, and Uganda). Maximum likelihood trees were inferred separately for the HA and NA datasets using RAxML, using a GTR model of nucleotide substitution with a gamma rate distribution. Five hundred bootstrap replicates were generated to assess the robustness of individual nodes. As further confirmation, a full dataset including all pandemic A/H1N1 influenza virus HA genes available in GISAID (n = 8805 sequences) was downloaded and a larger ML tree was inferred for these data. Results: The global population of A/H1N1 pandemic influenza viruses is highly spatially mixed, reflecting the rapid and continual global migration of this novel virus within a new host population. Accordingly, the phylogenetic positions of the majority (137/177; ≈ 77%) of influenza viruses collected in Africa are dispersed throughout the tree, indicative of frequent global viral migration into and out of Africa. However, in two notable exceptions, two monophyletic African viral lineages defined by high bootstrap values (> 90%) are detected over the course of multiple years, indicating that these viral lineages persisted within Africa. One of these lineages, the "West African clade," is continuously detected within West Africa for 2.5 years, from July 2010 (Ghana) to January 2013 (Cote d'Ivoire). This clade consists of 18 total West African isolates: 1 from Cameroon (2011), 3 from Cote d'Ivoire (2012-2013), 10 from Senegal (2010-2012), and 4 from Ghana (2010-2012). Additionally, a second lineage (n = 22 isolates), the "West-East African clade," also is detected within West Africa for 2.5 years, from January 2010 (Cameroon) to June 2012 (Burkina Faso). In addition to West African countries (Burkina Faso, Cameroon, Ghana, and Nigeria), this clade also spreads into Ethiopia in East Africa (2012), as well as a spillover event into Norway in 2013. Conclusions: The 2009 H1N1 pandemic represents one of the most intensively globally sampled and sequenced single disease events in history, providing an opportunity to study viral dynamics in regions that have typically been undersampled for influenza, particularly Africa. Given that air traffic volume within Africa is lower than in other global regions, it is not implausible that influenza virus lineages could persist for many years in Africa without spreading to other continents. Furthermore, the lack of synchrony among the variable seasonal patterns of geographically linked African countries may allow for persistence of influenza viruses even within geographically restricted regions, such as West Africa. Although the intensity of influenza surveillance in Africa still lags significantly behind that of other continents, these findings suggest that substantial viral diversity circulates within Africa, including viral lineages that are not detected elsewhere, warranting further research and surveillance in this understudied region.

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Clinical, immunological, and virological characterization of hospitalized influenza A patients during 2011-2012 in Chile

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Background: Influenza A virus affects mainly the upper respiratory tract and occasionally can cause viral pneumonia in some individuals. Disease outcome is modulated by both viral and host factors. The efficient transmissibility and the pathogenesis shown by the 2009 H1N1 pandemic strain (H1N1pdm2009) during its emergence emphasized the value of monitoring the clinical evolution of infected individuals to establish efficiency mitigation and clinical treatment strategies and to elucidate susceptibility factors in the general population. To date, no in-depth longitudinal studies have been conducted with influenza virus patients, integrating epidemiological data, pathogenesis, clinical findings (eg, presence of comorbidities or susceptibility factors), analysis of immune responses of the host, and a complete characterization of the antigenic properties, virulence, and genome of the virus. Materials and Methods: In this study, for the years 2011 and 2012, serial nasopharyngeal swabs and blood samples were collected through an approved clinical protocol from a total of 78 enrolled patients hospitalized with influenza A. Clinical data were obtained for each patient using the Centers for Excellence in Influenza Research and Surveillance Network Metadata fields (including symptoms, epidemiological history, morbidities, treatments, etc). Results: Current analysis showed that the majority of patients considered to be severe had risk factors of either preexisting comorbidities or belonging to high-risk age groups (individuals < 2 or > 65 years). We also identified that a large proportion (44.3%) of severely ill individuals had some level of immunosuppression, including individuals undergoing long-term medication treatment for unrelated health conditions (eg, arthritis, allergies). Subtyping by qRT-PCR or inhibition of hemagglutination (HI) test established that 87.5% of the 2011 samples corresponded to H1N1pdm09 and 64.7% of the samples of 2012 corresponded to H3N2. We identified 6 individuals who showed a decrease in seroconversion titers (day 21) against the homologous virus strain in comparison with the level of antibodies in preinfection sera (on day 1). Conclusions: Long-term immunosuppression for unrelated health conditions may play a role in human influenza A pathogenesis, which might lead to severity and/or decreased seroconversion. Alternatively, individuals with decreased seroconversion might be a result of antigenic drift of the virus during acute infection. To investigate genomic variation of the virus (quasispecies) during the acute phase of infection, we are currently sequencing 86 samples collected from 23 individuals during the first 7 days of infection. To evaluate immunosuppression, we are also investigating the status of the innate immune response of these patients by ELISA and qRT-PCR in serum and cells of the nasopharyngeal cavity, respectively.

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Identification of variants in influenza genome associated with severity of disease

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Background: Influenza A virus (IAV) can produce infections of varying severity in people. Mechanisms for developing mild, severe or even lethal influenza infections are not known, but it is likely that both viral and host genetic factors contribute to the outcome of infection. We aim to identify novel determinants within the IAV genome that influence severity of the infection and unveil their role in infections with different host genetic background. Materials and Methods: IAVs were obtained from nonhospitalized, hospitalized and intensive care treatment patients, whole viral genomes were sequenced with Illumina technology, and variants that may affect virulence were identified. In further functional studies of the isolates, we addressed gene expression, cytokine production and antiviral signaling cascade activation in peripheral blood mononuclear cell–derived macrophages in response to viral infection. Results: The pilot study was performed with six IAVs isolated from severely ill Finnish patients. Whole genome sequencing of IAV isolates allowed identification of unique variants in viral genomes that may affect virulence. The studied isolates induced differential cytokine responses in macrophages of a healthy individual. The strongest attenuator of cytokine responses in comparison to others appeared also to be a potent transcriptional inhibitor of antiviral and proinflammatory genes in human macrophages. Moreover, this isolate was also found to counteract activation of key protein kinases involved in antiviral signaling pathways. Sequencing data, together with functional studies, allowed us to identify variants in viral PB2, PB1, HA and NS1 proteins that may be essential for strong attenuation of host immune responses and are being further evaluated in our current studies. Conclusions: Here we set up a framework for study of IAVs isolated from patient samples allowing fast identification of IAV genetic variants associated with strong virulence. The pilot study allowed us to identify potential determinants of virulence in viral PB2, PB1, HA and NS1 proteins.

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The evolutionary dynamics of influenza A virus migration and competition in North American wild birds

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Background: The geographical separation of avian host species has structured the avian influenza A virus (AIV) gene pool into independently evolving Eurasian and American populations. Phylogenetic evidence has shown viral migration between these populations. The cocirculation of antigenically similar viruses creates suitable conditions to study viral competition in natural populations. In the mid 1990's, H6 subtype influenza viruses with hemagglutinin (HA) gene derived from Eurasian ancestors were detected in North American wild birds. Since 2003, the endemic H6-HA lineage has not been detected. The introduced lineage has displaced the endemic population. More recently, a Eurasian H3

lineage has been introduced and continues to circulate in North American birds. Recent studies of AIV in North American wild birds suggest bird migratory habits and the discontinuous distribution of susceptible hosts may produce long periods of cocirculation of competing virus strains before lineage extinction occurs. However, the relationship between viral migration and competition dynamics remains ambiguous. Materials and Methods: For this study, we sequenced the full genome of 700 H6 and H3 subtype subtype viruses collected from wild-bird systematic surveillance to investigate the evolutionary dynamics associated with competition between introduced and endemic viral lineages. We determine the most likely location of invasive viral lineage entry and detection, estimate rate of migration between discrete host populations as well as the location and duration of cocirculation of each lineage. We assume competitive exclusion is driven the immune status of the host population and the ability of the introduced lineage to effectively transmit. Results: Our results show repeated introductions of H6 subtypes into North America from Eurasia through *Charadriformes* detected in Delaware Bay. These introductions likely occurred in northern breeding habitats where Eurasian and North American *Charadriformes* comingle. However, lack of data prevents confirmation. Regardless, we estimate a wave front of viral invasion and the decreasing habitat of the displaced H6 lineage. In contrast, H3 viruses were recently introduced through Alaskan *Anserformes*. To date, this lineage has primarily been detected in western surveillance sites although recent evidence suggests the virus has occasionally migrated to several mid-western and eastern regions. Currently 3 H3 virus lineages (2 endemic lineages and 1 introduced Eurasian-origin) circulate in wild birds. It is unclear if these lineages are inducing any cross-reactive immunity to drive exclusion. Conclusions: The circulation and spread of highly pathogenic H5N1 viruses highlight the need to incorporate viral migration in pandemic preparedness planning. We show that despite the importance played by *Charadriformes* hosts in viral introductions and migration, the location of entry points for Eurasian viruses into this population remains enigmatic. Surveillance and control efforts targeted in *Charadriformes* may be important to control viruses introduced to North America from Western Europe. In contrast, Eurasian-origin H3 viruses entered North American via *Anseriformes* in Alaska. This lineage was initially restricted to the Pacific flyway, which suggests control measures focused in this region may be critical for containment of invasive viruses that enter through the Alaskan peninsula. The incorporation of viral migration and host habit can inform viral movement models and identify potential population bottlenecks for disease control efforts. This study highlights the need for targeted expansion of the current influenza surveillance network in wild bird populations.

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Co-circulation of two Influenza pandemic variants of the nonstructural gene in Brazil from 2009 to 2012

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Background: The 2009 H1N1 pandemic (H1N1pdm09) influenza A virus is a triple reassortant among European swine, North American classical swine and Eurasian avian-like lineages. The nonstructural (NS) gene, hemagglutinin gene and nucleoprotein belong to the classical swine lineage. The NS gene codes for the NS1 and NS2 proteins and is an interferon antagonist that allows efficient virus replication. A previous study divided strains of the H1N1pdm09 influenza A virus into two categories based on the presence of a V123I mutation in the NS1 gene: G1 (characterized as 123 Val) and G2 (characterized as 123 Ile). In our study we analyzed the frequency of these mutations in the NS gene sequences to investigate the association or lack of association between these genotypes and the geographic distribution or clinical outcome of the disease. Materials and Methods: We performed Sanger sequencing of the NS gene (1 to 834 nucleotides) from 28 samples collected during and after the 2009 pandemic event (April 2009 to December 2012) from different states of Brazil. In addition to the sequence data, we obtained 43 NS sequences from the GISAID database. We chose all South American sequences. A multiple alignment of nucleotide sequences was constructed using ClustalW from MEGA 5.1 software. Phylogenetic analysis of maximum likelihood (ML) was performed with the PhyML online tool, after determining the best model of nucleotide substitution through the jModelTest v2.1.1 software. The ML trees were reconstructed using the heuristic search algorithm "SPR branch swapping." The reliability of the topology obtained was evaluated by the approximate likelihood-ratio

test. Results: We found two NS variants circulating in Brazil; the V123I mutation was identified in 11 strains from the G2 genotype and 17 strains from the G1 genotype. The G2 genotype circulated in the southeast (n = 8) and south (n = 3) of Brazil only in 2009 (from May to August). The G1 genotype was detected in 11 sequences from 2009, 1 from 2010 and 5 from 2012 (from May 2009 to April 2012). The samples were obtained from patients who had classic flu syndrome, severe cases, or who died, and it was not possible to correlate mutations with disease severity. Throughout the period studied, it was also not possible to observe any geographic correlation within Brazil. Conclusions: The two variants of NS1 were present in Brazil, especially during 2009, in contrast with the other regions of the world, such as Argentina and Chile in South America, where only the G1 genotype was present. Studies with sequences from around the world are still necessary to further investigate the circulation of different genotypes globally and the possible correlation of variants with severity.

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Genotypic variation of contemporary swine influenza viruses circulating in Europe

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Background: As demonstrated by the recent swine-origin H1N1/2009 pandemic, pigs may play an important role in the pathogenicity and epidemiology of influenza A viruses. Pigs are permissive to both human and avian influenza viruses and can thereby function as a “mixing vessel” for the reassortment of genetically diverse avian, swine, and human influenza viruses to generate new, pathogenic strains potentially adapted for cross-species transmission. The European Surveillance Network for Influenza in Pigs 3 (ESNIP3) project is part of an EU-funded surveillance network seeking to gain insights into the epidemiology and evolution of swine influenza viruses (SIVs) circulating in Europe. The projects aims to define and reduce public health risks of influenza and thereby improve pandemic preparedness. Methods: We developed a robust, BLAST-based reference mapping approach to assemble influenza virus next-generation sequencing (NGS) data for large-scale genotypic identification. This method was applied to assemble quality-controlled NGS data sequenced on Roche/454 FLX and Illumina MiSeq platforms, and investigate the molecular evolution of 180 influenza-positive samples isolated from European swine as part of the ESNIP3 project. The consensus sequences generated above were combined with background influenza sequences representing the spread of genetic diversity found in swine to characterize the lineage of origin for each genomic segment. Maximum likelihood (ML) phylogenies were inferred with the GTR+Γ_4 substitution model and clade confidence estimated by bootstrapping with 1000 pseudoreplicates. Results: Full-genome sequencing and phylogenetic analysis reveal the dominant, established strains currently in circulation, as well as ongoing changes in the epidemiology of influenza in European pigs. Application of this whole-genome assembly method demonstrates improved identification of a broad range of influenza A virus genotypes, including novel reassortant strains of SIVs described here for the first time. Briefly, we detected novel reassortments including triple reassortments of human, pandemic H1N1, and swine viruses, and gained insights into the distribution of genotypic variation of SIVs across Europe. Conclusions: The results from this study can now be disseminated to the ESNIP partners through the network-wide database. This novel assembly approach can also be applied and evaluated for the genome assembly of other diverse influenza viruses. Our findings of the possible emergence of novel reassortant strains of influenza viruses highlight the necessity of further exploration and continued surveillance of swine influenza to minimize potential risks to public health.

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Aberrant alveolar macrophages in mice deficient for integrin β6 contribute to an altered response and resolution of influenza

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Background: β6 integrin (Itgb6) is a tightly regulated protein that is constitutively expressed at low levels in healthy lung and significantly up-regulated following injury or pathogenic insult. Absence of this integrin affects the expression of matrix metalloproteinase 12 (MMP12), transforming growth factor β (TGF-β), and collectins and plays a role in the response to pulmonary bacterial and viral infections, including the influenza virus. Given the putative role of β6 integrin in the lung during influenza A infection, we investigated the effect of Itgb6 on the lung's resident leukocyte populations. Materials and Methods: To determine the role of Itgb6 during influenza infection, Itgb6$^{-/-}$ KO and the appropriate age-matched C57BL/6 mice were intranasally inoculated with A/California/04/09 (H1N1) virus, and lungs and bronchoalveolar lavage (BAL) were harvested from infected and naive mice at different times postinfection and stained with fluorescent antibodies for cytometry and cell sorting. Lung tissue was fixed and stained for histology and immunohistochemistry. Magnetically sorted cells were lysed in Trizol reagent and RNA harvested for quantitative RT-PCR of specific cell populations. To detect serum cytokine and immunoglobulin levels, ELISA assays were performed. Results: In the absence of Itgb6 expression, there is a loss of the typical homeostatic CD11c^{positive} CD11b^{low} alveolar macrophage (AM) population and the development of spontaneous, bronchus-associated lymphoid tissue (BALT) in the lungs compared with wild-type animals. This ectopic tissue and the altered lung microenvironment that results from the absence of Itgb6 appear to have a protective effect as KO mice show significantly increased survival. We hypothesized that in the absence of the normal homeostatic cycle of alveolar macrophages, the lung remains more immunologically ready to deal with infection. Likewise, the presence of well-developed B- and T-cell follicles within the lung suggests that these animals are better equipped to mount a swift adaptive response to infection. The potentially protective effects of both BALT tissue and the altered AM population are currently under review. Conclusions: These data suggest a role for Itgb6 in the maintenance of homeostatic lung leukocyte populations through communication between the lung epithelium and the resident and inflammatory macrophages. A potential therapeutic role for selective ablation of Itgb6 is currently being investigated.

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The effect of influenza virus receptor tropism on lipid-surfactant production

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Background: It has been previously shown that avian influenza viruses can target α2,3-linked sialic acid (SA)-rich alveolar type II cells. One of the major functions of alveolar type II cells is the production of pulmonary surfactant, which is essential to life. Pulmonary surfactant is composed of a mixture of lipid and protein that lines the inner surface of the alveoli. Phospholipids such as disaturated phosphatidylcholine (DSPC) and hydrophobic proteins such as SP-B and SP-C play roles in lowering surface tension to prevent the alveoli from collapsing. Recent works on pulmonary surfactant in relation to influenza virus infection have focused on minor surfactant hydrophilic proteins such as SP-A and SP-D, which contribute to innate immunity. Clinically, surface active components of surfactant are important because their alteration can lead to severe pulmonary dysfunction. In this

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study, we tested the hypothesis that avian influenza virus targeting of alveolar type II cells will lead to a reduction in the levels of surface active components of surfactant. Materials and Methods: A/Hong Kong/1/1968 (R1-HK) and A/Hong Kong/1/1968 with hemagglutinin substitutions L226Q S228G (R2-HK) were used as models of human-like and avian-like influenza viruses, respectively. These viruses were a generous gift from Dr Mikhail Matrosovich. Eight-week-old female C57BL/6 mice were infected with 10^5 $TCID_{50}$ of influenza virus diluted in 30 µL of PBS at t = 0. On t = 3 days, mice were euthanized and bronchoalveolar lavage fluid (BALF) was obtained. Viral titers and DSPC, the major component of lipid surfactant, levels were determined. In addition, A549 human lung epithelial cells were infected with a multiplicity of infection of 5 for 18 hours before radiolabeling with ^{3}H-choline (1.5 µCi/1.5 mL). Cells were harvested at t = 1, 2, 4, 6, 9, and 12 hours for radioactivity and cell counts. Results: At three days postinfection, there was a 3.6-fold increase in the amount of DSPC in BALF in R1-HK-infected mice and a 1.8-fold increase in R2-HK-infected mice as compared with mock-infected animals. This result demonstrates an increased secretion of surfactant DSPC after infection with either virus, with the avian-like R2-HK having a blunted response. This difference in DSPC levels was independent of viral load, with each virus replicating to similar levels. To directly measure the impact of viral infection on PC synthesis in type II alveolar cells, we examined choline uptake (as a correlate of PC synthesis) in A549 cells. Infection of these cells by both viruses led to reductions in choline uptake, with a 30% reduction in R1-HK-infected cells and a 45% reduction in R2-HK-infected cells at t = 12 hours as compared with uninfected controls. Conclusions: These data showed that influenza virus infection of type II alveolar cells results in reductions in PC synthesis. As such, the in vivo targeting of these cell types by influenza viruses with avian-like receptor specificities is a likely explanation for the reduced lipid surfactant levels seen in the infected mouse lung. It is possible that the relative reduction in lipid surfactant levels may contribute to the severe disease seen following lung infection with avian-like viruses.

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Genetic analysis of low pathogenic avian influenza H4N6 and H9N2 viruses circulating in West Bengal, India, reveals genetic diversity

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Background: Our recent studies on avian influenza (AI) surveillance among wild, resident and migratory birds, and poultry during 2009-2011 in West Bengal revealed the presence of low pathogenic AI (LPAI) H4N6 viruses in domestic ducks and backyard chickens and LPAI H9N2 in chickens from both backyard and wet poultry markets. A total of 57 AI H9N2 and 15 AI H4N6 from various sites of the ten districts of West Bengal were identified by virus isolation, hemagglutination inhibition assay, molecular subtyping and partial sequencing of the hemagglutinin (HA) and neuraminidase (NA) genes. Materials and Methods: Complete analysis was performed by sequencing the HA gene of 15 isolates of AI H4N6. In addition, full genomes of 7 isolates of H4N6 and 6 isolates of H9N2 viruses were performed. Phylogenetic trees of each segment were generated using neighbour-joining and maximum likelihood methods. Results: The HA genes of both the LPAI H4N6 and H9N2 viruses showed the characteristic features of low pathogenic viruses. Phylogenetic analysis revealed that the LPAI H4N6 viruses clustered with the Eurasian lineage, but showed distinct divergence among viruses of different localities. They also showed closed relatedness with other LPAI subtypes like H4N3, H3N6, H7N6, and H5N2, predominantly of the duck species. On the other hand, the LPAI H9N2 viruses were similar to the G1-like virus sublineage with the closest relatives being H9N2 from Tripura, Pakistan, Saudi Arabia and Iran. The HA receptor-binding domain of all the H4N6 viruses retained the Q226 and G228 suggestive of preferential binding to the avian specific [2,3] linked sialic acids, but surprisingly all the H9N2 viruses possessed human-like residue 226L. Unique mutation in NA of AI H4N6 rendered an additional glycosylation site, while in AI H9N2 it resulted in a loss of glycosylation site. Several other mutations in HA, NP, NS, PA, PB_2 genes were observed suggestive of genetic diversification. Conclusions: Molecular characterization of the LPAI H4N6 and H9N2 viruses in this study indicates genetic diversity and is suggestive of a probable exchange of genetic pools among various LPAI viruses circulating the globe. Continuous surveillance and genetic

characterization of AI viruses are necessary to reveal the introduction of any reassortants in the region, as LPAI viruses possess the potential of acquiring increased virulence and species transfer.

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Assessing the biological significance of influenza mutation frequency with a fidelity variant selected with ribavirin

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Background: The mechanisms by which the influenza A virus (IAV) polymerase complex contribute to the emergence of mutation remain elusive because of the lack of structural data and relevant experimental models. An unresolved question is how populations of genetic variants generated through polymerase error during genomic replication facilitate IAV adaptation. We attempted to generate a virus with reduced mutation error, ie, a “high fidelity” virus. We conducted a mutagen resistance screen by passaging a human seasonal influenza strain A/Wuhan/359/95 (H3N2) in the presence of ribavirin to isolate a ribavirin-resistant variant and to identify possible mutations that may confer decreased mutational frequency to different IAV subtypes. Materials and Methods: Seventeen in vitro passages of six replicate cultures at a constant MOI of 0.01 were performed in the presence of 35-40 μM ribavirin (a purine analogue). Plaque-purified clones derived from two replicate cultures that consistently showed reduced sensitivity to ribavirin were selected and sequenced. The role of the observed mutations in the polymerase genes (PB2, PB1, PA, and NP) in ribavirin resistance was assessed by evaluating the ribavirin dose response on polymerase activity in a mini-genome system, as well as by using reverse genetics rescued viruses carrying the candidate mutations. To determine if the increase in ribavirin resistance can be explained by an increase in selectivity for purine nucleosides, another purine analogue, T-705 (favipiravir), was used to assess the T705-resistance levels of wild-type and mutant H3N2 and H5N1 viruses at the level of the polymerase complex. To confirm that the defined mutations conferring ribavirin resistance reduce viral mutational frequency, TOPO sequencing was performed to compare the mutational rates of the wild-type and mutant viruses in both H3N2 and A/Vietnam/1203/04 (H5N1) backbones. The role of the intrinsic error rate of the polymerase in generating antibody escape mutants when exposed to monoclonal antibody selection pressure was assessed by comparing the frequency of escape mutants in recombinant wild-type and mutant H3N2 viruses after passage in the presence of two monoclonal antibodies. Experimental infection of mice with wild-type and mutant viruses was done to determine the role of IAV diversity on pathogenesis. Results: We determined that the key mutation conferring ribavirin resistance phenotype was PB1-V43I. It conferred an EC50 value higher than wild-type by a factor of 1.73 in the mini-genome assay. Recombinant virus with the PB1-V43I mutation replicate to 10.8-fold higher virus titre compared with the wild-type virus in MDCK cells at 60 μM ribavirin. The mutation frequency of PB1-V43I virus was lower than that of wild-type H3N2 virus by a factor of 0.98-3.12. Monoclonal escape mutants were generated more readily by wild-type H3N2 viruses compared with the PB1-V43I mutant. We next assessed whether the PB1-V43I mutation conferred resistance to ribavirin when introduced into the highly pathogenic H5N1 virus in mini-genome assays and using reverse genetics rescued H5N1 viruses. The mutational frequency of the recombinant H5N1 virus carrying the PB1-V43I mutation was lower compared with the wild-type virus by a factor of 1.39-1.55. The high-fidelity H5N1 virus was comparable to wild-type virus in polymerase function and in one-step growth curves in cell cultures in vitro. It replicated to comparable titres in the lung of infected mice, but was 10-fold less lethal than wild-type virus and exhibited less neurotropism. Conclusions: We created IAV with reduced mutational frequency to provide experimental evidence for the role of quasispecies in antigenic drift and pathogenesis. This model can be applied to further investigate the role of viral genome diversity in other complex traits such as virus transmission and pathogenicity.

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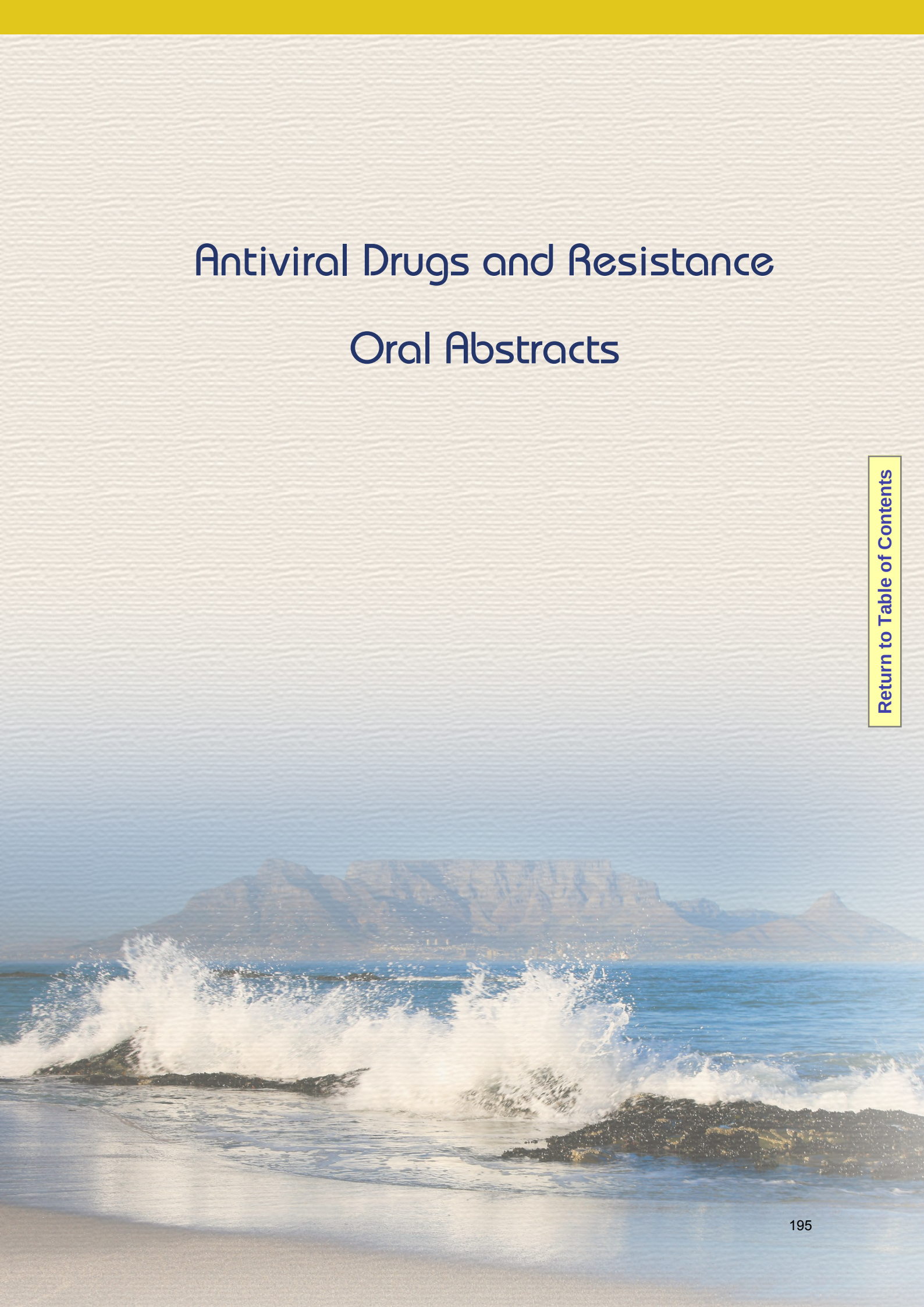

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Mechanism-based covalent inhibitors of influenza virus neuraminidases demonstrate broad spectrum antiviral efficacy *in vitro* and *in vivo*

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Background: While influenza vaccines play an important role in the prevention of disease, due to continual evolution of the viruses, the strains in the seasonal vaccine may not match those circulating by winter and do not provide 100% protection. Furthermore, when a new strain emerges, such as the swine pandemic H1N1 strain or the recently emerged H7N9 strain, it still takes months to prepare the new vaccines. Hence, broadly active antivirals can play an important role in modulating the severity of the influenza infection when vaccines are not available. Both Tamiflu and Relenza, which target neuraminidase, are based on a transition state analogue of the natural substrate sialic acid and are effective against all strains of influenza due to high conservation of residues within the neuraminidase active site. Both products are licensed globally for the treatment and prevention of influenza. Due to differences in their chemical structures, most mutations that have emerged confer resistance only to Tamiflu. However, some different mutations are now causing cross-resistance to Relenza. We describe here a new class of potent, specific, mechanism-based inhibitors of the influenza neuraminidase. They are based on the natural ligand sialic acid, with the substitution of fluorines at positions 2 and 3 of 2,3-difluorosialic acid (DFSA) and function by the transient formation of a covalent intermediate species. Materials and Methods: A series of DFSA compounds were synthesized with substitution of an amino or guanidino group on C-4 and the C-3 fluorine in either the equatorial or axial position. Inhibitors were tested, in enzyme inhibition assays, for inhibition of virus replication in cell culture in a lethal model of mouse influenza and by X-ray crystallography to explore possible formation of the covalent link. Results: The DFSAs inhibited a broad range of influenza virus neuraminidases and formed a covalent link to Tyr406 in the enzyme active site, demonstrated by NMR and by structural analysis. We demonstrated efficacy in enzyme inhibition assays *in vitro* and in cell-based plaque reduction assays, with IC_{50}s in the low nanomolar range. The C-4 guanidino substitution was more potent than the C-4 amino substitution. The equatorial 3-F improved efficacy over the axial 3-F. The DFSAs were also effective at protecting mice against a lethal infection with influenza at drug concentrations comparable with those of Relenza. Conclusions: Since all influenza neuraminidases use the same mechanism to catalyze hydrolysis of sialic acids, DFSAs should also be broadly active. Given the similarity of their structure to that of the natural substrate and their mechanism-based design, we predict that resistance is less likely to arise to this class of inhibitor, since mutations preventing binding of the DFSAs would affect the catalytic function of the neuraminidase enzyme. Importantly, we have demonstrated broad spectrum efficacy against viruses resistant to Tamiflu and Relenza, which indicates a different resistance profile. We believe that this class of compounds warrants further development.

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A novel I221L substitution in neuraminidase confers high-level resistance to oseltamivir in clinical influenza B virus isolates

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Background: We describe influenza B viruses, isolated from an immunocompromised patient after prolonged oseltamivir treatment, with novel I221L substitution in neuraminidase (NA) that confers high-level resistance to neuraminidase inhibitors (NAIs). Virus was first detected 8 days (D8) after onset of symptoms. Oseltamivir (75 mg orally, twice daily) was administered for 6 days (D9 to D14) and 17 days (D20 to D36), but virus was still detected in nasopharyngeal aspirates (NPAs). A progressive diminution of immunosuppression from D41 and the administration of immunoglobulins at D48 and D55 resulted in cessation of viral excretion. Viruses isolated in cell culture from D12 to D30 were sensitive to oseltamivir and zanamivir, with IC_{50} values of 14.7 and 2 nM, respectively, for the first isolate. However, viruses isolated from an NPA at D34 and a nasal swab at D40 were resistant to oseltamivir and zanamivir, with IC_{50} values of 1515 and 24.3 nM and 1776 and 27.3 nM, respectively. NA gene sequencing revealed mutation encoding an I221L substitution in NA at a level of 100% for these two isolates. Additionally, viruses bearing wild-type (wt) and a mix of wt (80%) and mutated (20%) NA genes were isolated from NPAs at D35 and at D37, respectively. Viruses isolated from a nasal swab at D40 and bronchoalveolar liquid at D42 had 100% and 80% mutated NA genes, respectively. Materials and Methods: To better understand this resistance profile, the NA (Km, Ki) of the isolated viruses was enzymatically characterized. The *in vitro* fitness in MDCK cells of viruses carrying wt or mutated NA genes was analyzed. We also quantified the proportions of wt (I221) and mutated (L221) NA genes directly in the patient samples with real-time RT-qPCR using specific probes. To understand how this I221L substitution induced this high-level resistance to NAIs, structural characterization of a wt and I221L variant NA was performed. Results: The Km and Ki revealed that the NA I221L variant had a 2.2 times lower affinity for MUNANA substrate and about 87.5 and 26 times lower affinities for oseltamivir carboxylate and zanamivir, respectively, than wt NA. Viruses with a wt or I221L variant NA had similar growth kinetics in MDCK cells. Five successive passages in MDCK (MOI 10^{-4}), without any selective pressure, revealed the stability of viruses bearing wt or variant NA and the absence of reversion of the I221L substitution. The quantification of wt or mutated NA genes in patient samples revealed a good correlation with the proportion of viruses isolated in cell culture: 100% mutated NA gene in the D34 and D37 samples and 100% wt NA gene at D35. However, the proportions of mutated NA gene were 89% and 99% in the patient samples at D37 and D42 but only 20% and 80% in the corresponding isolates, respectively, suggesting a possible selection for wt virus by MDCK cells for these samples. We used X-ray crystallography to understand the structural basis of resistance of the I221L variant NA to oseltamivir relative to wt. Crystals of the variant in complex with both inhibitors diffracted to high resolution, showing clearly defined density for bound oseltamivir and zanamivir. The structure of the I221L-oseltamivir complex showed that the leucine residue protrudes toward the hydrophobic pocket of the active site that accommodates the pentyloxy substituent of oseltamivir, thus causing a change in the conformation of the inhibitor such that the C81 carbon moves about 1.3Å from the wt NA bound position. Conclusions: Novel I221L NA substitution in influenza B viruses confers a high level of resistance to NAI while retaining good virus fitness. Enzyme kinetic analysis with crystal structures of the I221L variant NA provide a structural explanation of resistance of this variant to oseltamivir. Our structures show that, due to the I221L substitution, binding of the pentyloxy substituent of oseltamivir is not as favorable as in the wt NA.

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Characterization of the R292K neuraminidase mutation that confers resistance to the neuraminidase inhibitors in a novel H7N9 human isolate

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Background: From February 19 to May 30, 2013, the novel H7N9 influenza virus has caused 132 human infections with 37 fatal cases. The neuraminidase (NA) inhibitors are currently the front-line therapeutic options for the novel H7N9 influenza viruses, which possess an S31N mutation that confers resistance to the M2 ion-channel blockers. We characterized the A/Shanghai/1/2013 virus isolated from the first confirmed human case of A/H7N9 disease in China. Materials and Methods: The A/Shanghai/1/2013 and the A/Shanghai/2/2013 H7N9 influenza viruses provided by the China CDC have been passaged in eggs and MDCK cells before characterization. The A/Shanghai/1/2013 isolate contained a mixed population of R (65%; 15/23 clones)/K (35%; 8/23 clones) at the NA residue 292, as determined by clonal sequencing. The R292K mutation has been reported to confer resistance to NA inhibitors in N2 and N9 subtypes. Plaque purification was performed to derive the wild-type virus (NA^{R292}) or the mutant virus (NA^{K292}). We determined the sensitivity of the virus to NA inhibitors using the fluorescence-based NA inhibition assay and the dose-response curve in MDCK cells. Results: A/Shanghai/1/2013 with mixed R/K at residue 292 exhibited a sensitive phenotype to NA inhibitors by the enzyme-based NA inhibition assay. The plaque purified A/Shanghai/1/2013 with K292 showed decreased sensitivity to zanamivir by 62-fold, to peramivir by > 1000 fold, and to oseltamivir carboxylate by > 30,000 fold compared with its plaque purified wild-type counterpart possessing R292. In MDCK cells, the plaque purified A/Shanghai/1/2013-NA^{K292} virus exhibited no reduction in viral titer under increasing concentrations of oseltamivir carboxylate (range 0-1000 μM), whereas the replication of the plaque purified A/Shanghai/1/2013-NA^{R292} and the A/Shanghai/2/2013 viruses were completely inhibited at 250 and 31.25 μM of oseltamivir carboxylate, respectively. Although the plaque purified A/Shanghai/1/2013-NA^{K292} virus exhibited lower NA enzyme activity and a higher K_m for 2′-(4-methylumbelliferryl)-α-*D*-*N*-acetylneuraminic acid than that of the wild-type A/Shanghai/1/2013-NA^{R292} virus, the A/Shanghai/1/2013-NA^{K292} virus formed large plaques and replicated efficiently *in vitro*. Conclusions: Our results confirmed that the R292K NA mutation confers resistance to oseltamivir, peramivir, and zanamivir in the novel human H7N9 viruses. Detection of the resistance phenotype may be masked in the clinical samples containing a mixed population of R/K at NA residue 292 while applying the *in vitro*–based assay or the fluorescence-based NA inhibition assay. It is crucial to monitor the emergence of the R292K mutation among H7N9 patients by genotypic methods.

O-843

Neuraminidase inhibitor oseltamivir protects mice against lethal A/Anhui/1/2013 (H7N9) influenza virus infection

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Background: The high mortality rate among individuals infected with influenza A (H7N9) viruses is a public health concern. In the absence of a vaccine antigenically matching the novel human pathogen, antiviral drugs can play an important role at initial stages of an outbreak. Limited information is available about the effectiveness of specific anti-influenza drugs [eg, neuraminidase (NA) inhibitors (NAIs)] against H7N9 influenza viruses. Here, we determined the susceptibility of N9 viruses to NAIs

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in vitro, developed a mouse model, and investigated the antiviral activity of the NAI oseltamivir in vivo. Materials and Methods: The susceptibility of human and avian H7N9 influenza viruses to NAIs was determined in a fluorescence-based MUNANA assay. The pathogenicity of A/Anhui/1/2013 (H7N9) influenza virus was determined in BALB/c mice by intranasal inoculation with a range of virus doses (10^2-10^6 PFU/mouse). The antiviral activity of oseltamivir phosphate (oseltamivir) in 6- to 8-week-old BALB/c mice was assessed in (1) an early pre-exposure model (drug administered 4 h before virus inoculation) and (2) with delayed treatment (drug administered 24, 48, or 72 h after virus inoculation). In all regimens, mice were given different doses of oseltamivir (5, 20, or 80 mg/kg/day) twice daily for 5 days and challenged with 5 MLD_{50} of A/Anhui/1/2013 (H7N9) virus. Survival, weight changes, and virus replication in mouse tissues on days 3, 6, 9, and 12 after virus challenge were evaluated. Results: Phenotypic assays revealed that all 15 viruses of N9 NA subtype are susceptible to the NAIs oseltamivir carboxylate, zanamivir, and peramivir *in vitro*, with IC_{50} values ranging from 0.3 to 1.6 nM. These IC_{50} values were comparable with those for human influenza viruses of Group 2 NA. Influenza A/Anhui/1/2013 (H7N9) virus caused lethal infection in BALB/c mice without prior adaptation, with an MLD_{50} of $10^{2.5}$ PFU. Mice showed decreased activity, hunched posture, ruffled fur, dyspnea, progressive weight loss, and death on days 6-9 after virus challenge. Replication of the H7N9 virus was restricted to mouse lungs and did not spread systemically. Virus replication was characterized by a long duration and induced pulmonary expression of pro-inflammatory mediators. In the early pre-exposure model, treatment of mice with 5 mg/kg/day of oseltamivir was effective at providing 50% survival rates. Importantly, an oseltamivir regimen (20 mg/kg/day for 5 days), which is reported to produce a plasma concentration comparable with that of the recommended human oral dose (75 mg twice daily), completely protected mice from lethal H7N9 infection. Treatment delayed by 24 h with oseltamivir at 20 and 80 mg/kg/day protected 80% and 90% of mice, respectively. When administered 48 h after infection, 60% of mice survived in both groups. Similar lung virus titers were seen in oseltamivir-treated and control groups around the peak of infection (day 3) but were reduced at later time points. Analysis of arterial blood gases revealed hypoxemia and acute respiratory acidosis as early as 3 days after infection, with the resolution of hypoxemia observed in a dose-dependent manner. Sanger sequencing and clonal analysis showed no amino acid substitutions at conserved residues in the NA (E119, H274, R292, and N294; N2 numbering) known to be associated with a resistant phenotype. Conclusions: Human influenza A (H7N9) viruses cause severe lung damage as early as day 3 after inoculation, and virus exhibited longer persistence in a mammalian animal model. Influenza A (H7N9) viruses are comparable with currently circulating seasonal influenza viruses in susceptibility to the NAI oseltamivir *in vitro* and in a mouse model. Early initiation of NAI treatment is crucial to achieving high levels of protection.

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The compensatory role of non-neuraminidase mutations in replication or transmission of oseltamivir-resistant influenza virus

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Background: The emergence of seasonal oseltamivir-resistant (OR) A(H1N1) virus in 2007/2008 indicated that the transmission and rapid global circulation of such viruses was possible. Limited person-to-person transmission of OR A(H1N1)pdm09 virus was described in the winter of 2009/2010. In 2010/2011, OR virus from untreated patients was increasingly detected in several countries, albeit at low levels; in 2011, a cluster of unrelated OR cases was detected in Australia. All of these OR viruses had a single amino acid substitution (H275Y) in neuraminidase (NA). Accumulation of permissive mutations in NA is thought to compensate for the reduction in enzyme activity and/or expression caused by the H275Y mutation before the OR virus has efficient transmission capacity. Ferret models have demonstrated transmission of OR A(H1N1)pdm09 viruses, but they do not explain why widespread resistance has not been seen. We hypothesised that mutations in internal genes can

enhance the replicative capacity of OR strains and are likely to play an important role in sustained transmission of A(H1N1)pdm09 viruses. Materials and Methods: Whole genome sequencing of clinical specimens from a haematology ward outbreak in Wales, with documented person-to-person transmission of OR A(H1N1)pdm09 virus, identified a possible compensatory mutation in nucleoprotein at position 408 (NP-V408M). A panel of recombinant viruses with and without NA-H275Y and NP-V408M were created by reverse genetics using a 2009 A(H1N1)pdm09 backbone. Competition experiments between pairs of viruses in human airway epithelial (HAE) cells from healthy donors were performed. Pyrosequencing assays targeting the single nucleotide positions encoding the NP-V408M and NA-H275Y mutations were used for allelic quantification of time point samples, and relative growth curves were determined in MDCK cells. Results: HAE cells were infected with a 50:50 mix of paired recombinant viruses differing at one or more specifically incorporated amino acids. When viruses differed at the 275 position only, sensitive-H275 virus significantly outcompeted the resistant-275Y virus. However, resistant-275Y virus with an additional NP-V408M mutation replicated to a level similar to that for the sensitive-275H virus. Each HAE experiment was performed using cells from 5 different healthy donors. A single donor exhibited outlier behavior when infected with a mix of resistant-275Y+NP-V408M and sensitive-H275 viruses, yielding very high replication of the resistant-275Y+NP-V408M virus from an early time point; sequences showed an additional mutation at position 89 in PB2 (PB2-V89M). Further HAE competition experiments showed that virus with PB2-V89M out-competed all others, irrespective of oseltamivir sensitivity. A(H1N1)pdm09 viruses circulating in the winter of 2010/2011 in the UK harboured a number of sequence changes throughout the genome compared with the 2009 emergent virus. Using reverse genetics, the H275Y mutation was engineered into a 2010 pH1N1 backbone, and competition assays were performed in HAE cells. As before, OR virus was outcompeted by sensitive 275Y virus in vitro. Four donor ferrets were infected with a 50:50 mixture of sensitive-275H:resistant-275Y virus; on days 1-2 post-infection, a contact recipient ferret was co-housed with each donor, and a respiratory droplet sentinel was housed in an adjacent cage. Transmission occurred in all 8 sentinel ferrets; in 1 of the contact-exposed animals, OR virus was efficiently transmitted and shed for a sustained number of days. Conclusions: We demonstrated that amino acid changes in internal proteins of influenza viruses play a compensatory role in the replicative capacity of OR viruses, regardless of whether these viruses arose naturally *in vivo* (NP) or experimentally *in vitro* (PB2). Examining direct contact and respiratory droplet routes in ferrets, we found that recent (2011) A(H1N1)pdm09 OR strains (H275Y) still do not transmit as efficiently as their sensitive counterparts. OR virus was less able to transmit by the respiratory droplet route than by direct contact. The strains used contained 2 of the 3 amino acid changes hypothesised by Hurt et al to be important in supporting transmissible OR strains. Our data show the importance of vigilance for influenza virus genetic changes beyond the neuraminidase gene and suggest that certain routes of transmission may be more likely to sustain oseltamivir resistance.

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Drug susceptibility of zoonotic influenza viruses associated with outbreaks in humans: 2011-2013

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Background: In recent years, an increased incidence of human infections with emerging zoonotic influenza viruses has caused great public health concern. Between July 2011 and November 2012, several outbreaks of human infections with variant influenza A(H3N2) virus (A(H3N2)v) containing the influenza A(H1N1)pdm09 M gene occurred in the United States. These infections were primarily associated with swine exposure at agricultural fairs. Since February 2013, 132 human infections with a novel avian influenza A(H7N9) virus have been reported in China, where cases presented with rapidly progressing lower respiratory tract infections with a fatality rate of approximately 20%. In the absence of a vaccine to protect against such novel influenza viruses, antiviral drugs remain a key component of the public health response and are expected to play an essential role in the reduction of

morbidity and mortality due to infection with zoonotic viruses. However, the current pharmaceutical options for control of influenza infections are limited to neuraminidase inhibitors (NAIs), due to the high prevalence of resistance to the M2 inhibitors, which underscores the critical need for NAI susceptibility surveillance. Materials and Methods: A fluorescent neuraminidase inhibition (NI) assay was used to assess the susceptibilities of A(H3N2)v and A(H7N9) viruses to the licensed NAIs oseltamivir and zanamivir as well as to the investigational NAIs peramivir and laninamivir. IC_{50} values were determined using JASPR v1.2—a CDC-developed curve-fitting software. Fold changes in IC_{50} values were interpreted according to criteria recommended for the surveillance of influenza A viruses by the WHO GISRS Antiviral Working Group. Viruses with a < 10-fold increase in IC_{50} were characterized as exhibiting normal inhibition by the respective NAI, while those with 10-100-fold and > 100-fold increases were characterized as exhibiting reduced and highly reduced inhibition, respectively. Pyrosequencing and conventional sequence analyses to detect changes in the NA were performed on A(H3N2)v viruses showing elevated IC_{50} values for NAIs and on A(H7N9) viruses. Sequencing was performed on both the virus isolate and its matching clinical specimen, when available, to omit mutations arising as a result of propagation in cell culture. Recombinant NA proteins were synthesized to assess the role of identified mutations in reduced inhibition by NAIs. Viruses identified as showing reduced and highly reduced inhibition by NAIs were tested in cell culture–based assays in the presence of investigational anti-influenza compounds undergoing clinical development. Results: All A(H3N2)v isolates (n = 156) were interpreted as showing normal inhibition by oseltamivir and zanamivir compared with median IC_{50} values for the respective NAIs among A(H3N2)v viruses. The only exception was A/Ohio/88/2012, which demonstrated reduced inhibition by oseltamivir and zanamivir (35- and 70-fold increase in IC_{50}, respectively). This virus had two NA changes, S245N and S247P, and the role of the latter change in the altered inhibition by NAIs was demonstrated using recombinant NA proteins. Two A(H7N9) virus isolates, A/Anhui/1/2013 and A/Shanghai/1/2013—kindly shared by the China CDC—were tested, and both showed normal inhibition by NAIs in the conventional NI assay. The A/Shanghai/1/2013 virus isolate contained a subpopulation carrying the R292K NA mutation; when tested using a modified NI assay, the virus showed highly reduced inhibition by oseltamivir and reduced inhibition by zanamivir. The role of R292K in the altered inhibition by NAIs was confirmed using recombinant NA proteins. The A(H3N2)v and A(H7N9) viruses showing reduced/highly reduced inhibition by NAIs were tested with investigational anti-influenza compounds with broad-spectrum activity. In cell culture–based assays, these viruses showed susceptibilities to NT-300, favipiravir, and DAS181 comparable with those of seasonal CDC reference viruses. Conclusions: This study defines susceptibility profiles of influenza A(H3N2)v and A(H7N9) influenza viruses associated with human infections to FDA-approved antiviral drugs as well as to antiviral agents in development. These data are important in guiding the use of antiviral agents for the treatment and prophylaxis of emerging influenza viruses and also provide information essential for antiviral resistance monitoring and the development of detection assays.

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Antiviral Drugs and Resistance

Poster Abstracts

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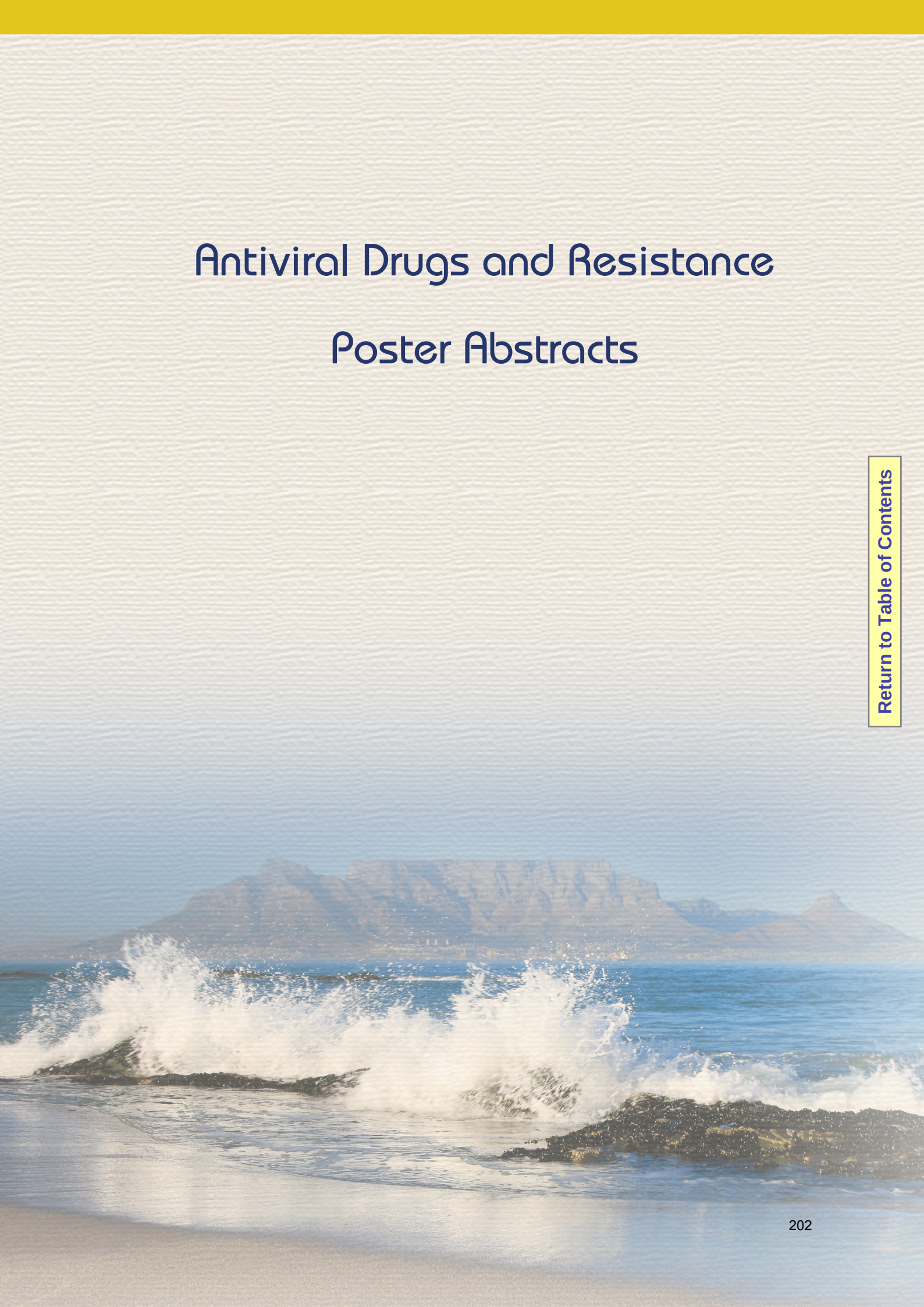

P1-255

Structural studies of influenza virus hemagglutinin and insight into antiviral drug target

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Background: The crystal structures of HA in pre- and postfusion conformations have been known, and those of the recombinant HA ectodomains from two 2009pdm isolates, A/California/04/2009 (CA04) and A/Darwin/2001/2009 (DA01), were reported. The crystal structure of recombinant HA protein from a 2009pdm isolate, A/Korea/01/2009 (KR01), showed a significant conformational difference between KR01 HA and other HAs. Conformational diversity of KR01 HA was found more pronounced in the complex structure of KR01 HA with the Fab fragment of a neutralizing monoclonal antibody GC0757 that binds to various H1 subtype strains. In this study, we present biochemical and structural features of KR01 HA and its complex with Fab0757. Docking simulation based on these HA structures led to a ligand that binds in a highly conserved pocket in the head domain at the interface between HA monomers. Materials and Methods: KR01, CU44, BR59, and Gy684 HAs were produced in insect cells using recombinant baculovirus expression vectors. Plasmids encoding each HA gene were amplified in *Escherichia coli* DH5α and used to cotransfect sf9 cells with linearized baculovirus chromosomal DNA. The recombinant baculovirus was harvested from the cell supernatant on day 5. Crystals of KR01, CU44, BR59, and Gy684 HAs were screened by the hanging drop vapour diffusion method. Small single crystals of KR01 and CU44 HAs were obtained in 100 mM HEPES (pH 7.5), 20% PEG 3350, and 0.2 M NaCl at 4°C, and in 100 mM Tris-HCl (pH 7.0), 22% PEG, 0.2 M calcium acetate at 24°C, respectively. The crystal structures of KR01 HA, CU44 HA and KR01 HA-Fab0757 were solved by molecular replacement. The initial solution was optimized by rigid body refinement. Manual adjustment of the backbone and side chains was conducted in Coot. Crystallographic refinement was carried out using the program refmac5 and PHENIX. R factor and R_{free} values are in the range of 0.23-0.24 and 0.27-0.29, respectively. Molecular docking was investigated for virtual screening. Results: We determined the structures of HA protein from a 2009 pandemic strain (KR01) and its complex with a Fab fragment derived from a broadly neutralizing H1-specific monoclonal antibody GC0757. Both native and Fab complex structures reveal a head-to-head arrangement of HA as well as different spatial orientation of HA1 relative to HA2, indicating that HA is flexible and dynamic at neutral pH. Considering the importance of the monomer-monomer interface of trimeric HA proteins, structure-based virtual screening and ligand docking were carried out to provide an insight into a novel antiviral drug target, which led to the discovery of PYR22 with high affinity to HA (K_d 5.94 nM). We determined the crystal structure of PYR22 bound to HA derived from a seasonal H1N1 influenza virus (A/Thailand/CU44/2006: CU44), which showed that the inhibitor binds in a pocket formed at an interface between HA monomers in the head region as well as in a pocket near the fusion peptide. Occupation of these sites by PYR22 can stabilize the HA structure, based on fluorescence-based thermal shift assay. Cell-based assay of PYR22 showed a significant antiviral activity against CU44, A/Korea/01/2009 (H1N1pdm), A/Gyeongnam/684/2006, and B/California/01/2007. Conclusions: KR01 HA on the virus surface adopts flexible conformations with substantially low energy barrier, as a loosely assembled trimer. We targeted several amino acid residues that are located at the monomer-monomer interface as well as HA1-HA2 interface, and structural modification and optimization of the lead compound are currently under way. This work was supported by grants from Mid-career Researcher Program (2010-0029242) through NRF funded by the MEST and from TEPIK (A103001) funded by the Ministry for Health, Welfare & Family Affairs, Korea.

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Amino acid change P431S affects neuraminidase affinity to sialic acid substrates in influenza virus A(H1N1)pdm09

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Background: Antiviral susceptibility by phenotypic assays of A(H1N1)pdm09 viruses isolated in Yucatan, Mexico, detected a cluster of three family members with reduced susceptibility to oseltamivir carboxylate. Sequence analysis of the full neuraminidase (NA) gene showed two mutations—N386K+P431S—in all three isolates with reduced NA activity. Residue N386K has been described and occurs at low frequency in viruses isolated from Russia, China, Kenya, Romania, and Mexico during and after 2009. A different amino acid change, N386S, has been described in oseltamivir-resistant strains from Australia. Interestingly, mutation P431S is a novel mutation reported for the first time in our study. In order to investigate the role of these mutations on influenza virus; single, double, and triple mutants were generated by reverse genetic techniques. Materials and Methods: Recombinant mutant viruses were generated using A/California/04/09 A(H1N1)pdm09. Because the substitution H275Y is well described as a cause of oseltamivir resistance, it was used in addition to the novel N386K and P431S mutations. The recombinant viruses generated were the single mutants N386K, P431S, and H275Y; the double mutants N386K+H275Y, N386K+P431S, and P431S+H275Y; and the triple mutant N386K+P431S+H275Y. Viruses were rescued in MDCK cells, titered by plaque assay, and were assessed for NA activity using the NA-Star Kit (Applied Biosystems). All viruses were sequenced to ensure the presence of the required mutations. To evaluate any differences on virus multicycle growth kinetics, MDCK cells were infected with wild-type and mutant viruses at a low multiplicity of infection, and viruses were harvested 12, 24, 36, and 48 hours post-infection. Results: Recombinant viruses with single-mutation H275Y were resistant to oseltamivir, as expected, with IC_{50} values > 500 nM; single-mutant N386K was susceptible, with IC_{50} values of 0.1 nM. However, when all mutants containing P431S were assessed, the NA activity was nearly 100-fold lower than that of the wild-type mutants. Similar results were obtained with different substrates (eg, MU-NANA).The double mutant H275Y+N386K had IC_{50} values similar to those of H275Y. The mutant N386K+P431S behaved as the original viral isolates from Yucatan, showing a reduced susceptibility to oseltamivir, IC_{50} values of 20 nM, and a fold increase of 60 times with respect to the susceptible virus. Interestingly, the recombinant viruses H275Y+P431S and H275Y+N386K+P431S showed the same result as the single mutant P431S, as no readable results were available from the phenotypic assays and no signal was detected, even with the MU-NANA substrate. The kinetics of viral growth in MDCK cells showed that the wild-type and single mutant N386K grew comparably over 48 hours, and the resistant H275Y and double mutant N386K+H275Y showed a similar pattern of growth. No significant difference between these and the wild-type mutants was obtained when analyzed using Tukey´s test. However, the double mutant N386K+P431S showed a decrease in viral titers at 24 hours post-infection with respect to the wild-type mutant. Meanwhile, the single mutant P431S showed significantly reduced viral growth at both 12 and 24 hours. In agreement with the lack of detectable NA activity in the NA-Star assay, H275Y+P431S and H275Y+N386K+P431S had the lowest viral growth. The triple mutant had significantly lower replication at all time points, and the double mutant was statistically different at 24, 36, and 48 hours post-infection. Conclusion: We isolated H1N1pdm09 viruses from a family cluster that contained two mutations (N386K+P431S) in the NA gene, and these viruses showed reduced susceptibility to oseltamivir carboxylate. We showed here that recombinant viruses containing the mutation P431S change NA activity against sialic acid–like substrates. However, viruses are able to grow in cell culture, suggesting that minimal NA function is retained. On the other hand, the mutation at residue N386K in combination with P431S restores the affinity for the substrate; however, rescued viruses with N386K+P431S show reduced susceptibility to oseltamivir carboxylate, as was identified in the family cluster of original isolates from Yucatan.

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FELLOWSHIP GATES Fellowship

Proteinase-inhibitor theory of pathogenesis of influenza

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Background: In the pathogenesis of viral diseases, the interaction of virus with the cell has not been sufficiently studied. The main point here is the virus intervention into a healthy cell with a mandatory deproteinization of the virus. However, deproteinization of viruses is still poorly understood. Materials and methods: To study the state and role of antiproteinase systems of a cell and virus in the development of influenza infection and get fundamentally new drugs on the base of inhibitors of human's blood trypsine - like proteinases. Infection and multiplication of influenza A virus (IAV) has been done on chickens' embryos and animals. For purification and concentration of IAV we used the method of ultacentrifugation. Extraction of trypsin-like proteinase inhibitor was done with ion – exchange and affinity chromatography. Results: A new theory of influenza pathogenesis with participation of proteinase-inhibitory system has offered. It has been established that purification and concentration of influenza viruses by different methods did not release the virus from cellular enzymes. In the experimental animals infected with the virus of influenza, disturbance of enzyme-inhibitory balance took place, especially during the first hours after the animals were infected. From the lungs of healthy mice, six isoforms of trypsin-like proteinases were observed. All of them got antiproteinase immune sera. Antiserum to the third isoform prevented the experimental animals fatality. From the industrial wastes of gamma-globulin manufacture they have extracted inhibitor of protein-like proteinases which prevented white mice fatalities in 80% of cases. Endogenous inhibitors of a human blood proteinases are perspective anti-influenza drugs for a human being. Conclusions: Endogenous inhibitors of human blood proteinases are prospective in producing anti-influenza drugs for humans.

P1-259

Novel class of low-molecular anti-influenza agents based on camphor pharmacophore

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Background: Due to a short-term life cycle and high rate of mutations, influenza virus is highly variable at escaping from the host immune response and the selection of drug-resistant mutants. Currently, all influenza isolates are resistant to the adamantane-based antivirals amantadine and rimantadine. Moreover, since 2007 to 2009, influenza viruses of the H1N1 subtype have developed 100% resistance to the second-generation antiviral oseltamivir followed by substitution with the oseltamivir-susceptible pandemic virus. With this in mind, the search and development of novel antivirals against influenza virus is an important challenge for medical science and health care around the globe. Here we present the results of the study of antiviral activity of a novel class of camphor derivatives. Materials and Methods: We have tested 33 imino derivatives of camphor for their inhibiting activity against pandemic influenza virus A/California/07/09 (H1N1)pdm09. The cytotoxic and virus-suppressing activity was determined for each compound with a microtetrazolium test, and virus yield was evaluated by hemagglutination reaction. The 50% cytotoxic dose (CTD50), 50% effective dose (ED50) and selectivity index (SI, ratio of CTD50 to ED50) were calculated by using the data obtained. The stage of viral life cycle containing a target for the antiviral activity of compounds was determined in time-of-addition experiments over 10 hours of virus replication in MDCK cells. Results: It was shown that, in general, derivatives of camphor are highly effective at decreasing viral titers. Of 33 tested compounds, 16 (48%) had an SI of 10 or more and 3 compounds had an SI of 500 or higher. EC50 values for active compounds were in the micromolar range, suggesting a high affinity of

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substances to their viral targets. Time-of-addition experiments revealed that the highest effect was reached when compounds were present in the culture medium 0-2 hours postinfection. The compounds of this class, therefore, counteract early stages of the viral life cycle, such as absorption, endocytosis, uncoating, membrane fusion and nuclear transport of RNPs. It should be noted that these compounds appeared to be active against the rimantadine-resistant strain of influenza virus, suggesting that the mechanism of their antiviral action is distinct from that of adamantane derivatives. Conclusions: Camphor derivatives should be considered a possibility for further development as potential antivirals that able to overcome the resistance to amantadine and rimantadine of currently circulating viruses. Further studies are necessary to optimize their structure and to decipher the exact mechanism of their virus-suppressing activity.

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Neuraminidase inhibitors as reflected in the Cochrane reviews

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Background: Shortly after the marketing authorisation of the two neuraminidase inhibitors (NIs) zanamivir and oseltamivir, the Cochrane Collaboration published their first meta-analysis on NI use for the prevention and therapy of influenza infection in otherwise healthy adults. Over the following years, this meta-analysis underwent several revisions. Each revision of the review generated enormous media – and subsequently public – interest. The aim of this presentation is to describe the evolution of the Cochrane reviews from a methodological perspective and also in terms of content. The results are compared with the past and current licensing status of the NIs in Europe and the United States. Materials and Methods: All Cochrane reviews on NI use in otherwise healthy adults were screened; those containing major revisions were analyzed further. Altogether, four reviews were selected – the initial meta-analysis from 1999 and the revisions dated 2006 (Issue III), 2010 and 2012. The evolution of the main methodologic features as well as the most relevant results of the reviews are described and compared with the product information for the NIs, as approved by the European Medicines Agency and the US Food and Drug Administration (FDA). Results: Over time, the results and conclusions of the reviews have changed, particularly with regard to the potential effects of NIs on the reduction of influenza-related complications. After the 2006 review, no new evidence has become available that could have led to the differing results. Instead, this evolution appears to be primarily based on the authors' decision to include or exclude certain data sources. At no time has there been a contradiction between the conclusions of the Cochrane authors and the assessment and licensing status of the neuraminidase inhibitors in Europe or the United States. Conclusions: From the perspective of drug regulation, these results are reassuring. However, the reasons for the great media interest each time a revision of this Cochrane review is published could benefit from further exploration.

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Pharmacokinetics of IV and PO oseltamivir in two cases of severe influenza B infection requiring haemofiltration and cardiac assist device/extracorporeal membrane oxygenation

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Background: In ICU patients with severe influenza infection requiring organ replacement therapy, IV oseltamivir (IV-OS) may be superior to PO administration. The currently recommended renal IV dosage (40 mg BID), however, may be insufficient for patients on continuous haemofiltration and/or mechanical circulatory support. This study aimed to investigate OS pharmacokinetics and pharmacodynamics in two influenza patients with myocarditis and/or renal failure requiring cardiac assist device/extracorporeal membrane oxygenation (ECMO) and/or haemofiltration. Materials and Methods: Patient 1 was a 9-year-old, previously healthy female infected with influenza B (Yamagata lineage) with rapid disease progression, malignant hyperthermia, rhabdomyolysis, generalized compartment syndrome and cardiac and renal failure. The patient initially received a biventricular cardiac assist device that was later converted to ECMO (day 19) and continuous haemofiltration (CVVH) from day 3 onward. OS treatment was initiated on day 3 of illness at 75 mg PO BID. Due to lack of gastrointestinal (GI) motility and severe hyperkalaemia, her antiviral regimen was switched to compassionate use IV-OS at a renal failure dose of 40 mg BID. On day 5, OS was increased to the regular IV dose of 100 mg BID. Extended serial PK sampling (including 8-9 time points covering 15 hours) was performed with the first and second 100-mg IV doses and again on day 11. Influenza viral loads from respiratory specimens were assessed every other day, and cytokine levels were followed prospectively. Patient 2 was a 27-year-old, previously healthy female presenting with influenza B virus infection (Yamagata lineage) and acute dyspnoea. She rapidly developed a pattern of cardiac failure strikingly similar to that of Patient 1. On day 3 of illness, Patient 2 developed cardiogenic shock requiring veno-arterial ECMO. PO-OS was started with 75 mg BID. The patient suffered massive intracerebral haemorrhage on day 10. During emergency placement of external ventricular drainage, ECMO was discontinued in an effort to control the bleeding. The patient continued to receive massive ICU support including catecholamines and multiple blood and plasma transfusions, raising questions regarding OS absorption. Serial PK analyses (8 time points covering 15 hours; arterial and venous) were performed on day 13. Influenza viral loads were assessed every other day. Results: OS plasma levels in Patient 1 remained below limits of detection, even after the first elevated dose of 100 mg IV-OS was administered. OS levels became detectable (peak: 1.64 ng/ml) only after the second 100-mg IV dose. After regular administration of 100 mg OS BID for 6 days, a plateau was reached with peak levels of 615, 566, 446 and 580 ng/ml and trough levels of 3.72, 4.08, 3.48 and 7.89 ng/ml for arterial, venous/pre-haemofilter, venous/post-haemofilter and ultrafiltrate, respectively. Oseltamivir carboxylate (OSC) levels were detected reliably on IV-OS, even with the first 100-mg IV dose. OSC peak levels reached 4270, 4300, 3490 and 5970 ng/ml, and OSC trough levels reached 3560, 3500, 1860 and 3290 ng/ml for arterial, venous/pre-haemofilter, venous/post-haemofilter and ultrafiltrate, respectively. No treatment-related adverse events were reported. Influenza viral loads remained unchanged around 4.8×10^4 copies/ml until day 5, when OS doses were increased to 100 mg IV. Viral loads began to decline to 1.6×10^4 copies/ml (day 5) and 1.2×10^4 copies/ml (day 7), dropping below levels of detection on day 9. Cytokine levels peaked on day 4: IL-6, 6.2 ng/ml; IL-8, 4.7 ng/ml; IP-10, 2.4 pg/ml; MCP-1, 3.2 pg/ml; G-CSF, 5.3 pg/ml; and IL-10, 1.3 pg/ml. Patient 2 showed no adverse events, but erratic OS levels with maximum peak levels of 114 ng/ml (arterial) and 84.3 ng/ml (venous) and minimum trough levels of 3.6 ng/ml (arterial) and 3.68 ng/ml (venous) were observed. OSC levels ranged from 450 and 414 ng/ml (trough, venous and arterial) to 750 and 761 ng/ml (peak, arterial and venous). Viral loads declined rapidly and became undetectable after day 3. Conclusions:

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In severe disseminated influenza infection with multi-organ failure and decreased GI motility, IV-OS may achieve superior and more reliable drug levels compared with PO administration. In patients on continuous haemofiltration, regular (as opposed to renal) IV-OS dosing may achieve a potent antiviral effect, with appropriate OSC levels well within the safety margin. Additional studies are needed to establish solid dosing recommendations for patients with multi-organ failure, decreased GI motility, mechanical circulatory support and/or continuous haemofiltration.

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A placebo-controlled randomized trial of oseltamivir treatment to reduce influenza illness duration and influenza virus shedding in a crowded urban setting in Bangladesh: assessing effectiveness when treatment was initiated within 5 days of illness onset

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Background: Randomized clinical trials have demonstrated that treatment with oseltamivir reduces illness duration and virus shedding among persons with uncomplicated influenza when initiated < 48 hours after illness onset. No clinical trial has evaluated the effectiveness of treatment initiated ≥ 48 hours after illness onset. We performed a double-blind, randomized, placebo-controlled trial in a crowded urban community in Dhaka, Bangladesh, to determine the effectiveness of treatment with oseltamivir to reduce patient illness and viral shedding among persons with illness onset < 120 hours after illness onset. Materials and Methods: From May 11, 2008 through December 31, 2010, we performed weekly surveillance for respiratory illness in households. Household members with one major symptom (fever, tachypnea, breathing difficulty, danger signs) or two minor signs (including cough, sore throat, chills, myalgia) and with illness onset ≤ 5 days had a nasal wash specimen collected. Patients aged ≥ 1 year with specimens with a positive rapid influenza test result were enrolled in the study. Study participants were randomized 1:1 to receive oseltamivir or placebo, provided a baseline nasal wash specimen at enrollment (day 0), were visited daily in their home to record symptoms, and returned to the clinic to have a specimen collected on days 2, 4, and 7 after enrollment. All specimens were tested for influenza with reverse transcriptase–polymerase chain reaction (PCR); virus isolation was attempted from PCR-positive specimens. We compared the duration of major symptoms and virus shedding after enrollment and stratified by whether treatment was initiated < 48 or ≥ 48 hours after illness onset. All analyses were intent to treat, except the virological outcomes, which were limited to those persons with a PCR-positive specimen at baseline and all three follow-up specimens (n = 1134). In post hoc analysis, we compared clinical and virological outcomes among patients enrolled on day 3 after illness onset. Results**:** Among 6544 households under surveillance, 5504 surveillance participants met the criteria for testing, and 1191 (22%) had a positive test result. Among 1190 persons enrolled in the study; 794 (67%) were enrolled < 48 hours and 396 (33%) were enrolled ≥ 48 hours after symptom onset. Characteristics of the oseltamivir and placebo group subjects were similar; most [1060/1190 (89%)] were children, and infecting viruses included influenza A(H3N2) [418/1190 (35%)], seasonal influenza A (H1N1) [131/1190 (11%)], A(H1N1)pdm09 [213/1190 (18%)], and influenza B [397/1190 (33%)]. The median duration of symptoms was shorter in the oseltamivir (3 days; interquartile range [IQR] 1, 5) compared with the placebo (4 days; IQR 1, 6; *P* = .01) group; among those enrolled ≥ 48 hours after illness onset, the median duration of symptoms was similar in both groups (oseltamivir: 3 days [IQR 2, 5]; placebo: 3 days [IQR 1, 5]; log-rank *P* = .04). There was a significant reduction in the percentage of participants from whom influenza virus was isolated on days 2 (15%), 4 (28%), and 7 (46%) after enrollment in the group treated with oseltamivir; similar findings were noted among those enrolled ≥ 48 hours after illness onset (day 2 [19%], day 4 [34%]). Among patients enrolled on day 3 after illness onset (n = 224), the median duration of symptoms was less in the oseltamivir group (3 days [IQR 1, 5]) compared with the placebo group (4 days [IQR 2, 6]; *P* = .02), and the proportion with virus

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isolated on days 2 (31%) and 4 (47%) after enrollment was significantly less among those receiving oseltamivir. Vomiting was more common among persons taking oseltamivir (5.9% vs. 3.2%; $P < .05$). Four viruses (3.9%) from 102 patients with H1N1pdm09 virus infection treated with oseltamivir developed resistance to oseltamivir during treatment. Conclusions: In a crowded low-income setting, oseltamivir treatment resulted in a modest reduction in the duration of symptoms and virus shedding among persons with uncomplicated influenza infections, including a reduction in virus shedding even when treatment was initiated ≥ 48 hours after illness onset. Post hoc analysis suggested that treatment initiated on day 3 after illness onset reduced the duration of symptoms and virus shedding.

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Clinical efficacy of laninamivir octanoate hydrate in the Japanese 2011-2012 and 2012-2013 influenza seasons

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Background: The antigenicity of the epidemic strains of influenza changes yearly. The susceptibility of epidemic influenza viruses to neuraminidase (NA) inhibitors can change markedly in a short time, so it is important to constantly monitor the efficacy of these drugs for each viral type/subtype. Laninamivir octanoate hydrate (laninamivir) is a long-acting, single-inhalation NA inhibitor that was approved in Japan in 2010 for the treatment of influenza A and B virus infection. We assessed the clinical effectiveness of laninamivir in the Japanese 2011-2012 and 2012-2013 influenza seasons. Materials and Methods: Patients with influenza virus infection diagnosed at 23 clinics and 1 hospital in 11 prefectures of Japan were enrolled in this study from November to April in the 2011-2012 and 2012-2013 influenza seasons. Patients positive by rapid diagnosis test kit and who had a temperature ≥ 37.5°C were registered after providing informed consent. Patients suspected of having other viral or bacterial infections were excluded. Laninamivir was administered according to the recommended dosage: a single inhalation of 20 mg for patients younger than 10 years of age and a single inhalation of 40 mg for patients aged 10 years or older. The duration of fever was defined as the time from the inhalation of laninamivir to afebrility. The duration of symptoms was defined as the time from inhalation until the patient noted improvement of all symptoms to a mild grade. The type and subtype of influenza A(H1N1)pdm09, A/H3N2, or influenza B were determined by RT-PCR using type- and subtype-specific primers and clinical specimens. We also isolated influenza viruses from clinical specimens at days 1 and 5 using Madine-Darby canine kidney cells, and the IC_{50} of four neuraminidase inhibitors (laninamivir, oseltamivir carboxylate, zanamivir, and peramivir) was determined with an NA inhibition assay using a fluorescent substrate. A specimen with a $\log_{10}$TCID50 (the 50% tissue culture infective dose) of 1.5/ml or more was defined as being influenza virus positive. Results: For 211 patients (A/H3N2: 190; B: 21) evaluated for clinical efficacy in the 2011-2012 influenza season, the median durations of fever of A/H3N2 and B virus patients were 33.0 and 50.0 hours, respectively ($P = 0.0989$). Fever was resolved within 72 hours after inhalation by 89.7% of the A/H3N2 patients and by 81.0% of the patients with B virus. The median durations of symptoms for A/H3N2 and B virus patients were 89.0 and 94.0 hours, respectively ($P = 0.5809$). On day 5, the influenza virus–positive rates for A/H3N2 and B virus patients were significantly different: 25.8% (40/155) and 70.6% (12/17), respectively ($P < 0.0001$). The median IC_{50} value for laninamivir for the virus isolates at day 1 was 3.65 nM for A/H3N2, while that for B was higher at 17.82 nM. No significant change in IC_{50} value was found between day 1 and day 5 isolates for any of the four tested NA inhibitors, and no IC_{50} value exceeded 50 nM. The incidence of adverse drug reactions was 1.28% (3/234), with no serious reactions reported. Conclusions: Laninamivir was effective against influenza A/H3N2 and B, with no safety issues. For patients treated with laninamivir, none of the residual viruses detected had increased IC_{50} values. Resolution of fever was more rapid for patients with A/H3N2 than for patients with type B virus. The virus shedding period after treatment was longer for influenza B, which may be related to the relatively higher IC_{50} values for B than for A/H3N2. The study is being continued during the 2012-2013 influenza season, and the pending results will be reported in our presentation.

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In vitro neuraminidase inhibitory activity of four neuraminidase inhibitors against influenza virus isolates in the 2010-2011, 2011-2012 and 2012-2013 seasons in Japan

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Background: Treating influenza with neuraminidase inhibitors (NAIs) has become the most popular treatment among primary care doctors in Japan. The early start of treatment with NAIs, within 48 h of the onset of the influenza symptoms, has contributed to mitigating symptoms and preventing severe disease. Four NAIs have been available for the treatment of influenza in Japan since 2010: oseltamivir phosphate (Tamiflu®), zanamivir (Relenza®), laninamivir octanoate (Inavir®), and peramivir (Rapiacta®). Since these various NAIs have been available in the market, drug resistance has become of important clinical concern. To study the extent of drug resistance in Japan, we surveyed the half maximal inhibitory concentration (IC_{50}) of four NAIs—oseltamivir, zanamivir, laninamivir, and peramivir—from influenza viruses isolated in the 2010-2011, 2011-2012, and 2012-2013 influenza seasons. Materials and Methods: A total of 30 clinics and 1 hospital from 14 prefectures participated in this study. Patients were enrolled from 1 November to 30 April in the several influenza seasons. Specimens were obtained from patients prior to treatment. The type and subtype of influenza—A(H1N1)pdm09, A/H3N2, or B—were determined by RT-PCR using type- and subtype-specific primers and clinical specimens. The viral isolation was done using Madine-Darby canine kidney cells, and the IC_{50} was determined with a neuraminidase inhibition assay using a fluorescent substrate. Results: A total of 269 and 325 influenza viruses were isolated from clinical specimens in the 2010-2011 and 2011-2012 seasons, respectively. Influenza A(H1N1)pdm09, A/H3N2, and B viruses were isolated from 185 (68.8%), 54 (20.1%), and 30 (11.2%) patients in the 2010-2011 season. A/H3N2 and B viruses were isolated from 283 (87.0%) and 42 (13.0%) patients, respectively, but no A(H1N1) pdm09 was isolated in the 2011- 2012 season. For the four NAIs, the IC_{50} of influenza B virus was higher than that of A(H1N1)pdm09 and A/ H3N2 in the 2010-2011 season and that of A/H3N2 in the 2011-2012 season. In the 2010-2011 season, IC_{50} values for oseltamivir carboxylate exceeding 50 nM were found for 2 of 185 A(H1N1)pdm09 (1.1%) viruses. The IC_{50} values to oseltamivir carboxylate were 600 and 840 nM, respectively, and these two virus isolates also showed an increased IC_{50} value to peramivir (19.0 and 24.0 nM). No isolates of A(H1N1)pdm09, A/H3N2, and B showed significantly high IC_{50} values for zanamivir or laninamivir. In the 2011- 2012 season, no isolates showed IC_{50} values exceeding 50 nM for any NAIs. The ratios of the geometric mean values of IC_{50} for influenza A/H3N2 in the 2011-2012 to 2010-2011 seasons were 1.06, 1.20, 1.08, and 1.26 for oseltamivir carboxylate, zanamivir, laninamivir, and peramivir, respectively. The increase in the IC_{50} values for zanamivir and peramivir was statistically significant ($P < 0.0001$), although the maximum IC_{50} values were not high: 4.50 nM for zanamivir and 1.70 nM for peramivir. For influenza B, there was a significant decrease in the IC_{50} values of the four NAIs during the 2011-2012 season compared with the 2010-2011 season, with ratios of 0.46, 0.65, 0.75, and 0.73 for oseltamivir carboxylate, zanamivir, laninamivir, and peramivir, respectively. Conclusions: Influenza A(H1N1)pdm09 virus isolates obtained in the 2010-2011 season from 2 of 185 patients showed significantly high IC_{50} values for oseltamivir carboxylate and peramivir. In contrast, all isolates were sensitive to zanamivir and laninamivir in the 2010-2011 and 2011-2012 seasons. The study is being continued during the 2012-2013 influenza season, and the pending results will be reported in our presentation.

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Antiviral treatment among older adults hospitalized with influenza, 2006-2012

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Background: Influenza has been estimated to result in more than 200,000 hospitalizations and between 3,300 and 49,000 deaths in the US each year, depending on the severity of the influenza season. Since 2009, US recommendations include prompt use of antiviral treatment for hospitalized patients with confirmed or suspected influenza as well as for patients with severe, complicated, or progressive illness and those at risk of severe influenza complications. Despite evidence that earlier treatment is associated with better outcomes, barriers exist to implementation of prompt antiviral treatment among hospitalized patients, including difficulty distinguishing influenza clinically from other acute respiratory infections (ARIs), lack of reliable low-cost rapid influenza diagnostic tests, late presentation of patients to care, and cost of antiviral medications. This study was designed to characterize the use of antiviral treatment among adults aged ≥ 50 years hospitalized with ARIs during six influenza seasons: 2006--2012. Materials and Methods: Population-based surveillance for laboratory-confirmed influenza was performed among adults aged ≥ 50 years presenting with ARIs to inpatient settings during six influenza seasons in Davidson County, TN. Eligible presenting symptoms included cough, non-localizing fever, shortness of breath, sore throat, nasal congestion, or coryza. Nasal/throat swabs were collected and tested for influenza by reverse transcriptase polymerase chain reaction (RT-PCR) in a research laboratory; results were not available to treating clinicians. Chart abstractions were performed after discharge and included demographic data, medical history, antiviral medication use, results of microbiologic and radiographic tests, hospital course, and outcome of illness at discharge. The proportion of patients who received antiviral treatment was calculated by year and patient characteristics, and predictors of antiviral use were examined. Results: The study population consisted of 1753 hospitalized adults with ARIs from four hospitals during times when influenza was circulating; 125 (7.1%) had RT-PCR–confirmed influenza (research laboratory, results not available to clinicians) and 38 (2.2%) had positive physician-ordered influenza test results (clinical laboratory). Clinical diagnostic testing was performed for 26% of all ARI patients, increasing from 21% during pre-pandemic years to 27% during pandemic years to 31% during post-pandemic years (P < .001). Of those with research-laboratory confirmed influenza, 54% (67/125) also had a clinical laboratory test performed. Of these, 80% (53/67) had only rapid tests performed, and 75% (40/53) had negative rapid test results. Overall, the proportion of inpatients who received antivirals during hospitalization ranged from 0.5% to 3.0% annually among patients with ARIs and increased from 0.9% during pre-pandemic years to 2.7% during pandemic years and to 2.8% in post-pandemic years (P = .046). Among those with research laboratory–confirmed influenza, antiviral use increased from 11.6% during pre-pandemic years to 20% during pandemic years, but then declined to 7% during post-pandemic years (P = ns). Of the 38 patients with clinical laboratory–confirmed influenza, 26.3% received antiviral treatment compared with 4.5% with a negative test result and 0.7% who were not tested (P < .001). Both research laboratory–confirmed influenza (adjusted odds ratio [AOR] 3.04; 95% CI 1.26-7.35) and clinical laboratory–confirmed influenza (AOR 3.05; 95% CI 1.07-8.71) were independent predictors of antiviral treatment among patients with ARI. Age, severity of disease, underlying high-risk condition, duration of symptoms, discharge diagnosis, and self-reported vaccination status were not associated with antiviral use. Conclusions: Despite recommendations for expanded use of antivirals for all hospitalized patients with confirmed or suspected influenza since the H1N1 pandemic (2009-2010), antiviral use was low. Although there has been a moderate increase in testing since the pandemic, testing remained low and the rapid tests used were insensitive for detecting influenza in hospitalized adults aged 50 years and older compared with RT-PCR testing. Antiviral treatment without a positive influenza test result was rare. More accurate point-of-care influenza tests could facilitate receipt of antivirals among hospitalized patients. Additional strategies are needed to improve appropriate antiviral treatment among hospitalized adults with influenza.

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Influenza antiviral surveillance in Argentina

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Background: Effective surveillance for influenza antiviral resistance is a key instrument for rapid detection of viral variants, allowing the establishment of control measures. Until recently, surveillance of antiviral susceptibility was poorly developed in South America. In 2010, the National Influenza Center (NIC) in Buenos Aires, Argentina, began a 2-year collaborative project with the Health Protection Agency (HPA), United Kingdom (UK), to establish a surveillance network in South America to monitor influenza antiviral drug resistance. Another 5 South American countries also participated in this project: Brazil, Chile, Colombia, Peru, and Uruguay. Since 2006 in Argentina, thanks to this project and the support of the WHO Influenza Collaborating Centres at CDC (Atlanta, USA) and NIMR (London, UK), it has been possible to obtain retrospective as well as current local data about the antiviral susceptibility of influenza A and B viruses. Materials and Methods: Phenotypic susceptibility to oseltamivir and zanamivir of 149 influenza A and B viruses isolated in MDCK cells during the period 2005-2010 was determined at the HPA (as a result of laboratory training) using the MUNANA fluorescence neuraminidase inhibitor (NAI) assay. Pyrosequencing of 46 A(H1N1)pdm09 clinical samples and 10 seasonal A(H3N2) isolates was also performed at the HPA to detect NAI resistance mutations. In addition, rapid genotypic screening of 739 influenza A(H1N1)pdm09 viruses (79 from 2011 and 660 from 2012) for the single-nucleotide polymorphism encoding the H275Y substitution was performed from clinical specimens using a real-time RT-PCR. This technique allows differentiation between wild-type and resistant viruses. In relation to resistance to the M2 ion channel blocking agents amantadine and rimantadine, 86 partial M2 genes of influenza A viruses—73 A(H3N2) isolated between 2001 and 2007 and 13 A(H1N1)pdm09—were sequenced at the NIC; this analysis was later expanded by pyrosequencing at the HPA on 42 seasonal A(H1N1) and A(H3N2) viruses isolated during the period 2005-2008. Results: In relation to the NAI assays, phenotypic analysis showed that 12 out of 35 seasonal A(H1N1) viruses were oseltamivir-resistant; the remaining influenza A isolates tested were sensitive to this antiviral. All isolates studied were sensitive to zanamivir. Four influenza B viruses were outliers, with decreased susceptibility, but for which no explanation was provided by NA sequence analysis. From the genomic studies, only 3 influenza A(H1N1)pdm09 viruses were identified as oseltamivir-resistant, harboring the H275Y substitution in NA. One of the samples had been recovered in 2009 from a transplant patient, another in 2011 from a 5-month-old child, and a third from a 1-year-old child in 2012; the latter two patients had not received antiviral treatment. All viruses tested by enzymatic assay were sensitive to zanamivir. In relation to adamantine-resistance studies, 20 out of 55 A(H3N2) viruses isolated during the period 2006-2007 harbored the S31N substitution characteristic of amantadine resistance. Pyrosequencing showed that 29 out of 33 A(H3N2) viruses isolated between 2006 and 2008 were amantadine-resistant, while all seasonal A(H1N1) viruses tested retained sensitivity. During 2011, a post-pandemic influenza season, all influenza A isolates studied [n = 31; 18 A(H3N2) and 13 A(H1N1)pdm09] were identified with the resistance-conferring substitution S31N. Complementary results will be presented in posters. Conclusions: Except for the three patients infected with influenza A(H1N1)pdm09-resistant virus, the lack of detection of resistant variants to NAI in community specimens indicates that the emergence of NAI-resistant viruses remains low in Argentina, as observed in the rest of the world. The high incidence of adamantane resistance registered in Argentina and other countries led to the discontinuance of the use of this category of antiviral. The establishment of local surveillance of antiviral susceptibility coupled with a dedicated database to share information provides a valuable contribution to a better understanding of antiviral resistance emergence in relation to influenza virus evolution worldwide.

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Population pharmacokinetics define dosing recommendations of oseltamivir in neonates and infants with influenza

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Background: Influenza morbidity and mortality in children is highest in those aged < 1 year, but until recently little data have been available on which to base optimal dosing for oseltamivir in this high-risk population, which has led to conflicting dosing recommendations in the US and Europe. Materials and Methods: Data for 133 children aged < 1 year from two prospective, open-label pharmacokinetic/pharmacodynamic (PK/PD) and safety evaluation studies – Collaborative Antiviral Study Group 114 (CASG114; US, 2006–10) and WP22849 (Europe, 2010–12) – were pooled to build a population pharmacokinetic (PPK) model. CASG114 was sponsored by the National Institute of Allergy and Infectious Diseases and enrolled 72 children aged < 1 year. WP22849, a Roche-sponsored study, enrolled 65 children aged < 1 year. Infants included in the analysis had influenza symptoms for ≤ 96 hours and a positive rapid diagnostic test result for influenza. In each study, infants received age-stratified weight-based doses of oseltamivir oral suspension twice daily for 5 days (CASG114 administered doses of 3.0 mg/kg for children aged 0–2, 3–5 and 6–8 months and of 3.0 or 3.5 mg/kg for ages 9–11 months; WP22849 administered 2.0 mg/kg for children aged 0–1 months, 2.5 mg/kg for those aged 1–3 months and 3.0 mg/kg for those aged 3–11 months). The analysis used non-linear mixed effects modeling in which several structural models simultaneously describing the disposition of oseltamivir and its carboxylate metabolite (OC) were tested. Weight was used to account for differences in body size across age groups, with clearance and volume parameters allometrically scaled using power exponents of 0.75 and 1, respectively. Covariates – including study, age, post-conceptual age, gestational age, body weight, sex, and ethnicity – were investigated using the full-model approach. Final model evaluation was performed using visual and posterior predictive checks and a non-parametric bootstrap. Putative targets were selected using either (i) an integrated PK/PD analysis or (ii) bridging exposure (Cmin, Cmax, and AUC at steady state) in infants and were compared with data observed in an earlier study in children aged 1–3 years who received the approved unit dose of 30 mg twice daily and in whom no antiviral resistance was demonstrated. Finally, model-based simulations were performed to identify optimal dosing regimens that met putative target criteria. Results**:** The pooled data set contained 604 oseltamivir and 648 OC quantifiable plasma samples from 133 infants aged 2 weeks to < 12 months. Thirteen infants (9.8%) were aged 13 days to < 1 month, 33 (24.8%) 1 to < 3 months, 23 (17.3%) 3 to < 6 months, 35 (26.3%) 6 to < 9 months, and 29 (21.8%) 9 to < 12 months. A two-compartment model with first-order absorption of oseltamivir and first-order conversion of oseltamivir to OC and a one-compartment model with first-order elimination of OC served as the base model. Apparent clearance (L/hr) and distribution volume (L) of OC increased linearly with age. All parameters were precisely estimated, and model diagnostics showed the ability to predict central tendency and spread of concentrations in the target population. Possibly due to the narrow exposure ranges studied, the PK/PD analysis showed no association between exposure and defined PD endpoints, including temperature, fever, viral load/shedding, resistance, and adverse events. As such, bridging of infant exposure to that seen in children aged 1–3 years and adults was used for dose selection. Simulations showed that OC exposure in infants of all age subgroups that received 3.0 mg/kg twice daily would achieve the *a priori*–determined target systemic concentrations that have demonstrated efficacy and are unlikely to produce antiviral resistance. Conclusions: A PPK model that incorporated allometric scaling of all parameters and a linear increase in OC clearance with age adequately described the disposition of oseltamivir and OC after adjustment for the developmental changes that occur in the first year after birth. Inter-individual variability in PK was highest in neonates, potentially reflecting differences in hepatic and renal maturation. Across all age subgroups, 3.0 mg/kg yields OC exposures that are well

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tolerated and should minimize the risk of underexposure and subsequent resistance and/or treatment failure.

P1-269

Phenotypic and genotypic analysis of influenza viruses isolated from adult subjects during a phase II study of intravenous zanamivir

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Background: Intravenous zanamivir (IVZ) is a neuraminidase inhibitor (NAI) suitable for the treatment of patients hospitalised with influenza. One of the main factors that can affect the efficacy of NAIs is resistance development. Viral susceptibility to zanamivir was evaluated by phenotypic and genotypic analyses. Materials and Methods: The study was initiated at the onset of the 2009 influenza A/H1N1 pandemic and included symptomatic, hospitalised patients with influenza. Patients received IVZ for 5 to 10 days. Throat swabs were conducted during treatment and at various time points depending on hospitalisation and duration of symptoms up to 23 days posttreatment (PT). Endotracheal (ET) samples were analysed when available. Phenotypic analyses were performed on cultured viruses by NA Star assay (Applied Biosystems). NA and HA genes were sequenced directly from clinical specimens. Results: Adult enrolment was completed in September 2011, with 130 subjects: 80% received prior oseltamivir treatment, 71% were infected with A/H1N1pdm09, 12% with A/H3N2, 11% with untyped influenza A, 2% with influenza B and 4% with an unknown subtype. A total of 50/611 samples from 39/129 subjects were culture positive: 37/128 Day 1 throat samples, 11/289 during-treatment throat samples, 0/168 PT samples and 2/26 ET samples. Matched Day 1 and during-treatment or PT samples were obtained from 9/121 subjects. Mean IC_{50} values (± SD) for influenza A/H1N1pdm09, A/H3N2 and influenza B were 0.20 ± 0.06, 0.26 ± 0.07 and 1.61 ± 0.35 nM, respectively. One A/H1N1pdm09 virus had a raised IC_{50} (0.44 nM); 185/513 NA sequences, 174 from throat and 11 from ET samples (153 influenza A/H1N1pdm09, 30 A/H3N2 and 2 influenza B) from 123 subjects were obtained. Most viruses analysed did not contain resistance mutations or mutations in the NA active site. Four influenza A/H1N1pdm09 viruses from four subjects harboured NA resistance mutations: Y155H (Day 1), D199G (Day 1), S247N (Day 1) and E119D/E (PT+5). Subjects with Y155H and D199G viruses received 2 and 1 days of prior oseltamivir treatment, respectively; therefore, it is unlikely that the mutations were selected during this time. Mutations were present on Day 1 and therefore did not arise by IVZ pressure. The S247N virus showed a shift in susceptibility to zanamivir of 2.6 and was not selected by IVZ or oseltamivir. E119D/E was not present at five other time points, before and after PT+5 days, from the same subject. Four subjects (A/H1N1pdm09) harboured NA mutations at the final time point—N63D (PT+16), V83A (Day 5), W190C (Day 10) and M269K (Day 3)—that were not present at Day 1. The emergent mutations were not associated with resistance but may have arisen due to selection pressure during IVZ treatment or by chance. A total of 180/496 HA sequences, 169 from throat and 11 from ET samples (148 A/H1N1pdm09, 30 A/H3N2 and 2 influenza B), from 123 subjects were obtained. Most viruses analysed did not contain resistance mutations. Two viruses (A/H1N1pdm09) from two subjects harboured resistance mutations at single time points: S162N (Day 5) and Q223R (PT+23). Five HA mutations detected in influenza A/H1N1pdm09 viruses from five subjects emerged during treatment: E81K (PT+16), V108L (PT+2), S164D (ET), D168N (ET) and S185N (PT+5). The HA mutations may have arisen due to selection pressure during IVZ treatment or by chance. Ten out of 92 A/H1N1pdm09 subjects harboured an HA mutation D222, which is associated with increased virulence of A/H1N1pdm09 viruses. Conclusions: Mean IC_{50} values for the influenza subtypes were within the expected range. Treatment-emergent NA resistance mutations were not detected. Four NA mutations emerged during IVZ treatment but were not in the NA active site and have not been associated with resistance. Two resistance-associated HA mutations, S162N and Q223R, in A/H1N1pdm09 viruses emerged during treatment. Both mutations may have arisen due to IVZ selection pressure or by chance. A total of 11% of A/H1N1pdm09 subjects harboured a mutation at D222—a determinant of virulence in severely ill patients with A/H1N1pdm09 infection.

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Detection of antiviral-resistant influenza viruses in Japan during pandemic and post-pandemic periods

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Background: In Japan, four neuraminidase (NA) inhibitors—oseltamivir, peramivir, zanamivir and laninamivir—are approved for chemotherapy against influenza and are prescribed with the highest frequency in the world. Therefore, Japan could be at high risk for the emergence and spread of antiviral-resistant influenza viruses. The spread of resistant viruses may change the chemotherapeutic strategy and limit the therapeutic options for influenza. Consequently, the nationwide monitoring of antiviral-resistant viruses is important for therapeutic and public health measures. To monitor antiviral-resistant viruses, we have screened clinical isolates from the 2008-2009 season through the 2012-2013 season in Japan. Materials and Methods: Clinical specimens were collected in 500 sentinel hospitals consisting of pediatric and internal medicine between May 2009 and May 2013. Influenza viruses were isolated from the specimens in 74 local public health institutes. Approximately 5% to 10% of total isolates were used for an allelic discrimination assay, NA gene sequencing and/or NA inhibition assay. To detect an H275Y substitution in the NA protein, which confers resistance to NA inhibitors, the allelic discrimination assay and/or NA gene sequencing were conducted. For the allelic discrimination assay, supernatants of A(H1N1)pdm09 virus–infected cells were directly added to the one-step duplex RT-PCR mixture without RNA purification. Fluorescent signals were collected during the annealing and extension steps, and amplification data and endpoint data were analyzed. For NA gene sequencing, the full-length NA gene was amplified from the viral RNA, and nucleotide sequences were determined. Chemiluminescent and/or fluorescent NA inhibition assays were performed to determine the susceptibility of viruses to the NA inhibitors oseltamivir, peramivir, zanamivir and laninamivir. The susceptibility of viruses was expressed as the drug concentrations required to inhibit NA activity by 50% (IC_{50}). Results: During pandemic (2008-2009 to 2009-2010) and post-pandemic (2010-2011 to 2012-2013) periods, we found that 159 (1.3%) of 12,099 A(H1N1)pdm09 viruses possessed the H275Y substitution and 2 (0.2%) of 902 A(H3N2) viruses possessed an R292K substitution in the NA protein. The mutant viruses were resistant to oseltamivir and peramivir, but not to zanamivir and laninamivir. The highest detection rate of the resistant A(H1N1)pdm09 viruses was found in patients who were 0-9 years old. The detection rate of the resistant viruses increased from 1.0% during the pandemic period to 2.0% during the post-pandemic period. Furthermore, among the cases with resistant A(H1N1)pdm09 viruses, the percentage of no known exposure to antivirals significantly increased from 16% during the pandemic period to 44% during the post-pandemic period. Conclusions: A(H1N1)pdm09 viruses resistant to oseltamivir and peramivir were sporadically detected in Japan, and they did not spread throughout the community. However, suspected human-to-human transmission of the resistant viruses gradually increased in the post-pandemic period. Consequently, sustained monitoring of resistant viruses is important to explore the possibility of human-to-human transmission of the resistant viruses. This study was conducted by collaboration with the influenza virus surveillance group of Japan.

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Clinical outcomes, viral load, and pharmacokinetics following treatment with IV zanamivir in hospitalized adults with influenza: results from a phase 2 open-label, multicenter, single arm study initiated during the influenza A H1N1 pandemic

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Background: There is a public health need for safe and effective treatments for influenza that are suitable for hospitalized patients. IV zanamivir (IVZ) is a parenteral neuraminidase inhibitor in clinical development for treatment of severe influenza and is active against influenza virus with the H275Y neuraminidase mutation. Materials and Methods: Study NAI113678 was initiated at the onset of the 2009 influenza A H1N1 pandemic; adult enrollment took place in 8 countries across the northern and southern hemispheres. Symptomatic, hospitalized patients with documented influenza by local diagnostic tests (rapid antigen test, PCR, or viral culture) were eligible if enrolled within 7 days of illness onset. Subjects received IVZ 600 mg twice daily for 5-10 days, adjusted for renal impairment or renal replacement therapy. Subjects were assessed during treatment and during 23 days of follow-up for safety, clinical outcome and virologic responses. Serial serum pharmacokinetics (PK) sampling was conducted with the first dose and during a repeat dose interval on day 3-5 (ClinicalTrials.gov NCT01014988). Results: 130 adults (43% female, including 4 pregnant women) were enrolled from 2009-2011. Median age was 48 years (range, 18-94), 75% were Caucasian, 34% were obese (BMI > 30), and 80% had received prior oseltamivir treatment (median duration 2 days). 77% had chronic medical conditions (respiratory disease 32%, immunocompromised 25%, gastrointestinal 22%, cardiovascular 20%). Median time from influenza symptom onset to IVZ treatment was 4.5 days (range, 1-7). At baseline, 89% had an infiltrate on chest X-ray consistent with viral pneumonia. While on study, 57% of subjects required mechanical ventilation and 3% extracorporeal membrane oxygenation. Median hospital stay was 15 days (range, 1-133); 83% required ICU care, median ICU stay was 11.5 days (range, 1-104). 92% of subjects were treated with antibiotics, 49% systemic corticosteroids and 26% vasopressors. Ninety-four percent of subjects had influenza A (71% H1N1pdm09, 12% H3N2, 11% subtype unknown), 2% influenza B and 4% not typed. Median time to virologic improvement (defined as 2 log drop from baseline or time to undetectable viral load by qPCR from nasopharyngeal samples) was 2 days. Subjects who started treatment within 5 days of symptom onset had higher median viral loads at baseline compared with those who started treatment ≥ 5 days after symptom onset (5.11 $\log_{10}$ copies/mL vs 3.89 $\log_{10}$ copies/mL, respectively). A total of 21 subjects had paired qPCR data in both endotracheal and nasopharyngeal samples with a median virus load of 4.89 and 3.60 $\log_{10}$ copies/mL (P = .004), respectively; of these, 17 (81%) had higher virus load in endotracheal samples reflecting lower respiratory tract disease. Fourteen and 28-day cumulative mortality was 13% and 17%. The most common causes of death were respiratory failure (5%), sepsis/septic shock (4%), cardiogenic shock (3%), and bacterial pneumonia (3%). None of the deaths was considered by the investigator to be attributable to zanamivir treatment. The effect of risk factors on mortality was explored using multivariate Cox regression modelling with stepwise selection method. Mortality was significantly associated with H3N2 (P = .0386), older age (P = .0462) and time from symptom onset to IVZ treatment (P = .0225). The adjusted hazard ratios (95% CI) are 2.910 (1.058, 8.007), 1.031 (1.001, 1.062) and 1.354 (1.044, 1.756), respectively. Steady state PK results from 76 subjects with normal renal function (mean Cmax = 35.3 µg/mL, AUC = 90.3 h*µg/mL, Cmin = 0.82 µg/mL) were as expected for 600 mg, and well above the mean reported IC_{50} for influenza viruses (0.08-1.01 ng/mL). PK data showed that dose adjustments for renal impairment yielded similar zanamivir exposures. There was no correlation between higher zanamivir exposure and serious adverse events, drug-related adverse events, and virologic responses. Conclusions: Clinical outcome, safety, viral load and pharmacokinetic data from critically ill patients treated with IVZ in this phase II study support further investigation of IVZ for hospitalized patients. A phase 3 study comparing IVZ with oral oseltamivir is ongoing (ClinicalTrials.gov NCT01231620).

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Antiviral resistance of influenza viruses isolated in Kenya, 2007 – 2011

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Background: Influenza viruses circulate in Kenya year-round. This, coupled with the continued threat of the emergence of novel influenza viruses with pandemic potential in humans, underscores the need for information on effective antivirals for the management of influenza infections. There are limited data on influenza virus susceptibility in sub-Saharan Africa. We present data on the antiviral susceptibility of influenza viruses circulating in Kenya from 2007 to 2011. Materials and Methods: We collected nasopharyngeal and oropharyngeal (NP/OP) specimens from patients with respiratory illness who presented to 12 national sentinel and population-based surveillance sites from January 2007 through December 2011. Specimens were tested at the Kenya Medical Research Institute/Centers for Diseases Control and Prevention laboratories by real-time reverse transcription–polymerase chain reaction for Influenza A and B, and influenza A–positive samples were subtyped. Influenza viruses, isolated from a subset of the influenza-positive specimens by cell culture, were assessed for susceptibility to neuraminidase inhibitors (zanamivir and oseltamivir) in neuraminidase inhibition (NI) assays. Virus isolates showing elevated IC_{50} values (drug concentrations inhibiting 50% of virus activity) in the NI assay were genetically analyzed by conventional sequencing and/or pyrosequencing to detect molecular changes in the neuraminidase protein associated with reduced susceptibility to neuraminidase inhibitors, such as the histidine to tyrosine substitution at position 275 (H275Y) in the N1 of the neuraminidase protein that confers resistance to oseltamivir. Influenza A isolates were also genetically characterized by pyrosequencing to detect markers of resistance to adamantanes, including the serine to asparagine substitution at position 31 (S31N) of the M2 protein. All functional and molecular characterization assays were carried out at the WHO Collaborating Center at the CDC (Atlanta, Georgia, United States). Results:
During 2007-2011, 55,208 NP/OP specimens were tested for influenza viruses, of which 5347 (9.7%) were positive for influenza A, 1719 (3.1%) were positive for influenza B, and 177 (0.3%) were positive for both influenza A and B. A total of 732 influenza isolates were tested with oseltamivir and zanamivir in the NI assays, including 135 influenza B isolates and 597 influenza A [121 A(H1N1), 250 A(H1N1)pdm09, and 226 A(H3N2)] isolates. All influenza A and B isolates tested (n = 732) were susceptible to zanamivir, while 668 (91.2%) were susceptible to oseltamivir. All influenza B, A(H3N2), and A(H1N1)pdm09 isolates tested were susceptible to oseltamivir. However, 64/121 (53%) A(H1N1) isolates, all collected between September 2007 and June 2010, were resistant to oseltamivir, and sequence analysis revealed the presence of H275Y substitution in their neuraminidase protein. A total of 350 influenza A isolates, including 121 A(H1N1), 58 A(H1N1)pdm09, and 171 A(H3N2), were characterized for the presence of markers that confer resistance to adamantanes. Of these 350 influenza A isolates, 224 (64%) had the most commonly detected marker for adamantane resistance, S31N, including 1 (0.8%) A(H1N1), 58 (100%) A(H1N1)pdm09, and 165 (96.5%) A(H3N2) isolates. A single A(H1N1)pdm09 isolate contained a combination of two markers of resistance to adamantanes: A30T and S31N. Further testing for adamantane susceptibility was discontinued due to widespread resistance to adamantanes among influenza viruses circulating globally. Conclusion: In spite of limited antiviral use in Kenya, more than half of the viruses characterized were resistant to adamantanes. However, more than 90% of the viruses circulating in Kenya from 2007 to 2011, including all the influenza viruses circulating from July 2010 to December 2011, were susceptible to oseltamivir and zanamivir. Thus, the use of these neuraminidase inhibitors for the management of influenza infections in Kenya would be beneficial. Antiviral resistance patterns in Kenya mirrored those seen elsewhere in the world.

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Zanamivir aqueous solution for treatment of influenza in a compassionate use program: results from a retrospective chart review study

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Background: Since the start of the 2009 H1N1 pandemic, investigational zanamivir aqueous solution (ZAS) for intravenous (IV) or nebulized administration has been available on a named patient compassionate use (CU) basis to treat critically ill patients with severe influenza for whom licensed influenza antivirals are not suitable. As of 8 May 2013, 1562 patients globally have received CU ZAS, and in an attempt to collect key safety and clinical data, treating physicians are asked to complete a brief data collection form (DCF), although provision of data is not a condition of ZAS supply. Study NAI115008 was a retrospective, multinational, noninterventional chart review study designed to collect more detailed safety and clinical outcomes data from a subset of treated patients, primarily pediatric (<18 years) and pregnant patients as these patients are more difficult to enroll in clinical trials. Materials and Methods: Sites where pediatric or pregnant patients (referred to as “Tier 1” patients) had been treated with at least 1 dose of CU ZAS between May 2009 and January 2011 were invited to participate in this study. Simultaneously, data from other adults (“Tier 2” patients) treated with CU ZAS at these sites also were collected. Site personnel reviewed each patient’s medical chart and recorded study data on a DCF. Information collected included influenza history and treatment, ventilation/oxygenation summary, underlying medical illnesses, hospital course, treatment emergent events, complications and death. Descriptive and exploratory statistical analysis methods were used. Results: DCFs were collected for 113 patients treated at 34 hospitals in 12 countries: Brazil, Canada, Denmark, Germany, Greece, Latvia, Netherlands, Norway, Portugal, United Arab Emirates, United Kingdom, and the United States. Of the 61 Tier 1 patients, 50 were children (median age 6 years; range, 1 day to 18 years) and 11 were pregnant or postpartum (median age 26 years; range, 15-38). Of the 8 patients pregnant at hospital admission, 4 were in the second and 4 in the third trimester of pregnancy. Time from delivery to ZAS administration for the 3 patients that were postpartum at hospital admission was 3 days, 37 and 38 days. The median age of the 52 Tier 2 patients was 50 years (range, 23-75). Many of the patients (94, 83%) received IV ZAS and 19 (17%) received ZAS via nebulizer; median time from influenza symptom onset to ZAS treatment was 10 days (range, 1-55 days). 12% received prior anti-influenza vaccine and 86% had prior oseltamivir treatment. Most patients had influenza A (73% H1N1pdm09, 8% subtype unknown), 2% influenza B and results were missing for 17%. 73% had at least 1 underlying condition, most frequently respiratory disease (36%), rheumatologic and immunologic (19%) and cancer (17%). The majority of patients (97%) required admission to an intensive care unit; median stay for pediatric patients was 17 days (range, 1-108), median stay for pregnant/post-partum patients was 18 days (range 7-84 days) and 22 days for Tier 2 adults (range, 2-75 days). All patients except one were ventilated or required supplemental oxygenation: 84% endotracheal mechanical ventilation and 27% extracorporeal membrane oxygenation. Exploratory analyses suggest shorter time from symptom onset to ZAS administration was positively associated with survival in adults including pregnant patients (Odds ratio 0.3, P = .0087). Overall, 21% experienced treatment-emergent adverse events, including renal failure (7%), circulatory collapse (5%), and paralytic ileus (4%). Altogether over half (55%) had recovered and 38% died (32% of children, 36% of pregnant or postpartum patients, 44% of other adults) and 7% had either not recovered or survival status was unknown. Overall, the most common causes of death were respiratory failure (10%), acute respiratory distress syndrome (7%), multiorgan failure (6%), and cerebral hemorrhage (5%). Conclusions: A high mortality rate was observed in this subgroup of patients treated with CU ZAS, consistent with the severity of illness of patients treated in this program. A global phase 3 controlled study is ongoing to determine clinical benefit of intravenous zanamivir versus oral oseltamivir in hospitalized patients with influenza infection (clinicaltrials.gov: NCT01231620).

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Oseltamivir exposure via breast milk is not associated with an increased risk of adverse outcomes in infants

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Background: The safety and tolerability of oseltamivir and its active metabolite oseltamivir carboxylate (OC) have been extensively investigated; however, limited information is available on the excretion of either compound in breast milk and the potential effects of exposure on the breastfed infant. To evaluate the safety of oseltamivir in this setting, we reviewed the existing literature and cases of oseltamivir exposure through lactation recorded in the Roche Global Safety Database. Materials and Methods: To identify publications describing oseltamivir use during lactation, specific search criteria were used to search the following online databases on 10 July 2012: MEDLINE®, Embase®, BIOSIS Previews®, Derwent Drug File, International Pharmaceutical Abstracts, PASCAL, and SciSearch. In addition, the Roche Global Safety Database was searched for reports of oseltamivir exposure during lactation, with a cutoff date of 30 April 2012. Results: In preclinical studies identified by the literature search, oseltamivir and OC were shown to be excreted in the milk of lactating rats, but there was no evidence to suggest a deleterious effect of oseltamivir exposure on the offspring. Review of a further 38 citations suggests that therapeutically effective oseltamivir exposure is unlikely in the breastfed infant and that no dose adjustment is required in the lactating mother. A total of 66 reports of oseltamivir exposure during lactation were identified in the Roche Global Safety Database: 14 of these occurred during lactation alone and 52 occurred both transplacentally and during lactation. Single-case review did not reveal any evidence suggesting a deleterious effect of oseltamivir exposure via breast milk. The majority of cases (57/66; 86%) were not associated with a maternal or neonatal event. One of the remaining nine cases involved a baby with transplacental and lactation exposure who was found to have pyelocaliectasis; the window of oseltamivir exposure in this case fell outside the critical period for congenital abnormalities associated with pyelocaliectasis (week 7 of gestation). In another case, a mother and baby both received oseltamivir treatment for influenza infection; the baby, who was also breastfed, developed urticaria after recovering from influenza. Another case concerned a baby with alopecia—an event not uncommon in infancy. Other reported events included suppressed lactation (2 cases), breast milk discoloration (2), rash in both mother and baby (1), and refusal to be breastfed (1). Conclusions: Evidence from the available literature and the Roche Global Safety Database suggests that oseltamivir is compatible with breastfeeding. Future cases of oseltamivir exposure in infants during lactation will continue to be monitored.

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Maternal exposure to oseltamivir is not associated with an increased risk of adverse pregnancy or birth outcomes

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Background: Influenza infection is associated with an increased risk of complications and poor outcomes in pregnant women. As a result, treatment guidelines recommend that pregnant women with influenza receive neuraminidase inhibitors, even in uncomplicated cases. A previously published review concluded that oseltamivir was unlikely to cause adverse pregnancy or birth outcomes. However, the rate of oseltamivir exposure in pregnant women has increased considerably in recent years, due in large part to the 2009–10 A/H1N1 pandemic. To re-evaluate the safety of oseltamivir in pregnancy, we performed a cumulative review of reports of exposure in pregnant women. Materials and Methods: To identify articles on pregnancy and birth outcomes among women and neonates exposed to oseltamivir, a search of the following online literature databases was conducted on 10 July 2012: MEDLINE®, Embase®, BIOSIS Previews®, Derwent Drug File, International Pharmaceutical Abstracts, PASCAL, and SciSearch. Cumulative data on cases of maternal exposure to oseltamivir up to 30 April 2012 were also extracted from the Roche Global Safety Database, which includes

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spontaneous reports of exposure during pregnancy, pregnancies that occurred during clinical trials of oseltamivir, and case reports from literature sources. Medical analysis of pregnancy cases assessed birth and pregnancy outcomes and adverse events (AEs). Results: The literature search identified 609 articles; only 12 contained relevant information. Most studies did not stratify results by antiviral use. Review of evidence from these articles did not suggest an elevated risk of adverse outcomes with oseltamivir exposure. A total of 2926 cases of maternal exposure to oseltamivir were identified in the Roche Global Safety Database, with 5660 AEs reported. Among the 2128 cases in which pregnancy outcome was known, 2003 (94.1%) reported live births; of these, the majority (1644/2003; 82.1%) were "normal" live births. There were 128 reports of abnormal pregnancy outcomes: spontaneous abortions, 61 cases; therapeutic abortions, 39; and stillbirths, 28. Rates of spontaneous abortions (61/2128; 2.9%), therapeutic abortions (39/2128; 1.8%), and pre-term deliveries (84/2128; 3.9%) were below background rates. In total, 1875 of 2926 cases (64.1%) had known birth outcomes, with 81 cases reporting birth defects. Of these, 53 cases had an alternative explanation for the defect and 17 had insufficient information for assessment. Of the remaining 11 cases (concerning 9 patients, as 2 reports were duplicated), 7 were anomalies that are not uncommon: ventricular septal defect (2 cases), Pierre-Robin syndrome and Dandy-Walker malformation (2 cases; events duplicated due to separate reporting in mother and baby), ear fistulas (2 cases) and congenital dacryostenosis (1 case). Fever associated with influenza infection may have been a contributory factor in a further four cases: anophthalmos, single atrium and ventricle/univentricular heart (duplicate reports), and atrial ventricular and ventricular arterial anomaly of pulmonary venous return. Conclusions: Based on the literature review and data from the Roche Global Safety Database, there is no apparent association between maternal exposure to oseltamivir and an increased risk of abnormal pregnancy or birth outcomes.

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Impact of different oseltamivir regimens on efficacy and resistance emergence: insights from a modelling study

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Background: Several studies have shown oseltamivir to be efficient at reducing influenza viral titre and symptom intensity. However, the usefulness of oseltamivir can be compromised by the emergence and spread of drug-resistant viruses. The selective pressures exerted by different oseltamivir therapy regimens have received little attention. Materials and Methods: We used a previous model of influenza H1N1 viral kinetics and symptom dynamics describing the interactions between the free virus, target cells, pro-inflammatory cytokines and NK cells. The model was fit to data from experimentally challenged volunteers and dealt with between-subject variability. We extended the model to explore the virological and clinical efficacy of oseltamivir through simulated pharmacokinetics: we included oseltamivir concentration as a time-dependent variable and we simulated random mutations conferring drug resistance. We explored the effect of treatment initiation time, dose, intake frequency and treatment duration on H1N1 influenza infection, illness dynamics and emergence of resistance. We simulated populations of 1000 subjects to describe the observed heterogeneity. A hybrid stochastic and deterministic approach was used to reduce the computational burden while capturing the inherent stochasticity of emergent drug resistance. Results: Clinical and virological efficacies were strongly associated with treatment initiation time. Efficacies were above 85% when treatment was initiated during the incubation period for every recommended regimen and fell below 5% when treatment was initiated 2 days after symptom onset. Without any treatment, the proportion of subjects shedding resistant virus, defined as the number of individuals with resistant viral load above the limit of detection, was less than 1%. In treated patients, the proportion of subjects shedding resistant virus was strongly associated with treatment initiation time: it was 23% when the recommended curative treatment was initiated during the incubation period and fell below 1% when treatment was initiated after symptom onset. Lower doses or premature treatment discontinuation led

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to lower efficacies and increased the proportion of subjects shedding resistant viruses. As an example, a 75-mg/day prophylaxis regimen initiated 2 days before inoculation and stopped 2 days after inoculation would decrease the efficacy to 18% and increase the proportion of subjects shedding resistant virus to 8%. Conclusions: Using a simulation model based on H1N1 infection data, we were able to reproduce drug-resistant emergence rates similar to those reported in the literature. Based on our simulation results, we recommend that current prophylaxis regimens should be avoided to limit resistance emergence. Initiation of treatment during the incubation period should be restricted to subjects prone to develop severe cases and should use high doses (at least 150 mg per intake) and frequent intakes (bid or tid) for a longer period (10-15 days) in order to decrease the risk of resistant virus emergence and to preserve high efficacies.

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Susceptibility of influenza B viruses to neuraminidase inhibitors: findings from the first 4 years (2008–2012) of the global Influenza Resistance Information Study (IRIS)

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Background: Type B influenza virus infections continue to account for a substantial proportion of clinical illness. Little is known about comparative disease profiles by virus lineage. A global observational trial (the Influenza Resistance Information Study or IRIS; NCT00884117) was initiated to study neuraminidase inhibitor (NAI) susceptibility and the clinical and virological course of influenza in treated and untreated patients. Materials and Methods: Patients in the northern and southern hemispheres (USA, France, Germany, Poland, Norway, Hong Kong, Australia) with influenza-like illness and/or a positive rapid influenza test result were enrolled. Throat/nasal swabs were performed on Days 1, 3 (self-swab), 6 and 10 and tested for influenza A and B viruses by RT-PCR. Influenza-positive samples collected on Days 1, 6 or 10 were cultured and subsequently sequenced (HA and NA) and phenotypically tested for NAI susceptibility. The lineage of B viruses was determined from sequencing. Clinical information, including the scoring of seven influenza symptoms (scale: 0 [absent], 1 [mild], 2 [moderate], 3 [severe]), was recorded on diary cards by the patient or the patient's legal guardian (Days 1–12). Symptoms were also assessed by the investigator at each visit. The decision to prescribe an NAI was left to the physician's discretion. Results: In the first 4 years of IRIS (December 2008 to March 2012), 2262 influenza-positive (RT-PCR) patients were enrolled, of whom 697 presented with a type B influenza virus infection (564 Victoria, 98 Yamagata, 35 undetermined lineage). Most type B patients (402; 58%) were children aged < 13 years. A total of 330 (47%) type B patients were treated with oseltamivir (as monotherapy) within 2 days of symptom onset; a further 26 started oseltamivir 2 days after symptom onset. Eleven patients received zanamivir, one received amantadine and another received rimantidine. A total of 328 (47%) did not receive any influenza antiviral. Symptoms were mild to moderate on Day 1 (mean total score: 12.8, treated; 12.9, untreated), and the mean temperature on Day 1 was 38.2°C. All viruses obtained at baseline or post-baseline were susceptible to NAIs: mean (SD) IC_{50} values for oseltamivir were 4.8 nM (2.5 nM) and 5.5 nM (2.3 nM) for the Victoria and Yamagata viruses, respectively; the corresponding values for zanamivir were 2.0 nM (1.4 nM) and 2.9 nM (1.6 nM), respectively. No known NAI resistance mutations were detected by NA or HA population sequencing. The proportion of RT-PCR–positive patients on Day 6 was 130/309 (42.1%) for patients treated with oseltamivir and 152/312 (48.7%) for untreated patients. In Kaplan–Meier analyses, no significant differences in median time to influenza RNA clearance were found between oseltamivir-treated and -untreated patients, either in adults or children. The time to symptom resolution (all symptom scores ≤ 1) was 5 days (95% CI, 4–5 days) in oseltamivir-treated children and 6 days (95% CI, 5–6 days) in untreated children (P = .026), but no significant difference in symptom resolution time was found in adults (Kaplan–Meier analysis). Conclusions: Analysis of type B influenza viruses obtained globally between 2008 and 2012 showed that all pre-treatment B/Victoria and B/Yamagata viruses were susceptible to oseltamivir and zanamivir. Moreover, no resistant viruses were detected during treatment. Given the non-randomised

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design of this study, no definitive conclusions can be drawn with regard to the clinical benefit of oseltamivir in patients infected with type B influenza viruses.

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Persistence of influenza A(H3N2) viruses following oseltamivir therapy in nursing homes in the Netherlands

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Background: Influenza viral load dynamics and emergence of antiviral resistance during oseltamivir therapy and prophylaxis have mainly been studied in previously health adults and children and immunocompromised individuals with influenza. This information is sparse on one of the main target populations for antiviral use if influenza prevention by vaccination fails: frail elderly residing in nursing homes. Therefore, we analysed specimens from nursing home residents in the Netherlands collected during oseltamivir therapy and prophylaxis. In addition, we aimed to assess transmission of (antiviral resistant) viruses by virus sequence analysis. Materials and Methods: The study was performed during the 2011/2012 and 2012/2013 winter seasons. Oseltamivir therapy was offered to residents with influenza-like illness (ILI) with or without laboratory diagnostic results. Oseltamivir prophylaxis was offered to residents without respiratory symptoms as soon as influenza virus infection in another resident was confirmed by laboratory testing. From residents on therapeutic use, specimens (combined nose and throat swabs) were requested at the start and end of therapy (day 5). From residents on prophylaxis, end-of-prophylaxis specimens were requested (day 10). RT-PCR–positive specimens were further characterised by virus isolation and phenotypic antiviral susceptibility assay; sequencing of the haemagglutinin (HA), neuraminidase (NA) and matrix protein genes directly from the clinical specimen; and single nucleotide polymorphism (SNP) real-time RT-PCR for detection of mutations resulting in oseltamivir reduced susceptibility–associated amino acid substitutions E119V and R292K in the NA of A(H3N2) viruses. Results: Of 49 residents with ILI, start-of-therapy specimens were obtained, 33 of which were positive for A(H3N2) influenza virus. An end-of-therapy specimen was obtained from 37 residents, 10 of which were positive for A(H3N2) influenza virus. Paired specimens were available for 28 residents, of whom 19 were confirmed to be infected with A(H3N2) influenza at the start of therapy. One of these 28 residents had an A(H3N2)-positive specimen only at the end of therapy. Twelve of nineteen residents (63%) had cleared the infection upon resampling, whereas 7 of 19 residents (37%) had A(3N2)-positive specimen at both the start and end of therapy. In five of these cases, the viral load decreased (mean Ct increase of 6.9; range 4.1-9.8), whereas in two cases the viral load increased (Ct decreases of 2.1 and 4.3). By the end of therapy, one resident with a decreasing viral load was shedding a virus containing the R292K amino acid substitution, whereas the start-of-therapy specimen contained 292R. NA sequence analysis, SNP detection and phenotypic analysis did not show emergence of viruses with a resistance marker or reduced inhibition by oseltamivir for the other six residents with positive end-of-therapy specimens. Of 32 residents who received oseltamivir prophylaxis, 1 was found positive for A(H3N2) influenza virus at day 5 following development of ILI and 2 were found positive for influenza virus type B lineage B/Yamagata/16/88 at day 10 (end of prophylaxis). Sequence analysis of the M protein of 17/43 detected A(H3N2) viruses showed a subset of viruses with the S31N and V27I amino acid substitution M2-blocker resistance markers, which is a unique combination. Uniqueness was confirmed by mutations in the NA and HA genes of these viruses compared with those of other sequenced A(H3N2) viruses. These unique viruses originated from two locations of one nursing home about 5 km apart. Conclusions: The high proportion of A(H3N2)-positive end-of-therapy specimens (37%) suggests that a 5-day course of oseltamivir therapy for nursing home residents may not be adequate to clear the influenza virus completely. Nevertheless, the R292K substitution in the NA of the influenza virus was found for only one resident receiving oseltamivir therapy, suggesting a relatively high barrier for the A(H3N2) virus to attain this or other substitutions associated with reduced

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inhibition. Finally, evidence of transmission of a strain with a unique M2-blocker resistance marker between two locations of one nursing home illustrates that a breach in first-line prevention mechanisms can lead to the inability of antivirals to be used for prophylaxis and therapy.

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Late treatment with oseltamivir does not reduce duration of influenza symptoms or shedding in persons with uncomplicated influenza

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Background: Influenza causes substantial morbidity and mortality each year, and treatment options are limited. Oseltamivir is a neuraminidase inhibitor that is widely used and effective if initiated within 48 hours after illness onset. Early treatment has been shown to mitigate severity and reduce illness duration by 1 to 3 days. During a pandemic, the logistics of drug delivery will be challenging, and it could be difficult to deliver antiviral therapy to all patients within 48 hours. Observational studies have suggested that initiation of antiviral treatment after 48 hours may be beneficial for hospitalized patients, but the benefits of late antiviral treatment have not been demonstrated for persons with uncomplicated illness who are seen in the outpatient setting. We conducted a double-blind, placebo-controlled trial to assess duration of influenza symptoms and viral shedding in outpatients who initiated therapy ≥ 48 hours after illness onset. Methods: Patients aged 1 to 79 years presenting to outpatient clinics with acute respiratory illness < 120 hours in duration were prospectively recruited during four influenza seasons (2007-08, 2008-09, 2009-10, and 2010-11). Recruitment occurred after patients had been evaluated and treated by a physician not involved in the study. Only patients who did not receive an oseltamivir prescription were eligible. After informed consent, a nasal (children) or nasopharyngeal (adolescents and adults) swab was obtained for influenza detection by real-time reverse transcription–polymerase chain reaction (RT-PCR). Results were usually available within 24 hours, and participants with laboratory-confirmed influenza were then immediately randomized to receive oseltamivir (standard dose for age) or placebo at a 2:1 ratio. Participants completed a symptom survey twice each day for up to 14 days after randomization. Additional swabs were performed for influenza detection at the time of randomization and 3-4 days post-randomization. Kaplan-Meier analysis was used to compare treatment groups with respect to time to symptom resolution and time to first negative RT-PCR result among those randomized 48 to 120 hours after illness onset. Unfortunately, the target sample size of 525 patients (including 350 randomized ≥ 48 hours after onset) was not achieved due to unforeseen circumstances, including the emergence of oseltamivir-resistant influenza viruses among study participants (2008-09) and the 2009 pandemic. All patients enrolled in the 2008-09 season were therefore excluded from the analysis. In 2009-10, the pandemic wave occurred before the procedures were in place to enroll and randomize patients, and influenza was essentially absent in the population during the subsequent months. In the final enrollment season (2010-11), enrollment ceased prematurely due to expiration of funding. Results: A total of 134 patients were randomized 48 to 120 hours after influenza symptom onset; 95 received oseltamivir and 39 received placebo. The median age of participants was 20 [interquartile range (IQR): 8-38] and 17 (IQR: 11-37) years for those who received oseltamivir and placebo, respectively. The mean interval from illness onset to randomization (first dose of study drug) was 83.0 hours (standard deviation: ± 20.1).The median time to symptom resolution did not differ between treatment groups [96 hours (95% CI: 84-120) for both the oseltamivir and placebo groups]. The probability of resolution by day 3 post randomization was 0.37 (95% CI: 0.27-0.47) for individuals who received oseltamivir and 0.32 (95% CI: 0.17-0.46) for individuals who received placebo (P = 0.5). There was no difference in duration of virus detection by RT-PCR. The probability that an individual was still RT-PCR positive through days 3-4 post randomization was 0.13 (95% CI: 0.06-0.20) and 0.11 (95% CI: 0.01-0.21) for individuals who received oseltamivir and placebo, respectively (P = 0.8). Similar results were observed when all randomized patients (< 120 hours of illness duration, n = 165) were included. Conclusions: In our study, late treatment with oseltamivir did not appear to reduce the duration of symptoms or viral detection among outpatients with influenza. However, we failed to reach our target

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sample size, which may have limited our power to detect clinically relevant differences between treatment groups. Early initiation of antiviral therapy is important for uncomplicated influenza.

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Combination effects of peramivir and favipiravir against oseltamivir-resistant 2009 pandemic influenza A (H1N1) infection in mice

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Background: Antiviral drugs are being used for prophylactic and therapeutic purposes against influenza illness in humans. However, the emergence of antiviral-resistant variants and a dearth in the effective countermeasures against them make managing the appropriate antiviral chemotherapies complicated, especially in immunocompromised patients. Combined medications, as seen in the treatment of cancers and other infectious diseases, have been suggested as an option for the control of antiviral-resistant influenza viruses. **Materials and Methods:** Here, we evaluated the therapeutic value of combination therapy against oseltamivir-resistant 2009 pandemic influenza H1N1 virus infection in DBA/2 mice. Mice were treated for 7 days with favipiravir and peramivir starting 48 hours before lethal challenge. **Results:** Compared with either monotherapy, combination therapy saved more mice from viral lethality and resulted in greater curative efficacy in the lungs of infected mice. Furthermore, the synergism between the two antivirals, which was consistent with the survival outcomes of combination therapy, indicated that favipiravir could serve as a critical agent of combination therapy for the control of oseltamivir-resistant strains. **Conclusions:** Our results provide new insight into the feasibility of favipiravir in combination therapy against oseltamivir-resistant influenza virus infection.

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Study of susceptibility of influenza viruses to neuraminidase inhibitors by phenotypic method: implementation process in Cote d'Ivoire

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Background: Since the recent influenza pandemic in 2009, many countries in sub-Saharan Africa have recognized the threat of influenza viruses, and, under the WHO initiative, set up surveillance systems at the national level. One of the objectives of this surveillance is to determine the resistance pattern of circulating viral strains. Two methods are available for this purpose: genotyping molecular-based assays and phenotyping neuraminidase (NA) inhibition assays based on calculation of IC50 values—the concentration of NA inhibitor that reduces NA activity by 50% of the virus–control. The present article aims to describe the implementation process of monitoring the susceptibility of influenza viruses to NA inhibitors (NAIs) by phenotypic method in Côte d'Ivoire. Materials and Methods: The implementation of this technique was carried out in four steps: training, procurement of equipment and analysis software, request for obtaining antiviral and reference strains, and the realization of antiviral testing within the laboratory. Training was held at the WHO Collaborating Centre in London and focused on the determination of susceptibility of influenza viruses to NAIs (oseltamivir and zanamivir) using the protocol of NA-XTD™ Influenza Neuraminidase assay by Life Technologies kit. The tests were performed on five strains from Côte d'Ivoire and four reference viruses. Multimode reader tristar LB 942 of Berthold technology with mikrowin 2000 software version 4.41 was acquired for luminescence reading. GraFit 7, commercially available software, was also acquired for calculation of IC50 values from NA enzyme inhibition assay data. The phenotypic method

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requires the use of a pure form of antiviral; 300 mg of RO0640802-002 tartrate-salt of oseltamivir carboxylate MW 386.44 was received from Roche Mannheim after sending a query using a form obtained on www.isirv.org and signing an MTA between Roche and the Institut Pasteur in Cote d'Ivoire, and 100 mg of zanamivir was obtained from GlaxoSmithKline by following the same procedure. Reference viruses are necessary for the quality control of phenotyping NA enzyme inhibition assays. After the training, we sent a request to WHOCC London and obtained six reference viruses: A/Denmark/524/2009 275H (oseltamivir and zanamivir sensitive), A/Denmark/528/2009 275Y (oseltamivir resistant and zanamivir sensitive), A(H3N2)119^{E} (oseltamivir and zanamivir sensitive), A(H3N2) E119V (oseltamivir resistant and zanamivir sensitive), B/ Perth/211:2001 197D (oseltamivir and zanamivir sensitive), and B/Perth/211:2001 D 197^{E} (oseltamivir and zanamivir) reduced susceptibility. Upon receipt, these viruses were grown on MDCK cells. Several aliquots of supernatant were obtained and stored at -80 °C. For this study, 50 viruses detected during sentinel surveillance of influenza in Cote d'Ivoire in 2012 and 2013, including viruses previously tested in London, were tested in duplicate with the corresponding reference viruses. The results were compared with IC50 values of the panel of influenza viruses in NA enzyme inhibition assays proposed by the isirv Antiviral Group. Results: All 50 virus isolates tested demonstrated NA activity in the NA activity assay. The IC50 values for sensitive and resistant reference viruses were within acceptable ranges compared with panel data proposed by the isirv Antiviral Group. We observed a good correlation between the IC50 values obtained with zanamivir for viruses tested both in London and in Côte d'Ivoire. IC_{50} values obtained for oseltamivir and zanamivir indicated the susceptibility of all isolates (100%) to both NAIs. Conclusions: The implementation of this technique will allow us to monitor the sensitivity of the virus to NAIs in the sentinel surveillance of influenza in Cote d'Ivoire. It will, however, be necessary to continue to improve it and to correlate the data obtained with data obtained with the genotyping molecular-based assay method.

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Quantifying the relative replication fitness of oseltamivir-resistant pandemic H1N1 influenza variants

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Background: During a 3-month period in 2011, H1N1pdm09 viruses resistant to the antiviral drug oseltamivir were isolated within the Hunter New England (HNE) region of New South Wales, Australia, with epidemiological evidence suggesting community transmission had occurred. These and similar viruses present a potential threat to public health. Here, through within-host modelling of *in vivo* virological infection data, we quantified the relative replication fitness of oseltamivir-resistant viruses compared with wild-type viruses. Materials and Methods: We assessed relative replication fitness (ie, relative growth dynamics) by analysing data from "competitive-mixtures" experiments. These experiments involve co-inoculation of a host (ferret) with mixtures of two different strains of influenza and observation of the relative growth dynamics of each strain. Here we analysed data from experiments comparing oseltamivir-sensitive and oseltamivir-resistant natural isolates gathered from the HNE outbreak. We also analysed experiments assessing the relative replication fitness of reverse-genetics–generated viruses with or without two potentially permissive mutations (PPMs) in the neuraminidase gene (V241I and N369K), which may improve the fitness of H1N1pdm09 H275Y viruses. We quantified relative *replication fitness* by fitting a mathematical model describing the within-host dynamics of each co-infecting strain to viral load data. Note that an assessment of overall fitness (including both replication and transmission phenomena) was beyond the scope of this quantitative analysis. Fitting our model to data enabled the estimation of model parameters that correspond to important biological processes that govern infection dynamics. We investigated several different hypotheses regarding which biological process is the primary driver of strain-dependent infection dynamics: (1) a strain-dependent production rate of infectious virus, (2) a strain-dependent average duration for the latent phase of infected cells, and (3) a strain-dependent production rate of *non-infectious* virus. An estimated difference in the biological parameter mentioned in either hypothesis 1

or 2 provides support for a replication fitness difference between strains. However, this was not the case for hypothesis 3, as a difference in that parameter does not generate strain-dependent replication fitness. Results: Direct observation of the strain-dependent viral load kinetics within these experiments *qualitatively* suggests that the naturally isolated HNE MUT viruses have comparable overall fitness relative to related WT viruses. Reverse-genetics viruses built from HNE isolates, with one or both of the PPMs removed, have qualitatively reduced overall fitness. Conversely, addition (through reverse genetics) of the PPMs into a demonstrably unfit H1N1pdm09 virus (isolated from Perth, Australia) indicates improvement in overall fitness. With focus on the within-host replication kinetics, our model-based *quantitative* analysis of the paired natural isolates provided no consistent indication of a within-host fitness advantage or disadvantage of the HNE MUT virus compared with the HNE WT virus. Depending upon the natural isolates compared and the hypothesised biological mechanism for generating a fitness difference, we obtained weak or no statistical evidence of a fitness difference. For the reverse-genetics experiments, under the two different hypotheses that a fitness difference is generated by either strain-dependent infectious virus production or strain-dependent latent phase duration, we found strong evidence that the two PPMs increased the replication fitness of both HNE and Perth oseltamivir-resistant H275Y viruses. Under the first of these respective hypotheses, we found that removal of the PPMs from the HNE virus decreased replication fitness (a ratio of 0.52 [0.15; 0.74]). Conversely, addition of the PPMs into the Perth virus increased replication fitness (2.85 [1.99; 7.20]). Finally, we were also able to fit these data without incorporating a fitness difference into the model under the hypothesis involving strain-dependent non-infectious virus production. Conclusions: We found mixed evidence of a replication fitness difference between WT and MUT isolates from the HNE outbreak, supporting the virological and epidemiological evidence that this event, while ultimately geographically isolated and short-lived, is potentially a source of widespread and sustained resistance. Our analysis provides strong evidence that the V241I and N369K mutations improve the replication fitness of H1N1pdm09 H275Y viruses, indicating that ongoing monitoring for these mutations in clinical isolates (oseltamivir-resistant or otherwise) is paramount.

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Drug resistance of influenza virus strains circulated in Russia during four epidemic seasons 2009-2010, 2010-2011, 2011-2012, and 2012-2013

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Background: Annual epidemics of human influenza A and, more variably, influenza B virus infections cause significant amounts of morbidity, economic losses, and mortality in the general population. Two classes of antiviral agents are also available for prevention and treatment of influenza, namely, the adamantane derivatives or M2 ion channel blockers, amantadine and rimantadine, and the neuraminidase inhibitors (NAIs), zanamivir and oseltamivir. One of the key concerns with regard to wide-scale use of influenza drugs in the population is the emergence of antiviral drug resistance. Materials and Method: Clinical isolates of influenza A and B viruses were recovered from ambulatory and hospitalized patients. Susceptibility to oseltamivir carboxylate and zanamivir was examined initially in a previously described fluorometric (MUNANA) neuraminidase assay. Susceptibility to rimantadine was examined on MDCK cells by virus yield decrease. The results of both tests were confirmed by sequencing. Results: In the 2009-2010 epidemic season, in the research institute of influenza, in the framework of the monitoring of drug resistance, 70 strains of the influenza virus from different regions of the Russian Federation were isolated. 49 strains belonged to the pandemic influenza virus subtype A (H1N1)pdm09, 7 were A (H1N1), and 15 strains to influenza virus A (H3N2). The isolated viruses were tested for resistance to rimantadine and the neuraminidase inhibitors—oseltamivir and zanamivir. Influenza A (H1N1)pdm09 and A (H3N2) were sensitive to zanamivir and oseltamivir, whereas among virus A (H1N1) 6 of 7 (86%) were resistant to oseltamivir. The average IC50 of zanamivir and oseltamivir for the virus A (H1N1)v was 0,26 nM and 0,13 nM, and for the virus A (H3N2) 0,10 nM and 0,14 nM, respectively. All tested viruses were resistant to rimantadine. During the epidemic season 2010-2011, 97 strains of influenza virus were isolated and tested for drug resistance. Of those, 80 strains belonged to the subtype A (H3N2), and 17 were influenza B. All

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viruses were resistant to rimantadine and sensitive neuraminidase inhibitors. The average IC50 of zanamivir and oseltamivir for the virus A (H3N2) was 0,98 nM and 0,6 nM, and for influenza B viruses (H3N2) 5,83 nM and 4,67 nM, respectively. During the epidemic season 2011-2012, 147 strains of influenza virus were isolated and tested for drug resistance. 109 strains were influenza A (H3N2) and 38 strains were influenza virus B. All viruses were sensitive to neuraminidase inhibitors. The average IC50 of zanamivir and oseltamivir for the virus A (H3N2) was 2,69 nM and 2,21 nM, and for influenza B viruses (H3N2) 4,19 nM and 3,24 nM, respectively. All viruses were resistant to rimantadine: it was shown that all strains carried S31N substitution in M2 and .rimantadine has no or little effect on viral reproduction on MDCK cells. During the epidemic season 2012-2013 239 strains of influenza virus were isolated and tested for drug resistance. Of those, 45 strains belonged to influenza A (H3N2), 82 strains were A (H1N1)pdm09, and 112 strains were influenza B. All viruses were sensitive to neuraminidase inhibitors. The average IC50 of zanamivir and oseltamivir for the viruses A (H1N1)pdm09 were 0,72 nM and 0,58 nM, and for the viruses A (H3N2) 0,84 nM and 0,52 nM, and for viruses B 1.10 nM and 2.36 nM, respectively. According to the results of sequencing of the viral gene M2, all the selected viruses were resistant to rimantadine. **Conclusions:** Since the 2010-2011 to the present time all influenza viruses have isolated in Russia are resistant to rimantadine and sensitive to neuraminidase inhibitors.

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Antiviral susceptibility of influenza A viruses isolated between 2009 and 2013 in Portugal

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Background: Monitoring influenza antiviral susceptibility has become an important issue in recent years. Influenza A viruses are naturally susceptible to neuraminidase (NA) inhibitors (NAIs) and M2 inhibitors (adamantanes). The increased resistance to oseltamivir and adamantanes (since 2006) and the availability and clinical use of influenza antivirals have enhanced the need for close monitoring of the loss of susceptibility. In 2009, the Portuguese National influenza Centre was developed under the scope of the National Influenza Surveillance Programme to systematically monitor antiviral (oseltamivir, zanamivir and adamantine) susceptibility. This study provides the results of antiviral susceptibility to NAIs and M2 inhibitors for influenza A viruses characterized during 4 winter seasons (2009 to 2013). Materials and Methods: A phenotypic method, the NA inhibition assay [considered the "gold standard" method for determining the susceptibility to oseltamivir and zanamivir, which determines the 50% inhibitory concentrations (IC_{50})], was performed during the study period for a selection of 119 influenza A viruses: 26 and 60 AH1pdm09 viruses from the 2009/2010 and 2012/2013 seasons, respectively; 33 AH3 viruses from the 2011/2012 season; and 2 AH3 viruses from the last season. Genotypic screening methods were performed for the most common NA inhibition–reducing substitution, H275Y in AH1pdm09 viruses (n = 340). Matrix gene sequencing for adamantane susceptibility surveillance and NA gene sequencing were performed for 64 and 119 strains, respectively. Results: In the study period, all AH1pdm09 and AH3 viruses carried the S31N substitution in the M2 protein, which confers resistance to the adamantanes to 100% of the isolates. For AH1pdm09, the IC_{50} for oseltamivir ranged from 0.45 to 2.82 (2009/2010) and from 0.15 to 3.27 (2012/2013). The resistance to oseltamivir was identified in 3 AH1pdm09 viruses that carried the H275Y substitution. Two of these viruses were isolated from patients with chronic diseases treated with oseltamivir: a pregnant 26-year-old woman with a fatal outcome and an 8-year-old child. One of the oseltamivir-resistant viruses had an IC_{5o} value 250-fold higher (IC_{5o} = 502.48) comparing with the susceptible viruses. The AH1pdm09 IC_{5o} values for zanamivir ranged from 0.32 to 3.88 (2009/2010) and from 0.29 to 2.99 (2012/2013). The median IC_{50} values for oseltamivir and zanamivir were stable between 2009 and 2013. None of the AH1pdm09 isolates presented the other substitutions (D119N, I223R and N295S) associated with reduced susceptibility to NAIs. The AH3 viruses analysed in the 2011/2012 season had IC_{5o} values for oseltamivir that ranged from 0.19 to 0.90 and for zanamivir from 0.39 to 0.84. The median IC_{50} values for oseltamivir and zanamivir were 0.41 ± 1.55 and 0.59 ± 1.21, respectively. In the last season (2012/2013), only two AH3 isolates were analysed and the IC_{5o}

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values were in the same range as for the 2011/2012 AH3 viruses. Since 2009, all AH3 viruses were susceptible to oseltamivir and zanamivir. None of the AH3 viruses had the amino acid substitutions known to reduce susceptibility to NAIs (E119V/I, R229K, N294S and H274Y). Conclusions: Influenza A viruses isolated in Portugal since 2009 showed resistance to adamantanes, which are no longer indicated for influenza A treatment. Otherwise, the resistance to oseltamivir was observed only in a reduced number of strains; all the viruses showed susceptibility to zanamivir. The use of conventional sequence analysis and genotypic screening methods to monitor the molecular markers of antiviral resistance to influenza A viruses provides a valuable tool for early detection of antiviral-resistant strains.

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Enhanced efficacy of a dimeric neuraminidase inhibitor against influenza virus infections in mice

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Background: Emerging drug resistance to antiviral therapies has become an increasing challenge for the effective treatment of influenza virus infections. This underlines the importance of continuous antiviral drug development. One such new antiviral compound is BTA938—a dimeric derivative of the viral neuraminidase inhibitor zanamivir. BTA938, functionalized at the C7 hydroxyl, contains a 14-carbon linker bridging two zanamivir moieties. Materials and Methods: Groups of 10 female BALB/c mice weighing 18-20 g (15 placebo) were treated by the intranasal route twice daily for 5 days with BTA938 or zanamivir, beginning 4 hours post-virus. Doses for all treatment and placebo groups were given 12 hours apart. The placebo group received sterile saline in a similar dosing regimen. All groups were observed for morbidity and weight loss through day 21. Kaplan-Meier survival curves were generated and compared by the log-rank test followed by pairwise comparison using the Gehan-Breslow-Wilcoxon test in Prism 5.0b (GraphPad Software Inc). Mean body weights were analyzed by one-way analysis of variance followed by Tukey's multiple comparison tests. Results: BTA938 was compared with zanamivir at a dose optimizing study against a challenge infection with influenza A/CA/04/2009 (H1N1pdm). Five concentrations of BTA938 (0.01, 0.03, 0.1, 0.3, and 1 mg/kg/day) were shown to improve survival following influenza virus challenge, while three concentrations of zanamivir (1, 3, and 10 mg/kg/day) were shown to improve survival of mice. These data demonstrate that BTA938 is approximately 10x more potent than zanamivir at improving survival following challenge with an influenza A H1N1pdm virus. A second study compared BTA938 and zanamivir against a challenge infection with influenza A/Victoria/3/75 (H3N2) virus. Five concentrations of BTA938 (0.03, 0.1, 0.3, 1, and 3 mg/kg/day) were shown to improve survival of mice following influenza virus challenge. Four concentrations of zanamivir (0.3, 1, 3, and 10 mg/kg/day) were shown to improve survival of mice. These data also demonstrated that BTA938 was approximately 10x more potent than zanamivir at improving survival following challenge with an influenza A H3N2 virus. We next compared BTA938 and zanamivir against a challenge infection with influenza A/Mississippi/03/2001 (H1N1) oseltamivir-resistant H275Y virus. In addition, the antiviral efficacy of BTA938 administered as a single dose was compared with zanamivir administered twice per day for 5 days. A significant difference from placebo was observed in the mean day of death for all treatment groups, demonstrating that a single dose of BTA938 is equivalent to comparable doses of zanamivir administered twice per day for 5 days against an oseltamivir-resistant influenza A H1N1 virus infection in mice. Finally, we evaluated the antiviral efficacy of a single 10-mg/kg dose of BTA938 when administered prophylactically 1, 3, or 7 days pre-virus challenge. A significant difference from placebo was observed in the mean day of death for all three prophylactic treatments. Conclusions: In vivo experiments clearly showed the high antiviral potential of BTA938 for the treatment of infections caused by influenza A H1N1pdm, H3N2, and oseltamivir-resistant H1N1 viruses in mice. Moreover, we demonstrated that administration of a single dose of BTA938 is sufficient for therapeutic and prophylactic protection in mice challenged with influenza A virus. *[Supported by Contracts N01-AI-30063 from the Virology Branch, HHSN272201000039I/HHSN27200005/A37 from the Respiratory Diseases Branch, NIAID, NIH, and NIH grant number U01AI070263].*

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Antiviral resistance: influenza B

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Background: Currently circulating influenza viruses are resistant to adamantanes and, except for a low number of sporadic cases, most are sensitive to neuraminidase inhibitors (NIs). Adamantanes are ineffective against influenza B viruses, and, although NI-resistant influenza B viruses have been rarely reported, one cluster of influenza B viruses with reduced susceptibility to NIs and with the I221V substitution in the active site of the neuraminidase was identified recently in the United States. Despite the low prevalence of oseltamivir-resistant influenza viruses, the constant evolution of influenza requires the monitoring of antiviral resistance among these viruses in the community. This is very important in the clinical management of severe influenza cases for the detection of novel genetic markers associated with antiviral resistance. This study reports the antiviral susceptibility to NIs of influenza B viruses isolated in Portugal during the 2010/2011, 2011/2011 and 2012/2013 seasons. Materials and Methods: Over the period of 3 influenza seasons, 146 influenza B viral strains were selected for phenotypic fluorescent assays in order to assess their susceptibility to the NIs oseltamivir and zanamivir. For this purpose, we determined the NI concentration required to inhibit 50% of the neuraminidase activity (IC_{50}) of each influenza virus. The IC_{50} baseline of influenza B viruses was calculated for both oseltamivir and zanamivir using the Robust Excel programme. The neuraminidase gene segments were also monitored for the presence of the main molecular markers associated with the resistance to NIs in influenza B viruses. Results: All influenza B strains analysed proved to be susceptible to oseltamivir and zanamivir. In the 2010/2011 season, the IC_{50} values ranged from 0 to 70 nM for oseltamivir and from 0 to 11 nM for zanamivir. The median IC_{50} value for zanamivir was about 8-fold lower than that for oseltamivir. Statistical analysis revealed the presence of one outlier (B/Lisboa/13/2010: 71.08 nM) for oseltamivir (2-fold reduction in susceptibility) and four minor outliers (B/Lisboa/15/2010: 9.52 nM; B/Lisboa/19/2010: 9.25 nM; B/Lisboa/51/2010: 10.94 nM and B/Lisboa/53/2010: 9.62 nM) for zanamivir (3-fold reduction in susceptibility) compared with the median IC_{50} value. During the 2011/2012 season, IC_{50} values ranged from 23.0 to 38.38 nM (oseltamivir) and from 2.97 to 9.07 nM (zanamivir). No minor or major outliers were found in this season. The IC_{50} values obtained in 2012/2013 ranged from 7.91 to 84.84 nM (oseltamivir) and from 1.48 to 5.88 nM (zanamivir). During this last season, 6 minor and 3 major outliers were found for oseltamivir and 13 minor outliers were found for zanamivir. Over the three seasons, the median IC_{50} values for both NIs were higher for the B/Victoria than for the B/Yamagata viruses. None of the actual known mutations associated with resistance of influenza B viruses to NI was found in the neuraminidase gene (R150K, D197E/N/Y, I221T/V, N294S, R374K and G407S). Conclusions: Influenza B viruses isolated in Portugal during the past three seasons were susceptible to the NIs evaluated. Portuguese influenza B strains revealed higher susceptibility to zanamivir than to oseltamivir, as observed in other countries. The IC_{50} values for oseltamivir were also different between viruses from the B/Victoria and B/Yamagata lineages; however, significance was not shown due to the small number of analysed viruses. Among the B/Yamagata strains, IC_{50} values for oseltamivir and zanamivir were higher in the 2011/2012 season than in the 2010/2011 and 2012/2013 seasons.

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Characterization of neuraminidase inhibitor susceptibility of influenza A isolates obtained in Kenya, 2008-2011

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Background: Vaccines and antivirals are the mainstay for mitigation and clinical management of influenza infections. However, due to the ever-changing antigenic profile, vaccine formulations are revised every year to make them efficacious. Neuraminidase (NA) inhibitors, mainly oseltamivir and zanamivir, function both as prophylactic and as treatment agents. In NA inhibition by antivirals, inhibitor molecules mimic NA's natural substrate and bind to the active site, preventing NA from cleaving host cell receptors and releasing new virus. Currently, there exist no data on antiviral susceptibility profiles of influenza A isolates circulating within the Eastern African region. Here we characterised the antiviral susceptibility of the 2008-2011 influenza A viruses circulating in Kenya by combining both the genotypic data involving known molecular markers in NA protein responsible for drug resistance and IC50 data generated from NA inhibition assays. Materials and Methods: Nasopharyngeal swab specimens from consenting outpatients aged ≥ 2 months were obtained and transported to the National Influenza Centre and screened by real-time RT-PCR using primers targeted at the matrix and haemagglutinin genes of influenza A subtypes. Positive specimens were inoculated onto MDCK monolayers to isolate virus. RNA was extracted from virus isolates followed by PCR amplification of NA gene segments. Nucleotide sequencing was performed using the BigDye chemistry prior to analyses using a suite of bioinformatics tools. Drug susceptibility was determined by enzyme inhibition assay using fluorescent substrate with known NA inhibitor–resistant and –sensitive viruses as controls. IC50 values were determined using curve-fitting software (Grafit 7.0), which is based on 50% of fitted upper asymptote. Results: Of 836 influenza A virus isolates obtained (2008-2011), 108 (13%) were analysed for markers of resistance to NA inhibitors: 64% (7/11) of the 2008 seasonal influenza A/H1N1 isolates analysed showed oseltamivir-resistant marker H275Y, while all 33 (100%) influenza A/H3N2 isolates obtained showed sensitivity to oseltamivir. Genetic analyses of the A (H1N1) pdm09 isolates obtained in 2009-2010 showed that all were sensitive to oseltamivir. All 14 influenza A/H3N2 isolates obtained in 2011 were also shown to be sensitive to oseltamivir. A total of 28 isolates were further subjected to phenotypic susceptibility assay. The mean zanamivir IC50 values were 1.75, 2.53 and 1.84 nM for the subtypes H1N1, pH1N1 and H3N2, respectively. Two of the 2008 sH1N1 and one of the sH1N1 obtained in 2009 showed normal sensitivity to oseltamivir in the NA inhibitor susceptibility assay (mean IC50 of 1.28 nM). The rest of the 2008-2009 sH1N1 analysed (n = 8) showed highly reduced sensitivity to oseltamivir. The IC50 values in the fluorescent assay ranged from 73 to 984 nM. Pandemic A/H1N1 strains obtained between 2009 and 2011 indicated oseltamivir IC50 values of 1.60 to 6.32 nM—categorised as normal sensitivity. All 8 candidate influenza A/H3N2 isolates obtained between 2008 and 2011 were sensitive to oseltamivir, with IC50 values ranging from 0.16 to 0.94 nM. The 2011 WHO ranges and median IC50 values for oseltamivir carboxylate were 0.4 to 10 nM and 0.5 nM, 0.1 to 5 nM and 0.2 nM, and 0.2 to 10 nM and 0.6 nM for wild-type sH1N1, sH3N2 and pH1N1, respectively. The 2011 WHO ranges and median IC50 values for oseltamivir carboxylate were 257 to 3455 nM and 458.2 nM and 132 to 2179 nM and 191.3 nM for mutant types sH1N1 and pH1N1, respectively. The WHO IC50 values for zanamivir, both for mutant and wild-type strains, ranged from 0.2 to 3 nM for all subtypes, with no significant differences between the mutant and wild-type strains for each subtype. Conclusion: Overall, our genotypic data demonstrate that there was oseltamivir resistance in seasonal influenza A (H1N1) viruses isolated in Kenya in 2008-2009. Most of the 2008-2009 sH1N1 isolates depicted highly reduced sensitivity to oseltamivir. This was due to the presence of the H275Y mutation in the NA protein sequence. H275Y mutation increased the IC50 value by 50- to 100-fold. Resistance to NA inhibitors was found to be specific to both drug and virus subtype. The drug susceptibility profile will be best informed using both elevated IC50 vales and known molecular markers of resistance.

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Resistance mutation R292K in A(H6N2) is induced by low levels of oseltamivir exposure in infected mallards

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Background: Resistance to neuraminidase (NA) inhibitors (NAIs) is problematic as these drugs constitute the major treatment option for severe influenza and are crucial in pandemic preparedness plans worldwide. The active form of oseltamivir, oseltamivir carboxylate (OC), is excreted via the urine, passes unchanged through sewage treatment plants and ends up in river waters where concentrations of up to 865 ng/L have been measured. Wetland birds — the natural reservoir of influenza A — reside in this environment. Our hypothesis is that, in this water environment, there is a risk of resistance development in the gut of influenza-infected wild birds because of their exposure to OC. It is well known that, from the large genetic pool of avian influenza, elements can be re-assorted or mutations introduced to create new pandemic strains. The phylogenetically different groups of NA, N1 and N2, have distinct binding sites and resistance characteristics with different mutations to NAIs. In a mallard model approximating a natural setting, we previously demonstrated that an A(H1N1) virus developed OC resistance through the mutation H274Y (N2-numbering) when birds were exposed to 1 µg/L of OC, and recent studies indicate persistence of the mutation without drug pressure. In this study, we assessed the resistance development in a virus from the phylogenetic N2 group, A(H6N2). Materials and Methods: Two male mallards (*Anas platyrhynchos*) were infected via esophageal introduction with A/*Mallard*/Sweden/50908/2006(H6N2) that was previously isolated from a wild mallard, propagated in embryonated hen eggs, sub-typed, NA sequenced and confirmed to contain no known resistance mutations. Every third day a new generation of two birds were introduced to the experimental room and allowed to be fecal-orally infected over two days; five days after introduction, each generation was euthanized. The experiment was repeated three times in four generations of birds (in total 14 days) — each continuously exposed to OC via their sole water source at concentrations of 120 ng/L, 1.2 µg/L and 12 µg/L respectively. Daily fecal samples were analyzed with RRT-PCR of the matrix gene to determine the presence of influenza, after RT-PCR and sequencing of the NA gene, and select samples of the HA gene were also analyzed. Phenotypic resistance evaluation was done with OC and zanamivir in a fluorescent substrate (MUNANA) assay to determine IC_{50} levels; prior to the assay, the samples had been propagated in embryonated hen eggs and re-sequenced. Results: After 4 days of exposure to 12 µg/L OC, the resistance mutation R292K in NA emerged and then persisted. Drug sensitivity was decreased ≈ 13,485-fold for OC (mean IC_{50} 2863 vs 0.21 nM, P = .000082) and ≈ 7.6-fold for zanamivir (mean IC_{50} 5.3 vs 0.7 nM, P = .000082). Viral shedding was similar when comparing R292K and wild-type virus, indicating sustained replication and transmission. Fitness loss with reduced NA activity and a decrease in recovered virus after propagation in embryonated hen eggs were observed in the mutated isolates. The initial, but not the later, R292K isolates reverted to wild type in the egg-propagation process, suggesting stabilization of the mutation over time, possibly through additional mutations in the NA (D113N or D141N) or HA (E216K) genes that appeared on the last days of the experiment. Conclusions: Our results indicate a risk of OC resistance development in different avian influenza viruses circulating among wild birds. If the resistance persists, there is a risk of transfer to humans via re-assortment or direct transmission of a resistant pandemic influenza virus. This would be of substantial public health concern as pandemic preparedness plans rely heavily on oseltamivir before vaccines can be mass-produced. This study also demonstrates that extensive use of one NAI can also reduce sensitivity to other drugs in the NA class. Prudent use of NAIs, increased efficiency of sewage treatment and alternative treatment and prophylaxis strategies are important measures to minimize the risk.

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Recent A(H1N1)pdm09 influenza viruses encode permissive NA mutations that improve the fitness of oseltamivir-resistant H275Y variants

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Background: Oseltamivir is relied upon worldwide as the primary drug of choice for the treatment of human influenza infection. During 2008, oseltamivir-resistant seasonal H1N1 influenza viruses spread globally, demonstrating the potential for influenza viruses to acquire oseltamivir resistance without compromising viral fitness. Since the emergence of the A(H1N1)pdm09 virus in humans in 2009, only limited transmission of oseltamivir-resistant A(H1N1)pdm09 virus has been observed. However, in 2011, widespread transmission of oseltamivir-resistant A(H1N1)pdm09 viruses was detected in the community in the Hunter New England (HNE) region of Australia. Using *in vitro* and *in vivo* models, we assessed the replication and transmission of these oseltamivir-resistant viruses and determined permissive NA mutations that improved the fitness of the oseltamivir-resistant H275Y variants. Materials and Methods: *In vitro* experiments were conducted to assess NA enzyme activity using the fluorescent substrate MUNANA and to examine cell surface expression of NA protein by flow cytometric analysis of cells transfected with NA plasmids containing a GFP tag. Viral replication and transmissibility were assessed using a competitive mixtures model whereby ferrets were infected with known mixtures of two viruses, and the relative proportion of those viruses was monitored over the course of infection and upon two rounds of transmission. Oseltamivir-resistant A(H1N1)pdm09 viruses from the HNE outbreak were compared with oseltamivir-sensitive strains from the same region and time. In addition, an earlier pair of oseltamivir-resistant and -sensitive A(H1N1)pdm09 strains from 2009 was tested. The full genomes of the HNE and early 2009 viruses were fully cloned and used to rescue a series of isogeneic reverse genetics viruses to allow the introduction or removal of key amino acids thought to alter the fitness of viruses with the H275Y NA mutation. Results: In vitro analysis demonstrated that two NA mutations (V241I and N369K) present in the HNE oseltamivir-resistant H275Y viruses contributed significantly to maintaining viral function, as their removal resulted in a significant reduction in both NA enzyme activity and NA protein expression. A third potentially permissive mutation, N386S, did not appear to contribute to either NA activity or NA expression. In ferrets, the HNE H275Y variant demonstrated comparable replication and transmissibility in ferrets compared with an equivalent oseltamivir-sensitive strain. However, using reverse genetics–derived viruses, we demonstrated that removal of the V241I and N369K permissive mutations from the HNE H275Y variant substantially decreased the replication and transmissibility of the virus. Conversely, we showed that introduction of these substitutions into an early 2009 A(H1N1)pdm09 virus that did not naturally contain these mutations substantially improved their replication and transmissibility in the ferret model. Conclusions: Our findings demonstrate that H1N1pdm09 viruses containing the NA V241I and N369K amino acid mutations are substantially more permissive to the acquisition of NA H275Y than are viruses without these mutations. It is of concern that these permissive mutations are now present in virtually all currently circulating A(H1N1)pdm09 viruses; therefore, the potential for widespread oseltamivir resistance may now be greater than when the A(H1N1)pdm09 virus first emerged.

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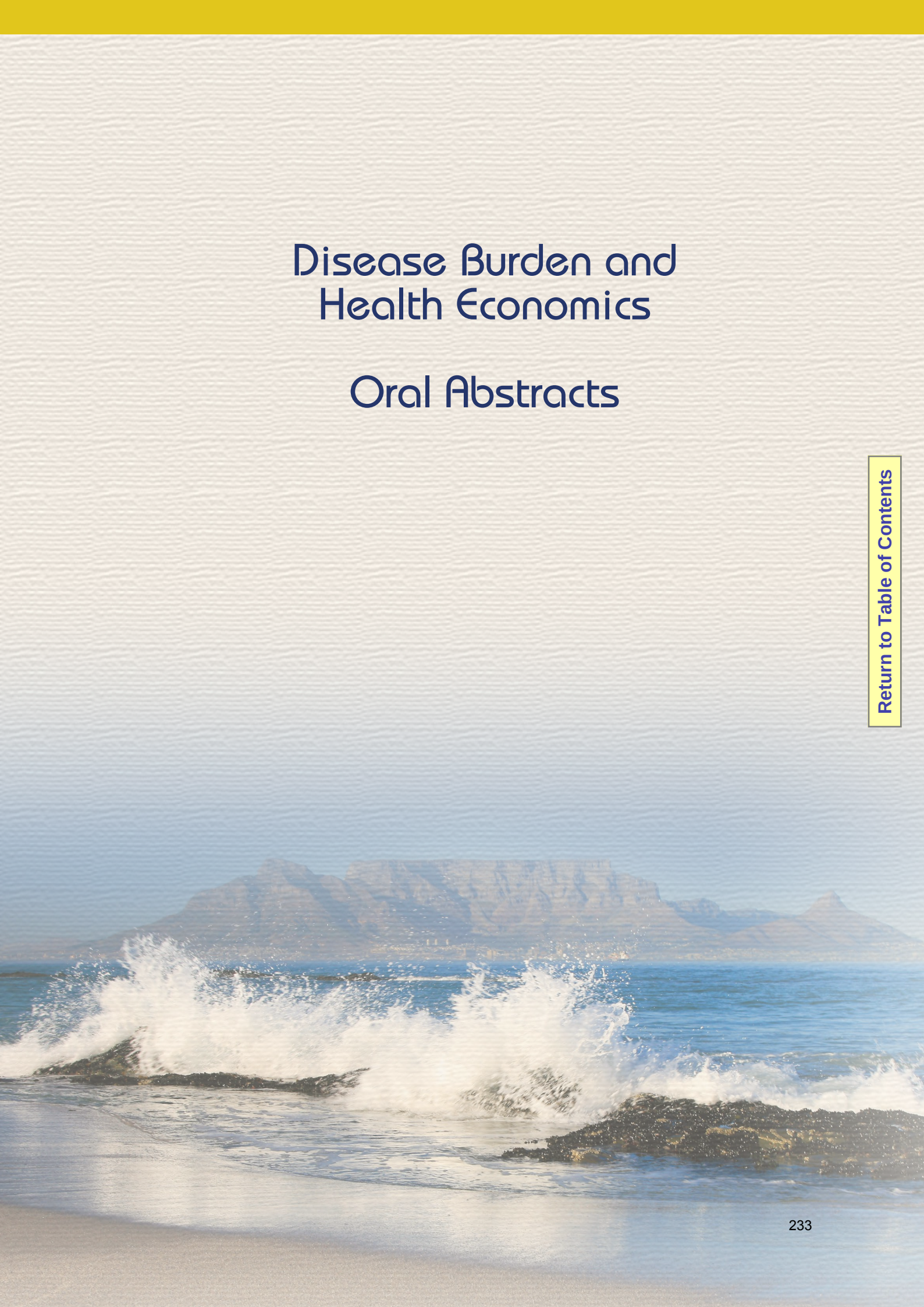

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Routine PCR testing of respiratory samples from coronial autopsies increases the recognition of influenza and other respiratory viruses as causes of death

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Background: Influenza and other respiratory viruses are known to be important causes of severe illness and death, but many of these go undiagnosed. In this study, we assessed the value of routine testing of postmortem samples from patients with undetermined or suspected infectious deaths in improving the detection of deaths due to respiratory viruses. Materials and Methods: Samples were collected from coronial autopsies between July 2007 and June 2011, where there was an undetermined or possibly infectious cause of death and tested by PCR and/or culture for influenza A, influenza B, adeno, respiratory syncytial, human metapneumo, parainfluenza 1,2&3, entero, and rhinoviruses. In 2007, influenza viruses were not routinely subtyped, and these are reported as unsubtyped influenza A. Results: Respiratory samples from 1611 cases were tested, at an average of 1.5 samples per case. One or more respiratory virus was detected in 8.3% of cases, the highest rate being 28% (42/151 cases) in children under 10 years of age. Influenza virus was the commonest respiratory virus (50 cases, 3%), followed by respiratory syncytial virus (RSV) (30 cases, 2%). Children had a wide range of respiratory viruses, while influenza A and RSV predominated in adults. RSV was found in 18 cases aged 60 years or over, compared with only 12 influenza cases. Influenza or RSV detection was associated with, bronchitis or bronchiolitis in 7 (9%) of the 80 cases and caused pneumonia in 14 (0.8%) deaths overall. Cases with A/H1N1 pdm, untyped influenza A or influenza B were younger than those with influenza A/H3N2 or prepandemic seasonal H1N1 (aged 44, 38, and 42 years old vs aged 60 and 61years old), and there was strong male predominance for A/H1N1 pdm and untyped influenza A cases (M:F = 24:7). Histological changes of diffuse alveolar damage were seen in 6/17 cases where influenza A/H1N1 pdm was detected and 5/13 RSV cases, while the other influenza virus cases were dominated by changes indicating secondary bacterial infection (15/18). When multiple samples were collected, influenza virus was detected in all samples in 85%. Only 10% of the influenza positive cases were diagnosed prior to death. Conclusions: There is a very significant underestimation of the contribution of influenza to deaths in the community. The routine PCR testing of a single lung sample from autopsy cases where death is unexplained or possibly infectious would substantially increase the detection rates.

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SCHOLARSHIP International Research Scholarship

The burden of influenza and RSV among inpatients and outpatients in rural western Kenya, 2009-2012

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Background: In Kenya, few data are available on the burden of influenza and RSV. Such data are needed to identify those most in need of limited vaccination and treatment resources. Materials and Methods: Since August, 2009 the Kenya Medical Research Institute and the Centers for Disease

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Control and prevention (KEMRI/CDC) has conducted hospital-based surveillance for severe acute respiratory illness (SARI) at Siaya District Hospital (SDH) and outpatient surveillance for influenza-like illness (ILI) at Tingwang'i Health Centre (THC) in western Kenya. Both facilities are located in Karemo division. Karemo, included in a Health Demographic Surveillance System (HDSS), has 7 outpatient facilities, 1 hospital, and a population of ≈ 80,000. SARI was defined as an acute onset of cough or difficulty breathing or pleural chest pain within the last 14 days requiring hospital admission. We defined ILI as axillary temperature ≥ 38°C and cough or sore throat in an outpatient. We collected a nasopharyngeal (NP) and oropharyngeal (OP) swab from SARI and ILI patients, and administered a questionnaire, including information on the patient's village of residence. NP and OP swabs were tested for influenza A and B and respiratory syncytial viruses (RSV) by rt–PCR. To estimate the burden of disease we restricted our analysis to patients admitted at SDH, or seen at THC, who were residents in the HDSS area of Karemo division. Incidence rates were estimated by dividing the number of cases with laboratory-confirmed virus infections by the mid-year population residing in Karemo. Rates were adjusted for those that met the SARI/ILI case definitions, but not tested. We reviewed medical records in all 7 outpatient facilities in Karemo division for a 2-year period and determined 10% of outpatient visits among Karemo residents occurred at THC. This was used to make further adjustments on the ILI rates. For influenza-associated SARI we compared rates for residents living within 5 and 10 kilometers of the hospital with those for Karemo division. This was intended to assess whether rates of hospitalized influenza and RSV declined with inclusion of persons who lived within Karemo division but farther from the hospital. Outcome data (discharged alive or died) was collected on hospitalized patients at SDH. Results: From August, 2009 to July 2012, we enrolled 5507 SARI patients at SDH, and 1632 ILI patients at THC. Most (SARI = 75%, ILI = 77%) were children < 5 years; median age was 1.6 years and 2.4 years for SARI and ILI patients, respectively. The average annual incidence per 1000 persons of influenza-associated SARI was 4.8(95% CI 3.0-7.6) among children < 2 years; 1.4(95% CI 0.7-2.8) in children aged 2-5 years; and 0.3 (95% CI 0.2-0.4) among persons ≥ 5 years. The incidence of influenza-associated medically attended ILI per 1000 was 32.6 (95% CI 19.2-55.4) among children < 2 years; 19.0 (95% CI 11.3-31.8) in children aged 2-5 years; and 3.8 (95% CI 2.6-5.7) among persons ≥ 5 years. Incidence rates of influenza-associated SARI were not significantly different among residents living within 5 or 10 KM of the hospital or in Karemo division. Rates per 1000 persons of SARI associated with RSV were 10.7(95% CI 7.9-14.6) among children < 2 years; 2.0 (95% CI 1.1-3.4) in children aged 2-5 years; and 0.1 (95% CI 0.0-0.2) among persons ≥ 5 years. Rates of ILI associated with RSV were 29.4 (95% CI 16.8-51.4) among children < 2 years; 21.6 (95% CI 13.3-35.1) in children aged 2-5 years; and 0.8 (95% CI 0.3-1.9) among persons ≥ 5 years. The case fatality proportions were 13/348 (4%) and 14/437 (3%) among SARI patients with laboratory confirmed influenza and RSV, respectively. Conclusions: Influenza and RSV produce a significant burden of disease in western Kenya. These estimated rates of the inpatient and outpatient burden of influenza in Kenya are higher than published direct estimates from the US. Our estimates of the incidence of hospitalized influenza-associated SARI are also consistent with other studies in western Kenya. Additional work will further estimate the burden of non-medically attended influenza and RSV in this context.

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Mortality associated with influenza and respiratory syncytial virus among children less than 5 years of age in a high HIV-prevalence setting—South Africa, 1998-2009

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Background: Influenza and respiratory syncytial virus (RSV) infections are common causes of pneumonia and are responsible for substantial global morbidity and mortality, in particular among individuals < 5 and ≥ 65 years of age. Available data suggest that the burden of influenza and RSV infection may be higher in developing countries and among HIV-infected individuals. Understanding the burden of influenza and RSV would assist policy makers to prioritize interventions. However, data on mortality due to influenza and RSV infection in low- and middle-income countries and particularly from high HIV-prevalence settings remains scanty. We applied modeling approaches to estimate influenza- and RSV-associated mortality among South African children < 5 years of age from 1998 through 2009. Materials and Methods: We estimated the influenza- and RSV-associated mortality among children < 5 years of age in 2 age categories (< 1 year and 1-4 years of age) using national vital statistics data coded according to the *International Classification of Diseases, Tenth Revision*. We modeled the excess mortality attributable to influenza and RSV infection by applying Poisson regression models to monthly all-respiratory and pneumonia and influenza (P&I) deaths, using national influenza and RSV laboratory surveillance data as covariates. Since the diagnosis of AIDS is rarely coded on the death certificate, we indirectly estimated the annual influenza- and RSV-associated deaths among HIV-infected and -uninfected children using Poisson regression models that incorporated year- and age-specific HIV prevalence and highly active antiretroviral treatment (HAART) coverage as covariates.Results: Over the study period, the influenza season peaked between May and August with 10 of the 12 years experiencing peak activity in June-July. RSV peak activity was observed between March and April in 8 of the 12 years with early or late peaks observed in February or May in the remaining years. The mean annual number of influenza-associated deaths in children < 5 years of age was 452 (8 per 100,000 person-years) and 398 (7 per 100,000 person-years) among all respiratory and P&I deaths, respectively. In the same age group, the mean annual RSV-associated mortality was 546 (10 per 100,000 person-years) for all-respiratory deaths and 482 (9 per 100,000 person-years) for P&I deaths. Among all-respiratory deaths, the mortality rate was higher among infants < 1 year of age for both influenza (22 per 100,000 person-years) and RSV (35 per 100,000 person year) as compared with children 1-4 years of age (5 per 100,000 person-years for influenza and 4 per 100,000 person-years for RSV). A similar trend was observed for P&I associated deaths. In children < 5 years of age the influenza-associated mortality rate among all respiratory deaths was greater in HIV-infected (83 per 100,000 person-years) as compared with HIV-uninfected (6 per 100,000 person-years) individuals (age-adjusted relative risk (aRR): 11.5; 95% CI: 9.6-12.6). In the same age group, the RSV-associated mortality rate was also higher in HIV-infected (138 per 100,000 person-years) as compared with HIV-uninfected (8 per 100,000 person-years) individuals (aRR: 8.1; 95% CI: 6.9-9.3). A similar trend was observed for P&I influenza- and RSV-associated deaths and across age groups. Overall among HIV-uninfected children, RSV was associated with 1.2 times as many all respiratory deaths as influenza; 1.6 and 0.8 among children < 1 year and 1-4 years of age, respectively. Similar trends were observed among P&I deaths related to RSV and influenza virus infections. Conclusions: The mortality burden was substantial for both influenza and RSV and the mortality rates were higher among HIV-infected as compared with HIV-uninfected children across age groups and underlying cause of death. While the mortality rates were greater among HIV-infected children, the burden remained considerable among HIV-uninfected individuals and was highest in infants as compared to older children. These findings support increased efforts for control of seasonal influenza in children in South Africa and other low- and middle-income countries. In countries with high HIV-prevalence such as South Africa, early antiretroviral therapy against HIV and prevention of mother-to-child transmission programs will remain an important intervention to reduce the mortality associated with influenza and RSV infection.

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Monitoring mortality for public health action in Europe, EuroMOMO: an important tool for influenza surveillance and impact assessment

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Background: Mortality data from vital statistics have been used for over 100 years to estimate influenza attributable mortality; however they are rarely available on a real time basis. EuroMOMO is a European network that operates timely monitoring of all-cause excess mortality using a standardized approach across participating countries in order to monitor and assess the impact of public health threats and to enable comparison between countries. The EuroMOMO system was applied to determine the pooled all-cause mortality in five influenza seasons from 2008/2009 to 2012/2013. Materials and methods: Since 2009, EuroMOMO has published weekly up-to-date observed, expected and excess mortality data from a maximum of 18 partners in 14 European countries. Weekly pooled outputs are available in the public domain (www.euromomo.eu). The EuroMOMO system uses Poisson regression to model weekly expected all-cause mortality, including a trend and a seasonal component. The model is fitted on spring and autumn periods of at least 5 years of historical mortality data. Standardized indicators (z-scores) enable comparison across countries and age groups. Winter excess is defined as the sum of the weekly positive and negative variations around the baseline of expected deaths from week 40 to week 20 the following year. Pooled analysis included data from 15 countries/regions representing an underlying population of 258 million. Results: Over the winter seasons 2008/2009 to 2011/12, the pooled average seasonal total number of excess deaths in participating countries was 33,957 (range, 20,181 to 53,803), representing an excess mortality rate of 13.2/100,000. During seasons 2009/2010 and 2010/2011, mortality was increased in 5-14 and 15-64 age groups, but low in the 65+ age group. In the 2012/2013 winter season, the estimated pooled total excess mortality was 40,254 (95% CI: 37,268-43,242), representing a mortality rate of 15.6/100,000 population (14.4-16.7): excess mortality in 2012/2013 mainly affected persons aged 65 and above, which had the highest excess since the 2008/2009 winter season. The magnitude of excess varied across countries in all 5 winter seasons. Conclusions: EuroMOMO has, over the past 5 years, demonstrated its usefulness for early impact assessment of public health treats, eg, in-season and timely end-of-influenza season mortality estimates. Analysis of age group specific mortality gave an early indication of the age shift in A(H1N1)09pdm related mortality in the pandemic and postpandemic era. Comparisons of excess mortality between countries revealed diverging country results that were valuable for risk assessment. The winter season 2012/13 was characterized by a marked excess mortality among older persons. However, apart from influenza, which showed a diversity of circulating viruses across Europe, other factors such as other respiratory viruses or cold weather may have contributed to the overall excess. Future plans include the expansion of the network and the addition of a standard methodology to estimate influenza attributable mortality across Europe.

O-850

Case fatality risk in the United Kingdom during the first two waves of the 2009 H1N1 pandemic – results from the Flu Watch Study

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Background: The case fatality risk (CFR) for influenza is an important measure of severity and is used in the planning and response to seasonal and pandemic influenza. Cases can be defined as infections (including those with and without clinical disease) or the subset of infections with laboratory-confirmed illnesses. The Influenza CFR is challenging to estimate given the difficulties in measuring accurate population-level denominators (number of infected or ill) and numerators (number of deaths attributable to influenza). Population level denominators should ideally come from representative prospective community level studies where either rates of serological infection are measured among unvaccinated individuals or else rates of laboratory confirmed influenza illness are measured regardless of medical consultation. Material and Methods: Denominator data was derived from the MRC/Wellcome funded Flu Watch study, a community level, household-based cohort study running from 2006-2011. The data in this analysis relate to active follow up over the summer and winter waves of the 2009 pandemic and the 2010/11 postpandemic season. Household members completed a baseline health and demographic survey as well as weekly surveys of illness. They also submitted nasal swabs on the second day of any respiratory illnesses for RT-PCR analysis of influenza. For our denominator, we estimated the number of symptomatic influenza cases in the UK during the pandemic in different age groups by applying rates of age and region standardised rates PCR confirmed influenza estimated from Flu Watch to national population estimates from the census. Using these denominators, we estimated risk of death if ill (CFR) using the number of reported deaths during the pandemic from a mandatory national influenza death reporting system created during the pandemic. Results: During the first 2 waves of the pandemic, the estimated risk of death in PCR confirmable influenza cases (CFR) was 5 (3-8) per 100,000 cases in those aged 0-4 years, 4 (3-5) in those aged 5-14 years, 19 (12-27) for those aged 15-64 years, and 271 (41-1898) for those aged 65 and above. Only 12% (7%-20%) of Flu Watch PCR-confirmed pandemic influenza cases had underlying chronic illness; however, the corresponding national percentage among those who died was 85%, suggesting underlying chronic illness greatly increases the risk of death. Conclusions: Using robust population-level denominators of influenza illness and nationally reported deaths due to influenza, we estimated the risk of death if ill with pandemic influenza was very low but increased dramatically with age. As expected, chronic illness also appears to increase risk of death. Competing biases include the fact that not all influenza illnesses will be PCR confirmed (which would tend to lead to an overestimate of CFR) and that not all influenza related deaths are identified (which would tend to lead to an underestimate of CFR). Even so, the case-fatality from the 2009 pandemic was still considerably lower than the best estimates from previous pandemics, including those from 1918 when the case fatality in young adults was particularly high.

O-851

Increased risk of swine influenza in pig industry workers compared to the general population in the UK

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Background: Pigs are mixing vessels for influenza, as they are susceptible to infection with swine, avian and human influenza viruses, and if simultaneously infected with two different viruses, there is an opportunity for viral assortment. H1N1, H1N2 and H3N2 subtypes have been circulating in pigs

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worldwide for years, and recent findings in the UK suggest H1N2 is the most commonly observed and H1N1 most commonly isolated. The 2009 pandemic virus (H1N1pdm09) comprised genetic components from swine-adapted viruses including the North American triple reassortant H3N2 viruses and a Eurasian virus, involving genomic segments originally derived from both avian and human influenza viruses. Thus there is a growing case for systematic monitoring of transmission between pigs and humans, but quantified data on the extent of such transmission remain very limited. This study looked at swine influenza virus (SIV) infection in pig industry workers in England to assess pig-human transmission. Serologic data on SIV infection in pig workers was compared with a comparison group from the general population. Materials and Methods: We recruited people with occupational exposure to pigs in two ways. Pig vets were recruited at the Pig Vet Society national meetings in winter 2009 and spring 2010. We also recruited farmers from swine farms who participated in a study of swine influenza viruses in English pigs in the autumn of 2010. These farms were mostly in North-Yorkshire and East-Anglia, regions with the highest density of pig farms. All pig industry workers provided blood samples for serologic analysis and completed questionnaires on baseline demographics and influenza vaccination history. Participants from the Flu Watch study, a community level, household-based cohort study of influenza in England, formed the population comparison group. Flu Watch participants were frequency match based on age, region, bleed month and gender (in decreasing priority order). They also provided blood samples and data on demographics and vaccination. All blood samples were batch tested for the presence of antibodies against a panel of H1N1, H1N2 and H3N2 strains of SIV, as well as human H1N1 and H3N2 strains, using haemagglutination inhibition (HI) tests. Antibody titres ≥ 40 were considered to be indicative of previous infection. We built separate logistic regression models for each virus strain to determine if occupational pig exposure was associated with infection. We also ran a model for all swine H3 viruses combined as well as all swine H1 viruses combined. Results: We tested 71 pig workers and 71 controls. Adjusted results show that pig industry workers had 20.4 times the odds of infection (95% CI 2.2-186.4) with the human pandemic strain H1N1pdm09 than the general population. They also had 4.3 times the odds of zoonotic infection (95% CI 1.4-13.5) with a swine H1N2virus (A/sw/Eng/438207/94). Both pig workers and controls commonly tested positive for swine H3N2 viruses, but there wasn't strong evidence in support of an increased risk of these viruses among pig industry workers. Conclusions: The H1N1pdm09 result is consistent with the findings that the virus became quickly established in UK pigs (presumably from a human source) and then transmitted between pigs and humans. Results from the H1N2 swine virus indicate cross-species transmission from pigs to humans. Our findings indicate that cross-species transmission of SIVs into humans is common in the UK. Robust, active surveillance systems are needed to help public health authorities understand the epidemiology of zoonotic SIV and monitor newly emerging strains in pigs. The findings suggest routine influenza immunization of pig workers should be considered to minimize the risk of interspecies transmission and viral reassortment.

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Disease Burden and Health Economics

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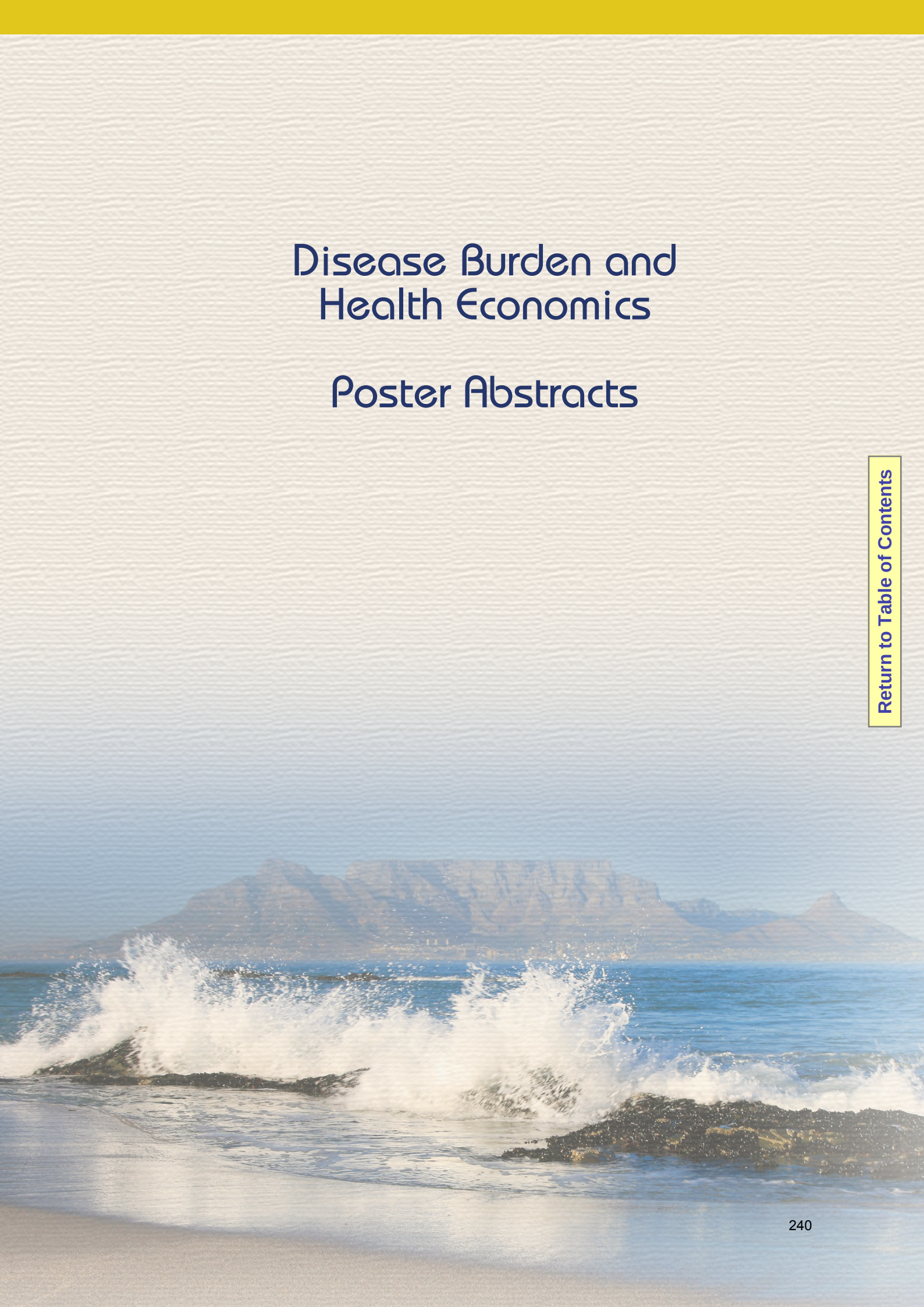

P1-293

Regional variation in mortality impact of the 2009 A(H1N1) influenza pandemic in China

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Background: Laboratory-confirmed deaths grossly underestimate influenza mortality burden, so that reliable burden estimates are derived from indirect statistical studies, which are scarce in low and middle income settings. Objectives: Here we used statistical excess mortality models to estimate the burden of seasonal and pandemic influenza in China. Methods: We modeled data from a nationally-representative population-based death registration system, combined with influenza virological surveillance data, to estimate influenza-associated excess mortality for the 2004-05 through 2009-10 seasons, by age and region. Results: The A(H1N1) pandemic was associated with 11.4-12.1 excess respiratory and circulatory (R&C) deaths per 100,000 population in rural sites of Northern and Southern China during 2009-2010; these rates were 2.2-2.8 times higher than those of urban sites (P < .01). Influenza B accounted for a larger proportion of deaths than pandemic A(H1N1) in 2009-2010 in some regions. Nationally, we attribute 126,200 (95% CI, 61,000-248,400) excess R&C deaths (rate of 9.4/100,000) and 2,323,000 YLL (1,166,000-4,533,000 YLL) to the first year of A(H1N1)pdm circulation. Conclusions: The A(H1N1) pandemic posed a mortality and YLL burden comparable to that of interpandemic influenza in China. Our high burden estimates in rural areas highlight the need to enhance epidemiological surveillance, and health-care services, in under-developed and remote areas.

P1-294

Cost-effectiveness of alternative strategies for annual influenza vaccination among children aged 6 months to 14 years in 4 provinces in China

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Background: We developed a model to evaluate the cost-effectiveness of alternative strategies to increase the influenza vaccination coverage rate among children aged 6 months to 14 years in 4 provinces in China. Materials and Methods: We built a spreadsheet-based model for cost-effectiveness analysis of the current seasonal influenza vaccination policy, as well as alternative strategies to increase influenza vaccination coverage rate, among children aged 6 months to 14 years in the 4 provinces (Shandong, Henan, Hunan, and Sichuan). We took a health care system perspective with government as the payer. We used data from 2005/6 to 2010/11, excluding 2009/10. Costs are reported in 2010 US dollars. Results: In comparison with no vaccination, the mean (range) of medically attended cases averted by the current pay-out-of-pocket policy for the 2 age groups (6 to 59 months and 60 months to 14 years) was 1465 (23~11,132) and 792 (36~4247), respectively, during the studied 5 influenza seasons. The total influenza vaccination program cost to government for the 2 age groups was $6983 and $17,918, and the cost-effectiveness ratios (CERs) were $0 ($-

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11-51) and $37 ($6-125) per case adverted, respectively. In comparison with the current policy, the incremental cost effectiveness ratio (ICER) of alternative strategies, OPTION One-telephone reminder and OPTION Two-comprehensive package, decreased as influenza vaccination rate increased. The ICER for children aged 6 to 59 months was lower than that for children aged 60 months to 14 years. Conclusions: The model we developed is a useful tool in identifying elements for evaluating alternative strategies to increase influenza vaccination coverage rate. However, more data are needed to produce more accurate cost-effectiveness estimates of potential vaccination policies.

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Incidence of influenza-associated mortality in Bangladesh, 2010-12

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Background: Influenza-associated morality estimates can help assess the burden of disease, identify high-risk groups and assist policy makers determine if an annual influenza immunization should be a prioritized public health intervention. The objective of this study was to estimate the incidence of influenza-associated mortality in Bangladesh during 2010-12 by combining national influenza surveillance data from 11 tertiary hospitals across the country and community surveys conducted in the catchment areas of these hospitals. Materials and Methods: During July to December 2012, we randomly selected 22 unions, the smallest administrative units in Bangladesh, from the catchment areas of 11 tertiary hospitals and collected mortality data in the unions. We employed a social networking approach to identify people who died in these unions in May, 2010 to April 2012. We interviewed the household members, who had taken care of the decedents during their illness and asked about decedent's demographics, medical history, symptoms, and health seeking 14 days before death. For children aged < 5 years, we classified a death as being associated with acute respiratory illness (ARI) if caregivers reported that decedents developed sudden onset fever and cough or difficulty breathing within 14 days of death. For people aged ≥ 5 years, we classified a death as being associated with ARI if caregivers reported that decedents developed sudden onset fever and cough or sore throat within 14 days of death. As part of the ongoing national hospital based surveillance in 11 hospitals, physicians routinely collected throat and nasopharyngeal swabs from case-patients. We only considered swabs from children < 5 years who presented with fever and cough or difficulty breathing with 1 danger sign; and from patients presented with fever and cough or sore throat if aged ≥ 5 years. We tested the samples for influenza and its subtypes. To calculate the incidence of influenza-associated mortality, we multiplied the monthly proportion of samples from the hospitals that were laboratory-confirmed for influenza in May 2010 to April 2012 by the number of decedents in the community with a history of sudden onset of ARI 14 days prior to death, and divided this numerator by the age-specific 2011census population of the unions. Results: We identified 10,043 deaths; 1191 (12%) were associated with ARI. Of those who died with ARI, 150 (13%) were children < 5 years, 30 (2%) were aged 5-19 years, 211 (18%) were aged 20-59 years and 800 (67%) were aged ≥ 60 years; 738 (62%) were males. Of the ARI-related deaths, 884 (67%) received treatment from certified physicians or hospitals and 399 (33%) were treated by uncertified providers or drug sellers. Among the deceased, 939 (79%) had comorbidities, including 614 (65%) from persons aged ≥ 60 years. We tested 2618 samples for influenza viruses from hospitalized patients who came from the catchment areas. A total of 360 (13%) patients were laboratory-confirmed for influenza; the proportions of samples that were confirmed for influenza among people aged ≥ 5 years were 19% and 4% among children < 5 years. The annual cumulative incidence of influenza-associated mortality was 11.1 (95% CI 10.4-11.7) per 100,000 population for all ages. The annual incidence of influenza-associated mortality among children < 5 years was 3.7 (95% CI 3.1-4.3); for people aged 5-19 years it was 1.3 (95% CI 0.8-1.8); for 20-59 years it was 5.3 (95% CI 4.6-6.1) and for persons aged ≥ 60 years the incidence was 108.5 (95% CI 101.1-116.0) per 100,000 persons. The annual mortality per 100,000 persons attributable to A (H1N1) pdm09 was 3.7 (95% CI 3.6-4.0); 4.1 (95% CI 3.9-4.4) for influenza A/H3 and 3.4 (95% CI 3.2-3.6) for influenza B. After extrapolating the incidence rate to the 2011 census population of Bangladesh, the estimated deaths associated with influenza were 15,989

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(95% CI 14,981-16,853) per year among all ages; it was 12,503 (95% CI 11,650-13,367) among ≥ 60 years annually. Conclusion: The burden of influenza mortality was highest among elderly persons aged ≥ 60 years. Vaccination for persons aged ≥60 years or for patients with comorbidities may reduce the burden of influenza.

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Economic burden of seasonal influenza b in France during winter 2010-2011

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Background: In France, 4%-10% of the population is annually affected by influenza. This represents direct medical expenses and indirect costs, which varies according to the dominant influenza virus strain circulating. Policy makers are interested in its burden, for better allocating resources for prevention and control measures. As international literature regarding seasonal influenza B is rare and not entirely applicable in France, our study aims to assess its costs in 2010/2011 under the French Health Insurance perspective. Materials and Methods: Cases—patients of all ages, consulting for an acute respiratory infection a physician member of an influenza surveillance network in France (GROG network), completing the routine clinical form and whose nasopharyngeal swab was lab confirmed positive for influenza B. Physicians completed follow-up questionnaires 7 and 28 days after swabbing. Costs (consult, drugs, absenteeism, exams, hospitalization) were assessed for each patient. Treatments costs were modeled using linear, tobit and probit regressions. Total costs estimation for the French Health Insurance were calculated by multiplying total costs per patient, flu attack rate, and population. Results: N = 201 patients were included. Influenza B mean cost was 90.62€ SD 132.76 per patient. Risk factors or influenza vaccine status did not impact the mean cost. In children and older people, these costs were very similar (0-4 years old = 76€, 5-14 years old = 75€, ≥ 65 years old = 72€) with greater follow-up consults and antibiotics use causing the largest impact. For adults, costs almost doubled, reaching approximately 141.25€ per patient, due to work absenteeism. Total influenza B costs for the National Health Insurance were estimated to be more than 500 million Euros in France during 2010/11. Conclusions: The results show that in a season where influenza B is dominant, it causes an important economic impact. Further investigations of strategies (vaccines) for reducing influenza B cases, providing evidences for policy-makers decisions are in progress.

P1-297

Excess mortality associated with influenza A(H1N1pdm09) in Hong Kong during two epidemic waves in 2009-2011

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Background: Most studies documenting the burden caused by multiple epidemic waves following the 2009 influenza A(H1N1pdm) pandemic were based on laboratory-confirmed cases that could underestimate the true impact of influenza. Hong Kong experienced multiple waves of influenza A(H1N1pdm) since 2009. We examined the patterns in excess mortality associated with pH1N1

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during the first epidemic wave in 2009-10 and the second wave in 2011. Materials and Methods: Age-specific all-cause and cause-specific mortality rates in Hong Kong from 1998 through 2011 were fitted in multiple linear regression models. The models allowed for activities of different types/subtypes of influenza virus and cocirculating respiratory syncytial virus, and adjusted for environmental temperature and absolute humidity, and temporal trends in mortality rates. The influenza-associated excess mortality during the epidemics of interest was measured as the differences between estimated mortality rates in the presence or absence of influenza activity for influenza A(H1N1pdm). Results: The influenza A(H1N1pdm) associated excess mortality risk in the first wave of the epidemics in 2009-2011 was 1.94 (95% CI: -8.21, 11.63) and 1.23 (95% CI: -3.19, 5.37) per 100,000 population from all-cause and respiratory diseases, respectively. During the second epidemic wave in early 2011, the excess mortality risk of A(H1N1pdm) was estimated to be 4.60 (95% CI: -0.97, 10.39) and 4.00 (95% CI: 1.64, 6.40) per 100,000 population for all-cause and respiratory deaths, respectively. The seasonal influenza A(H3N2) virus that cocirculated with A(H1N1pdm) in the summer of 2009 was associated with an excess all-cause mortality risk of 3.53 (95% CI: 0.24, 6.76) per 100,000 population. In 2010, the same virus was associated with an excess all-cause mortality risk of 6.23 per 100,000 population. Conclusions: Substantial mortality burden associated with influenza A(H1N1pdm) was observed during the first wave of the pandemic in 2009-10 and the second wave in 2011 in Hong Kong, although the reasons for substantial impact in the second wave remain to be clarified. More than half of the estimated excess all-cause deaths were attributable to respiratory causes in each epidemic. The excess mortality burden associated with influenza A(H1N1pdm) was comparable to that caused by the cocirculating seasonal influenza A(H3N2) virus.

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Sex-specific influenza-associated excess mortality in Hong Kong, 1998-2009

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Background: Influenza-associated excess deaths estimated from statistical models have been used to measure the burden of mortality attributable to influenza virus. Given the discrepancies that have often been observed in the prevalence and burden of major diseases between males and females, we examined the patterns in influenza-associated excess mortality among men and women in Hong Kong in the past decade. Materials and Methods: Multiple linear regression models were fitted to age-specific all-cause and cause-specific mortality rates in Hong Kong from 1998 through 2009 for males and females separately. The models allowed for activities of different types/subtypes of influenza virus and cocirculating respiratory syncytial virus were adjusted as the product of influenza-like illness rates from sentinel surveillance and laboratory detection rates of the specific viruses (Wu P, et al. *J Infect Dis.* 2012). Environmental temperature and absolute humidity, and periodic temporal trends in mortality rates were also adjusted for in the models. The influenza-associated excess mortality was measured as the differences between estimated mortality rates in the presence or absence of influenza activity. Attributable fractions were estimated for each cause. Results: The annual influenza-associated all-cause excess mortality rates were 9.6 and 12.6 per 100,000 person-years for men and women from 1998 through 2009. The attributable fractions of all-cause deaths were 2.0% and 2.1% in men and women respectively. More than 90% of the influenza-associated all-cause excess deaths were estimated to occur in the elderly both for men and women. Cardio-respiratory diseases accounted for more influenza-associated excess deaths in men (80%) than in women (70%). Conclusions: Influenza virus infections were associated with a higher number of excess deaths among men than women each year in Hong Kong, but the attributable fractions were very similar for men and women. Most of the influenza-associated excess deaths occurred in the elderly for both sexes. Cardio-respiratory diseases accounted for the majority of the influenza associated excess deaths in both sexes, in particular among men.

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Epidemiology and economic burden of Influenza among children < 5 years of age hospitalized in Suzhou, China

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Background: The disease burden and economic impact of influenza-associated hospitalization in children has been rarely reported in mainland China to date. We performed a prospective surveillance to describe the epidemiology, clinical characteristics, and economic burden of laboratory-proven influenza hospitalized children in Suzhou, China. Methods: A prospective study of laboratory-confirmed influenza among children hospitalized in Suzhou Children Hospital (SCH) with severe acute respiratory illness (SARI) was performed from March 2011 through March 2013. Nasopharyngeal aspirates were collected for detecting influenza viruses by real-time reverse transcriptase-polymerase chain reaction. Data on demographics, clinical characteristics, therapy, direct medical and other relative costs, day care absence, parental work loss, and similar respiratory symptoms occurred in other members of the family were collected by chart review, face to face interview, telephone survey, and billing data requirement. Results: In total, 1950 SARI cases were enrolled and 1398 (71.7%) of them had been collected nasopharyngeal aspirate specimens for virus detection. There was no significant difference on demographics and clinical characteristics between the sampled and nonsampled SARI cases. Overall, 13.2% of SARI cases were infected with influenza viruses (55.4% were influenza A and 44.6% were influenza B). The influenza positive rate increased with age. Similar respiratory symptoms were more likely developed in the family contacts of influenza positive children than others (34.9% vs 28.4%). A total of 61.5% SARI cases had over-the-counter medicine before physician visit and 51.4% had visited other hospitals before admission to SCH. In all influenza infected cases, pneumonia (85.1%) was the most common complication, followed by sinusitis (13.2%), diarrhea (10.3%), and asthma (2.9%). In terms of therapy, 37.6% of SARI cases required oxygen treatment, and the influenza positive children tended to require oxygen treatment (48.0% vs 36.0%, P < .05). The median length of hospital stay was 7 days (IQR: 6-8). The median of direct medical cost for SARI hospitalizations was US$706.2 (IQR: 553.1-893.8), there was no significant difference between influenza positive children (US$760.2, IQR: 598.7-929.5) and influenza negative children (US$727.4, IQR: 595.5-908.5). However, the direct medical costs of influenza A hospitalizations (US$691.8, IQR: 542.0-886.7) was lower than that of type B cases (US$839.3, IQR: 659.8-1006.3) (P < .05).The relative nonmedical costs for each SARI case was US$55.6 (IQR: 25.4-111.1) (influenza positive vs negative: US$63.5 vs US$55.6). Daycare absence in influenza-positive children aged > 3 years was 7 days (IQR: 3-15), and 6 days (IQR: 5-14) in influenza negative children. In addition, the parents' work loss in both influenza positive cases and negative cases was 7 days (IQR: 5-8), resulting in a median lost income of US$158.7 (IQR: 88.9-238.1). Overall, the median total costs (including direct and indirect costs) was US$1090.4 (IQR: 863.3-1225.6) for influenza hospitalizations, and US$1010.5 (IQR: 826.6-1267.1) for hospitalizations without influenza. Conclusion: Influenza was an important cause to hospitalization in Suzhou children. The relatively high cost of hospitalization in children led to a heavy economic burden to local families. These results indicate the use of influenza vaccine in children > 6 months of age especially in those with ACIP high-risk condition may have the potential effectiveness to reduce the health and economic impact of influenza.

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Direct economic burden of medically attended influenza in western Kenya from the parental perspective, 2009-2011

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Background: The economic costs of influenza in tropical Africa remain under explored. Calculating the economic burden of influenza is crucial to estimating the costs and benefits of vaccine implementation. We estimated the direct costs of medically-attended influenza disease in Kenya from the parental perspective. Materials and Methods: We analyzed medical records for 7388 inpatients and outpatients at Lwak Mission Hospital (LMH) to identify persons with severe acute respiratory illness (SARI) or influenza-like illness (ILI) during January 1, 2009-December 31, 2011. LMH is located in a population-based infectious disease surveillance site in western Kenya. We examined medical chart data for prior outpatient consultations and medications taken for the current illness, routine diagnostic tests, medications administered to patients during the current visit, and length of stay (for hospitalized patients). We determined costs for consultation, routine diagnostic tests, medications, and hospital admission from the medical facility catalogue of prices. Cost estimates for consultations prior to any hospital visit were taken from published literature from western Kenya. Influenza diagnostics are not routine and were not included in direct cost estimates. We estimated the total cost of treatment per visit by adding the cost of consultation, over-the-counter drugs, routine diagnostics, medication, and admission (where applicable). We then estimated the mean direct cost of an episode of influenza-associated illness for outpatients and inpatients aged < 5 and ≥ 5 years old. We extrapolated the annual cost of influenza to Nyanza Province using published rates of hospitalized SARI, and nonhospitalized SARI in western Kenya. We also estimated rates of influenza-associated medically attended ILI in western Kenya using unpublished data. The population of Nyanza Province from the 2009 census (5.4 million) was multiplied by national age-specific population proportions to estimate the population of Nyanza Province that was under and over age 5. The direct costs were also calculated as a percentage of average monthly income in Kenya as a measure of impact on the family (Kenya National Bureau of Standards, 2012). Results: Out of 7388 patients, 4295 had SARI and/or ILI and were swabbed for influenza; 1639 had nonhospitalized SARI, 576 were hospitalized for SARI and 2080 were medically attended ILI patients. Of these, 419 (26%), 51 (9%) and 201 (10%) tested positive for influenza, respectively. The mean (SD) patient cost in Kenyan shillings (KSH) of influenza-associated outpatient ILI, outpatient SARI, and hospitalized SARI in children under age 5 was 1254 (1129.1-1378.5), 937 (307.6-1566.9), and 2609 (2152.2-3064.8), respectively. Extrapolations of existing rates of influenza-associated outpatient ILI, outpatient SARI, and hospitalized SARI from population-based surveillance sites in western Kenya to Nyanza Province suggested an estimated 33,424-70,115 influenza-associated outpatient ILI, 28,512-48,142 influenza-associated outpatient SARI, and 1,843-3,937 influenza-associated hospitalized SARI cases among children under age 5 in Nyanza Province annually. The total annual direct economic burden of influenza in children < 5 in Nyanza Province was estimated to be from 68.6 to133.0 million KSH (US$847,000-$1,642,000) for outpatients (ILI and SARI); and 4.8 to10.3 million KSH (US$59,000-$127,000) for inpatients. The mean (SD) patient cost in KSH of influenza-associated outpatient ILI, outpatient SARI, and hospitalized SARI in persons aged 5 and older was 1155 (45.4-2265.5), 1997 (1937.9-2055.5), and 3036 (2681.3-3389.9), respectively. Among persons aged 5 years and older, we estimated there to be 26,709-59,405 influenza-associated outpatient ILI, 106,674-133,617 influenza-associated outpatient SARI, and 921-2,303 influenza-associated hospitalized SARI cases in Nyanza Province annually. The total annual direct economic burden of influenza in persons aged 5 and older in Nyanza Province was estimated to be from 243.8 to 335.4 million KSH (US$3,010,000-$4,141,000) for outpatients (ILI and SARI); and 2.8 to 7.0 million KSH (US$35,000- $86,000) for inpatients. The mean direct costs per outpatient visit and hospitalization for influenza of 1400 and 3000 KSH are approximately 5% and 11% of average monthly income (Kshs. 28,000), respectively. Conclusion: The direct costs of inpatient and outpatient care for influenza-associated illness are substantial. Further work should estimate the indirect costs of influenza, costs from the governmental perspective, and then the cost-effectiveness of influenza vaccination.

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Expected public health impact of high-dose trivalent influenza vaccine compared with quadrivalent influenza vaccines in US elderly

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Background: Numerous seasonal inactivated influenza vaccines are available for use in persons aged 65 years and older. With the availability of quadrivalent influenza vaccines (QIVs), trivalent influenza vaccines (TIVs) and a high-dose trivalent vaccine (TIV HD), healthcare providers must decide which vaccine to administer. Recently, Reed and colleagues developed an influenza epidemiological model and forecasted significant expected public health benefits of QIV over standard-dose TIV in the United States. The objective of our study was to assess the relative public health benefit of using TIV HD versus QIV for routine influenza vaccination of US elderly adults. Materials and Methods: We developed an epidemiological model to explore the comparative effectiveness of these 2 vaccines in reducing influenza-related health outcomes in US elderly patients. The model assumed strain-specific incidence rates based on US epidemiology from 1999/2000 through 2008/2009 seasons. We used the model developed by Coudeville and colleagues to estimate the expected strain-specific efficacy of TIV HD. Other epidemiological parameters were consistent with the Reed model, but we enhanced it by incorporating data on cross protective immunity of TIV HD against mismatched influenza B-lineage strains. Results**:** The probability of influenza infection in individuals vaccinated with QIV and TIV HD was estimated at 3.31% and 2.69%, respectively. This represented a reduction of 620 cases per 100,000 individuals vaccinated with TIV HD instead of QIV. TIV HD can be expected to prevent an additional 26 influenza-related (IR) hospitalizations and 9 IR deaths per 100,000 vaccinated persons compared with QIV. At a population level (37,997,762 people aged 65 or older), assuming 67% coverage rate of each vaccine, the model estimates TIV HD could lead to a reduction of 157,963 influenza cases, 6,593 IR hospitalizations, 2,301 IR deaths, and 42,431 IR workdays lost per season compared with QIV. **Conclusions:** Although some limitations exist, our model suggests a significant expected public health benefit from using TIV HD over QIV in US elderly adults. The validity of these findings will be revisited once the results of an ongoing relative efficacy study of TIV HD and standard dose TIV are available. Funding: Funding for this study was provided by Sanofi Pasteur.

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Incidence of medically attended influenza-associated respiratory illness in children aged < 5 years in Bangladesh, 2011-2012

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Background: Influenza is a common viral etiology of acute respiratory illness-associated morbidity and mortality in children. Most influenza infections are self limiting; however, some children will require medical attention, including hospitalization. Data on incidence of medically attended influenza-associated illness in different risk groups can help policy makers assess the need for influenza specific interventions such as maternal immunization or routine influenza vaccination for children in Bangladesh. We combined influenza surveillance and health utilization survey data to estimate the incidence of medically attended influenza-associated respiratory illness among children aged < 5 years in Bangladesh. Methodology: During January 2011-December 2012, we conducted surveillance in 4 tertiary level hospitals located in 4 diverse locations of the country. One day every 2 weeks in the out-patient department (OPD) and 1 day every week in the in-patient department (IPD), surveillance

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physicians collected nasal and throat swabs from all children aged < 5 years, who presented with any 2 symptoms, including fever, cough, or difficulty breathing within 7 days of symptom onset and resided in the primary catchment areas of the hospitals. Respiratory specimens were tested for influenza viruses using real-time reverse transcription polymerase chain reaction. Investigators identified the primary catchment areas of the hospitals by reviewing the hospital log books and identifying the areas where majority of the patients resided. During 2008-2009, we conducted a health utilization survey in the primary catchment areas of the 4 hospitals to determine the proportion of all episodes of respiratory infection among children aged < 5 years meeting the case definition (any 2 of fever, cough , or difficulty breathing) in the preceding 12 months that received outpatient or inpatient care at the surveillance hospitals. We estimated the incidence of medically attended and hospitalized influenza-associated illness by dividing the estimated number of children who were laboratory confirmed for influenza in the OPD and IPD by the population aged < 5 years of the catchment areas and adjusted for the proportion of children who sought care at the surveillance hospitals. Results: In 2011, 569 children were enrolled from the OPD and 209 from the IPD. In 2012, 709 children were enrolled from the OPD and 153 from the IPD. In 2011, 9% (50/569) of respiratory specimens from children attending OPD and 3% (6/209) from children admitted in the IPD tested positive for influenza. Similarly in 2012, 6% (43/709) of respiratory specimens from children attending OPD and 2% (3/153) from children admitted in the IPD tested positive for influenza. Of the 93 specimens that were positive for influenza infection from OPD in both years, 48% were laboratory confirmed for influenza B, 27% for influenza A/H3 and 25% for A(H1N1)pdm2009. Among the 9 respiratory specimens that were laboratory confirmed for influenza infection from IPD, 7 (78%) were influenza B; 1 was influenza A/H3 and the other was for influenza A(H1N1)pdm2009. The median age of influenza positive children attending OPD was 22 months (IQR 13-36 months) and that of influenza positive children admitted in the IPD was 12 months (IQR 6-22 months). From the health utilization survey, we identified 744 children who required ambulatory care for respiratory symptoms and 18 (2.4%) of them sought care at the OPD of the surveillance hospitals. Similarly 60 children required hospitalization due to respiratory symptoms in the preceding year and 21 (35%) of them were admitted in the IPD of the surveillance hospitals. The estimated incidence of ambulatory influenza illness per 100 children per year was 13.3 (95% CI 10-20) in 2011 and 11.3 (95% CI 9-19) in 2012. Similarly, incidence of hospitalized influenza illness per 1000 children per year was 0.5 (95% CI 0.09-1.2) in 2011 and 0.3 (95% CI 2-4.3) in 2012. Conclusion: Data from our surveillance suggest that influenza is associated with a high incidence of illness requiring medical attention in very young children. Though influenza vaccine is not routinely recommended in the country, children in Bangladesh may benefit from influenza-specific interventions.

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Incidence of influenza like illness among children under 5 years of age in Suzhou community, China: multiple cross-sectional telephone surveys

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Background: Influenza surveillance systems could not identify the true burden of illness caused by influenza in the community because they are restricted to consulting cases. A telephone survey was conducted to estimate the incidence and the burden of self-defined influenza like illness (ILI), and to describe healthcare seeking behavior in Suzhou children population. Methods: From January 2012 to April 2013, we conducted 4 (January 2012, October 2012, January 2013 and April 2013) random, population-based cross-sectional telephone surveys on children under 5 years of age. Each survey would not last longer than 1 month. The sampling frame was Suzhou immunization database stratified by region and age group. The sampling proportion was basing on the population size. Each survey would not last longer than 1 month. Results: In total, there were 14711 children had been sampled to

telephone survey, 7975 (54.2%) of them can be reached by phone at most 3 calls, with 7258 (91.0%) who agreed and finished the survey and 717 (9.0%) rejected. Of the 7258 children who finally finished the survey, 3820 (52.6%) were boys and 3438 (47.4%) were girls; and 24.7% were under 1 year old, 23.8% of them were 1 year old, 18.9% were 2 years old, 16.5% were 3 years old and 16.1% were 4 years old. The sample was representative in terms of gender, region, age, and population size. Overall, there were 3096 children reported with onset of fever, cough, sore throat, vomit, and/or diarrhea in the past month, and 1395 (45.1%) of them had visits to hospitals. In detail, there were 1699 (23.4%) children had onset of runny rose, 1665 (22.9%) children had onset of cough, 660 (9.1%) children had fever, 290 (4.0%) had diarrhea, 211(3.9%) with sore throat, and 145 (2.0%) had vomiting. The consultation rates were different when onset of different symptoms (fever 78.2% > sore throat 75.2% > vomiting 64.0% > diarrhea 63.7% > cough 55.5% > runny nose 36.3%). Of those who felt sick but didn't visit hospitals, most of them (56.3%) were treated by themselves, and 41.3% of them stayed at home to recover. The child reported with onset of fever and cough or sore throat in the past month, we would count as one ILI case. The incidence rate of self-reported ILI in children under 5 years old was 5.6 per 100 children (95%CI: 5.0-6.1). The highest ILI incidence was 7.0 per 100 children (95%CI: 5.9-8.2) in January 2012, the lowest ILI incidence was 3.5 per 100 children (95%CI: 2.6-4.4) in April 2013. There was no difference between boys and girls on ILI incidence. Of the 404 self-reported ILI cases, 327 (80.9%) children sought health care in hospitals. The consultation rates of ILI varied from 73.7% to 85.0% by each survey, and the mean consultation rate was 80.9% (95%CI: 77.1%-84.8%). The children who had ILI preferred to go to the local hospitals (26.0%), the tertiary children hospital (22.8%), and community health service centers (13.6%) to seek health care. Conclusion: The self-defined ILI causes a significant burden of illness in the children in Suzhou community and it is a frequent cause for consultation. These results are complementary to the influenza surveillance data in interpretation of the influenza disease burden.

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The burden of influenza-associated respiratory disease in South Africa: a preliminary estimate

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Background: South Africa, like many countries, has ongoing surveillance for respiratory disease in a select number of sentinel sites. However, apart from national estimates of excess mortality associated with influenza, there is limited information on the complete disease burden of influenza in South Africa. The objective of this study was to create the first, preliminary estimate of the annual number of cases of influenza-associated respiratory disease in South Africa. Materials and Methods: Based on data from a sentinel severe acute respiratory illness (SARI) surveillance programme conducted in 6 hospitals in 4 provinces in South Africa, we used a multiplier model to estimate the annual national number of influenza-like illness cases, respiratory hospitalizations, and in-hospital deaths associated with influenza in South Africa from 2009-2011. We measured incidence rates of hospitalizations at 1 large hospital in Soweto with population denominators. To estimate the number of deaths, we applied the in-hospital influenza-associated SARI case-fatality rate from all 6 hospitals in the surveillance programme to the influenza-associated SARI incidence from the 1 hospital. We accounted for deaths of SARI patients who were not enrolled in the surveillance system using data from two hospitals that have monitored the deaths of all patients hospitalized with respiratory illness at the hospital, whether enrolled in SARI surveillance or not. To estimate the number of influenza-like illness cases, we used the published literature to determine the ratio of cases to hospitalizations. To estimate the national

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number of cases, hospitalizations, and deaths, we applied the age-specific incidence rates by age and HIV infection status from the one hospital with population denominators to the total population of South Africa using census data. Results: From 2009-2011, an annual mean of 2,986 (range 2890-3155) SARI cases were enrolled, and a mean of 254 (8.5%) were laboratory-confirmed influenza. There were 24 deaths among the 790 influenza-associated SARI hospitalizations (case-fatality ratio 3.0%). The mean crude annual incidence rate of influenza-associated SARI hospitalizations was highest in HIV-infected children < 5 years of age (452/100,000), followed by HIV-infected adults 20-64 years of age (216/100,000) and HIV-uninfected children < 5 years of age (214/100,000). Over the 3 years, the estimated mean incidence rate per 100,000 population was 13,149 (range 11,203-16,885) cases of influenza-like illness, 63 (range 55-77) hospitalizations, and 3 (range 3-3) deaths. Extrapolating data from this hospital to national census data, we estimate that there were approximately 6.6 million (range 5-6-8.4) influenza-associated cases of influenza-like illness, 31,000 (range 27,740-38,491) hospitalizations for influenza-associated pneumonia, and 1500 (range 1353-1674) in-hospital deaths from influenza-associated pneumonia annually in South Africa. The highest number of annual hospitalizations and deaths were among children < 5 years of age (mean 11,992 hospitalizations and 158 deaths annually) and HIV-infected adults 25-64 years of age (mean 10,975 hospitalizations and 938 deaths annually). The disease burden was highest in 2009, the year of the influenza A(H1N1)pdm09 pandemic. Overall, an estimated 16.9%, 11.3%, and 11.4% of the general population had influenza-associated respiratory disease in 2009, 2010, and 2011, respectively. The years of potential life lost to age 60 years were 67,276 in 2009, 54,822 in 2010, and 47,963 in 2011. Conclusions: These estimates suggest that influenza causes a substantial burden of respiratory disease in South Africa. The highest burden in Soweto and extrapolated nationally is among children < 5 years of age and HIV-infected adults. Influenza-associated deaths occur primarily among HIV-infected adults. These disease burden estimates are likely an underestimate because patients with pneumonia in South Africa may not be hospitalized, SARI surveillance only monitors respiratory infection, and our mortality estimates are based on a small number of deaths identified through hospital-based surveillance. Extrapolating national estimates from one surveillance site may lead to bias if the site is not representative of the country as a whole. To reduce the burden of influenza in countries like South Africa, young children and adults with HIV infection should be targeted for interventions such as influenza vaccination.

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Two levels of the influenza severity pyramid caught with one system

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Background: Most concepts of risk assessment concerning influenza (seasonal and pandemic) include at least 2 factors, transmission and severity. Usually a pyramid with different levels of severity is used showing symptomatic cases that do not need medical consultation at the base followed by outpatient influenza-like illness (ILI) or acute respiratory illness (ARI) surveillance, the hospital level, intensive care unit level (ICU) and fatal outcomes at the peak. Sentinel systems in primary care (outpatient surveillance) are established in European countries for about 20 years and provide valuable information about the time course and burden in the population. However, establishing respective systems at the hospital level is an unsolved challenge in many countries. Here we present an approach that uses data from primary care reporting data of the next higher level of the pyramid. Materials and Methods: About 550 sentinel primary care physicians of the Working Group Influenza (Arbeitsgemeinschaft Influenza) provide weekly data on ARI patients in different age groups, but also the weekly number of ARI patients that need hospitalisation due to their illness. With a negative binomial logistic regression model we estimated the excess of medically attended ARI (MAARI) due to influenza in five age groups and multiplied these estimated weekly influenza consultations with the age-groupspecific proportion of reported hospitalised ARI-Patients during the influenza season. Results: Between seasons 2001/02 and 2012/13 influenza consultations in Germany ranged from 1 to over 7 million per season (1300-9500 per 100,000 population), with considerable differences between age groups. Estimated influenza hospitalisations ranged from 4000 to over 37,000 per season (5-45 per 100,000 population). For the 2012/13 season, 7.8 million outpatients and 30,000 inpatients were

estimated for influenza. Case-hospitalisation rate was 0.4% and had an age dependent inverted L-shaped curve (preliminary data). In comparison, the mandatory notification system (where only laboratory confirmed cases are notified) reported 71,200 influenza cases of whom 11,400 were hospitalised (case-hospitalisation rate 15%). Conclusions: We used an indirect approach to get information about the number of hospitalized influenza patients and the case/hospitalisation ratio through the primary care physician sentinel. Although there is a risk of underestimating the number of influenza hospitalisations and only aggregated data in five age groups are available, valuable, age-specific information about the severity of the influenza season was obtained in a timely manner. In combination with a system that focuses on the ICU this hospital "surveillance gap" could be closed.

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Acute respiratory infections and influenza-like illnesses: how many does a person have in a year?

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Background: Since 2011, the Robert Koch-Institute has been running the internet-based syndromic monitoring system GrippeWeb to measure acute respiratory infections (ARI) and influenza like illnesses (ILI) directly within the population. Usually, the incidence of ARI or ILI is determined by calendar week in a given population; however, the number of ARI or ILI per year in a given individual has been measured rarely, but are important for example for person-based socio-economic, and burden of disease calculations. Materials and Methods: Any person aged 14 years or older may register with GrippeWeb, parents can report for their children below the age of 14 years. Participants receive weekly reminders to report if they had a new onset respiratory illness in the previous weeks. We defined ARI as an acute respiratory infection with new onset and 1 of the symptoms fever, cough, or sore throat. ILI is defined as ARI with fever and cough or sore throat. We excluded the first 4 reports from each participant, ignored ARI or ILI if they occurred immediately after a week when an ARI or ILI was reported, and restricted analysis to those who reported at least 90% of the 52 weeks between weeks 35/2011-34/2012. Results: 62% of GrippeWeb participants reported at least 90% of all weeks. Data from 780 participants could be analysed. The median number of annual ARI was 5.5; 3; 3; 2 and 1 among participates aged 0-4, 5-14, 15-34, 35-59, and 60+ years. The median number of annual ILI was 2; 1; 0; 0; and 0 in the same age groups. Nine (1%) participants had between 10 and 12 ARI per year and 4 (44%) of these were older than 14 years. During the analysed period 27% and 16% of all participants included in this analysis attended health care at least once for an ARI or ILI, respectively. Conclusions: The presented results show that adherence of GrippeWeb participants to regular reporting is astonishingly good allowing cohort analyses, such as those presented. Numbers of ARI per year are strongly age-dependent. Frequent ARI among 0-4 year-old children, including possible sequelae, such as otitis media, are likely to have substantial socio-economic impact. Results may be used for burden of disease calculations.

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P1-308

Towards more specific and transparent research and development costs: the case of seasonal influenza vaccines

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Background: The ability to calculate the development costs for specific medicines and vaccines is important to inform investments in innovation. Unfortunately, the literature is predominated by nonreproducible studies only measuring aggregate level drug research and development (R&D) costs. We describe methodology that improves the transparency and reproducibility of primary indication expected R&D expenditures. Methods: We used publically accessible clinical trial data to investigate the fate of all seasonal influenza vaccine candidates that entered clinical development post year 2000. We calculated development times and probabilities of success for these candidates through the various phases of clinical development. Clinical trial cost data obtained from university based clinical researchers were used to estimate the costs of each phase of development. The cost of preclinical development was estimated using published literature. Results: A vaccine candidate entering preclinical development in 2011 would be expected to achieve licensure in 2022; all costs are reported in 2022 Canadian dollars (CAD). After applying a 9% cost of capital, the capitalized total pretax R&D expenditure amounts to $474.88 million CAD. Conclusion: Clinical development costs for vaccines and drugs can be estimated with increased specificity and transparency using public sources of data. The robustness of these estimates will only increase over time due to public disclosure incentives first introduced in the late 1990s. However, preclinical development costs remain difficult to estimate from public data. Competing Interest and Funding: Ayman Chit is a current employee of Sanofi pasteur (SP) and a former employee of GlaxoSmithKline (GSK). Jayson Parker has worked in the pharmaceutical industry and advises a hedge fund which invests in life sciences. Scott Halperin has received research contracts and grants and has served on ad hoc advisory boards for vaccine manufacturers. Manny Papadimitropoulos is an employee of Eli Lilly & Company. Paul Grootendorst has provided expert testimony and/or reports on behalf of both generic and branded drug companies and has received financial and in-kind research support from pharmaceutical companies. Data base access to *Trialtrove* was provided through the GSK subscription. Other than Ayman Chit's direct contribution, GSK and SP had no role in the study design, interpretation of results, or decision to publish. All opinions expressed are solely those of the authors.

P1-309

Will QIV be more cost-effective than TIV in preventing influenza in Taiwan?

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Background: An influenza pandemic occurrence could lead to heavy economic burden and social disorder, and heavily strain the existing public health services. In Taiwan, the prevalence rate of influenza increased from 4.9 in 2009 to 6.4 per 100,000 in 2011 with the attack rate being 10%-20% among the general population and 40%-50% in densely populated areas such as schools, nursing homes, and hospitals. The mortality rate caused by influenza was 9.0% in 2011/2012. Vaccination is recommended by the WHO as the most effective way to prevent infection and severe outcomes caused by influenza viruses. Among healthy adults, influenza vaccine can prevent approximately 70% to 90% of influenza illness. Since 1998, Taiwan has initiated a government-funded trivalent influenza vaccine (TIV) vaccination program to priority groups as the first-line strategy to prevent seasonal influenza. These priority groups include the elderly, children aged 6 months to 6 years-old, school-aged children, residents of nursing care facilities, health care workers, livestock breeding workers,

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and animal epidemic prevention workers. The coverage rates of influenza vaccine were 61.9% in children aged 6 months to 6 years and 37.3% in the elderly. The annual TIV is composed of 2 influenza A virus strains, A/H1N1 and A/H3N2, and 1 influenza B virus strain. The B-lineage in the vaccine is either from the Yamagata or Victoria lineage. When the B-lineage strain that is included in the vaccine does not match the circulating B-lineage virus, the vaccine will not provide optimal protection against influenza B. Between 2000 and 2010, the circulating strains of influenza B compared with A has varied from 1.3% to 56.2%. However, in 2004/2005, the proportion of influenza B was over 70% and with 51% of the circulating influenza B strain not being covered by the vaccine. An alternative strategy is to provide a quadrivalent influenza vaccine (QIV) to increase the protection against influenza. A QIV adds a second influenza B strain representing the other influenza type B-lineage which could eliminate a mismatch in lineage between the vaccine and circulating strains. The objective of the study is to assess the cost-effectiveness of QIV vs TIV as an influenza vaccine strategy from the perspective of the Department of Health (DOH) in Taiwan. Materials and Methods: A multicohort Markov model was applied to assess the costs and effects of QIV in Taiwan. Data related to medical resource use, efficacy of vaccine were derived from existing literature. Direct costs data were obtained from the National Health Insurance claims database released by the Collaboration Center of Health Information Application (CCHIA) of the Department of Health. An Excel-based program was used to conduct the simulation. Outcomes included computed life-years, quality-adjusted life years (QALYs), influenza cases avoided, medical costs averted and incremental cost-effectiveness ratios (ICERs). The perspective of the Department of Health is assumed and costs are expressed in US dollars (US$). The discount rate of costs and effect is 3.5% and the time horizon used in the model is 100 years. Results: Under a universal mass vaccination strategy utilizing QIV instead of TIV will avoid 3590 deaths and bring an additional 10,556.7 QALYs at an extra cost of US$429.4 million, yielding an ICER of US$40,670.5 per QALY gained. When herd effect is taken into consideration, the ICER was US$30,854.5 per QALY gained. The cocirculation of influenza A and B virus strains had the greatest impact on the uncertainty of the base case ICER. When setting the willingness-to-pay threshold at 3 times per capita GDP (US$61,134 in 2012), the probability that QIV is cost-effective when compared with TIV was 72% (with herd effect assumption) and 54% (without herd effect assumption). Conclusions: These results would aim to provide decision makers with health economic information about the cost-effectiveness of implementing QIV in a mass vaccination program as compared to TIV. From this perspective and in a public health context in Taiwan, the QIV could be considered a cost-effective strategy.

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Influenza-related mortality in the United Kingdom (UK): a linked General Practice Research Database (GPRD) analysis 2001-2009

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Background: This study aimed to determine the mortality burden associated with seasonal influenza in the United Kingdom (UK) using linked: individual patient, hospital and family doctor records, including mortality stratified by: age, risk factors and complications. Materials and Methods: A cross-sectional observational study was carried out using data derived from the linked General Practice Research Database (GPRD), between January 2001 and March 2009. Included subjects were all those with family doctor episodes of care coded for acute respiratory events related to infection (influenza, upper and lower respiratory tract infections [LRTI], and influenza-like illness [ILI]). Analyses were stratified by age and by “at-risk” categories (in the UK, people with certain conditions are recommended to be vaccinated against influenza). All-cause mortality, with and without hospitalisation was analysed. This included a period of 28 days post-discharge and stratified mortality by risk status, complication (cardiovascular, central nervous system, cerebrovascular, gastric bleed, renal, chronic obstructive pulmonary disease [COPD], upper/lower respiratory related) and by age groups (≤ 5, 5-18, 19-49, 50-64, and ≥ 65 years). Extrapolation of mortality data from this study to UK population was also considered. Results: 156,193 influenza-like episodes were recorded during the

study period (12.5 million population), of which 1,289 (0.8%) had an associated death which occurred either in primary or secondary care (within 28 days postdischarge). Of these, 87% of deaths were not associated with a hospital admission, 3% occurred in children aged ≤ 18 years and 60% in persons aged ≥ 65 years. The mean age of overall mortality was 70.6 years (SD = 22.6). For deaths that occurred in hospital, the mean age was 79.5 years (SD = 11.2) and it was noted that 10% of all hospitalisations were associated with a death. 89% of all hospital deaths occurred in subjects aged ≥ 65 years and none in children. Analysis of all-cause death hospitalised and 28 days-post discharge increased the overall hospital related deaths by 7.7%. Mortality rates per 100,000 persons by study age group (including 28 days postdischarge) were 1.2, 1.3, 3.8, 5.3, and 46.7, respectively. The risk of mortality was lower in those vaccinated; odds ratio 0.78. With respect to comorbid conditions, chronic heart disease and diabetes were the most commonly recorded comorbidities in the at-risk cohort and were present in 49% and 28%, respectively, of all high risk deaths. Renal disease and immuno-compromised subjects had the highest likelihood of death, with 25% of all renal subjects getting influenza dying, as well as 11% of all immuno-compromised subjects. In subjects who died with a complicated influenza case, the major complications present were LRTI, COPD and cardiovascular complications. Highest mortality rates were in patients aged ≥65 years. When the burden and mortality demonstrated by this study are extrapolated to the UK population, it is estimated that 760,000 cases of ILI occur each season and that the mortality associated with influenza could be approximately 6250 each year. Conclusions: This study, the first of its kind in the UK, demonstrates that seasonal influenza, even at a time of low overall activity, is associated with substantial mortality and illness, which is potentially preventable by vaccination. This study is likely to underestimate the true impact of influenza due to only capturing individuals who visited a physician, as most affected individuals self-diagnose, and due to the incompleteness and coding errors inherent within administrative databases.

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Age and gender differences in hospitalization associated with influenza in Hong Kong, 2004-2010

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Background: Influenza is associated with substantial hospitalization burden around the world. Identifying the high-risk groups of influenza-associated hospitalization is critical for optimal allocation of medical resources. Children, elderly and those with underlying conditions have long been recognized as high-risk groups for seasonal influenza and listed as priority groups for vaccination. However, few studies have explored the gender difference in influenza associated hospitalization risks, although men and women differ in their susceptibility of influenza virus infection, immune response to influenza virus infection, and vaccination and health seeking behavior. Moreover, these gender discrepancies likely have different patterns across age. In this study, we assessed the age-gender-specific hospitalization burden associated with seasonal H1N1 (sH1N1), H3N2, pandemic H1N1 (pH1N1), and B viruses. Methods: We applied the quasi-Poisson regression model to weekly numbers of hospitalization from 2004 to 2010 in Hong Kong. Hospitalization from 2004 to 2010 in Hong Kong were aggregated into gender-specific weekly numbers for the following disease categories: acute respiratory disease (ARD) and pneumonia and influenza (P&I) for the age groups of 0-17, 18-64 and 65+ years, and gender-specific cardiovascular disease (CVD), ischemic heart disease (IHD), cerebrovascular disease (stroke), and diabetes mellitus (diabetes) for the 65+ age group. Age- and gender-specific weekly proportions of positive specimens were respectively calculated for influenza A seasonal subtypes (sH1N1, H3N2), pandemic strain pH1N1, and influenza type B viruses, which were simultaneously entered into the Poisson models as virus proxy variables. Cocirculating respiratory syncytial virus, adenovirus and parainfluenza virus, as well as the long term and seasonal trend, temperature and relative humidity were adjusted in the model as confounding factors. Age- and gender- specific excess hospitalization numbers associated with each influenza

virus type/subtype were calculated by subtracting the baseline from the observed hospitalization. Here the baseline hospitalization for each influenza virus type/subtype was predicted from the models by setting the corresponding virus proxy to zero, ie, assuming no such virus was circulating. Excess hospitalization rate was obtained by dividing the excess hospitalization number with the population size. The 95% confidence interval of excess hospitalization rate was calculated by bootstrap method. The male/female ratio (M/F ratio) of excess hospitalization rate for each disease category was then calculated and its 95% confidence interval was estimated by delta method. Results: For ARD and P&I, excess hospitalization rates associated with seasonal and pandemic influenza type/subtype showed a U shape for the age groups of 0-17, 18-64 and 65+, but pH1N1-associated hospitalization rates were slightly elevated for adults aged 18-64 for both men and women. In the 0-17 age group, men showed higher excess hospitalization rates than women, with the M/F ratios ranging from 1.2 to 1.8. These M/F ratios were statistically significant ($P < .05$) only for P&I hospitalization associated with sH1N1, H3N2, and pH1N1, respectively. In the 18-64 age group, the excess hospitalization rates were comparable between men and women, and none of the M/F ratios were significant. In the 65+ age group, men showed higher risk than women for seasonal influenza viruses, with the highest M/F ratios for H3N2 (1.9 for ARD and 2.1 for P&I), followed by sH1N1 and B. Men generally showed lower hospitalization rates associated with pH1N1 than women, although the M/F ratio was not significant. For CVD, IHD, stroke, and diabetes in the 65+ age group, men tended to have higher excess hospitalization rates associated with all seasonal influenza viruses than women. But men showed a lower risk than women for pH1N1 associated hospitalization rates of CVD, IHD, and diabetes. Conclusions: In children and the elderly, men had higher excess hospitalization rates associated with seasonal influenza viruses than women. But the gender effects were different between age groups for the pandemic virus, with a higher rate for men in children but for women in the elderly. In adults below 65 years of age, the hospitalization burdens of seasonal and pandemic influenza are generally comparable between men and women.

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A Bayesian method to estimate the disease burden of multiple respiratory viruses

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Background: Although the disease burden of influenza and respiratory syncytial viruses (RSV) has been intensively studies, few attempts have made on other respiratory viruses such as adenovirus and parainfluenza. Poisson models with virus proxies have become a commonly used modeling approach in disease burden studies, but it failed to derive reliable estimates simultaneously for multiple respiratory viruses. In order to improve the estimates of the disease burden respiratory viruses, here we introduced a Bayesian inference method in Poisson models and also assessed, its validity and reliability using empirical data. Materials and Methods: Poisson models were first fitted to weekly numbers of hospitalizations diagnosed with acute respiratory diseases in children aged less than 18 years in Hong Kong Island from 2004 to 2010. Virus proxy variables of weekly age-specific positive numbers for influenza A (seasonal subtypes sH1N1, H3N2 and pandemic strain pH1N1), influenza B, RSV, parainfluenza, or adenovirus, together with seasonal confounders of time and temperature, were simultaneously added into these models. A Bayesian inference method based on Gibbs sampling (BUGS) was then applied to estimate the parameters of each virus proxy. The baseline in assessing hospitalization associated with each virus was separately derived by setting the corresponding virus proxy to zero. Excess hospitalizations were calculated as the difference between observed and baseline rates. The performance of these models was quantified by the mean absolute percentage difference (MAPD) between estimates of excess hospitalization rates and true rates with laboratory confirmed infections in a cohort of pediatric patients. The model with smaller MAPD was regarded as the model with better performance. The classical maximum likelihood inference method

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and a variety of virus proxies (age-specific and all-ages positive proportions of specimens) were also compared with the new Bayesian inference method. Results: In the models using the same virus proxies, the estimates from the Bayesian inference had smaller MAPD from the observed rates than the classical likelihood estimates, particularly for RSV, parainfluenza, and adenovirus. Compared with the proxy variables of all-ages and age-specific positive proportions, the proxies of age-specific positive counts provided the estimates closer to the observed hospitalization rates for influenza A subtypes, influenza B, RSV, parainfluenza, and adenovirus, regardless of inference methods. The combination of Bayesian inference and age-specific count proxies provided the better estimates than the other combinations, with smaller absolute percentage difference from the observed rates. This new modeling approach also successfully captured the change in the effects of seasonal influenza viruses and noninfluenza viruses during the 2009 pandemic period. Conclusions: The Bayesian inference combined with the proxies of age-specific positive numbers provided the most optimal estimates for excess hospitalization associated with multiple respiratory viruses in both the 2009 H1N1 influenza pandemic and interpandemic period. This modeling approach could be applied to other warm and cold regions.

P1-313

Influenza-associated mortality in Thailand, 2006-2011

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Background: Influenza related mortality in subtropical or tropical regions particularly in developing countries remains poorly quantified and often underestimated. The results for wealthy subtropical countries (eg, Hong Kong and Singapore) may not be comparable with the impact of influenza in low or middle income tropical countries in Southeast Asia. We analyzed data in Thailand, a middle-income tropical country with good vital statistics and influenza surveillance data. Methods: Weekly mortality data for all-cause and 3 underlying causes of death—cardiovascular (*International Classification of Diseases, Tenth Revision [ICD-10]*, codes I00-99), respiratory diseases (ICD-10 codes J00-99) and pneumonia and influenza (P&I) (ICD-10 codes J10-18) from 2006 to 2011 were obtained from Bureau of Policy and Strategy, Ministry of Public Health (MoPH), Thailand. Weekly numbers of positive influenza A viruses and influenza B viruses, as well as the total number of specimens tested, were obtained from influenza sentinel surveillance system at the National Institute of Health, MoPH. A negative binomial regression model was used to estimate deaths attributable to influenza in 2 age groups (< 65 and ≥ 65 years) by incorporating influenza viral data as covariates in the model. Of all-cause deaths among person aged < 65 and ≥ 65 years, ill-defined deaths accounted for 25% (58% of these occurred in community) and 54% (87% occurred in community), respectively. To account for the high proportion of ill-defined cause of death, we reapportioned ill-defined deaths by age (< 65 and ≥ 65 years) to the various categories (P&I, respiratory, circulatory) so that the final proportion in each category was equivalent to those found in the in-hospital deaths. Results were presented as a range with and without the reapportioned ill-defined deaths. Results: From 2006 to 2011, the average annual number of influenza-associated deaths from underlying respiratory/circulatory and from underlying P&I ranged from 1926 to 3115 (3.03-4.91 per 100,000), and from 567 to 965 deaths (0.89-1.52 per 100,000), respectively. Three quarters of underlying respiratory/circulatory deaths and 90% of underlying P&I deaths occurred in hospital. Deaths among persons aged ≥ 65 accounted for 63% and 52% of all influenza-associated deaths with underlying respiratory/circulatory and P&I causes, respectively. For underlying respiratory/circulatory coded deaths, the estimates of annual influenza-associated excess mortality ranged from 1.21 to 1.47 for person aged < 65 and from 24.53 to 45.71 per 100,000 for person aged ≥ 65. For underlying P&I coded deaths, the estimates of annual rates of excess deaths range from 0.46 to 0.66 and from 5.90 to 11.63 per 100,000 for person aged < 65 and ≥ 65, respectively. Conclusions: In Thailand, these data suggest that influenza-associated mortality is more than 10 times greater than previously

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appreciated from surveillance data. The year-round presence of influenza viruses made it difficult to fit a model to the data, but our initial approach did provide an important step for developing better models in tropical regions.

P1-314

Mortality associated with influenza and respiratory syncytial virus among individuals ≥ 5 years of age in a high HIV–prevalence setting—South Africa, 1998-2009

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Background: Available data suggest that the burden of influenza and respiratory syncytial virus (RSV) infection may be higher in developing countries and among HIV-infected individuals. Understanding the burden of influenza and RSV would assist policy makers to prioritize interventions. However, data on mortality due to influenza and RSV infection in low- and middle-income countries and particularly from high HIV–prevalence settings remains scanty. Materials and Methods: We estimated the influenza- and RSV-associated mortality among individuals ≥ 5 years of age in 5 age categories (5-19, 20-44, 45-64, 65-74, and ≥ 75 years of age) using national vital statistics data coded according to the *International Classification of Diseases, Tenth Revision*. We modeled the excess mortality attributable to influenza and RSV infection by applying Poisson regression models to monthly all-cause, all-respiratory, all-circulatory, and pneumonia and influenza (P&I) deaths, using national influenza and RSV laboratory surveillance data as covariates. Since the diagnosis of AIDS is rarely coded on the death certificate, we indirectly estimated the annual influenza- and RSV-associated deaths among HIV-infected and -uninfected children using Poisson regression models that incorporated year- and age-specific HIV prevalence and highly active antiretroviral treatment (HAART) coverage as covariates. Results: Over the study period, the influenza season peaked between May and August with 10 of the 12 years experiencing peak activity in June-July. RSV peak activity was observed between March and April in 8 of the 12 years with early or late peaks observed in February or May in the remaining years. The mean annual number of influenza-associated deaths in individuals ≥ 5 years of age was 9093 (21.6 per 100,000 person-years), 3613 (8.5 per 100,000 person-years), 3156 (7.5 per 100,000 person-years), and 2186 (5.2 per 100,000 person-years) among all-cause, all-respiratory, all-circulatory, and P&I deaths, respectively. The influenza-associated mortality rate per 100,000 person-years for all-cause deaths was 0.8 in the 5-19 year age group, 10.7 in the 20-44 year age group, 37.3 in the 45-64 year age group, 115.4 in the 65-74 year age-group, and 379.2 in the ≥ 75 year age group. Among individuals ≥ 5 years of age the influenza-associated mortality rate for all-cause deaths was greater in HIV-infected as compared with HIV-uninfected individuals (age-adjusted relative risk aRR: 7.9; 95% CI: 7.1-8.9). The increased risk of influenza-associated mortality among the HIV-infected individuals was greatest in the 5-19 (RR: 46.6; 95% CI: 20.9-104.3) and 20-44 (RR: 72.8; 95% CI: 38.1-138.9) year age groups, while no increased risk of influenza-associated mortality due to HIV infection was identified in individuals ≥ 65 years of age. A similar trend was observed for all-respiratory and P&I influenza-associated mortality, while no excess risk associated with HIV infection was identified among all-circulatory influenza-associated deaths. Among individuals ≥ 5 years of age, the annual RSV-associated mortality was 511 (1.2 per 100,000 person-years) for all-cause deaths, 429 (1.0 per 100,000 person-years) for all-respiratory

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deaths and 292 (0.7 per 100,000 person-years) for P&I deaths. Nonsignificant RSV-associated mortality was identified among circulatory deaths only in the 5-19 age group (mean annual deaths: 8). The RSV-associated mortality rate per 100,000 person-years for all-cause deaths was 0.4 in the 5-19 year age group and 2.4 in the 20-44 year age group, while no RSV-associated mortality was identified in individuals ≥ 45 years of age. Among individuals < 45 years of age, where the HIV burden is highest, the RSV-associated mortality rate for all-cause deaths was much higher in HIV-infected as compared with HIV-uninfected individuals (aRR: 66.1; 95% CI: 26.0-163.2). Similar trends were observed for the RSV-associated mortality among all-respiratory and P&I deaths. Conclusions: Among individuals ≥ 5 years of age, the mortality burden associated with influenza infection was substantial especially among elderly individuals and HIV-infected older children and adults. Conversely, mortality associated with RSV infection was observed only in individuals aged < 45 years, and it was mainly associated with HIV-infection.

P1-315

Can excess mortality models estimate the mortality burden of multiple pathogens when their seasonality is synchronous? South Africa as a case study

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Background: Since many influenza and respiratory syncytial virus (RSV) infections are not confirmed by laboratory diagnosis, and related deaths may be attributed to other comorbid conditions or secondary infections, modeling techniques have been used to estimate the mortality burden associated with these pathogens. In recent years, Poisson and negative binomial regression models have become a popular tool to simultaneously estimate the relative burden of mortality or hospitalization for several pathogens that have similar seasonal patterns. In countries and settings like the United States of America and England, where the influenza and RSV seasons are synchronous, both influenza- and RSV-associated mortality has been reported across age groups (including elderly individuals) and underlying causes of death. Using data from South Africa where the RSV season precedes the influenza season in most years, we assessed the behavior of the Poisson regression model over different timing of the RSV season from 1998 through 2009 to determine if synchronous RSV and influenza seasons confound estimates of mortality from these pathogens. Materials and Methods: We estimated the influenza- and RSV-associated mortality among individuals ≥ 45 years of age using national vital statistics data coded according to the *International Classification of Diseases, Tenth Revision*. We modeled the excess mortality attributable to influenza and RSV infection by applying Poisson regression models to monthly all-cause, all-respiratory, all-circulatory, and pneumonia and influenza (P&I) deaths, using national influenza and RSV laboratory surveillance data as covariates. We then implemented a sensitivity analysis whereby we applied a 1 month incremental shift (1 to 5 months) of the RSV season while keeping the influenza season constant. This allowed us to model synchronous RSV and influenza seasons and compare the results with those from the observed asynchronous seasons. We then refitted the Poisson model for each underlying cause of death to obtain estimates of influenza- and RSV-associated mortality under each scenario whereby the RSV season progressively approaches and then diverges from the influenza season. Results: Over the study period, the influenza season peaked between May and August with

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10 of the 12 years experiencing peak activity in June-July. RSV peak activity was observed between March and April in 8 of the 12 years with early or late peaks observed in February or May in the remaining years. The estimated mean annual all-cause RSV-associated deaths in individuals ≥ 45 years of age was 0 when actual data with asynchronous seasons were used (ie, no incremental shift of the RSV season was applied). However, the mortality associated with RSV increased progressively as we shifted the RSV season to make the influenza and RSV seasons more synchronous. We found a peak mean annual RSV-associated mortality of 3661 deaths when the RSV and influenza seasons coincided in most years (2 months incremental shift). Thereafter, the estimated mean annual RSV-associated mortality decreased again to 0 when the peak circulation of the 2 pathogens were further apart (5 months incremental shift). This trend was observed for all underlying causes of death evaluated in this study. Conversely, the influenza-associated mortality remained comparable across the analyses implemented over the monthly shifts of the RSV season. The mean annual all-cause influenza-associated deaths ranged between 5811 (2 months incremental shift) and 6635 (4 months incremental shift) and was 6442 when no incremental shift of the RSV season was applied (actual data). Conclusions: Modeling indicated that no RSV-associated mortality was identified in South African individuals ≥ 45 years when no incremental shift of the RSV season was applied. However, the model would indicate substantial RSV-associated mortality when the RSV and influenza seasons were made artificially more synchronous. This suggests that careful interpretation of the results of such excess mortality models should be applied when used to simultaneously estimate the mortality attributable to pathogens that cocirculate. Ecological studies conducted in settings similar to ours, where influenza and RSV peak activities are not synchronous, may assist in better differentiating the relative burden of these pathogens in adults.

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Evaluation of the burden of acute respiratory infections and healthcare utilization behaviour in rural and urban populations of Madagascar

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Background: Data on the burden of influenza-like illness (ILI) and severe acute respiratory infections (SARI) are scarce in developing countries. In Madagascar, hospital-based surveillance of SARI has been effective since November 2010 in 2 hospitals in Antananarivo and Moramanga. Our study aimed at evaluating the burden of ILI and SARI in urban and rural population settings in Madagascar. We also describe health utilisation behaviours in our study area. Methods: A cross-sectional community-based study was designed, targeting 19 administrative units in and around the town of Moramanga. A 1-stage stratified cluster design was used with a sampling rate of 1:2 urban and 2:3 rural houses. Structured interviews used case definitions of sudden fever and cough for 2 weeks for ILI, and sudden fever, cough, and dyspnoea during 1 year for SARI. Socio-demographic data, medical history, and healthcare utilisation were also collected. Results: A total of 21,754 individuals were interviewed, with a global yearly incidence of SARI estimated at 5.5%, and an averaged 2 weekly ILI incidence estimated at 6%. Children were most affected, and cost of treatment most often cited as a barrier to care. In multivariate random effects models, living in urban areas, being < 5 years old, not being a farmer, being a professional, and living close to health facilities were all independently associated with improved access to health care facilities. Residual household effect on healthcare access behaviour remained significant (P < .0001). Rural populations were less likely to access care, more often visiting informal care structures. Urban populations were more likely to access private healthcare. Conclusions: The estimates of disease burden described demonstrate the importance of acute respiratory infections and suggest a larger toll on population health than previously documented. Future research into health care utilisation behaviours and ILI incidence will further improve public health planning and allow more targeted strategies.

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Health facility-based surveillance for influenza in a health and demographic surveillance site in Ghana

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Background: Influenza and other respiratory viruses cause substantial morbidity and mortality in developed countries. Recent data suggest that burden may be higher in less developed settings, such as Africa, where lower healthcare access and higher prevalence of certain coinfections such as HIV, malaria, and tuberculosis and other comorbidities exist. Among African children influenza has been shown to cause 22% and 10% of out-patient and in-patient respiratory illnesses respectively, and in Ghana, 30% of outpatient visits for respiratory disease during rainy seasons from 2008 through 2010 were positive for influenza. However these data are from sentinel surveillance, and few data exist in Africa, especially west Africa, on the rate of mild and severe disease in different groups and what specific risk factors may be important for severe disease. This study seeks to collect comprehensive data to determine seasonal baseline rates of influenza-like illness (ILI) and hospitalization due to severe acute respiratory illness (SARI) in a population of approximately 130,000 persons within an existing health and demographic surveillance system (HDSS) in Ghana. Materials and Methods: Surveillance for ILI and SARI was initiated in 17 health facilities serving the Dodowa HDSS population in February 2013. The case definition for ILI was an out-patient with a measured fever of at least 37.5^0 C (axillary) and a cough, and for SARI was an in-patient with measured fever of at least 37.5^0 C (axillary) or a history of fever, and cough with onset in the last 7 days. In 9 of 17 facilities, epidemiologic data and nasopharyngeal (NP) and oropharyngeal (OP) samples are gathered from all patients meeting the SARI case definition and the first 5 ILI cases per week. The remaining 8 sites collect only epidemiological data on patients meeting the ILI case definition without collecting respiratory samples. Respiratory specimens are tested for influenza viruses at the National Influenza Center at the Noguchi Memorial Institute for Medical Research. The total number of hospitalizations and out-patient consultations are also collected from surveillance sites to assess the proportion of visits for respiratory illness, and the proportion of sampled patients who test positive for influenza viruses. Results: From February to April 2013, the total out-patient attendance for HDSS residents at 9 health facilities was 7690 with 1177 (15.3%) visits for respiratory illnesses of which 136 (11.6%) were enrolled in ILI surveillance. Among 649 hospitalized HDSS residents, 87 (13.4%) had symptoms of respiratory disease, of which 56 (64.4%) met the SARI case definition and 47 (54.0%) were sampled. Additionally, epidemiological data from the remaining 8 health facilities found 2966 total outpatient visits among HDSS residents, of whom 612 (20.6%) had respiratory symptoms, and 132 (21.6%) of these cases met the ILI case definition. To date, a total of 183 respiratory samples, 136 (74.3%) from ILI and 47 (25.7%) from SARI patients have been tested for the presence of influenza virus. Seven (5.1%) ILI patients and 1 SARI case (2.1%) were positive for influenza A/H3N2 infection. Conclusions: February, March and April are some of the hottest and driest months in Ghana. Despite this, influenza continued to circulate. Nearly year-round circulation of influenza viruses has been noted in other tropical climates with peak incidence following the rainy season. In such climates the burden of influenza may be even higher than in temperate countries, which makes documentation of influenza burden important in Ghana. In the initial 2 months of surveillance for SARI and ILI we have overcome challenges such as generalized classification of various admissions as respiratory related diagnosis and hard copy questionnaires preferred over personal digital assistants by clinical staff. Enhanced surveillance activities in these health facilities on the established platform will enable an estimation of influenza disease burden in Ghana.

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Cost of all-cause and influenza-associated acute respiratory infections in north India

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Background: Acute respiratory infections (ARI) are important causes of mortality and morbidity, especially among children. Data on the economic burden of ARI are important to guide policy decisions about options for ARI prevention, such as national recommendations for available vaccines against pneumococcal and influenza. However, the economic burden of ARIs is not well documented in India where a large proportion of the population incurs out of pocket expenditure for medical care and the annual per capita income is US $1375. We evaluated costs to patients for medically attended (outpatient and inpatient) episodes of ARI in select public and private hospitals in and around Delhi, India. Materials and Methods: A cross-sectional survey was conducted among patients attending the outpatient departments or hospitalized at 3 public and 8 private hospitals from September 2012 to March 2013. Participants were asked about direct medical costs (costs of consultation, investigations, and medications), nonmedical costs (travel, food, etc), and indirect costs (loss of wages) for treatment of ARI episodes. Participants were initially surveyed at the time of the outpatient consultation or during hospitalization and then followed up till the end of the episode. Nasopharyngeal swabs were taken only from hospitalized children (< 10 y) and elderly (> 60 y) patients and tested for influenza viruses by reverse transcription polymerase chain reaction. Data on the cost of outpatient services and hospital beds for public hospitals were obtained from the World Health Organization–Choosing Interventions that are Cost Effective (WHO-CHOICE, 2011) and these costs were added to costs reported by patients to calculate total direct medical costs of hospitalization; all costs were converted to 2013 US dollars using the Wholesale Price Index (WPI) of India. Median and interquartile range of costs per ARI episode were calculated by type (public vs private) and level of facility (Primary/Secondary/Tertiary); the private nursing homes were also categorized as secondary or tertiary based on availability of ICU care for the study. Results: A total of 523 patients were enrolled, including 309 outpatients and 214 hospitalized patients. Of the 309 (149 aged < 5 y) outpatients, 232 were seen at public and 77 at private health facilities. The median direct and indirect costs to the patient per outpatient ARI episode were US$3.8 (IQR 2.70-9.69) and US$1.9(IQR 1.9-3.8) for public facilities, and US $9.5 (IQR 4.0-28.0) and US$3.8 (IQR 1.9-3.8) for private facilities. Among the 214 (63 aged < 5 y) hospitalized patients, 88 were admitted to public and 126 to private facilities; the median length of hospitalization was 5 days (IQR 4-7). Compared with hospitalizations at public facilities, total costs (medical and nonmedical) were significantly higher at private facilities (US$117.6 IQR 70.9-283.5 versus US$236.31, IQR 155.0-375.0, P < .001); medical costs alone were also significantly higher (US$92.4, IQR 56.2-198.3 versus US$203.5, IQR 140.4-336.8, P < .001). In contrast, caregiver's median wage loss due to hospitalization was lower at private facilities (US$19.1; IQR 15.2-25.2) than at public facilities (US $28.8; IQR $19.1-61.1), likely due to shorter hospitalization in private hospitals. Of the 112 hospitalized patients, 6 (5.4%) had respiratory specimens positive for influenza viruses. Conclusion: The economic impact of ARI in India is substantial, with a single hospitalization costing more than the monthly per capita income in India. The impact may vary if measured in the public or private sector. Data from other parts of the country are needed to obtain an accurate picture of the cost of ARI in India. This study was supported in part by cooperative agreement U01 IP000492 from the Centers for Disease Control and Prevention, Atlanta, United States.

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Systematic review and meta-analysis of influenza-associated hospitalizations among children in China

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Background: Children < 5 years old are recommended by WHO for influenza vaccination. Immunization coverage for recommended vaccines is very high among the approximately 80 million children < 5 years old in China. Uptake of new influenza vaccine recommendations would likely be very high in this age group. As influenza surveillance has increased nationally, there have been an increased number of outbreaks reported among school-aged children in recent years. However, there are limited national data on influenza-associated severe outcomes in young and school-aged children (< 5 and 5-18 years). We reviewed the literature to assess the role of influenza in respiratory disease hospitalization in children in China. Materials and Methods: We conducted a systematic search of biomedical databases (PubMed, Embase, Global Health, CINAHL, WHOLIS, Web of Science, LILACS, IndMed) for English articles and China National Knowledge Infrastructure (CNKI) for Chinese articles, published from 1996 and 2012 reporting influenza testing among children under 18 hospitalized for respiratory disease. The review of the literature followed PRISMA guidelines. Search terms included key words from 2 categories: respiratory infection (respiratory infection/respiratory disease, pneumonia, bronchiolitis, bronchitis, influenza-like illness, ILI, SARI, ARI, ALRI, LRTI, CAP), and viral etiology (influenza, influenza virus, influenza A, influenza B, influenza C, viral etiology). We used "or" to combine each key word to maximize sensitivity in identifying eligible papers. The inclusion criteria were: 1) original data; 2) hospitalizations for community-acquired respiratory disease; 3) laboratory-confirmed influenza infection; 4) includes and describes criteria for testing; 5) study spans at least 1 year (12 continuous months of influenza testing); 5) at least 50 individuals tested for influenza; 6) influenza diagnostic assay specified; 7) total number of individuals tested and total positive for influenza specified. We extracted data on study year, case definition, sample type, test method, total tested number tested for influenza, and total number positive for influenza from each eligible paper. We also stratified the data by 3 age groups, < 2 year old, < 5 year old, and < 18 year old. We analyzed reasons for heterogeneity and then estimated the pooled result with a random effects regression model in Stata12. Results: We identified 2551 potentially relevant articles, and 219 warranted full text review. 79 studies met inclusion criteria. The 79 studies cover 22 provinces/areas: 11 areas from northern China (29 papers) and 11 from southern China (50 papers). The 79 studies were conducted from 1996 to 2012. The diagnostic methods used included: polymerase chain reaction (PCR) (n = 13 studies), immunoassay (n = 56), serology (n=1), culture (n = 1), multiple tests not including PCR (n = 6); other methods: (n = 3). The pooled percentage of influenza positive among inpatient children < 2 years old was 4.7 % (95% CI 4.0%-5.4%), for northern China was 7.1 (95% CI 5.3%-8.9%), and for southern China was 3.8% (95% CI 3.1%-4.6%). For the < 5 year age group, the overall proportion was 7.3% (95% CI 6.4%-8.1%), northern China is 10.4 (95% CI 8.3%-12.4%), southern China is 5.9% (95% CI 5.0%-6.9%). For children < 18 years old, the overall proportion is 7.9% (95% CI 7.1%-8.7%), northern China is 9.8% (95% CI 8.2%-11.5%), southern China is 7.1% (95% CI 6.1%-8.1%). Studies using PCR for influenza diagnosis had a higher percentage positive than those using other assays, and this difference was statistically significant for the < 5 and < 18 years old groups (P < .04 and P < .02 respectively). Among this subset using PCR, the pooled percentage of influenza positive among children < 2 years hospitalized with respiratory disease is 7.0% (95% CI 4.2%-9.8%), among < 5 years is 8.9% (95% CI 6.8%-11%), under < 18 years is 8.8% (95% CI 7.0%-10.7%). Conclusions: Influenza is an important cause of respiratory hospitalizations among children in China. Our results are consistent with burden studies conducted in other countries. Our results also support the use of PCR for influenza testing, as the use of other diagnostic methods may result in an underestimate of the true influenza burden. These data suggest that influenza vaccination should be considered as a routine intervention at least for children < 5 years old in China.

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Estimating the provincial burden of influenza-associated severe acute respiratory illness in South Africa

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Background: South Africa has few estimates of the annual national number of cases of influenza illness, and the rates may vary by province based on differences in risk factors for severe influenza-associated hospitalizations. This study was a secondary analysis of severe acute respiratory illness (SARI) surveillance data from South Africa and the published literature to estimate the provincial burden of influenza-associated SARI in South Africa. Materials and Methods: We used a multiplier model to estimate numbers of individuals with hospitalized and nonhospitalized influenza-associated SARI for each province in South Africa for 4 age groups (< 5, 5-24, 25-44, and ≥ 45 years) for 2009-2011, stratified by HIV serostatus. Surveillance in 1 large public hospital in Soweto, Gauteng Province was used to estimate incidence rates of SARI in the population (base incidence). These rates were adjusted for the provincial-level prevalence of known risk factors for pneumonia (from the South Africa Demographic and Health Survey and the Actuarial Society of South Africa) to obtain an estimate of SARI incidence for the other 8 provinces in South Africa. Risk factors included indoor air pollution, crowding, malnutrition, low birth-weight, and nonexclusive breastfeeding; relative risks were determined from the published literature. The incidence rates were subsequently multiplied by the percent of SARI associated with influenza virus from sentinel surveillance. These were then further adjusted for health-seeking behavior for pneumonia from a healthcare utilization survey to get the rate of hospitalized and nonhospitalized influenza-associated SARI. Results: The relative risks for SARI for exposure to indoor air pollution and crowding used in this model were 1.8 and 1.4, respectively. Nonexclusive breastfeeding, malnutrition, and low birth-weight had relative risks of 1.9, 1.8, and 1.4, respectively. The mean annual base incidence rates of influenza-associated SARI (per 100,000) in HIV-positive individuals from 2009-2011 were 453, 119, 203, and 232 for the < 5, 5-24, 25-44, and 45+ year age groups, respectively. The mean annual base incidence rates of influenza-associated SARI (per 100,000) in HIV-negative individuals from 2009-2011 were 215, 11, 13, and 36 for the < 5, 5-24, 25-44, and 45+ year age groups, respectively. From 2009-2011, the estimated annual number of hospitalized and nonhospitalized influenza-associated SARI cases in South Africa ranged from 27,047 to 43,378, and 6295 to 8073, respectively. The number of hospitalized cases of influenza-associated SARI was highest in 2009 with 43,378 cases; when pandemic influenza A(H1N1) circulated. The number of hospitalized and nonhospitalized cases was highest among children under 5 years of age in 2009 with 21,614 cases. In subsequent years, the 25-44 year old age group had the highest number of hospitalized and nonhospitalized cases with 13,652 cases in 2010 and 16,020 cases in 2011. The number of hospitalized cases in Gauteng Province (population of 11 million and HIV prevalence of 11.2%) ranged from 7553 in 2009 to 4907 in 2010. In adults aged 25-44 (highest HIV prevalence) the average incidence of hospitalized cases was 62 per 100,000. Kwa-Zulu-Natal Province (population of 10.3 million and HIV prevalence of 15%) consistently had the highest estimated number of cases, ranging from 11,729 in 2009 to 8169 in 2011. Northern Cape Province (population of 1.1 million and HIV prevalence of 7%) consistently had the lowest number of cases ranging from 971 in 2009 to 569 in 2010. The average incidence of hospitalized cases in 25-44 year olds in Kwa-Zulu-Natal and Northern Cape were, respectively, 113 and 57 per 100,000 adults. Conclusion: These results indicate that influenza morbidity varies by province and the groups at higher risk include HIV-positive individuals and young children under 5, similar to other published studies. The magnitude of influenza burden in each province is related to the prevalence of risk

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factors in the population, in particular HIV prevalence. The high HIV prevalence in Kwa-Zulu-Natal drives the number of cases up in this province. Understanding the relative burden of influenza in different groups and regions can help guide more effective use of resources to prevent and control influenza.

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All-cause and respiratory mortality in a rural community in northern India: a comparison of prepandemic, pandemic, and postpandemic influenza periods

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Background: The 2009 Pandemic mortality were estimated to be 42,000-159,000 deaths in Asia during first year of circulation, although few of these were laboratory-confirmed. Accurate pandemic mortality estimates for India may be challenging because of inadequate vital statistics and limited availability of testing. Therefore we used verbal autopsy and death reporting data from a rural community in Northern India to assess impact of 2009 pandemic influenza on all-cause and respiratory mortality. Materials and Methods: Ballabgarh Comprehensive Rural Health Services Project has conducted health research in Northern India since 1967, with a current population under surveillance of 90,240. Ballabgarh Health and Demographic Surveillance System (HDSS) assigns a unique identification number to each member of the community, and routinely gathers information on vital events, including births, deaths and cause of death. A validated verbal autopsy questionnaire is used to interview family members (between 2-4 weeks after a death) to elucidate the cause of death. Two physicians assign cause of death on the basis of verbal autopsies. Since 2003, the crude annual death rate in Ballabgarh HDSS has ranged from 6.1 to 7.4 per 1000 population. We conducted a retrospective analysis of HDSS death reports and verbal autopsy data with population denominator from to calculated the mean monthly rate of all-cause and respiratory mortality for prepandemic (July 2008-August 2009), pandemic (September 2009-October 2010 as defined by confirmed cases of pH1N1 during this period in CHRSP area), and postpandemic (November 2010-December 2011) period. We also calculated age-stratified average monthly rate of these mortality indicators for < 5 years (U5), 5-14, 15-65, and ≥ 65 years. Kruskal Wallis test was used to assess any significant difference between the prepandemic to that of pandemic and postpandemic periods for each mortality indicator. Results: Of the 2221 total deaths recorded during July 2008-December 2011, all-cause mortality were similar during prepandemic (n = 757), pandemic (n = 736), and postpandemic (n = 728) periods. Likewise, of the 289 respiratory cause deaths, 97 (33.6%) and 90 (31.1%) deaths were recorded during pre- or postpandemic periods, with slight increase (102; 35.2%; NS) during pandemic period. The average monthly crude death rates were 0.62, 0.59 and 0.57 per 1000 population (P = .64), and respiratory death rates were 0.79, 0.82 and 0.71 per 10000 (P = .54) respectively, for prepandemic, pandemic, and postpandemic periods, respectively. Age stratified all-cause mortality was higher among 15-64 years (n = 859; 39%) and > 65 years (811; 37%), as compared with children U5 (493; 22%) and least among children ages 5-14 (58; 3%). The average monthly all-cause U5 mortality rate was 1.36/1000 and 1.17/1000 during pre- and postpandemic period respectively, with slightly higher during pandemic period at 1.44/1000 (P = .49). No difference in mortality rates were observed for adult age groups; 0.40, 0.34, and 0.34 per 1000 among persons aged 15-64 years (P =.07); and 4.3, 4.3, and 4.4 per 1000 among persons ≥ 65 years (P = .94). Age stratified respiratory mortality was highest among > 65 years (146/289; 50.5%), followed by children U5 (73; 25.2%), ages 15-64 years (66; 22.8%) and least among children 5-14 years (4; 1.3%). Similarly on age stratification, U5 respiratory mortality rates were 1.24, 1.93, and 2.68 per 10,000 (P = .44); in 15-64 years were 0.39, 0.23, and 0.22 (P = .76); ≥ 65 years 8.4, 9.3, and 5.8 (P = 0.07) during pre-, pandemic and postpandemic periods. Overall mortality indicators did not differ significantly during the pandemic period compared with the pre- and postpandemic periods. Conclusions: A statistically significant difference in crude and respiratory mortality rates was not detected between the prepandemic, pandemic, and postpandemic periods, although a trend towards increased crude mortality in U5 children was observed during the pandemic period. While detection of excess mortality in the current

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study was limited by the size of the population under surveillance, the short time period for defining baseline mortality patterns, and the possible contributions of seasonal influenza viruses and other pathogens to mortality in the area; however, routine collection of population-based mortality data at HDSS sites can serve as a potential platform for describing excess mortality associated with influenza and other pathogens.

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Strain-specific influenza burden of disease among hospitalized Canadian adults during the 2011/12 season: a Public Health Agency of Canada/Canadian Institutes of Health Research Serious Outcomes Surveillance Network Study

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Background: In Canada, publicly funded seasonal trivalent inactivated influenza vaccine (TIV) is recommended for all adults aged 65 years or older, children under 60 months and all others with medical comorbidity placing them at increased risk of influenza complications. The goal of Canada's influenza immunization program is prevention of hospitalization and death. As policy-makers anticipate the availability of quadrivalent influenza vaccines (QIV), assessment of the contribution of influenza A and B and of the contribution of the B lineages to serious outcomes will be critical to assess potential disease and economic impacts of quadrivalent vaccines. Sentinel surveillance for influenza among hospitalized adults provides a platform for assessment of strain-specific influenza burden of illness. Materials and Methods: In 2011/12, the PCIRN Serious Outcomes Surveillance Network conducted active surveillance for influenza among hospitalized adults from 1 Nov to 25 May in 40 acute care facilities in 6 provinces, encompassing ≈ 15,000 beds. A nasopharyngeal swab (NP) for influenza polymerase chain reaction (PCR) testing was obtained from all patients admitted with community-acquired pneumonia, exacerbation of chronic obstructive pulmonary disease or asthma, unexplained sepsis, any respiratory diagnosis or symptom, and, on one day per week, acute coronary syndrome, stroke, or other cardiac diagnoses with temperature ≥ 37.5°C. Positive NP swabs were sent to the PCIRN central lab in Halifax for subtyping of influenza strains. Ribonucleic acid was extracted from NP swab material using an automated system, and subsequently tested by real-time Reverse Transcriptase-PCR (RT-PCR). A primary screening RT-PCR assay targeting the Matrix gene differentiated the influenza strains as A or B, thereby determining which of two secondary assays directed at the HA gene was used to subtype the virus. The influenza A subtyping identified pH1N1 and sH3N2 strains, and B subtyping determined the Victoria and Yamagata B lineages. Results: In 2011/12, the PCIRN SOS network enrolled 540 lab-confirmed influenza cases; viral subtyping was available in 460 (85%) cases. Among patients for whom subtyping was available, mean age was 67y (19-100 y); 41.7% of cases were > 75 y and 45.4% were male. 35% of hospitalizations were caused by influenza A strains (61.5% by H1N1, and 37.9% by H3N2; 0.6% were untypeable), and 65% were caused by B strains. Among the B strains, 29.8% were antigenically related to the 2011/12 vaccine strain B/Brisbane/60/2008 (Victoria lineage) and 68.2% were similar to B/Wisconsin/1/2010 strain (Yamagata lineage); in 2% the lineage was not determined. Overall, the non-vaccine Yamagata lineage-like strain was responsible for 44.3% (n = 204) of the total hospitalizations. The mean age

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was 61 y (SD ± 20) and 70 y (SD ± 19) for those infected with A and B strains, respectively; mean age was highest in patients with B/Yamagata infection (73.6y ± 17.2 y) followed by those with A/H3/N2 (71.2y ± 20.1 y). Mean length of stay ranged from 8.8-11 days and did not vary by influenza subtype. Overall, 11.3% of patients required admission to ICU and rates of ICU admission were not significantly different by infecting strain (A/H1N1 15.2%, A/H3N2 11.5%, B/Victoria 13.5% and B/Yamagata 8.8%, p=). Overall the mortality rate was 7.2% and was similar for patients with influenza A compared to influenza B (6.2% vs 7.7%, respectively) but was higher amongst those with A/H3N2 than those with A/H1N1 (11.5% vs 3.0%, respectively; P = .04) and amongst those with B/Yamagata than those with B/Victoria (9.8% vs 3.4%, respectively; P = .06). Conclusion: In 2011/12, almost half of hospitalized influenza cases in Canadian adults were caused by a B/Yamagata-like influenza strain alone, the only circulating strain not represented in the vaccines used in Canada that season. Mortality associated with influenza B was similar to that observed with influenza A but interestingly, appeared to be lower with the vaccine-strain influenza B (B/Victoria) than the non-vaccine strain influenza B (B/Yamagata) suggesting attenuation of disease by vaccination. Influenza B is an important contributor to serious outcomes in adults in Canada, suggesting that use of a quadrivalent vaccine may offer benefits over TIV in supporting the goal of the Canadian influenza immunization program, reduction of hospitalization and death. Funding: This study was funded by the Public Health Agency of Canada, the Canadian Institutes of Health Research and GlaxoSmithKline Biologicals SA.

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Estimation of seasonal influenza disease burden in Pakistani population using sentinel site data during 2011-12

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Background: Acute respiratory disease including influenza related severe acute respiratory infection (SARI) constitute a major cause of morbidity and mortality globally. Influenza A (H1N1) pdm09 pandemic highlighted the need for reliable disease burden estimation from lower and middle income countries like Pakistan to better understand the impact of this vaccine preventable disease. These estimates would enable governments to make evidence based decisions for priority allocation of limited health resources where needed. This retrospective study was designed to estimate age-specific incidence of influenza related SARI in Islamabad region of Pakistan over a 2-year period (2011-12). Materials and Methods: The incidence rates were estimated according to the WHO manual for disease burden estimation of Influenza. The catchment area was mapped based on SARI data from one designated influenza sentinel site and other healthcare facilities in Islamabad region. A proportion of respiratory samples were tested using CDC real-time PCR protocol. From the calculated number of Influenza associated SARI from 1 sentinel hospital, proportion of lab confirmed influenza cases was inferred for the catchment population (denominator) after adjustment of sampling fraction. The proportion of SARI-associated hospitalizations reported outside the catchment area was determined for the total population under demographic surveillance and incidence rates were adjusted accordingly. The incidence rate was calculated as per 100,000 for each age group with 95% confidence interval. Results: The catchment population for sentinel site was 0.7 million against the total denominator of 1.1 million and incidence rates were accordingly adjusted. During January 2011 to December 2012, among 2491 hospitalizations, 2136 (86%) patients were enrolled; 422 of these (19.7%) were positive for influenza. During 2011, A (H1N1) pdm09 dominated with 48% detection rate followed by seasonal influenza including A/H3 (22%), A/Un-typed (18%) and influenza B (12%). In comparison, during 2012 A/H3 subtype was detected (64%) more frequently followed by Influenza B (21%). Influenza A(H1N1)pdm09 was detected in only 7% and 8% of Influenza A isolates remained un-typed. Influenza B-associated hospitalization rates were almost 2-fold higher during 2012 as compared with 2011. During both years, male patients reported more frequently (55.6%) than females across all age groups. Furthermore during the study period, 15 to < 50 year age group had the highest number of hospitalized SARI and influenza positive cases. The proportion of influenza positive hospitalized patients with acute medical illness was significantly higher during 2011 than 2012

(194/842 vs 228/1649 patients, P = .0001). The Incidence rates of all cause respiratory and influenza related SARI were highest among children under 5 years during 2011 and 2012; highest incidence was found in 0 to < 1 year group with 301/100,000 cases (95% CI: 200-453) followed by 153/100,000 in children aged 1 to < 5 years (95% CI: 79-259), and lowest in, 5 to < 15 years (18/100,000 in 95% CI: 15-23). During both years, influenza associated hospitalization rates were highest among persons aged 15-50 years, followed by children aged 5-14 years and lowest in persons over 65 years. The estimated average annual influenza-associated hospitalization incidence during the study period was 84.4/100,000 persons. Conclusion: Our study found the maximum incidence rates for influenza associated hospitalizations in children under 5 years. In our population, 48% of the burden of influenza associated hospitalizations was accounted for by A(H1N1)pdm09 influenza virus in 2011 with other seasonal influenza A viruses accounting for 40% of the burden. In contrast during 2012 A/H3 predominated and resulted in high hospitalization rates. Based on these findings, we understand that influenza accounts for a significant proportion of respiratory morbidity and hospitalization. These estimates from 1 sentinel site can serve as a first step towards accurate estimation of national influenza-associated disease burden. Furthermore, it is necessary to test for other respiratory pathogens such as respiratory syncytial virus, human metapneumovirus and rhinoviruses in influenza-negative samples for more clear disease burden estimation. Reliable disease burden estimates will assist public health managers and policy makers to prioritize allocation of health resources for surveillance, prevention and control, and plan immunization strategies for the high-risk groups.

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Excess deaths and excess hospitalizations associated with common respiratory viruses in Hong Kong, 2004-2010

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Background: Respiratory viruses cause acute respiratory illnesses in populations at any age group, and lead to a substantial burden of hospitalizations and deaths every year. Laboratory-confirmed cases are usually an underestimate of the impact of respiratory virus infections, given the difficulty in clinical settings to identify all the cases potentially associated with a virus. Comprehensive studies involving assessment and comparison of the severity of different respiratory virus infections, especially for noninfluenza viruses are limited. This study aimed to estimate excess all-cause and cause-specific hospitalizations and mortality attributable to common respiratory viruses in Hong Kong, and thereby assess the impact of virus infections in 2004-2010. Materials and Methods: Weekly age-specific deaths and hospitalizations by principal discharge diagnosis (pneumonia and influenza/respiratory diseases) from 2004 to 2010 were obtained from the Hospital Authority in Hong Kong. Weekly positive proportions of specimens tested for common respiratory viruses, including influenza A(H1N1), A(H3N2), A(H1N1pdm09) and B, respiratory syncytial virus (RSV), parainfluenza virus, and adenovirus, and weekly consultation rates of influenza-like illness (defined as fever > 38°C and cough/sore throat) for sentinel surveillance at public outpatient clinics and private general practitioner clinics were obtained from the Department of Health of Hong Kong SAR. Linear regression models were applied to investigate the association between type/subtype specific virus infections and all-cause/ cause-specific hospitalization or mortality rates based on the assumption that increase in the virus's activities would be associated with additive increase in mortality and hospitalizations. The activity of each virus was estimated by the weekly proportion of the ILI consultation rates multiplied by the weekly positive detection rate of each virus. The excess hospitalization/death rates were estimated by subtracting the predicted rates estimated from the fitted regression model by setting virus activity to zero from the predicted rates from the model based on the reported weekly virus activity. The 95% confidence intervals for excess hospitalization/mortality rates were estimated with a bootstrap approach. Results: The annual (2004 to 2010) all-cause excess mortality rate associated with the 7 respiratory viruses ranged from 1.18 to 7.07 per 100 000 person-years, except for parainfluenza that was not associated with substantial excess mortality. For most viruses investigated, the highest excess all-cause and respiratory mortality occurred among the elderly aged > 65 y. The all-cause excess hospitalization rate associated with 3 subtypes of influenza A virus, RSV, and adenovirus ranged from 30 to 100 per 100 000 person-years. Most these

respiratory viruses were associated with relatively higher excess hospitalization rate in young children 0-4 years and in that age group the excess hospitalization burden associated with RSV and parainfluenza was similar to that attributable to influenza A viruses during the study period. We found that the ratios of excess deaths to excess hospitalizations were estimated to be 0.02-0.07 for RSV and influenza A(H1N1), A(H3N2), and the ratios were higher among > 65 y age group compared with younger age groups. Conclusions: Influenza virus, RSV, and adenovirus were associated with most of the excess deaths caused by common respiratory virus infections among the elderly, while the highest excess hospitalization rates associated with influenza viruses, RSV, and parainfluenza were estimated in the youngest age group (0-4 y). Our results were also consistent with increasing seriousness of infection with age. Compared with other common respiratory viruses, seasonal influenza A (H3N2) virus was associated with the highest burden of hospitalizations and mortality in Hong Kong in 2004-2010.

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Healthcare utilization for influenza-like illness and severe acute respiratory infection in a province in northern Vietnam

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Background: Influenza-like illness (ILI) and severe acute respiratory infection (SARI) surveillance have shown that seasonal influenza viruses circulate year-round in Vietnam. However, the disease burden posed by influenza in the population is poorly defined since the sentinel surveillance system only identifies cases that sought healthcare at selected sites and the population served by these sites is not well quantified. We conducted a household survey in Thai Binh Province in northern Vietnam to ascertain retrospectively the number of self-reported ILI and SARI episodes and associated health-seeking behaviors in order to estimate the incidence of self-reported ILI and SARI in this population. Methods and Materials: A sample size requirement of 6500 people was calculated based on a previous report of the incidence of acute respiratory infections in a community in Vietnam. Thirty clusters were selected by using the probability proportional to size method. In each cluster, 70 households were selected for investigation. For the selection of the first household of a cluster, we randomly selected a household to begin the survey. The next houshold was selected following the “door to door” principle until the sample size was reached. Household socio-demographic, economic, and health data were collected using a standardized questionnaire. When a household member reported having an episode of ILI in the previous 30 days or SARI in the previous 12 months, an additional standardized questionnaire was used to collect data on healthcare utilization. Data were entered into a Microsoft Access 2007 database and analyses will be performed by using Stata Version 10.1 (StataCorp, College Station, Texas, United States). Results and Conclusions: A total of 2100 households with 6804 household members were enrolled in the study. From the 6804 household members, 662 (10%) self-reported having had an episode of ILI (541 episodes) or SARI (121 episodes) in the previous 30 days or 12 months, respectively. Six (0.9%) people with ILI or SARI did not seek any healthcare. Of the 656 patients who sought healthcare, 249 (37.9%) went to commune health centers, 224 (34.1%) went to hospitals, 41 (6.2%) went to private clinics, and 76 (11.6%) went to pharmacies. Fifty-two (7.9%) ill subjects reported self-treatment, 7 (1.1%) sought care from unlicensed providers, and 7 (1.1%) went to traditional healers. Further analysis of the data is ongoing. Detailed results and conclusions will be done by August 2013 and added to the abstract.

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SARI/ILI in the north of Vietnam: burden, economic impact and healthcare utilization

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Background: The burden of disease, economic impact and health care utilization related to influenza viruses is not well described for hospitalized severe acute respiratory infection (SARI) patients in the north of Vietnam. Pandemic A/H1N1 2009 is circulating in Vietnam and may pose patients at critical condition in ICU or at risk to die. Influenza diseases were mild most of the cases, but still there were some cases that were hospitalized in ICU or dead due to pandemic A/H1N1 and other seasonal influenza viruses. Our study proposes to describe the burden of human influenza illness in terms of morbidity, mortality, socioeconomic impact, and understand healthcare utilization at hospitals in Thai Binh province, north of Vietnam. Materials and Methods: A modified WHO case definition of SARI was used. Adults and children meeting the definition were enrolled at three hospitals (a provincial hospital, a pediatric hospital, and a district hospital) in Thai Binh, north of Vietnam. For each patient, the direct and indirect costs, work days lost, and school absenteeism associated with hospitalization and influenza treatment, and a nasal or throat swab were collected for influenza virus detection by real-time reverse transcription polymerase chain reaction at the National Influenza Centre. Results: From January 2013 through August 2013, of 9346 hospitalized patients at enrolled hospitals in Thai Binh, 2826 (30%) were identified as SARI. Of 750 SARI cases tested, 83 (11%) were positive for influenza viruses, including A/H3N2 (37%), B (32%), and pA/H1N1 2009 (31%). Both normal and low white blood cell counts were found in influenza-related SARI patients. Of influenza related SARI patients, 15% required treatment in ICU. More severe cases were found in the pediatric hospital and provincial hospital in Thai Binh. There were 4 SARI deaths at the 3 district hospitals. The median hospital stay was 5 days (IQR 4-8). Of 750 survey respondents, 3 (< 1%) reported receiving an influenza vaccination during the past 12 months. Socioeconomic impact included direct and indirect costs per patient of 1,500,00 VND ($US70), an average of 7 work days lost and 5 school days lost, and care giver 6 days lost. Conclusions: No deaths data due to influenza causes were available at the moment; the complete data will be manageable and reportable in August 2013. By preliminary results, we found the most frequent influenza viruses identified were A/H3N2 and B. A low rate of influenza vaccination was found in the study population. The costs due to SARI case hospitalization are important to patients with limited resources (per capita monthly income = 1,387,000 VND ($66)), even for only 1 week of the treatment.

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The duration of illness of laboratory confirmed influenza A and B in the community in France and Turkey: 2010-2012

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Background: Knowledge about the burden of illness is essential for rational policy decisions on cost effective management in healthcare. The introduction of influenza vaccines containing 2 B lineages has created a need for more information regarding the burden of disease of influenza B. The influenza B study in General Practice (IBGP) was undertaken to provide comparative information about clinical and socio-economic aspects of laboratory confirmed influenza B and A patients in France and Turkey

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in the seasons 2010/11 and 2011/12. Materials and Methods: We used routine influenza surveillance networks to compare influenza B ("cases") and influenza A (which we defined as "controls") detected in France and Turkey in seasons 2010/11 and 2011/12 (defined as season 1 and 2). Established sentinel surveillance networks in France (570 physicians) and Turkey (515) participated. Case recruitment was from willing persons with laboratory confirmed diagnoses. Subjects were recruited by sentinel physicians who were responsible for follow-up and data collection. Specimens taken and an accompanying simple investigation request form were sent (mostly by post) to the National Influenza Centres (NIC) for diagnosis (including subtyping of influenza A and lineage characterization of influenza B viruses). Validation of the testing procedures was undertaken at the Mill Hill Influenza reference centre using a 10% sample. Potential controls were identified by the national coordination team in each country using reports provided by the NIC. Clinical data were collected when the swab was taken and subsequently 7 and 28 days later at telephone contact follow-up using the same questions and similar forms in each country. The respective cohorts were compared in each country with respect to age, gender, symptom presentation, therapeutic interventions, and frequency of consultation. Duration of illness (defined as the number of days from reported date of first symptoms to recovery defined by the practitioner) was measured as the primary outcome variable in a multivariate regression analysis. Results: The dominant A and B circulating viruses differed between countries and between seasons. In season 1 in France, 95% of those characterized were B Victoria whereas in Turkey, 98% were B Yamagata. In Season 2 in France, both B strains circulated in similar proportions whereas in Turkey all were B Victoria. In France 231 cases (215 influenza A controls) and in Turkey 216 influenza B cases (153 influenza A controls) were recruited for the IBGP study. The symptom profile of cases and controls in both countries was similar. Few recruits consulted their GP more than once: in France around 73% consulted once for influenza B (66% in Turkey) and 67% for influenza A (77% in Turkey). There were age and gender differences between countries (eg, 67% of cases were aged 0-14 in France compared with 29% in Turkey), reflecting the differing delivery of health services, as well as the differences in circulating viruses and in surveillance networks. Based on this finding, we carried out country-specific analyses. In the multivariate regression analysis, after controlling for potential confounders (eg, gender, age, and season), we found no significant differences in the duration of illness for influenza A and B in both countries. Conclusions: The IBGP study shows that in 2 very distinct settings the presentation of illness and the duration of illness due to influenza B (both B Victoria and B Yamagata) do not differ from influenza A. This finding is set against a background of very different circulation of B lineage viruses in the 2 seasons considered. These findings will help determine the public health value of introducing influenza vaccines containing 2 B lineages.

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P1-329 G FELLOWSHIP GATES Fellowship

Assessing the burden of influenza in relation to age, its impact on out-patients and hospitalized patients; a case study in Uganda

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Background: The World Health Organization has emphasized assessing the severity of influenza virus in hospitalized patients. In regards to out-patients and hospitalized individuals, there is risk of spread of influenza in areas where relative contact between persons is high which in turn affects the burden of disease. Influenza virus is highly associated with age of the patient with younger individuals believed to be more likely infected with influenza than adults. This is of importance in targeting the most suitable group for vaccination. Method: Demographic and clinical data, together with nasal pharyngeal and or throat swabs were collected from enrolled patients using 2 case definitions; influenza-like illness (ILI) for out-patients and severe acute respiratory infection (SARI) for hospitalized patients. Samples were screened at the National Influenza Center (NIC)/Uganda Virus Research Institute (UVRI) for influenza A and B using real-time PCR. Data were statistically analyzed using SPSS. The impact was assessed by evaluating the number of days lost from school at hospital among school attending children. Results: A total of 2123 specimens from enrolled patients were tested for ILI and SARI on real-time PCR. 684 (32%) were hospitalized SARI patients and 1439 (68%) were ILI out-patients. 254 patients were SARI and ILI positive for influenza; with 189 (74%) ILI and 65

(26%) SARI. The results showed that in out- patients (ILI), there was a 1-time more likelihood to have Influenza (OR 1.44, 95% CI: 1.069-1.939) than hospitalized patients (SARI) and this was statistically significant (P .018). 1940 (91%) of the patients were from children aged < 17years, and 183 (9%) patients were from individuals aged > 17 years. 242 (12.5%) patients of the children aged < 17 years were positive to influenza compared with 12 (6.5%) in individuals aged > 17 years. The results showed that children aged < 17 years were twice more likely to be infected with influenza (OR 2.031, 95% CI: 1.114-3.703) compared with individuals aged > 17years. The study had P 0.017, and hence, statistically significant. There is no substantial statistical significance of burden of influenza in individual case definitions of ILI and SARI in relation to age with P 0.223 and P 0.67 respectively. Children aged < 17 years lost an average of 3 school days in hospital. Conclusion: There is more influenza among ILI out-patients (74%) than among SARI (26%), indicating a high burden of influenza in ILI than SARI patients. Influenza virus is associated with age of an individual. There is loss of days at school among school attending children.

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Estimating the incidence and severity of seasonal influenza in the United States using population-based surveillance data

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Background: Surveillance for influenza is complicated by the fact that only a fraction of all infected persons will seek medical care and an even smaller group will be tested and confirmed to have influenza. This limits our ability to understand the full incidence and severity of an epidemic. Moreover, death certificates and hospital discharge records which traditionally have been used to estimate excess hospitalizations and deaths and characterize the annual variation in the incidence of severe influenza are not generally available in a timely manner. During the 2009 H1N1 pandemic, to provide more timely estimates of the burden of influenza-related illnesses, hospitalizations and deaths in the United States, CDC developed a model to correct surveillance data for biases in influenza detection using a series of probabilistic multipliers. Postpandemic, we sought to further explore this approach for the annual estimation of the incidence of seasonal influenza-related health outcomes. Materials and Methods: To estimate influenza disease burden for the 2010-11 and 2011-12 influenza seasons, we used surveillance data on population-based rates of laboratory-confirmed influenza hospitalization from the Influenza Hospitalization Surveillance Network (FluSurv-NET). Additional data were collected to characterize influenza detection probabilities during hospitalization and deaths occurring outside the hospital. We explored two different methods to estimate hospitalizations and deaths in the US population. First, we used the probabilistic model developed in 2009. We estimated a correction-factor to adjust the observed rate of influenza-associated hospitalization based on the frequency and sensitivity of hospital influenza testing and then extrapolated rates of hospitalization to the US population. We calculated deaths using a ratio of deaths to hospitalizations and the probability of death occurring outside of the hospital. In a second analysis, we used the data on rates of hospitalization and detection of hospitalizations and deaths, and combined with prior information and associated uncertainty in a Bayesian evidence synthesis framework. After fitting the model, posterior estimates of model parameters were used to estimate the number of hospitalizations and deaths in the US population. Results: Using the multiplier method, we estimated 2.7-4.9 times the rate of hospitalization than reported in surveillance across both years. This multiplier varied by age group, with the lowest in children aged < 18 years and the highest in adults aged ≥ 65. In the second season, the multiplier remained similar for children and older adults, but increased for adults aged 18-64 years. For the 2010-11 influenza season, we estimated a total of 198,600 hospitalizations (95% CI: 121,153-342,306) and 8,684 deaths (95% CI: 4176-18,280); for the 2011-12 season, 77,681 hospitalizations (95% CI: 40,992-136,497) and 3885 deaths (95% CI: 1911-7688). Persons aged ≥ 65

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years of age accounted for 47%-58% of deaths and 39%-49% of hospitalizations, varying by season. Using the Bayesian method, we estimated that surveillance detected 31%-50% of influenza-associated hospitalizations and 14%-46% of deaths in the surveillance catchment area, varying by age group and season. For the 2010-11 season we estimated a total of 175,870 influenza-associated hospitalizations (95% credible interval: 164,030-188,960) and 7932 (95% CI: 6177-10,147) deaths; for the 2011-12 season, 66,950 influenza-associated hospitalizations (95% CI: 59,390-75,980) and 2519 (95% CI: 1647-3787) deaths. Future plans involve expanding the models to estimate illness incidence and include outcomes such as medically attended illness and intensive care admission. Conclusions: Using these probabilistic approaches, we were able to produce estimates of annual influenza disease burden in a more timely fashion. Multiplier values and detection probabilities can vary over time if changes in public awareness of seasonal influenza lead to different levels of influenza detection and reporting. Data collected in two post-pandemic seasons, in multiple sites and age groups, improved our understanding of influenza detection and reporting and can allow for better estimates of the annual disease burden of influenza. Exploring different methods to synthesize surveillance data and correct for biases can further refine estimates of the annual influenza disease burden to provide public health officials with necessary information to evaluate annual programs and allocate resources effectively.

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Public health impact of applying QIV as seasonal influenza vaccine

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Background: Vaccination is currently recommended by the WHO as the most effective way to prevent infection and severe outcomes caused by influenza viruses. Trivalent influenza vaccine (TIV) is composed of 2 influenza A viruses strains and 1 of the 2 influenza B lineages. The strains which selected in the annual vaccine are based on viral surveillance data. When the B-lineage strain that is included in the vaccine does not match the circulating B-lineage virus, the vaccine will not provide optimal protection against influenza B. An alternative strategy is to provide a quadrivalent influenza vaccine (QIV) to increase the protection against influenza. A QIV adds a second influenza B strain representing the other influenza type B-lineage which could eliminate a mismatch in lineage between the vaccine and circulating strains. However, it remains unclear how additional public health benefit could be gained by applying QIV as seasonal influenza vaccine. The objective of this study was to estimate the public health impact of applying QIV instead of TIV on influenza-related outcomes over ten influenza seasons in Taiwan. Materials and Methods: Two vaccination strategies were compared accounting for vaccine coverage, circulation of influenza A and 2 influenza B viruses and vaccine effectiveness. The baseline strategy was use of the existing TIV as occurred during the influenza seasons. The alternative strategy is the hypothetical use of QIV during these same seasons. We estimated the hypothetical impact over the previous 10 influenza seasons, from the 2000/2001 season through 2009/2010 season. We calculated the net difference for rate of illness, hospitalization and influenza-related death by gathering data from observed data, literature and government statistical report. First, we adjusted these rate by estimates of annual vaccine coverage (VC) and vaccine effectiveness (VE) to back-calculate the hypothetical rate which would occur without TIV. Second, we applied VC and VE estimate for QIV to calculate the expected rate of influenza-related health outcomes. Third, we calculate the difference of observed rate of influenza-related health outcomes from TIV compared to QIV. We identified the distribution of influenza A, influenza B and subtype of 10 influenza season in Taiwan. We also estimated the mismatching rate between influenza B lineage and annual TIV vaccine and incorporated the vaccine coverage rate and vaccine effectiveness by different subtype. Data on annual VC was obtained from government statistical report. Data on VE was collected from previous published studies. All calculations were performed by Microsoft Excel worksheets. Results: Applying QIV as an alternative vaccine strategy result in fewer influenza cases, hospitalizations, and death. The average avoided illness was 154,497 in the 10 seasons with a rate of 371.6 per 100,000 (range: 2-1627 per 100,000). The average avoided hospitalizations were 50,984 with a rate of 223.0 per 100,000 (range: 1-976 per 100,000). The average avoided death was 1117 with a rate of 4.9 per 100,000 (range: 0-22 per 100,000). The

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additional health benefit is more significant when the mismatching of annual TIV vaccine and the circulation of B virus. In the 4 of the 10 seasons, the avoided influenza case increased from 188.5 to 1334.6 per 100,000 and the avoided hospitalization and influenza-related death were also higher during these seasons. Conclusions: In recent years, public health authorities have great interest in the influence of including both type B lineages in the season influenza. The effectiveness of TIV against influenza B viruses could reduce when the B-lineage strain that is included in the vaccine does not match the circulating B-lineage virus. Using a simple model, we demonstrate that the use of QIV is expected to result in an annual reduction in influenza-related health outcomes when compared to TIV. The potential net impact of QIV on influenza-related health outcomes is expected to vary among seasons, which is dependent on the annual circulation of the 2 influenza B lineages, vaccine coverage and effectiveness. Including a second lineage of influenza B in the annual seasonal vaccine could result in a modest additional protection.

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A study of health outcomes in vaccinated and unvaccinated subjects with reported respiratory infections in the United Kingdom

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Background: The present study aimed to determine the burden of seasonal influenza in the United Kingdom, by vaccination status, and to assess whether the characteristics of vaccinated patients differed from unvaccinated patients. Materials and Methods: A cross-sectional observational study was performed using data derived from the linked General Practice Research Database (GPRD), between January 2001 and March 2009. Subjects were included if they had an episode of care coded by their family doctor for acute respiratory infections (ARI) related to: influenza, upper and lower respiratory tract infections, and influenza-like illness. Analyses were stratified by age, by at-risk categories and by vaccination status. The at-risk status of the subjects was assessed using the National Institute's for Health and Care Excellence guidelines (UK Department of Health); these guidelines identify subjects who are at high risk of infection and at high risk of subsequent complications, ie, fall in 1 or more of the following clinical risk groups: chronic respiratory disease, chronic neurological conditions, diabetes, chronic heart disease, pregnant, chronic liver disease, chronic renal disease, immunosuppressed, and anyone aged 65 years or older. Means were compared using independent-samples t-test, assessing each age group separately, using age and the number of risk conditions as test variables. Results: In the at-risk group, the mean age of those vaccinated was 65.1 years (SD 16.5), vs 48.2 years (SD 20.4) in those unvaccinated; the difference between the means was 16.9 years (95% CI 16.4-17.4; P < .001). The mean number of risk conditions in the vaccinated group was 1.29 (SD 0.60), vs 1.15 (SD 0.45) in the unvaccinated group; the difference between means was 0.14 (95% CI 0.12-0.15; P < .001). Similar results were seen in the not-at-risk group; the difference between the mean ages was 27.9 years (95% CI 27.7-28.2; P = .003). The majority of ARI episodes occurred in unvaccinated patients, only 16.0%-22.1% of subjects with an ARI episode were vaccinated annually. By age, the proportion of vaccinated subjects increased to 74.6%-82.6% for at-risk subjects aged 65 years and older, and decreased to 0.0%-2.2% for not-at-risk subjects younger than 5 years of age. 58.2% of subjects at-risk were vaccinated. More resources were consumed by unvaccinated not-at-risk patients, while in at-risk patients the opposite was true. Conclusions: The majority of patients suffering an acute respiratory event were not vaccinated. This analysis also confirmed that vaccinated subjects had more at-risk conditions than unvaccinated subjects which were generally younger than the at-risk group and therefore the two groups are not directly comparable. A considerable number of those unvaccinated were still at risk and should have received vaccine.

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Influenza pneumonia surveillance among hospitalized adults substantially underestimates the burden of severe influenza disease

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Background: Studies seeking to estimate the burden of influenza among hospitalized adults often use case definitions that require presence of pneumonia. This requirement neglects the fact that influenza can also present with nonpneumonia respiratory manifestations or exacerbate chronic nonrespiratory illness such as congestive heart failure. We sought to assess the extent to which restricting influenza surveillance to only those adults hospitalized with pneumonia could underestimate the total burden of influenza disease. Materials and Methods: We conducted a retrospective cohort study from January 2003 through March 2009 using hospitalization databases from the Healthcare Cost and Utilization Project (HCUP) and regional influenza surveillance data from the US Centers for Disease Control and Prevention. We used data from all inpatient discharge abstracts for adults aged 18 years and older from community hospitals in the HCUP State Inpatient Database from Arizona, California, and Washington during the study period. Our objective was to estimate the proportion of influenza-associated events that would be missed by requiring a pneumonia diagnosis to be part of a surveillance case definition. Our exposures of interest were positive laboratory tests for influenza A (H1N1), influenza A (H3N2), and influenza B from 2 contiguous US Federal Regions: Region 9 encompassing Arizona and California and Region 10 encompassing Washington. The 2 outcomes of interest (with ICD-9-CM diagnosis and procedure codes) were: 1) respiratory and circulatory hospitalizations (390-519); and 2) severe illness hospitalizations—defined as acute respiratory failure (96.7 and any of 518.5, 518.81, or 518.82), severe sepsis (995.92, 785.52, or any codes for infection and organ dysfunction), and in-hospital death. We linked the hospitalization datasets with the virus surveillance datasets by geographic region and month of hospitalization. We used negative binomial regression models to estimate the number of influenza-associated events among patients hospitalized with the two outcomes of interest. We sub-categorized the 2 outcomes of interest to include: 1) all outcomes; and 2) all outcomes that listed an ICD-9-CM code for pneumonia (480-486). Results: During the study period, there were 28,176,969 hospitalizations in the 3 states; 16,044,193 (57% of total) hospitalization records contained a code for a respiratory or circulatory diagnosis, while 2,066,072 (7% of total) met the definition of a severe illness. Using multivariate binomial regression models, we estimated that there were 80,793 influenza-associated respiratory and circulatory hospitalizations and 26,666 influenza-associated severe illness hospitalizations. We repeated these analyses using only events that listed a diagnostic code for pneumonia. When a pneumonia diagnosis was required, the estimated number of influenza-associated respiratory and circulatory hospitalizations was 24,770, or 31% of 80,793 hospitalizations. The estimate for the number of influenza-associated severe illness hospitalizations decreased to 8,113 cases (30.4% of total) after requiring a pneumonia diagnosis. Within adult age groups, we estimated that requiring pneumonia diagnoses to define influenza-associated events would capture from 14.5% (for persons aged 75 to 84 years) to 39.9% (for persons aged 18 to 49 years) of total influenza-associated respiratory and circulatory hospitalizations. For severe illnesses, requiring a pneumonia diagnoses would result in capture of only 23.0% (for persons aged 75 to 84 years) to 38.3% (for persons aged 50 to 64 years) of influenza-associated severe illness hospitalizations. Conclusions**:** If case definitions for events used to model influenza-associated hospitalizations require the presence of pneumonia, we estimate that ≈ 70% of the likely burden of influenza hospitalizations will be missed. Our study has limitations, as it excludes children and the modeling-based estimates of influenza-associated hospitalizations have not been validated with season-specific results from studies with laboratory-confirmation of influenza hospitalizations. However, we believe that our results do have implications for influenza burden of disease studies. Studies which only consider hospitalizations that include a diagnosis of pneumonia will underestimate the total burden of influenza hospitalizations, and often to a substantial degree.

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P1-334

Burden of outpatient visits due to influenza infection and cases averted by vaccination – United States, 2011/12 influenza season

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Background: Public health policy makers need estimates of the burden of illness caused by influenza to allocate resources for prevention and treatment. They also need to evaluate the success of vaccination programs by estimating the reduction in disease burden associated with those programs. We used the United States Influenza Vaccine Effectiveness (US Flu VE) Network to estimate the incidence of outpatient visits due to laboratory-confirmed influenza infection and the reduction in incidence associated with influenza vaccination at 4 sites across the United States. Methods: We used administrative databases from health care payers and providers at 4 of the US Flu VE Network sites to enumerate cohorts of health plan enrollees and health care patients (subjects) for the 2011/12 influenza season. To ensure that all cohort members were eligible for influenza vaccination, eligible subjects were restricted to those born prior to March 1, 2011. We used administrative databases to determine age, dates of influenza vaccination, and dates of medically attended acute respiratory illness (MAARI) defined by International Classification of Diseases, Version 9 (ICD-9) codes. We then identified the subset of cohort members who had been prospectively enrolled in the 2011/12 US Flu VE study. These participants were a subset of all cohort members who sought care for an acute respiratory illness, defined as fever and/or cough of less than seven days duration. Influenza VE participants were tested for influenza infection by rRT-PCR using probes developed by the US Centers for Disease Control and Prevention. We stratified influenza VE participants based on age, influenza vaccination, and number of MAARI visits (as 0, 1, or 2+), and assigned each participant in the influenza VE cohort a sampling weight. Sampling weights were the ratio of the total cohort size in the participant's age/vaccination/MAARI visit stratum to the total number of influenza VE participants in that stratum. We used these weights to extrapolate the proportion of positive influenza tests among influenza VE enrollees to the cumulative incidence of laboratory-confirmed, medically attended influenza in the cohorts. Incidence was calculated overall and stratified by age group and vaccination status. We calculated cases averted per 1000 vaccinees as the difference in cumulative incidence between vaccinated and unvaccinated cohort members, assuming differences in incidence between vaccinated and unvaccinated persons are attributable to vaccination. Results: The study cohorts included 424,733 subjects, of whom 10.6% were children < 10 years of age, 9.4% were children 10-17 years of age, and 39.2% were adults 50 years of age and older. Overall vaccination coverage ranged from 17.9% to 46.4% across the 4 sites. Across the 4 sites, the overall cumulative incidence of laboratory-confirmed, medically attended influenza ranged from 5.0 to 16.5 cases per 1000 population. Cumulative incidence ranged from 13.7 to 39.8 per 1,000 for children < 10 years of age; from 5.2 to 30.1 per 1,000 for children 10-17 years of age; from 6.4 to 13.2 per 1000 for adults 18-49 years of age; and from 3.6 to 13.4 per 1000 for adults 50 years of age and older. Influenza cases averted by vaccination ranged from 0.7 to 5.1 cases per 1,000 vaccinees across the 4 sites. Cases averted by vaccination ranged from 6.4 to 16.8 per 1000 vaccinees among children < 10 years of age; from 1.4 to 15.4 per 1000 vaccinees among children 10-17 years of age; and from -3.6 to 4.0 per 1000 vaccinees among adults 50 years of age and older. Conclusions: The 2011/12 influenza season was mild in the United States. The cumulative incidence of medically attended influenza ranged from 5 to 16 per 1000 population across our study sites. This low incidence of laboratory-confirmed infection parallels low rates of outpatient visit for influenza-like illness and pneumonia/influenza mortality observed from national US influenza surveillance. In this mild seasonal epidemic, influenza vaccination showed modest impact at the US Flu VE Network sites, with between 1 and 5 cases of medically attended influenza prevented per 1000 persons vaccinated.

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P1-335

Excess mortality associated with influenza among tuberculosis deaths in South Africa, 1999-2009

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Background: Data from the 1918 influenza pandemic suggests that many individuals who died had active tuberculosis (TB), and that underlying TB infection may have contributed to the elevated mortality observed in young adults. Increased mortality associated with seasonal influenza virus infection has been described for a number of underlying causes of death including chronic respiratory and cerebrovascular diseases and diabetes. However, little is known about the mortality burden associated with seasonal influenza virus infection among patients with TB. The lung damage induced by pulmonary TB (PTB) may result in increased risk of mortality subsequent to influenza virus infection. In South Africa, approximately 80% of TB cases are HIV-infected and HIV is a known risk factor for influenza-associated mortality. We aimed to estimate the influenza-associated mortality among individuals who died of TB in South Africa from 1999 through 2009. In addition, we compared influenza-associated mortality among deaths with underlying TB to HIV-infected and -uninfected non-TB respiratory related deaths. Materials and Methods: We estimated the influenza-associated mortality among individuals < 65 and ≥ 65 years of age using national vital statistics data coded according to the *International Classification of Diseases, Tenth Revision*. We modelled the excess mortality attributable to influenza by applying Poisson regression models to monthly PTB, extrapulmonary TB (EPTB) and all non-TB respiratory deaths, using national influenza laboratory surveillance data as a covariate. Since the diagnosis of AIDS is rarely coded on the death certificate in South Africa, we indirectly estimated the annual influenza-associated deaths among HIV-infected and-uninfected non-TB respiratory deaths using Poisson regression models that incorporated year- and age-specific HIV prevalence and highly active antiretroviral treatment (HAART) coverage as covariates. Results: From 1999 through 2009 a mean of 550,769 (range 418,729-621,002) deaths occurred annually among South African individuals. Of these, 118,995 (22%), 63,596 (12%), and 9722 (2%) deaths were attributable to non-TB respiratory, PTB, and EPTB, respectively. Over the study period, the influenza season peaked between May and August with 9 of the 11 years experiencing peak activity in June-July. PTB deaths were seasonal with increased mortality in winter each year, coinciding with the period of influenza virus circulation. Our model estimates that, among individuals of any age the mean annual influenza-associated mortality rates per 100,000 person-years were 164 (n = 437) among PTB, 27 (n = 1125) among HIV-infected non-TB respiratory, and 5 (n = 2367) among HIV-uninfected non-TB respiratory deaths. No influenza-associated mortality was found among EPTB deaths. Among individuals < 65 years of age, the mean annual influenza-associated mortality rates per 100,000 person-years were 141 (n = 374) among PTB, 27 (n =1 125) among HIV-infected non-TB respiratory, and 2 (n = 937) among HIV-uninfected non-TB respiratory deaths. In this age group, individuals with PTB experienced a greater risk of influenza-associated mortality as compared with HIV-infected individuals with non-TB respiratory disease (relative risk (RR): 5.2; 95% CI: 4.6-5.9) and HIV-uninfected with non–TB respiratory disease (RR: 61.0; CI: 41.4-91.0). Among individuals ≥ 65 years of age the mean annual influenza-associated mortality rates per 100,000 person-years were 836 (n = 64) among PTB and 64 (n = 1430) among HIV-uninfected non-TB respiratory deaths (RR: 13.0; 95% CI: 12.0-14.0). In this age group where the HIV burden is lowest no influenza-associated mortality was found among HIV-infected non-TB respiratory deaths. Conclusion: Our models indicate that mortality associated with influenza infection among individuals with PTB was substantial, whereas no peak in EPTB mortality was observed during the influenza epidemics. Individuals < 65 years of age with PTB experienced an increased risk of influenza-associated mortality as compared with non–TB respiratory disease, irrespective of the HIV status. An increased risk associated with PTB was observed also among individuals ≥ 65 years of age where the HIV

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burden is lowest. If confirmed in other settings, this may support recommendations for active inclusion of patients with TB for influenza vaccination and empiric antiviral treatment of patients with TB presenting with acute respiratory symptoms during influenza epidemics.

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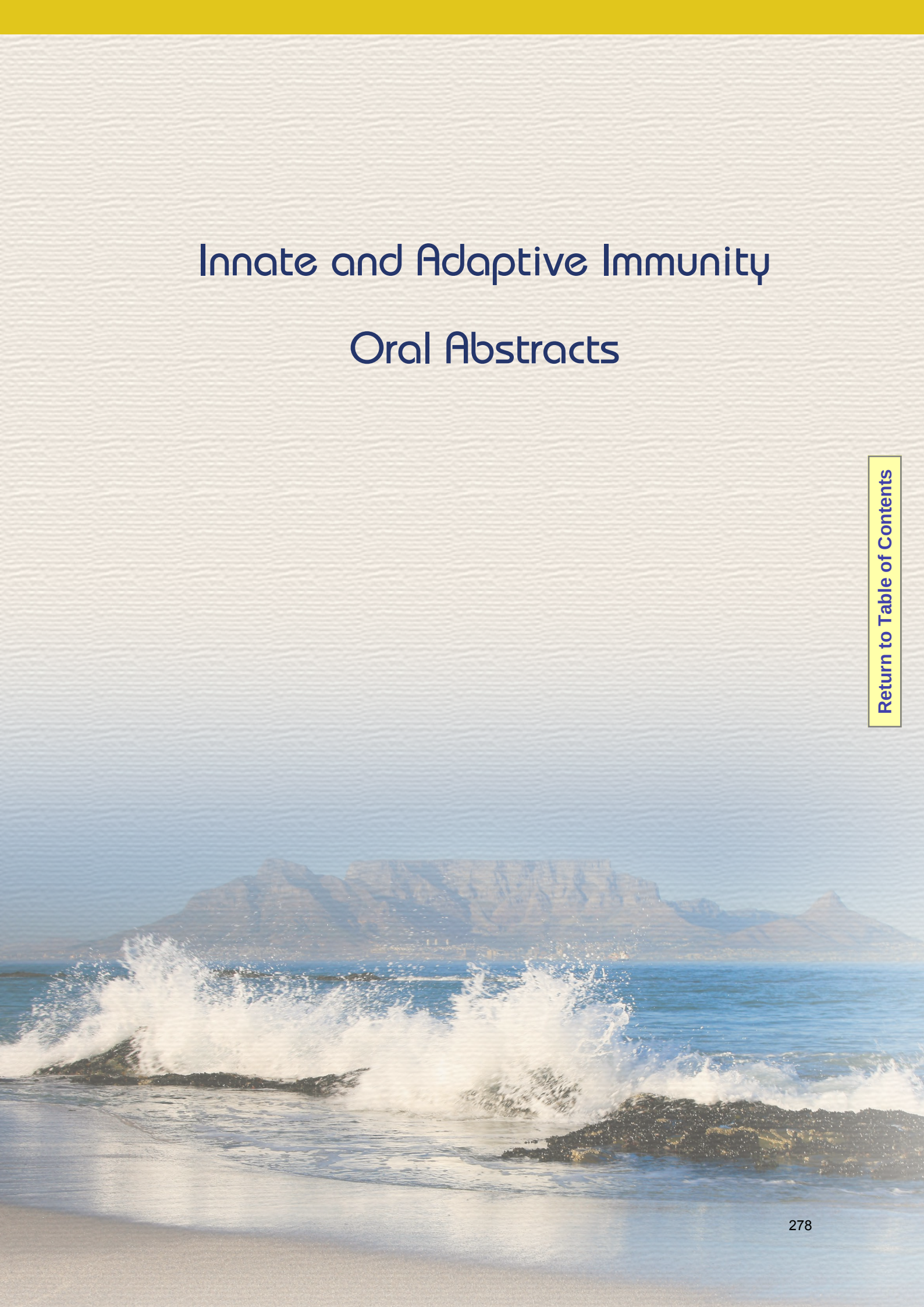
Innate and Adaptive Immunity

Oral Abstracts



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O-852

A potent cytotoxic T-cell epitope in the extracellular domain of influenza B virus NB promotes virus clearance

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Background: Influenza B viruses are a serious threat to human health since they are responsible for about one out of three influenza epidemics. Influenza B viruses drift easily, and two antigenically different lineages co-circulate, with one usually being dominant over the other. Surprisingly, little is known about the type and contribution of influenza B virus-specific T-cell responses in human or experimental hosts. Materials and Methods: We generated recombinant proteins comprising fusions of the conserved ectodomain of the NB protein (NBe) of influenza B virus with a leucine zipper-based tetramerizing domain resulting in NBe-tGCN4-Flag. NBe-tGCN4-Flag was used to immunize laboratory mice with different adjuvants (alum, LTR129G, water-in-oil). Antibody and T-cell responses directed against NBe were quantified by peptide ELISA, ELISPOT, intracellular cytokine staining, and in vivo killing assays. Protection studies were performed with B/Memphis/10/1997 virus and using body weight and lung virus titers as readouts. Results: NBe-tGCN4-Flag was purified from an *E. coli*–based expression system and behaved as a stable, soluble tetrameric protein. Immunization of mice with this NBe-fusion protein induced strong H2b-restricted NBe-specific cytotoxic T-cell responses, which, upon challenge, were associated with stronger influenza B virus clearance from the lung when compared with challenged control immunized mice. Such specific T-cell responses were also observed following infection of mice with influenza B virus and were restricted to the $H2^b$ haplotype (C57/Bl6 mice). These cellular responses were specific for the $CD8^+$ T-cell compartment and mapped to a highly conserved, membrane-distal T-cell epitope in NBe. In vivo killing assays confirmed the induction of strong cytotoxic activity following immunization with NBe-fusion protein. These T-cell responses could be induced by vaccination with NBe-tGCN4-Flag adjuvanted with alum, administered via the mucosal route or by using a water-in-oil adjuvant. In addition, we demonstrated that Flumist® containing B/Brisbane/60/08 virus induces NBe-specific T-cell responses in mice. Conclusions: This is the first identification of immune protection provided by the ectodomain of the NB protein and, to our knowledge, the first record of a protective $CD8^+$ T-cell epitope for influenza B in the mouse.

O-853 SCHOLARSHIP Promising Investigator Scholarship

Human cytotoxic T lymphocytes directed to seasonal influenza A viruses cross-react with the newly emerging H7N9 virus

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Background: Since the first human case of infection with the avian influenza A(H7N9) virus in China in March 2013, 132 cases have been reported, of which 37 had a fatal outcome. Due to the absence of H7N9 virus–specific antibodies in the human population, the virus may have the potential to cause a pandemic, although at present (May 2013) there is no evidence of efficient human-to-human transmission. It has been suggested that cross-reactive cell-mediated immunity may afford protection against infection with antigenically distinct influenza viruses. Virus-specific CD8+ cytotoxic T lymphocyte (CTL) responses induced after infection with seasonal H3N2 (sH3N2) and H1N1 (sH1N1) viruses or the pandemic H1N1 (pH1N1) virus are mainly directed to conserved internal proteins. Therefore, these CTLs may contribute to cross-protection against influenza A of novel subtypes, including H7N9. In this study we tested the cross-reactivity of CTLs specific for sH3N2, sH1N1, and pH1N1 viruses with the H7N9 virus. Materials and Methods: The amino acid sequence of known CTL epitopes was compared with their sequence in the H7N9 viruses, and variant epitopes that were

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identified were synthesized. Polyclonal CTL populations were obtained from HLA-typed donors (2008-2012) after *in vitro* stimulation with sH3N2, sH1N1, or pH1N1 viruses. Cross-reactivity of these T cells with the H7N9 virus was assessed by determining IFN-γ production and their capacity to lyse H7N9 virus–infected cells. Results: Sequence comparison of 20 known influenza A CTL epitopes revealed that 12 were fully conserved in the H7N9 virus, which indicates that CTLs elicited after infection with seasonal influenza A viruses are likely to cross-react with the avian H7N9 virus. Cross-reactivity of polyclonal CTL populations with four of the eight variant epitopes was assessed by IFN-γ ELISpot assay. Furthermore, the cross-reactivity with H7N9 virus–infected cells was assessed by determining the frequency of CTL producing IFN-γ or their lytic activity, which showed a large extent of cross-reactivity of CTL specific for sH3N2, sH1N1, or pH1N1. Conclusions: Apart from some individual epitopes displaying amino acid variation, CTLs elicited after sH3N2, sH1N1, and pH1N1 virus infections display considerable cross-reactivity with the avian influenza A(H7N9) virus. The pre-existing cross-reactive CTLs in the human population may afford some level of protection and may dampen the impact of a potential pandemic of the H7N9 virus.

O-854

Induction of broadly neutralizing influenza virus group 2 HA antibodies through natural infection and novel vaccination strategies

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Background: Influenza caused by pandemic and epidemic influenza virus strains remains a public health concern worldwide. Current influenza virus vaccines contain H1N1 (group 1 hemagglutinin), H3N2 (group 2 hemagglutinin), and influenza B virus components. Their narrow breadth of protection frequently renders them ineffective against the continuously drifting seasonal influenza viruses. Also, predicting the subtype of the next pandemic virus is still impossible. Indeed, the recent H7N9 outbreak in China as well as reports of novel H3N8 strains circulating among harbor seals, human infections by swine-origin H3N2 variant strains, and sporadic human infections with H4, H7, and H10 avian influenza viruses have raised concerns about future influenza virus pandemics. Hence, a vaccine with broader protective activity toward newly emergent strains would be a major improvement to the present strategy. Recently, the highly conserved stalk domain of the hemagglutinin protein has attracted increasing attention as a potential candidate for rational design of a broadly protective influenza virus vaccine. Many functional epitopes in the stalk domain of group 1 hemagglutinins have been described through characterization of a large number of broadly neutralizing stalk antibodies. However, only three stalk antibodies directed against group 2 influenza hemagglutinins have been isolated so far, providing very little information about the epitopes found in these domains. Also, very little is known about the general level of induction of these antibodies by influenza virus vaccination or infection. Materials and Methods: We performed a quantitative and qualitative characterization of the anti-stalk humoral response in the mouse model as well as in humans by using chimeric hemagglutinin constructs previously developed in our group. Furthermore, we tested the breadth of the humoral response elicited in mice by a vaccination strategy based on the stalk domain of group 2 HAs. Results: Influenza virus infection induced significant titers of stalk-reactive antibodies in both humans and mice, while immunization with inactivated influenza virus vaccines failed to do so. Furthermore, the hemagglutinin stalk-directed antibodies induced in mice and humans had broad reactivity and neutralizing activity *in vitro* and *in vivo*. We also demonstrated that a novel vaccination strategy based on the stalk domain of the H3 hemagglutinin is able to induce broadly neutralizing anti-stalk antibodies in mice that are highly cross-reactive to various H3, H7 (including the novel Chinese H7N9), H10, H14, and H15 hemagglutinins. Conclusion: These observations are relevant for the development of universal influenza virus vaccines and are encouraging, as they suggest that broadly neutralizing stalk-reactive antibodies against group 2 HAs can be induced in humans.

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O-855

Type I interferon antagonistic properties of IAV polymerase proteins determine pathogenicity

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Background: Infection with influenza A viruses (IAVs) results in an early and strong activation of cellular innate immune responses, dominated by the most prominent host antiviral defense mechanism—the type I interferon (IFN) system. To ensure efficient replication, IAVs have evolved several strategies counteracting these host immune responses. Two well-known IFN antagonists of IAVs are the NS-1 and PB1-F2 proteins. However, besides the well-characterized functions in replication and transcription of vRNA, the polymerase complex of IAVs also seems to play a crucial role in the inhibition of the immune response of host cells. In the current study, we discovered new virulence-determining amino acid positions within the viral polymerase proteins PB1 and PA, which are crucial for type I IFN inhibitory properties. Material and Methods: Specific mutant strains carrying alterations in the depicted amino acid positions of the PB1 or PA protein were created using standard reverse genetic approaches. Activation of the type I IFN response was analyzed at four different steps in the pathway: (i) analysis of the activation of interferon regulatory factor 3 (IRF3) using the pTATA-luc-4x-IRF-3 reporter gene construct; (ii) analysis of the transcriptional activity of the IFNβ promoter using the pGL-IFNβ-luc reporter gene construct; (iii) measurements of the mRNA levels of *ifnβ* and the interferon-dependent genes (ISG) *mxa*, *oas*, and *rig-I* in qRT-PCR; and (iv) analysis of phosphorylation of STAT1 in Western blot analysis, which is provoked by viral-induced and -secreted IFNα and β via their receptor IFNAR. To assess virulence and replication fitness in vivo, BALB/c, C57Bl/6 wt, and C57Bl/6 IFNAR$^{-/-}$ mice were used. Body-weight loss and lethality upon infection were monitored for 15 days. Additionally, accumulation of infectious progeny virus in the mouse lung was analyzed in a standard plaque titration assay. Results: We identified six amino acid positions within the viral polymerase proteins PB1 and PA, which are crucial for regulation of innate immune responses. Mutation of these amino acids led to dramatically reduced virulence due to an increase in type I IFN induction, as evidenced by experiments using IFNAR$^{-/-}$ mice. In addition, our data show clear differences in the induction of the type I IFN response in human lung epithelium, but no differences in the accumulation of viral RNA, suggesting an interplay of the viral polymerase proteins and virulence regulation on the level of type I IFN inhibition. The interferon antagonistic properties of these virulence-determining amino acids were also verified in the H5N1 virus system. Moreover, deeper analysis of the impact of the depicted sites in PB1 and PA suggested a corresponding interplay of the identified amino acids within these two proteins, leading to a strong reduction in replication. In summary, our data revealed the molecular basis of the interferon antagonistic properties of the viral polymerase proteins PB1 and PA—a function that contributes to the virulence of IAV. Conclusions: In the current study, we identified six amino acid positions within the polymerase proteins PB1 and PA that strongly affect virulence and significantly inhibit the host type I IFN response. These data shed new light on viral strategies for counteracting the host type I IFN response, since PB1 and PA—like NS1 and PB1-F2—seem to be part of the type I IFN counteracting strategy evolved by IAVs.

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O-856 SCHOLARSHIP International Research Scholarship

Protection against influenza infection was associated with neutralizing antibodies detected in the absence of HI antibodies for pandemic H1N1 but not for H3N2 in a natural infection cohort

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Background: Defining components of natural acquired immunity could provide insights into the development of cross-protective or universal influenza vaccines. We have been conducting an influenza cohort study since 2007 in a community that has never had influenza vaccination and found that haemagglutination inhibition (HI) antibody titer is associated with protection against H3N2 but not H1N1 infection. In this study we compared pre-pandemic serum from influenza-infected versus likely exposed but non-infected community cohort members using assays to detect neutralizing antibodies. Materials and Methods: The Ha Nam community cohort comprises 270 households under active ILI surveillance. Blood is collected before and after each peak in RT-PCR–confirmed influenza transmission for HI assay. Seroconversion is defined as a four-fold or greater rise in HI titer. Participants with an RT-PCR–positive swab sample and/or seroconversion are considered to be infected. Households with at least one symptomatic influenza case or 2 asymptomatic cases were selected for further assessment of sera from infected versus non-infected but likely exposed community members. Pre- and post-transmission and some acute sera were tested at dilutions from 1:5 to 1:640 by microneutralization (MN) assay. Results: A total of 110 members of 32 households were eligible for investigation of pre-pandemic H1N1 2009 sera, of whom 71 were infected and 39 were non-infected. The median (IQR) age of the infected population was 14 (8-33) years compared with 36 (27-44) for the non-infected population. The majority of children were infected: half of the mothers and 30% of the fathers. MN titers in pre-pandemic sera were above 5 in 14 participants, including 12 that were not infected, and geometric mean titers were significantly higher in non-infected compared with infected persons in univariate analysis (P = .002) and in a multivariate analysis including age, relationship, sex, and HI titer (P = .008). Twenty percent of participants who MN converted did not HI convert, including 5 with PCR-confirmed infection, and titer increases were lower for HI compared with MN (r^2 = 0.682). The non-HI converters were significantly older (P = .004). A total of 54 members from 19 houses were eligible for investigation of sera from the pre-H3N2 season; 33 were infected and 21 were not infected, both groups had a median ages of about 30 years. A substantial proportion had pre-season HI and MN titers of 20 or more, and both were associated with infection in univariate analysis, whereas only the HI titer was associated with infection in multivariate analysis. H3N2 HI and MN conversion was concordant, and titer changes were very similar (r^2 = 0.826). Conclusions: H1N1 reactive antibodies that can be detected by MN but not HI assay were found in both pre- and post-pandemic sera and may account for some of the protection against novel H1N1 infection that is associated with increasing age and prior H1N1 infection, whereas protection against H3N2 infection was largely due to HI antibody with no clear detection or influence of non-HI neutralizing antibody. Further investigations are planned to characterize the extent of cross-reactivity, isotype, and epitopes and the conditions required for induction of these antibodies.

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Inhibition of cellular p38 MAP kinase impairs influenza virus–induced primary and secondary host gene responses, thereby protecting mice from lethal H5N1 infection

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Background: MAP kinases are major signal transducers in the cell. All four thus far known MAP kinases (ERKs, JNKs, p38 MAPKs, ERK5) are activated by influenza A virus (IAV) infection. While the JNK MAPK pathway promotes the antiviral cytokine response, we have shown previously that the MEK/ERK MAPK pathway is exploited by IAV to support viral RNP export. Accordingly, we have demonstrated that inhibition of this signaling cascade blocks virus replication in vitro and in the animal model, representing a suitable target for a novel antiviral strategy. Materials and methods: Here we have investigated the role of p38 MAPK in the antiviral response against IAV in cells and animals. Results: Global gene expression profiling of HPAIV-infected cells in the presence of the p38 inhibitor SB202190 revealed that inhibition of p38 leads to reduced expression of type I interferons (IFNs) and other cytokines after H5N1 and H7N7 infection. More than 90% of all virus-induced genes were either partially or fully dependent on p38. This was confirmed by siRNA-mediated knock down of p38 isoforms or expression of a dominant negative mutant of the p38 upstream kinase MKK6. The broad impact of p38 could be attributed to the fact that inhibition of the kinase not only affects primary gene expression responses to infection but also impairs the secondary gene expression response by interference with the JAK/STAT pathway. Specifically, p38 coregulates NF-κB and AP1 transcription factors in the initial response to accumulation of 5´PPP vRNA. In the secondary response to virus-induced IFNs, p38 specifically phosphorylates STAT1 at serine 727 and thereby enhances STAT1 transcriptional activity. *In vivo* inhibition of p38 leads to a nearly complete shutdown of virus-induced cytokine expression, thereby protecting mice from lethal H5N1 IAV infection. These observations show that p38 acts on two levels of the antiviral type I IFN response: initially the kinase regulates IFN induction and later p38 controls IFN signaling by STAT phosphorylation and thereby promotes expression of IFN-stimulated genes. Conclusion: In summary, these data show for the first time that p38 is a crucial regulator of the innate cytokine and type I IFN responses and that this broad suppression of the virus-induced cytokine burst by p38 inhibition is sufficient to protect mice from lethal influenza virus infection.

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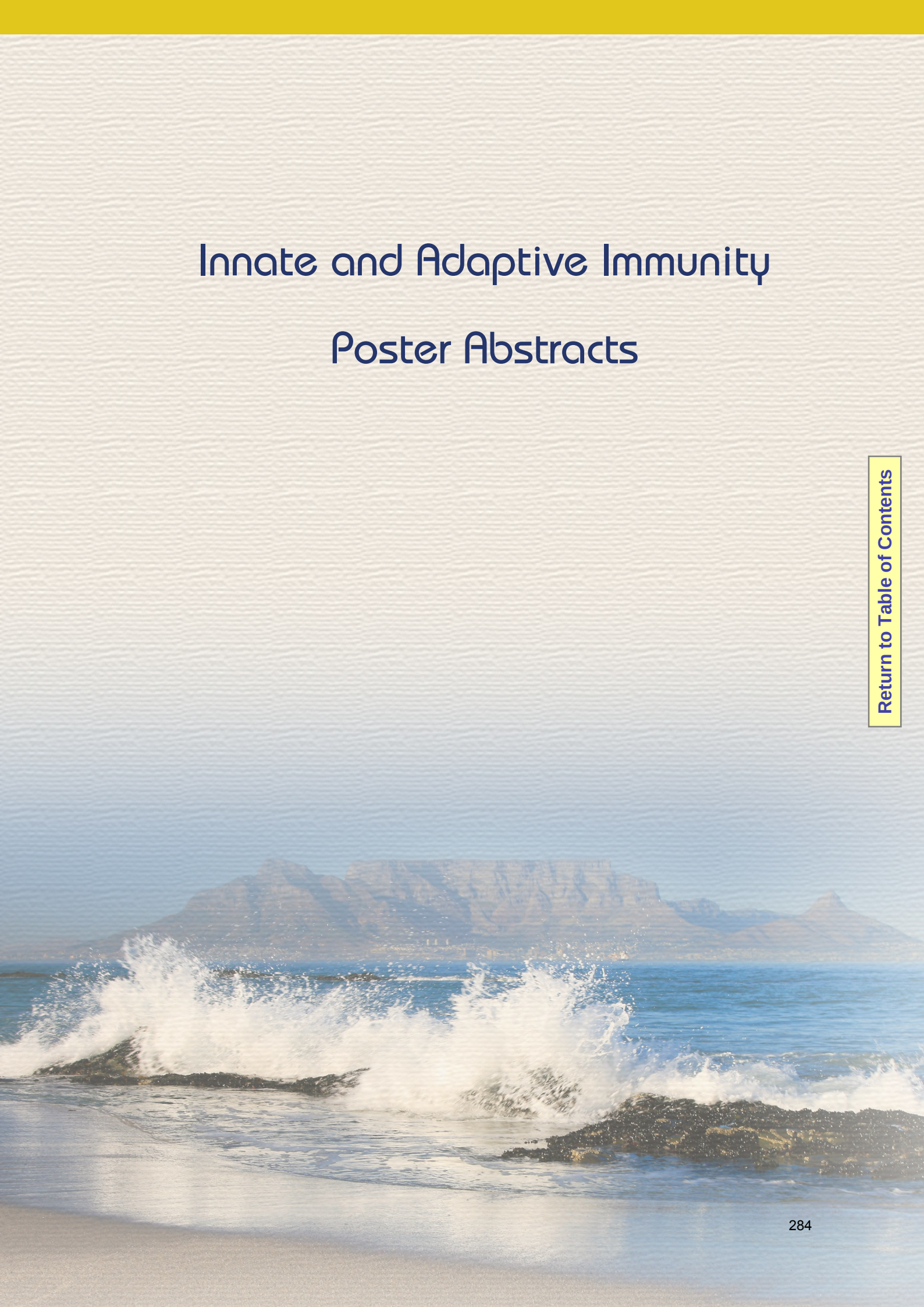
Innate and Adaptive Immunity

Poster Abstracts



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Local antibody immune response to seasonal, pandemic and potentially pandemic (avian) live attenuated influenza vaccines in human nasal swab and saliva samples

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Background: Local immunity of upper airways is a key factor of protection against influenza viruses. In contrast with inactivated influenza vaccines, which stimulate mainly serum antibody response, live attenuated influenza vaccines (LAIVs) induce pronounced mucosal antibody immunity, so local immunity studies are of great importance in the development of LAIV immunogenicity criteria. For this reason, detailed analysis of local antibody production to different vaccine strains is required. Local immune responses can be assessed by quantification of virus-specific IgA in nasal swabs. Saliva is a promising material for anti-influenza immunity studies due to the possibility of very easy and rapid saliva collection using simple non-invasive technique. Our previous studies showed a direct correlation between anti-influenza protection and IgA levels in both nasal swabs and saliva. However, more extended studies of salivary antibody response to LAIVs are necessary as is an analysis of the interaction of antibody levels in saliva, nasal swabs and serum. The objective of this work was to study and compare local immune responses to seasonal, pandemic and potentially pandemic (avian) LAIVs in human nasal swab and saliva samples. Materials and Methods: Trivalent seasonal LAIVs and monovalent A/17/California/2009/38 (H1N1), A/17/Duck/Potsdam/86/92 (H5N2), A/17/Mallard/Netherlands/00/95 (H7N3) and A/17/Turkey/Turkey/05/133 (H5N2) LAIVs were used for single-dose (for seasonal) or two-dose (for monovalent) intranasal vaccination of young healthy volunteers. Nasal swab, saliva and serum samples were collected before vaccination, 28 days after vaccination and 28 days after revaccination. Serum virus-specific antibody titers were measured using HAI (haemagglutination inhibition) assay. Local virus-specific IgA levels in nasal swab and saliva samples were estimated by ELISA. A urea test was used for an antibody avidity study. Total protein was measured with a BCA assay. Results: Analysis of nasal swab samples from volunteers vaccinated with *seasonal* LAIVs showed a reduction in the percentage of vaccine-induced antibody conversions during the last 10 years. At the same time, geometric mean titers of local preexisting antibodies before vaccination rose progressively from year to year. However, the percentage of vaccine-induced antibody conversions in persons with low background antibody titers remained high: 43% – 88%. Preexisting levels of local antibodies to A(H1N1)pdm2009 and avian strains A (H5N2) and A (H7N3) were low or absent. In some volunteers, cross-reactive antibodies to A(H1N1)pdm2009 were detected in nasal swab, saliva and serum samples. For avian strains only, local preexisting cross-reactive antibodies were observed. After two-dose vaccination with A(H1N1)pdm2009 IgA, conversions were observed in 54% of nasal swab and 38% of saliva samples. Avian strains induced 20% – 54% of conversions in nasal swab and 20% – 38% in saliva samples. Geometric mean antibody titers increased after both first and second vaccinations for avian strains and only after the first dose for A(H1N1)pdm2009. LAIVs were also able to magnify local antibody avidity. Preexisting immunity was shown to significantly affect local immune response intensity to both seasonal and monovalent LAIVs. Inverse correlation was observed between antibody levels before and after vaccination for both nasal swabs and saliva. Results obtained with non-invasive techniques immunogenicity matched those obtained by HAI assay in 40% – 67% of cases and were able to provide additional information about LAIV immunogenicity. Conclusions: Seasonal, pandemic and avian LAIVs were able to induce marked antibody production in nasal swab and saliva samples of young healthy volunteers. Local immune responses in both nasal swab and saliva samples depended on preexisting antibody levels and were most evident in volunteers with low preexisting antibody titers. There are low levels of cross-reactive local antibodies in humans that can partially neutralize avian viruses. Non-invasive techniques of LAIV immunogenicity estimation provide additional information to serum antibody tests. Saliva is a promising material easily accessed for evaluation of antibody production and for rapid measurements of preexisting antibody levels before vaccination.

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Cross-reactive antineuraminidase antibodies against pandemic A(H1N1) and avian A(H5N1) influenza in sera of healthy volunteers

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Background: Humoral immunity induced by influenza virus neuraminidase (NA) can reduce viral shedding and decrease the severity of influenza illness. NA-specific antibodies have been previously demonstrated to provide cross-protection against viruses of the same NA-serotype in animal challenge experiments. These antibodies are believed to be of particular importance in cases of possible new antigenic shift of influenza virus hemagglutinin (HA). However, limited information is available on the presence of cross-reactive antineuraminidase antibodies against pandemic and potentially pandemic strains in humans. Materials and Methods: In the present study we examined serum antibodies cross-neutralizing NA activity of pandemic virus A/California/07/09(H1N1) (H1N1/2009pdm) among volunteers 1-82 years old before and 1 year after pandemic strain appearance. The role of prior seasonal influenza A(H1N1) infections in induction of NA-specific antibodies against H1N1/2009pdm was studied. We also evaluated preexisting serum antibodies against avian influenza A/Vietnam/1203/04(H5N1) (H5N1/2004) among healthy people aged 1-91 years, examined between 2005 and 2011. A total of 465 serum samples collected in the fall of 2005, 2010, and 2011 were analyzed using a peroxidase-linked lectin micro-procedure previously reported by Lambre et al. To avoid non-specific inhibition in the enzyme-linked lectin NA inhibition assay, A(H7N1) reassortant influenza strains containing A/equine/Prague/1/56(H7N7) HA and NA of H1N1/2009pdm or H5N1/2004 virus were generated and used to establish N1-specific titers. Results: Before pandemic strain appearance, in 2005, preexisting NA-inhibiting (NI) anti-H1N1/2009pdm antibodies in titers > 1:20 were detected among 7.1% (4 of 56) of volunteers aged 18-20 years; only one person demonstrated NI antibody titer against H1N1/2009pdm higher than 1:40. A year after the pandemic A(H1N1) virus first emerged in Russia, 27.0% (44 of 163) of volunteers aged 1-82 years expressed NI anti-H1N1/2009pdm antibodies in titers ≥ 1:40. To determine a possible origin of revealed NI antibodies against H1N1/2009pdm, we separated volunteers into several groups on the basis of HA-inhibiting (HI) serum antibody levels against A(H1N1) strains circulated between 1934 and 2009. Volunteers presumably exposed to seasonal A(H1N1) viruses according to HI assay but with low (≤ 1:10) HI anti-H1N1/2009pdm titers had a 2.1-fold higher mean NI antibody titer against H1N1/2009pdm than subjects unexposed and completely seronegative to various seasonal and pandemic A(H1N1) strains ($P < .05$). Our data suggest that exposure to seasonal A(H1N1) viruses may have contributed to the induction of NI antibodies against H1N1/2009pdm. Since homology between amino acid sequences of H5N1/2004 and H1N1/2009pdm NAs is higher than between avian A(H5N1) strain and epidemic A(H1N1) viruses circulating until 2008 (87.9% vs 82.6%), we aimed to reveal changes in levels of preexisting NI antibodies against H5N1/2004 caused by H1N1/2009pdm-like strains. According to NI assay, the mean antibody titer against H5N1/2004 virus among volunteers aged 18-20 years had increased 2.9-fold over the period from 2005 to 2011 ($P = .04$). In 2005, only 7.1% (4 of 56) of young people had antibodies of this specificity in titers ≥ 1:40, while in 2011 the percentage was more than 4-fold higher ($P = .015$): 30.8% (8 of 26). In general, 17.9% (95% CI, 13.6-23.2%) of 246 volunteers of a wide age range examined in the fall of 2011 had pre-existing NI antibodies against A(H5N1) influenza virus in titers ≥ 1:40. The mean NI anti-H5N1/2004 antibody titer among subjects who were seropositive to H1N1/2009pdm, according to NI assay, was 4.5-fold higher than that among seronegative volunteers (1:22.3 vs 1:5.0; $P < .0001$). The results of this study suggest that H1N1/2009pdm appearance and circulation have led to increase in titers of NI antibodies cross-reacting with potentially pandemic avian influenza A(H5N1) due to presumable common NA epitopes. Conclusions: Existing cross-reactive NI antibodies against highly pathogenic avian influenza A(H5N1) in sera of some part of human population alone or in association with another correlate of immune cross-subtype protection may moderate the severity of a possible pandemic or explain the relative insusceptibility to infection of some age groups.

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Cell-mediated and antibody responses to MF59®-adjuvanted and non-adjuvanted seasonal influenza vaccines in children under three years of age

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Background: Annual seasonal influenza epidemics result in around 3 to 5 million cases of severe illness and around 250,000 to 500,000 deaths worldwide. Influenza infection is especially dangerous for individuals at higher risk for complications of influenza disease, such as the very young, the elderly, or chronically ill individuals, where infection may result in hospitalization and death. Nonadjuvanted, trivalent, seasonal influenza vaccines (TIV) are suboptimal in young children and infants. This phase 2, randomized clinical trial was conducted (between May 2011 and February 2012 across 2 study sites in Belgium) to evaluate cell-mediated and antibody responses to an MF59®-adjuvanted (Novartis Vaccines and Diagnostics), trivalent, influenza vaccine (ATIV) compared with TIV in children under 3 years of age. Materials and Methods: Previously nonvaccinated children (6 to < 36 months) received 2 doses of either ATIV (n = 43) or TIV (n = 41) 4 weeks apart (days 1 and 29). The antigen content of ATIV and TIV were identical; one 0.25 mL vaccine dose contained ≈ 7.5 µg of haemagglutinin surface antigen from each of the 3 strains, A/California/7/2009 (H1N1), A/Perth/16/2009 (H3N2), and B/Brisbane/60/2008. Blood samples were collected prevaccination (day 1) and 3 weeks after administration of the second dose (day 50) for immunogenicity analyses. Purified peripheral blood mononuclear cells were restimulated overnight in vitro with either A/H1N1, A/H3N2, or B strain subunit antigens, then permeabilized, stained intracellularly for cytokine, and analysed by flow cytometry. Antibody titres were measured by haemagglutination inhibition (HI) assay and assessed according to European licensure criteria. Solicited local and systemic adverse events were recorded for 7 days after each vaccination. Unsolicited adverse events were recorded throughout the entire study period (day 1-50). Results: In agreement with the results of previous studies in this age group, the most commonly reported solicited local adverse event after the first vaccine dose was tenderness at the site of injection. There were no reports of severe local reactions in response to ATIV or TIV. The frequencies of systemic reactions were similar in both ATIV and TIV groups. No serious adverse events (SAEs) occurred in the ATIV group, whereas 2 children in the TIV group experienced a SAE. No cases of high fever (≥ 40°C) occurred in ATIV recipients after the first or second doses; 3 subjects in the TIV group experienced high fever after the first dose, and 1 subject after the second dose. Both vaccines induced strong increases in HI antibody titres. Geometric mean antibody titres (GMTs) against the 3 viral strains were much higher in response to ATIV than TIV. All 3 of the European (CHMP) licensure criteria were met against both A strains in both vaccine groups. Against the B strain, all criteria were met for the ATIV group, while the criterion for seroprotection against the B strain was not met for the nonadjuvanted TIV group. Higher cross-reactive antibody responses against heterologous A/H3N2 and B strains were observed in response to ATIV compared with TIV. Both ATIV and TIV induced an increase in influenza antigen-specific T cells. Overall, the cytokine secretion profiles of $CD4^+$ T cells in response to ATIV and TIV were similar and responses were dominated by IL-2 and TNF-α. IL-13 was not detected, demonstrating that MF59 adjuvantation did not result in any Th2-type bias. Conclusions: These data demonstrated enhanced immunogenicity of the ATIV, Fluad® (Novartis Vaccines and Diagnostics) compared with nonadjuvanted TIV in children under 3 years of age. The MF59-adjuvanted vaccine was well tolerated, and induced higher antibody titres and $CD4^+$ T cell responses compared with TIV. Differences between antibody responses to ATIV and TIV were more pronounced against the heterologous strains. The presence of MF59 did not affect the quality of the cellular response to influenza antigen, nor did MF59 bias the immune response towards a Th2 functional phenotype.

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Effect of ts mutations in polymerase genes of influenza A virus on its virulence, immunogenicity, and protective efficacy in a mouse model

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Background: Live attenuated influenza vaccines (LAIVs) are administered intranasally and provide protection and significant mucosal and cell-mediated immune responses, mainly due to their ability to mimic natural infection. Vaccine strains for LAIVs have cold-adapted (ca), temperature-sensitive (ts), and attenuated phenotypes that are guaranteed by the presence of specific mutations from master donor virus (MDV) in their internal genes. Mutations responsible for ts/ca phenotype of Russian MDV A/Leningrad/134/17/57 (H2N2) were recently established, but the impact of these mutations on the width and intensity of vaccine-induced immune responses has been poorly studied. Materials and Methods: We used a number of reverse genetics–derived mutant viruses based on virulent virus A/PR/8/34, which contained different combinations of one or more ts/ca mutations specific for MDV: PB2–V478L; PB1–K265N,V591I; and PA–L28P,V341L. Groups of female CBA mice were infected intranasally with one dose of 100 MID_{50} of tested mutant viruses. Infectious viral titers were determined 3 dpi in mouse lung and nasal turbinates to assess the virulent potential of the mutants. Organ homogenates were titrated in chicken embryos by limited dilutions and expressed as $log_{10}EID_{50}$/ml. Antibodies against whole A/PR/8/34 virus in mouse sera and upper respiratory tract washes were analyzed at 7 and 28 dpi by HAI assay and/or ELISA. To assess protective efficacy of the studied mutants, mice were challenged with 100 LD_{50} of A/PR/8/34 virus 30 dpi. Survival rate, weight loss, and the reduction of virus titers in lungs compared with the mock-immunized group were the measures of protection. Results: The ts phenotype of the mutant viruses, established in our previous study, did not always correlate with their virulence in mice. Thus, mutations in the PA gene did not alter the ability of the virus to replicate at high temperatures, but addition of the mutations in the PB2 and/or PB1 genes slightly reduced the virus titer in mouse lung. PB2-V478L had the greatest impact on virus attenuation, followed by PB1–K265N and PA–L28P,V341L. Addition of a single PB2 mutation reduced the virus titer in lung by 2.3 log_{10}, and subsequent inclusion of the PB1–K265N mutation additionally reduced the lung titer by 1.7 log_{10}. Inclusion of all five mutations in polymerase genes of A/PR/8/34 resulted in fully attenuated virus with a lung titer as low as 1.7 log_{10}. Study of the humoral immune responses revealed a reduction in HAI antibody titers of 28 dpi after inclusion of a single PB2 mutation (GMT value: 63.5 vs 142.5 for A/PR/8/34 virus). Sequential addition of mutations in the PB1 and PA genes showed a stepwise decrease in HAI titers, and we found good correlation between virus attenuation (ie, virus titers in lung) and the level of serum HAI antibody titers. In general, this correlation was confirmed by ELISA of serum virus-specific IgG and IgA antibody levels. Thus, the mutant virus bearing all five mutations was the most attenuated for mice from one side, but from the other side it induced the lowest levels of anti-virus serum antibody titers. However, when two mutations in the PA gene were excluded from this mutant, the virus was significantly more immunogenic, but still attenuated for mice. Interestingly, immunization with all mutant viruses protected mice from subsequent challenge, despite the variations in their serum antibody levels. Mice survived the challenge and did not exhibit weight loss, despite the fact that some virus titer was detected in mouse lungs from groups with low antibody levels. These data support previous findings that the protection induced by LAIVs is mediated by a wide range of factors, not only by serum antibodies. Conclusions: In summary, we showed that individual mutations in polymerase genes of influenza A virus are involved and/or responsible for changes in virus virulence and in humoral adaptive immune responses. In addition, we demonstrated that it may be possibility to modify the immunogenic potential of LAIV strains by varying the combinations of tested mutations in order to improve vaccine efficacy. This work was supported by RFBR Grant №12-04-31364.

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A longitudinal analysis reveals an increase in neutralizing antibodies to both previously encountered seasonal as well as pandemic influenza A virus strains

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Background: Antigenic diversity shapes humoral immunity in unique and unexpected ways. This is particularly true of the antibody response generated against influenza A viruses. While it is known that immunologic memory developed against previously encountered influenza A virus strains impacts the outcome of subsequent infections, exactly how sequential exposures to antigenically variant viruses shape the humoral immune response in humans remains poorly understood. Materials and Methods: To address this important question, a longitudinal analysis of antibody titers against various pandemic and seasonal strains of influenza virus spanning a 20-year period (1987-2008) was performed using samples from 40 individuals obtained from the Framingham Heart Study. Results: Longitudinal increases in neutralizing antibody titers were observed against previously encountered pandemic H2N2, H3N2 and H1N1 influenza virus strains. Antibody titers against seasonal strains encountered later in life also increased longitudinally at a rate similar to that against their pandemic predecessors. Titers of cross-reactive antibodies specific to the hemagglutinin stalk domain were also investigated, as they are known to be influenced by exposure to antigenic diversity. These titers rose modestly over time, even in the absence of major antigenic shifts. No sustained increase in neutralizing antibody titers against an antigenically more stable virus (human cytomegalovirus) was observed. Conclusion: The results herein describe a unique role for antigenic variation in shaping the humoral immune compartment, and provide a rational basis for the hierarchical nature of antibody titers against influenza A viruses in humans.

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Chicken IFITM3 restricts influenza viruses and lyssaviruses *in vitro*

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Background: Interferon inducible transmembrane protein 3 (IFITM3) is an effector protein of the innate immune system that confers potent, cell intrinsic resistance to infection by diverse enveloped and non-enveloped viruses both in vitro and in vivo, including influenza viruses, West Nile virus, dengue virus and reovirus. IFITM3 prevents cytosolic entry of these viruses by blocking complete virus fusion with cell endosome membranes. Materials and Methods: Assessment of avian IFITM3 was initially carried out in a stable over-expression system using HA-tagged proteins in human A549 cells. The stable cell lines were established using lentiviruses produced by a triple plasmid transfection in HEK293T cells. Endogenous expression was subsequently assessed by qRT-PCR in a range of chicken tissues, and viral restriction was tested by flow cytometry in chicken DF-1 fibroblast cells. Results: Although the IFITM locus, which includes *IFITM1, 2, 3 and 5*, is present in mammalian species, this locus has not been unambiguously identified or functionally characterised in avian species. Here we showed that the IFITM locus exists in chickens and is syntenic with the IFITM locus in mammals. Using lentiviruses expressing IFITM genes, we obtained human cell lines expressing the IFITM proteins with a C-terminal HA tag. Using this model we showed that the chicken IFITM3 protein

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restricts cell infection by influenza A viruses and lyssaviruses to a level similar to that of its human orthologue. Furthermore, we showed that chicken IFITM3 is inducible in chicken DF-1 cells by quantitative RT-PCR and that knock-down of constitutive expression in chicken fibroblasts results in enhanced infection by influenza A virus. Chicken *IFITM2* and *3* are constitutively expressed in all tissues examined, whereas *IFITM1* is expressed only in the bursa of Fabricius, gastrointestinal tract, caecal tonsil and trachea. Conclusions: Despite being highly divergent at the amino acid level, IFITM3 proteins of birds and mammals can restrict replication of viruses that are able to infect different host species, suggesting that IFITM proteins may provide a crucial barrier for zoonotic infections.

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Outcome of 2009 pandemic H1N1 virus infection in pregnant ferret model

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Background: Pregnant woman are at increased risk of mortality and morbidity due to influenza virus infections, as highlighted during the 2009 pandemic. The mechanisms that affect influenza virus infection outcome in this population, however, are not well defined. In the present study, we investigated the consequence of 2009 pandemic H1N1 influenza virus (H1N1pdm09) infection during pregnancy using the ferret model. Materials and Methods: To understand the outcome of infection during early gestation, we analyzed viral shedding and histopathological and immunological changes in pregnant ferrets infected by H1N1pdm09. Results: We found quantifiable differences in viral titers in the respiratory tracts of infected pregnant and non-pregnant ferrets, with higher viral loads detected in the former. We observed that H1N1pdm09-infected pregnant ferrets had elevated levels of numerous cytokines in the upper respiratory tract, while no significant changes in cytokine levels were found in the lung or spleen tissue. Interestingly, the total numbers of $CD8^+$ peripheral blood T lymphocytes were significantly lower in pregnant ferrets than in non-pregnant ferrets following infection. Similarly, influenza-specific B-cell ELISPOT responses in H1N1pdm09-infected pregnant ferrets were lower than those of non-pregnant ferrets. Conclusions: These results suggest that pregnancy alters the immune response, which in turn increases susceptibility to an influenza infection during pregnancy.

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Study of the innate immunity in chickens infected with low pathogenic avian influenza viruses of different subtypes

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Background: Today, it is important to study innate immunity in birds infected with various infections (bacterial, viral, parasitic) to elucidate the mechanisms of natural protection that will allow developing more effective means of specific prevention and treatment. The objective of our research was to study the natural immune response in chickens infected with low pathogenic "wild" avian influenza viruses (LPAIV). The second objective of our study was to investigate the immunostimulatory effect of the phytonutrient Vitastim on the immune response in birds after infection with LPAIV. Materials and

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methods: In our study, we used LPAIV H5N2, H4N6, H13N5, H6N4 isolated from wild birds in Ukraine. Infection was introduced intranasally in the dose of $10^{6,0}$-$10^{6,5}$ EID_{50}. On days 1, 3, 5, 7, 10, 14 postinfection, we studied clinical state, the presence of histopathologic changes, the presence of the virus in the inner organs, the level of antibodies in the serum as well as the level of cell immunity. We investigated the immune response in chickens after infection with LPAIV followed by administration of Vitastim added in their drinking water using labeled streptavidin-biotin method (LSAB). Results: Low pathogenic "wild" influenza viruses did not cause clinical disease in birds during 14 days. There was no virus detected in inner organs. Specific antibodies were found in the serum of 20% to 40% of infected birds in 7-14 days after infection in low titers by ELISA and HAI. Suppression of immune response on CD4, IgM, IgG, IgA, macrophages was registered during first 5 days. A sharp increase of activity of these cells was observed later on starting from the seventh day and till the last day of investigation ending on 21st day. At the same time their level in chickens infected with the low pathogenic avian influenza virus was higher than in intact bird. The same situation was observed in lungs. However, the results of studying CD4 showed depressed state during all period of observations. The state of cell immunity in spleen of the infected birds as compared with the control group of birds in terms of CD4 and macrophages was more active than the state of humoral immunity. As far as the lungs were concerned, unlike the spleen, the former demonstrated the state of humoral immunity in terms of IgM, IgG, IgA levels was more active. Increase of the number of macrophages was registered in earlier stages than in other subpopulations that is on the third day through the seventh day. Percentage of all investigated cell subpopulations in caeca and trachea of infected chickens was higher than that in healthy birds. Though suppression of immune response was registered on the first day, nevertheless, the cell level immunity in infected chickens was higher starting from the first day. It was determined that Vitastim had a more substantial effect on humoral immune response for norm and for LPAI (caeca, trachea, lungs), which was an evidence of a more intensive formation and accumulation of B-lymphocytes that produce immunoglobulins. When studying spleen amplified proliferation of T-lymphocytes, macrophages was determined, which is characteristic of the activation of cell immune response. The investigation of 7 subpopulations of immune cells allows determination of immune response at the stage of early direct influence on pathogen—CD8, at the stage of cooperation and transmission of antigenic products—CD4, at the stage of its processing and presentation—macrophages and IgG-, IgM-, and IgA-producing cells. Conclusion: As can be seen from above low pathogenic wild viruses isolated from wild birds in Ukraine did not cause clinical disease in chickens, but at the same time, we registered an immune response at the level of cell and humoral immunity. The findings suggest the need for further research. We have established immunostimulating effect of Vitastim that can be recommended for application in order to stimulate the immune response of poultry.

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Cellular and humoral immune responses to a high-dose influenza vaccine

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Background: Immunesenescence, or immunologic changes associated with aging, increases the risk of serious complications of influenza infection in older adults and decreases the response to vaccination. Internal viral proteins in the split-virus influenza vaccines are key proteins that will likely induce or recall T-cell responses. We investigated whether the new high-dose split-virus vaccine stimulated both higher antibody responses and greater cellular responses than the standard-dose split-virus vaccine. Materials and Methods: We conducted a double-blind, randomized controlled trial to compare the immune responses of high-dose with standard-dose influenza vaccine in adults ≥ 65 years during the fall of 2010. Subjects had blood drawn on days 0, 7, 14, and 28 post-vaccination for both peripheral blood mononuclear cells (PBMCs) and for serum for hemagglutination inhibition (HAI) testing. A randomly selected subset of PBMC samples underwent testing. To assess antigen (Ag)-specific T-cell responses, PBMCs were infected with viruses at a multiplicity of infection of 1 for 1 hr, followed by viral neutralization and overnight incubation. Cells were then treated with Brefeldin A (10

µg/ml) for 5 to 6 hours prior to antibody staining. Cells were first stained with Dead Cell Stain dye and then surface stained with fluorochrome-conjugated CD4, CD8, and CD69 and permeabilized for intracellular staining of the cytokines TNF-α, IL-2, and IFN-γ for flow cytometric analysis. T-cells studies were restricted to influenza A due to difficulty growing influenza B. The number of plasmablasts was assessed by staining cells with fluorochrome-conjugated CD3, CD20, IgD, CD27, and CD38. The number of Ag-specific plasmablasts (IgG+ or IgM+) derived from pre-existing memory B cells was measured by Ag-specific ELISPOT assay. PBMCs (10-15 x 10^5 cells/well) were plated onto ELISPOT plates coated with either 5 µg/mL anti-human IgG or IgM or 10 µg/mL of monovalent influenza vaccines. Spot-forming units were counted using ImmunoSpot® and were expressed as % Ag-specific IgG or IgM B cells out of the total IgG- or IgM-secreting B cells. Results: During the fall of 2010, 105 adults aged ≥ 65 years were randomized to receive either high-dose or standard-dose seasonal trivalent split-virus influenza vaccine. Sera were collected from 103 subjects for HAI testing, and 48 subjects were randomly selected for collection of PBMCs to evaluate cellular immunity. Seroprotection (HAI titer ≥ 40) values against 3 vaccine strains (A[H1N1]pdm09, A[H3N2], and B) at day 28 were 80%, 82%, and 80%, respectively, among those who received high-dose vaccine compared with 57%, 45%, and 61% ($P < .05$), respectively, for those who received the standard dose. Of the samples from the 48 participants tested for cellular immune response, there were no differences in age, sex, or underlying health problems among those who received high-dose (n = 26) vs standard-dose (n = 22) vaccine. Percentages of activated T helper cells (CD4+CD69+) at day 28 for H1N1 and H3N2 viruses were 51.4 ± 18.3 and 40.9 ± 17.7, respectively, in the high-dose group and 54.1 ± 17.5 and 44.0 ± 16.9 in the standard-dose group. Percentages of activated T-cytotoxic cells (CD8+CD69+) at day 28 for H1N1 and H3N2, respectively, were 71.4 ± 17.4 and 61.1 ± 17.3 in the high-dose group and 73.5 ± 12.5 and 64.4 ± 14.2 in the standard-dose group. Pre-vaccination response to stimulation was the only significant predictor of activity of activated polyfunctional T cells—ie, cells that secrete more than one cytokine (IFN-γ, IL-2, or TNF-α) ($P < .0001$)—in a model that included age and vaccine type. The percentage of plasmablasts at 7 days post-vaccination was higher in the high-dose group than in the standard-dose group (0.350 ± 0.291 vs 0.142 ± 0.105; $P = .03$). IgG+ plasmablasts for influenza A(H1N1) and IgM+ plasmablasts for both influenza A(H1N1) and H3N2 were more frequently found in the high-dose group compared with the standard-dose group ($P < .05$). Conclusions: High-dose vaccine resulted in higher rates of seroprotection for all three vaccine strains and increases in the percentage of Ag-specific plasmablasts. Pre-vaccination response to stimulation strongly predicted T-cell immune response to influenza vaccination. However, we found no significant differences in T-cell immune responses between high-dose and standard-dose vaccines.

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Phospholipase D inhibition alters intracellular trafficking kinetics and protects against productive influenza infection

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Background: Despite advances in understanding influenza virus pathogenesis and replication, new antiviral therapeutics remain elusive. Clinically useful inhibitors of virus-specific processes can rapidly lose efficacy because of the virus' unparalleled ability to mutate the target. The host cell lipid metabolic response to infection provides a potential target to block viral entry and restrict infectivity. Here we demonstrate that phospholipase D (PLD) catalytic activity is initiated during entry of influenza viruses, and chemical inhibitors of this enzyme are highly effective at protection against the pathologic consequences of infection. Focusing on virus-dependent host cell processes expands the inventory of therapeutic targets and reduces the likelihood of viral resistance. Materials and Methods: A549 cells

were used for all in vitro experiments. The PLD2-selective inhibitor VU0364739 was always used at 10 µM, and cells were pretreated for 1 hour before infection. Viruses were used at indicated MOI, and at 0.01 MOI for trafficking experiments. Lipids were extracted and analyzed by LC/MS. Microscopy data were analyzed using Nikon Elements and ImageJ. All data were analyzed using GraphPad Prism. Results: To assess if influenza infection can stimulate PLD activity, A549 cells were cultured in the presence of a primary alcohol, n-butanol. PLD will use n-butanol to make phosphotidylbutanol. Six hours after infection with A/California/04/2009 (H1N1), we observed a more than two-fold increase in phosphotidylbutanol levels compared with uninfected cells, indicating that PLD activity increases during influenza infection. RNAi was used to knockdown each isotype of PLD. By counting the number of infected cells in culture, we determined that knockdown of PLD2 led to more potent protection from A/Brisbane/59/2007 (H1N1). Using traditional virologic assays, A549 cells were drugged with 10-µM VU0364739 and/or 1-µM oseltamivir carboxylate 1 hour before infection with 0.01 or 5 MOI A/Brisbane/10/2007 (H3N2), and supernatant was titrated using $TCID_{50}$ assay. Regardless of MOI, PLD2 inhibition resulted in a dramatic decrease in infectious particle production. Additionally, PLD2 inhibition was more efficacious than oseltamivir carboxylate treatment, and the two treatments may have some synergistic effect during a low MOI infection. Knowing that proper PLD function is important in many receptor recycling models, we hypothesized that inhibiting PLD would alter endocytosis kinetics. We measured the accumulation of endosome trafficking proteins during 0.01 MOI A/California/04/2009 (H1N1). Less clathrin accumulated 50 and 80 minutes after infection when PLD2 was inhibited. In cells treated with VU0364739, early endosomal marker Rab5 and late endosome marker CD63 also accumulated to much lower levels 30 and 90 minutes after infection, respectively. The innate immune response to influenza is critical for overcoming infection. MxA has been identified as a key effector protein that can neutralize incoming influenza particles. We measured accretion of MxA during infection when PLD2 was inhibited. Ninety minutes after a 0.01 MOI A/California/04/2009 (H1N1) infection, significantly more MxA accumulated in cells treated with VU0364739. Conclusions: This study has demonstrated influenza infection stimulates an increase in PLD activity, and that isotype PLD2 plays a critical, beneficial role in the pathogenesis of early influenza infection. Inhibition of PLD2 by small molecule reduces cell-cell spread of multiple influenza strains and leads to fewer infectious particles being released from those cells that are infected. The mechanism of protection appears to be tied to viral endosome trafficking. PLD2 inhibition changes the kinetics of intracellular trafficking, and this slowdown perhaps gives the cell more time to sense the infection and mobilize antiviral effector proteins to protect against infection. Using a lipidomic approach combined with high-throughput drug screens, we've identified a host protein critical to influenza infection and built a small molecule therapeutic that protects the host from infection with little or no toxicity.

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The human antibody response to 2009 H1N1pdm neuraminidase

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Background: Epidemiologic and human virus challenge studies have demonstrated a correlation between neuraminidase (NA) inhibition (NI) antibody titers and influenza disease. The NA-specific response following vaccination and infection is poorly characterized and was of particular interest during the 2009 H1N1 pandemic because potential cross-reactivity between antibodies specific for the NA of seasonal and pandemic H1N1 viruses may have contributed to protection against severe disease. Materials and Methods: NI antibody titers were measured by enzyme-linked lectin assay (ELLA) in human sera collected before and after vaccination with a monovalent 2009 H1N1pdm vaccine and in acute and convalescent sera from individuals with confirmed influenza infection in the fall of 2009. The IRB-approved vaccine and natural history studies were conducted at the Center for Immunization Research, Johns Hopkins University and the NIH Clinical Center, respectively. We also

identified NA-specific human monoclonal antibodies (mAbs) in a mAb panel that had been generated from plasmablasts of 2009 H1N1pdm-infected patients and characterized their specificity and biological activity using virus-coated plates or NA-transfected cells by ELISA. The biological activity of these human mAb was assessed by determining the mAb concentration that inhibited 50% of NA activity using fetuin as a substrate in the ELLA. Results: Pre-existing NI antibodies against the 2009 H1N1pdm virus A/California/7/2009 (CA/09) were evident in the elderly and correlated with increased NI titers following vaccination. There was evidence of antigenic competition between hemagglutinin (HA) and NA in both vaccinated and infected individuals, with the greatest HA inhibition titers elicited when pre-existing NI titers were low. NI antibody titers that increased in response to 2009 H1N1pdm infection often also increased to the previously circulating seasonal H1N1 virus, supporting the idea of antigenic sin in which recall responses to conserved antigenic domains predominate. This idea was further verified by examining the fine specificity of NA-specific human mAbs. An NA-specific mAb isolated from a patient likely to have had a primary infection specifically inhibited the enzyme activity of only the infecting strain. Asn at residue 369 was essential for the binding of this mAb to NA. Another 5 NA-specific mAbs were reactive with the NA of CA/09 in addition to a variety of seasonal strains, including the NA of A/Brevig Mission/1/1918. This suggests that these mAbs were the result of a recall response. These mAbs did not bind to the NA of an H5N1 virus. Sequence analysis showed numerous somatic mutations in the hypervariable regions of these mAbs, confirming that they resulted from the expansion of pre-existing memory B cells. We identified amino acids 249 and 273 as residues essential for the binding of these mAbs. Two human mAbs were identified that could bind to all N1-containing viruses tested, including A/Vietnam/1203/2004 (H5N1). Only one of these mAbs, 1009-3A02, whose epitope included Asn 248 at the lip of the enzyme active pocket, inhibited NA activity. Conclusions: Our results show that NA is an important 2009 H1N1pdm virus and vaccine antigen, with evidence of antigenic sin in both vaccine and infectivity studies. Examination of the reactivity of NA-specific mAbs selected from plasmablasts in the circulation of patients infected during the 2009 H1N1 pandemic provides further evidence of a recall response to conserved antigenic domains—in most instances these mAbs are cross-reactive with the NA of previously circulating seasonal H1N1 viruses. Further studies to identify NI antibody titers that protect against disease are warranted, as this is essential for evaluating NA-specific immunity in different population groups.

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Evolution helps us understand the mystery of childhood and adult mortality in the 1918 influenza pandemic

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Background: In the 1918 influenza pandemic, there was remarkably high mortality among younger adults, but much lower mortality among children. To influenza virologists, the cause of this high adult mortality has remained a mystery. Many have sought an explanation for this exceptional virulence by studying the molecular features of the 1918 pandemic virus. Results: Clinicians and epidemiologists have identified the different conditions that are associated with increased risk of mortality due to seasonal and pandemic influenza. Regardless of patient age, these high-risk conditions share one feature in common – chronic low-grade inflammation that is characteristic of what cardiovascular scientists call “metabolic syndrome.” In these patients, the “immune rheostat” seems to be set at a different and perhaps more precarious level. Thus, intrinsic features of the host response are responsible for the increased risk, which helps us understand the pattern of mortality seen in the 1918 influenza pandemic. Some investigators believe the high mortality rate in young adults was due to secondary bacterial pneumonia, but this explanation is incomplete and unsatisfactory: children were infected more frequently than adults with the pandemic virus, and they likely had higher rates of nasopharyngeal colonization with the same bacteria that were associated with pneumonia deaths in young adults, yet few children died. It is more likely that adults died *with* bacterial pneumonia, not necessarily *because of* it. In other words, influenza induced changes in the innate and adaptive immune responses of young adults but not of children. These changes set the stage for secondary bacterial pneumonia that was seen in young adults who died. Lower mortality in children compared with adults is not unique to influenza. It is also seen in first infections with several viruses (eg, mumps,

polio, varicella) and in bacterial infections (eg, invasive pneumococcal disease, staphylococcal and streptococcal bacteremia). It is also seen in the acute lung injury that occurs in severe malaria and other conditions, such as sickle cell chest syndrome and extensive soft tissue trauma and burn injury. Thus, across a broad range of infectious and non-infectious conditions, children tolerate acute lung injury much better than adults. The dividing line between the two occurs in the second decade of life, around the time of puberty. Evolutionary biologists have dramatically confirmed this mortality pattern by documenting steep increases in the annual probability of death during the second decade of life in different human populations over historical time (Burger O, et al. *Proc Natl Acad Sci USA.* 2012;109:18210). Surprisingly, few investigators have sought explanations for the difference in pre- and post-pubertal mortality in animal models of acute lung injury, and, until recently, no one had studied it for influenza. In a new report, mortality in PR8-infected C57BL/6 mice was much lower in the period immediately before puberty compared with the period after puberty (Suber F, et al. *Am J Respir Crit Care Med.* 2013;187:A1704). When mice were either castrated or ovariectomized to delay the onset of puberty, the low influenza mortality rate was maintained, but when they were treated with estrogen, mortality rates rose to the level seen in control post-pubertal mice. This model should allow influenza virologists to undertake experiments that discriminate between host-related and virus-related factors that determine influenza-related survival and death. Conclusions: An increased risk of influenza-related mortality is seen in patients with chronic low-grade inflammation characteristic of metabolic syndrome, and these changes—not secondary bacterial pneumonia—underlie the higher mortality rates of young adults compared with children seen in the 1918 influenza pandemic and in other infectious and non-infectious conditions associated with acute lung injury. The risk of death rises steeply at the time of puberty, and this increase appears to be the heritage of human evolution. A new model of influenza in pre- and post-pubertal mice should allow influenza scientists to undertake experiments to help us better understand the mystery of childhood and adult mortality in the 1918 influenza pandemic.

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The role of antigen presenting cells induced by influenza and pneumococcal coinfections: an *in vitro* model

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Background: Viral and bacterial coinfection is frequently observed in children with severe acute respiratory infections and accounts for much of the morbidity and mortality during seasonal and pandemic influenza. We set up an in vitro model to simultaneously challenge immune cells (macrophages and dendritic cells [DCs]) with *Streptococcus pneumoniae* (*Sp*) and Influenza A/H1N1 virus to mimic human respiratory coinfection. We hypothesized that the synergistic production of specific pro-inflammatory cytokines and chemokines released by macrophages and DCs during concurrent influenza virus and pneumococcal infection affects pathogenesis and disease severity. Materials and Methods: Human monocyte-derived macrophages (MDM) and a monocytic leukemia cell line (THP-1) were seeded in a 96-well plate (1×10^5 per well) and exposed to viable or heat-inactivated Influenza A/H1N1 virus at MOI 0.5 (IAV; A/Solomon Island/3/06) for 1 h in serum-free conditions and for a subsequent 3 h in the presence of fetal bovine serum. Cells were washed, medium was removed, and fresh medium containing viable or heat-inactivated *Sp* (1×10^3, 1×10^4, 1×10^5 CFU) was added. After a 2-h incubation, gentamicin 100 µg ml^{-1} was add to kill extracellular bacteria and maintained in the cell culture until the end of the experiment. Culture supernatants were harvested 4H/8H/24H post-coinfection and used for measurement of 27 cytokines, chemokines, and growth factors that mediate immunity and inflammation. Cell viability was monitored by Annexin/PI staining analysis, and total mRNA from coinfected cells was extracted for transcriptional analysis. Viral RNA and bacterial DNA loads were quantified by in-brew molecular assays. Results: We established a coinfection system involving human macrophages, which play a pivotal role in mounting effective innate and adaptive responses to IAV and *Sp* coinfections. We found a significantly synergistic production of TNF-α, IL-6, IL-1ß, and IP-10 at 24 h post-coinfection in IAV-infected THP-1

and MDM challenged with high-dose *Sp* (1 x 10^5 CFU). IAV or *Sp* single infection or challenge of IAV-infected cells with a lower dose of *Sp* (1 x 10^3, 1 x 10^4 CFU) induced low or no soluble immunomodulators. In the earlier time (4 h/8 h) post-coinfection, a weak and non-synergistic response was observed. The priming of activated macrophages to promote the immunomodulation, in our coinfection system, was bacterial viability–dependent but viral replication–independent. It seems that it is not the virus viability infection itself but the cellular response to the coinfection that is responsible for the increased cytokine release. Interestingly, we observed that IAV-infected THP-1 cells exposed to heat-inactivated *Sp* secreted higher amounts of interferon gamma-induced protein 10, which is currently described in the literature as a potential biomarker of several respiratory infections. Moreover, preliminary results showed that, in viable *Sp* single-infected human monocyte-derived DCs, the production of soluble immunomodulators was identical to that released by human macrophages. As recently reported elsewhere with infected DCs, we also observed a time- and dose-dependent production of IL-12p70. Furthermore, *Sp*-infected immune cell viability sharply decreased, suggesting an effect of bacterial virulence factors on the immune system. Conclusions: The host cytokines response mounted by immune cells following viral and bacterial coinfections strongly influences the nature of innate and adaptive immune responses. Our preliminary results show a specific, synergistic, time- and dose-dependent modulation of the immune response orchestrated by macrophages and DCs during viral and bacterial coinfection. Taken together our results suggest that pathogen entry into the antigen presenting cells seems to be the key to the immunomodulation but not the replication itself. This *in vitro* approach is being complemented by experiments involving respiratory epithelial cells to evaluate the complex cross-talk between host and pathogens, mimicking a human respiratory physiopathology, and then validation in an animal model. This work was performed within the framework of the LABEX ECOFECT (ANR-11-LABX-0042) of Université de Lyon, within the program "Investissements d'Avenir" (ANR-11-IDEX-0007) operated by the French National Research Agency (ANR).

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The NS1 protein of seasonal influenza A viruses inhibits ISG15 induction in human cells

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Background: Influenza A virus (IAV) infection provokes an antiviral immune response, including activation of interferon (IFN) and IFN-stimulated proteins. One of these proteins is the interferon-stimulated gene product 15 (ISG15), which is a ubiquitin-like polypeptide that can be covalently attached to target proteins. ISG15 was shown to have antiviral activity against a variety of DNA as well as RNA viruses, including influenza viruses, but its precise mode of action is not well understood. The aim of this study was to elucidate the impact of ISG15 on IAV propagation as well as on species specificity. Materials and Methods: The following influenza virus strains were used in this study: A/Panama/2007/1999 (H3N2), A/New Caledonia/20/1999 (H1N1), A/duck/Alberta/60/1976 (H12N5), A/mallard/Germany/439/2004 (H3N2), and A/Puerto Rico/8/1934 (H1N1). The lung epithelial cell line A549 was used for infection experiments as well as growth curve analyses. Knock down of ISG15 in A549 cells was established using siRNAs. Expression of ISG15, other ISGs, and virus proteins such as NP or NS1 was determined by fluorescent-activated cell sorting (FACS). Transfection-based experiments were performed in human embryonic kidney 293T cells. More than 10 NS1 proteins originating from human as well as avian virus strains were investigated with regard to their ability to suppress ISG15 expression. In brief, 293T cells were transfected with the different NS constructs, stimulated with interferon at 24 hours post-transfection, and analyzed for NS1 as well as ISG15 expression via FACS. Results: We devised a FACS-based assay to assess expression of viral antigen and IFN-stimulated genes in infected cell cultures in a quantitative manner. Analysis of human cell cultures infected with seasonal IAV at low and medium multiplicity revealed an unexpected distinction of cell populations expressing either viral antigen or ISG15, but not both. This was in contrast with infections with Newcastle disease virus or influenza B virus, which generated large numbers of infected and ISG15- double positive cells, suggesting that seasonal IAV is capable of suppressing ISG15 expression during infection. In order to determine whether ISG15 was suppressed

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by other IAVs, we compared seasonal with avian virus strains. The proportion of both infected and ISG15-positive cells was 3-4–fold higher after infection with low-pathogenic avian IAV in comparison with seasonal IAV, suggesting that these non-human viruses are debilitated in inhibiting ISG15-expression. The proportion of double-positive cells increased up to 6 times upon infection with a mutant virus lacking the NS1 gene, indicating a role for NS1 in the suppression of ISG15 in infected cells. Along this line, transfection-based experiments demonstrated that the presence of an active viral polymerase was sufficient to induce ISG15, which was inhibited by co-expressed NS1 protein. In a similar transfection assay, NS1 proteins of seasonal IAV significantly reduced subsequent IFN-stimulated ISG15 induction, whereas this was not observed for NS1 proteins of low-pathogenic avian IAV. Notably, the NS1 protein of the newly emerged H7N9 virus also did not inhibit ISG15 induction. Finally, knock down of ISG15 had no effect on replication of seasonal IAV but led to 2-6–fold higher virus titers of low-pathogenic avian IAV at different time points. Conclusions: Our study demonstrates on a single-cell level that NS1 proteins of seasonal IAV strains are able to suppress ISG15 induction in infected or NS1-transfected cells. On the contrary, NS1 proteins expressed by low-pathogenic avian IAV or the novel H7N9 virus are unable to inhibit IFN-induced ISG15 expression. Preliminary data suggests that the suppression of ISG15 by NS1 is most likely due to a mechanism on the post-transcriptional level, which is currently being investigated. In conclusion, we propose that the ISG15 system contributes to limiting the IAV host range.

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Localized antibody responses in influenza virus-infected mice

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Background: Traditionally, vaccine-mediated protective responses were quantified by measuring the level of increase of influenza virus–specific antibodies circulating in blood. However, virus-specific antibodies in serum do not necessarily correlate with protection in vaccinees receiving intranasally administrated live attenuated influenza vaccines (LAIVs). Local mucosal and cellular immune responses are believed to be the protective mechanism induced by LAIVs. Recently, antibody secreting cell (ASC) responses derived from peripheral blood mononuclear cells (Cherukuri A, et al. *Vaccine.* 2012;356:685-696) of ferrets and antibody obtained via human nasal washes, but not the systemic serum (Barria MI, et al. *J Infect Dis.* 2013;207:115-124), were found to better correlate with B-cell responses induced by LAIV. ASCs are found in the upper and lower respiratory tract in influenza infections and play an important role in combating influenza infections. Analyses of antigen-specific B-cell receptors on these ASCs were limited by cell-based assays such as ELISPOT or FACS probe by hapten or B-cell tetramers. Although the localized mucosal and systematic ASC responses of Influenza A virus–infected mice are different (Joo HM, et al. *Vaccine*. 2010;28:2186-2194), direct comparisons of antibodies secreted by ASCs at these locations are lacking. Here, we isolate antibodies secreted by ACSs at multiple anatomical sites and characterize the epitope specificity and other properties of these antibodies systematically. Materials and Methods: Mice intranasally infected with influenza virus (A/HK/68) were used as a model. Lymphocytes from different nodes (eg, cervical lymph nodes [CLNs], which drain the upper respiratory tract, and mediastinal lymph nodes [MLNs], which drain the lower respiratory tract) and from the spleen of infected mice were harvested for cell cultures at days 3, 7, and 28 post-infection. In addition, supernatants of nasal washes, bronchoalveolar lavage fluid, and serum of the mice were harvested. Antibodies secreted by the cultured cells and antibody presented in the harvested body fluids were characterized by influenza A–specific isotyping ELISA, micro-neutralization assay, as well as fine epitope mapping assay using a yeast surface display library for H3 hemagglutinin. Results: Antibody in lymphocyte supernatants (ALS) from cultured cells of MLNs and spleen and antibody in serum were found to be positive for influenza virus–specific IgM at day 3 post-infection. Nasal washes, bronchoalveolar lavage fluid, and ALS from CLNs and MLNs were found to be IgA-positive at day 7 post-infection. High IgG1 and IgG2a responses were detected in ALS from MLNs at day 7 post-infection. The control ALS from cells derived from iliac lymph nodes, which drain the mouse tail but not the respiratory tract, was negative for influenza-specific IgA, IgG1, IgG2a, and IgM throughout the study. Bronchoalveolar lavage fluid collected at day 28 post-infection and ALS from MLNs collected at day 7 post-infection demonstrated

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a weak neutralization property in a standard micro-neutralization assay. Antibody repertoire mapping of ALS from MLNs collected at day 7 and of serum collected at day 28 post-infection was also performed. The antibody repertoire mappings were comparable and both identified a major immunodominant antigenic site in HA1 and a weaker antigenic site located in HA2. However, two additional antigenic sites were identified in the mapping of ALS from MLNs collected at day 7 when compared with the mapping of the serum obtained at day 28 post-infection. Conclusions: This study illustrated the feasibility of recovering ASC specificity at different localizations after influenza A challenge. With the use of cell-free supernatant, the properties of the ASC-secreted antibodies can be further characterized by various methods traditionally used for serum. The method described will provide information about influenza A–induced antibody responses early post-infection, ie, at the time when the virus was cleared.

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A comparison of human influenza A H1N2 nucleoprotein T-cell epitopes with circulating influenza A viruses from 1918 to 2003

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Background: In 2000, an H1N2 influenza virus arose in the human population and was reported as a sporadic cause of illness across the globe for approximately 3 years. Its greatest impact was in the UK, where it accounted for 54% of influenza A viruses isolated during the 2001-2002 season. The greatest number of infections occurred in the 5-14–year age group (39%) followed by the 14-44–year age group (32%). This H1N2 virus did not contain novel surface antigens or genes because it was a reassortant of the H1N1 and H3N2 viruses that were circulating in the population at the time. Natural influenza infection induces production of neutralising antibodies and stimulates long-lived CD8+ memory T-cell immunity. T cells are targeted directly at the conserved internal proteins of the influenza virus, thereby providing a level of cross-protective immunity against type A influenza viruses. This study examined the T-cell epitopes for all HLA types found in the H1N2 nucleoprotein (NP) for evidence of drift or novel epitopes that might explain its emergence as the dominant circulating strain in the UK over a season. Materials and Methods: H1N2 NP was used as the reference data set and consisted of 46 H1N2 NP sequences originating worldwide, with 10 originating from the UK. The NP epitopes were compiled using Immune Epitope Database and Analysis Resource. Epitopes were identified in the data sets using the Identify Short Peptides feature available in the Influenza Research Database. Data sets of H1N2, H2N2, H1N1 and H3N2 viruses isolated from 1918 to 2003 were sourced from GISAID EpiFlu™. The full spectrum of reference epitopes available for each site in combined H1N1, H2N2 and H3N2 NP data sets was identified and characterised as either a perfect match or otherwise with the reference epitope data set. The percentage of strains achieving a perfect match for each epitope sequence was recorded in 5-year blocks to allow assessment of the potential for exposure of population cohorts over time to viruses antigenically related to the emergent H1N2. Results: The reference H1N2 data set had a high degree of similarity in all 55 epitopes expressed in the NP, except for 3 viruses—from Asia (2) and North America (1)—with sporadic point mutations expressing novel epitopes not seen elsewhere. These were not considered further, restricting the reference data set to 43 strains. The extent of H1N2 epitope match with the aggregated historical virus data set was as follows: 100% match (20% of epitopes), 75%-99% match (20%), 505%-74% match (49), 255%-49% match (4%) and 15%-24% match (7%). Four epitopes circulated from 1918 until the mid 1960s, re-emerging again in the late 1970s, and epitope NP_{17-25}, which had circulated before 1950 and re-emerged in the late 1990s; 14% of epitopes found in the H1N2 virus emerged as novel epitopes in the early 1970s, and a further 16% of epitopes also emerged for the first time in the mid 1990s. A further two epitopes that had been predominant in the

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H2N2 viruses during 1956-1968 and continued to circulate sporadically were no longer seen in viruses other than the H1N2 by the late 1990s. Conclusions: Amino acid substitutions in both epitope anchor points and residues, which interact with the T-cell receptor, have an impact on recognition by specific T cells. This study showed that, although some NP epitopes have been highly conserved over the past century, a number of novel epitopes emerged in the late 1990s. These epitopes were incorporated from parent strains into the reassortant H1N2 viruses examined in this study. H1N2 viruses were predominately isolated from individuals up to the age of 44 years in the UK in 2001-2002, many of whom were anticipated to have had prior exposure to both H1- and N2-containing viruses. We hypothesise that the relative novelty of a number of the NP epitopes may have contributed in part to the success of this strain.

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Pregnant women infected with the pandemic influenza A(H1N1)pdm09 virus show a differential cytokine and chemokine response related to disease severity

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Background: The innate immune system is the first line of defense against viruses inducing cytokine and chemokine expression. During pregnancy, immunological and hormonal alterations place women at increased risk of certain infections and associated complications. Pregnant women represent a disproportionately higher percentage of severe cases of influenza with an increased risk ranging from 4- to 10-fold that of the general population. Increased morbidity and mortality in pregnant women were reported based on data from seasonal influenza. The impact of pregnancy on the immune response is not well known. Although A(H1N1)pdm09 infection resulted in increased disease severity in pregnant women, the precise mechanisms responsible for this risk are not yet defined. The present study aimed to investigate the host cytokine profiles in A(H1N1)pdm09 infection regarding disease severity in pregnant women. Materials and Methods: This study included 41 pregnant women with confirmed A(H1N1)pdm09 infection during the 2009 pandemic: 12 died as a consequence of the infection (ID), and 29 survived the infection (IS) Samples of 17 non-infected pregnant women were included as control (NI). Respiratory samples, tracheal aspirates (TAs), as well as nasopharyngeal (NP) secretions were obtained and collected from July to September 2009. Samples were sent to the National Influenza Reference Laboratory, which houses the WHO National Influenza Center (NIC) for A(H1N1)pdm09 diagnosis. Specimens have been kept at -80°C since then. The A(H1N1)pdm09 infection was confirmed by real-time RT-PCR using the CDC protocol. Total RNA from cells was extracted using the QIAamp® Viral RNA Mini kit QIAGEN™. cDNA was synthesized with Oligo-dT primers and Superscript III reverse transcriptase (Invitrogen) and quantified by real-time quantitative PCR analysis. The gene expression profiles for cytokines (IFN-β, TNF-α, IL-6, IL-12, TGF-β, IL-17), chemokines (IL-8, RANTES, MCP-1), and viral Matrix (M1) gene were quantified and normalized using the house-keeping gene product β-actin mRNA. We used a SYBR green PCR MASTER MIX (Applied Biosystems). The M1 gene copy number was measured to evaluate viral replication in each sample. Statistical analysis was performed using the GraphPad Prism software by ANOVA. This study was approved by the Independent Ethics Committee. Results: IL-6 mRNA expression in ID was significantly higher than in IS (5.424 ± 0.9513 vs 1.370 ± 0.4333, $P < .01$) or NI (1.578 ± 0.3566, $P < .001$), expressed as copies of IL-6 mRNA/β-actin mRNA, respectively, while both ID and IS showed increased expression of TNF-α and IL-8 in comparison with NI. Conversely, TGF-β mRNA was lower in both groups with influenza infection than in NI. RANTES, MCP1, and IL-12 levels were not significantly different among all groups. Interestingly, the expression of INF-β in ID was significantly lower than in the other two groups (0.6000 ± 0.08528 vs 1.275 ± 0.1058 [$P < .0001$] and 1.293 ± 0.1147 [$P = .000$], respectively) in relation to the increased M1 gene transcription in ID compared with IS. Conclusions: The present study shows that the expression of several cytokines and chemokines

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may play a role in the pathogenesis of the severity of the respiratory disease in pregnant women during the A(H1N1)pdm09 virus infection. The decreased expression of TGF-β may contribute to disease progression inducing proinflammatory cytokines and chemokines in ID and IS. The highly elevated expression of IL-6 and low INF-β in ID probably allowed an increase in viral replication, indicated by M1 gene expression. These results may serve as important biomarkers for the identification of patients at risk of severe complications during the A(H1N1)pdm09 infection, but the underlying mechanism awaits further detailed investigations. Addressing these issues will be necessary to ensure that pregnant women receive appropriate guidance and health care in the event of influenza pandemic or epidemic. The importance of a comprehensive preventive vaccination campaign in pregnant women is highlighted.

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The presence of bacterial LPS inhibits influenza virus infection of human macrophages and the consequent induction of CD8+ T-cell immunity

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Background: It is well established that infection with influenza A virus (IAV) facilitates secondary bacterial disease. However, there is now a growing body of evidence that the microbial context in which IAV infection occurs can affect both the innate and adaptive responses to the virus. For example, recent studies in mice suggest that exposure to bacterial species and ligands, such as those derived from the normal bacterial flora, can facilitate the development of an effective anti-viral immune response and reduce the severity of an IAV infection. To date, these studies have been restricted to murine models of disease, and the relevance of these findings in primary human cells remains to be elucidated. Of particular interest is how bacterial pre-stimulation affects IAV infection of human macrophages. Macrophages are a key leukocyte present in the nasal cavity—an area of high bacterial density and diversity as well as the first site of infection for IAV. Moreover, macrophages are long-lived and crucial for tissue homeostasis as well as protection against IAV. Training of these cell types by bacterial ligands in the nasopharyngeal mucosa could therefore have a lasting influence on the immune response toward a viral infection. Materials and Methods: We used primary human monocyte-derived macrophages (MDMs) to investigate the effect of pre-stimulation with bacterial ligands on IAV infection of MDMs. Results: We showed that pre-stimulation with the bacterial ligand lipopolysaccharide (LPS), but no other bacterial ligand, reduced the ability of IAV to infect MDMs. Specifically, LPS blocked virus transcription in MDMs by inducing an anti-viral/type I interferon response. We then demonstrated that this reduced rate of viral infection is associated with a reduced ability to present a model (non-IAV derived) antigen to autologous CD8+ T cells. Conclusions: Taken together, these data provide the first evidence that exposure to bacterial ligands such as LPS can play an important role in modulating IAV infection of primary human cells, which may then have important consequences for the development of the host's adaptive immune response.

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Severe immunopathology induced by influenza A virus is due to PB1-F2–mediated activation of the NLRP3 inflammasome

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Background: The ability of a host to recognize infection is critical for virus clearance, and the anti-viral response often begins with induction of inflammation. It has been demonstrated that PB1-F2 expressed by pathogenic influenza A viruses (IAVs) contributes to the enhancement of early inflammation in an infected host, although the mechanism responsible is unclear. Activation of the NLRP3 inflammasome has been linked to induction of inflammation and implicated in responses to IAV infection, but whether identified virulence proteins can be activators of this complex is unknown. Therefore, we investigated PB1-F2–mediated activation of the NLRP3 inflammasome as a mechanism responsible for overt inflammatory responses to IAV infection. Materials and Methods: Immortalized wild-type, ASC$^{-/-}$, caspase1$^{-/-}$, and NLRP3$^{-/-}$ C57BL/6 macrophages; primary cells; and human peripheral blood mononuclear cells (PBMCs) were infected with wild-type or genetically modified virus unable to produce PB1-F2 (delPB1-F2 X31) or exposed to PB1-F2 peptide matching amino acids 61-87 from either A/Puerto Rico/8/34 (PR8) or A/Wuhan/359/1995 (Wuh) IAVs. Phagolysosomal uptake of peptide and ASC speck formation were visualized using pHrodo labeling and real-time confocal microscopy. Secretion of cytokines IL-1β and TNFα were detected by ELISA. Activation of caspase-1 was detected via immunoblot. Wild-type and NLRP3$^{-/-}$ mice were infected with wild-type or delPB1-F2 X31 virus or exposed to the PR8 or Wuh peptides, and enhancement of the inflammatory response was evaluated via quantitative assessment of the bronchoalveolar lavage fluid for neutrophil, macrophage, and lymphocyte cellular contents via flow cytometry and for inflammatory IL-1β and TNFα cytokine content by ELISA. Results: Using wild-type and NLRP3$^{-/-}$ mice, we showed that PB1-F2 induces secretion of pyrogenic cytokine IL-1β by activating the NLRP3-inflammasome, contributing to inflammation triggered by pathogenic IAV. Compared to infection with wild-type virus, mice infected with delPB1-F2 X31 virus had decreased IL-1β secretion and cellular recruitment to the airways. Moreover, mice exposed to PB1-F2 peptide derived from the pathogenic PR8 IAV had enhanced IL-1β secretion compared to mice exposed to the seasonal IAV derived PB1-F2 Wuh peptide. Implicating the NLRP3-inflammasome complex specifically, we show PB1-F2 peptide induced IL-1β secretion was Caspase-1 and NLRP3-dependent in mice, as well as *in vitro* using human PBMCs and murine macrophages. Importantly, we demonstrate PB1-F2 is incorporated into the phagolysosomal compartment, and upon acidification, induces ASC speck formation. Furthermore, NLRP3-deficient mice exposed to PB1-F2 peptide had reduced airway cellular recruitment and decreased IL-1β and TNFα production compared to wild-type mice. Conclusions: Elucidating immunostimulatory PB1-F2 as a mediator of NLRP3-inflammasome activation provides further understanding of disease attributable to induction of exuberant inflammatory responses to IAV infection. Many IAV strains evolving in mammals become truncated for PB1-F2 protein production. We have begun exploring whether genetic modification of the naturally truncated PB1-F2 of seasonal isolates to encode a full-length protein modifies the inflammatory response to infection and activates the inflammasome complex. Our novel findings may significantly contribute to knowledge of activators of inflammasomes and their contribution to host inflammatory responses to infection. This work was supported by funding from NHMRC grants 566780 and 606976, and the Victorian Government's Operational Infrastructure Support Program. J McAuley's attendance at the conference is supported by NHMRC Program Grant 567122.

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Evaluation of immunogenicity and cross-reactivity of influenza A viruses with different receptor-binding specificity in a mouse model

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Background: Influenza pandemics are usually caused by influenza viruses that acquire receptor binding preference to human-like α2,6-sialic acid (SA) receptors. Some avian influenza viruses with α2,3-SA receptor specificity were able to infect humans with a high mortality rate. These viruses are recognized as potentially pandemic, and specific vaccines have been extensively developed and studied. It was recently shown that the immunogenicity of influenza vaccines varies depending on the receptor specificity of virus hemagglutinin (HA), and study of the impact of the mutations affecting receptor binding preferences on the induction of adaptive immune responses is of great interest. In addition, there is a need to move from egg-based vaccine production to more reliable and well-established cell-based production. Egg-derived influenza A viruses can acquire mutations after replication in MDCK cells, and the impact of these changes on virus immunogenicity is not fully understood. Materials and Methods: A pair of highly related influenza viruses derived from an A/Singapore/1/57 (H2N2) heterogeneous population was used in this study. These viruses differed by three amino acids in their HA: E156K, Q226L, and G228S (H3 numbering); the E–Q–G variant displayed α2,3-SA receptor binding specificity, whereas K–L–S had α2,6-SA receptor specificity, which was determined by their agglutination of various red blood cells. Egg-grown viruses were used to immunize groups of 12 female CBA mice intranasally at a dose of $10^{5.0}EID_{50}$. Serum samples, saliva, and washes of upper airways were collected 14 days after inoculation to determine virus-specific antibodies by HAI assay and IgA, IgG, and IgM antibodies by ELISA. Adaptation of H2N2 viruses to MDCK cells was performed by serial passage at MOI = 0.001. HAs of MDCK-adapted viruses were sequenced to find adaptive changes, and these MDCK-grown viruses were also used as antigens for ELISA assay to assess cross-reactivity of antibodies raised by egg-grown variants. Results: Two viruses with E–Q–G and K–L–S residues at positions 156, 226, and 228, respectively, induced equal homologous serum HAI titers, but differed in their ability to induce cross-reactive immune responses. Antibodies induced by the E–Q–G variant cross-reacted well with the K–L–S variant, but not vice versa. The same trend was noted in serum IgA and IgG antibodies: while homologous GMT values differed by only 2 to 3 times (with K–L–S variant being more immunogenic), E–Q–G–specific antibodies were 30 to 500 times more cross-reactive than K–L–S–specific ones. Both homologous and heterologous IgM antibody levels differed significantly between variants: K–L–S was more immunogenic, but E–Q–G was more cross-reactive. Interestingly, local IgA antibody levels correlated well with HAI titers: both viruses induced equal homologous titers, whereas E–Q–G–induced antibodies cross-reacted better than K–L–S–specific ones by 8 times. Adaptation of K–L–S variant to MDCK cells resulted in acquisition of two mutations in HA1 subunits: G158E and L321P, H3 numbering; the 158th residue is located in the receptor binding site, and receptor specificity of the MDCK-adapted variant changed significantly. These two mutations decreased homologous and heterologous IgG antibody titers induced by egg-grown viruses by 4 and 8 times, which confirms the impact of adaptive changes on virus antigenicity. L321P change was also noted in MDCK-adapted E–Q–G variant. In addition, the virus acquired P221S and A96V mutations in HA1 and HA2 subunits, respectively. As a result, K–L–S–specific IgG antibodies cross-reacted well with the MDCK-adapted E–Q–G variant, while they were unable to recognize the egg-grown E–Q–G variant. The immunogenicity and cross-reactivity of the MDCK-grown variants are being studied. Conclusions: Mutations affecting receptor-binding specificity of influenza A viruses can occur during virus propagation in cell culture. These changes can affect virus immunogenic and cross-reactive potentials, which is important in terms of producing cross-protective influenza vaccines targeted to potentially pandemic avian influenza viruses.

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Production of interferon α and β and pro-inflammatory cytokines and the expression of suppressor of cytokine signaling (SOCS) in obese subjects infected with influenza A/H1N1

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Background: During the 2009 H1N1 influenza pandemic, obesity was recognized as an independent risk factor for increased influenza morbidity and mortality. Mechanisms involved in the high mortality risk from obesity during influenza A virus infection have been described in murine models and include delayed pro-inflammatory response, aberrant type I interferon expression, impaired DC antigen presentation, and altered antigen-specific $CD8^+$ T cell responses and T-cell memory, which lead to a higher rate of morbidity and mortality. Additionally, obese individuals have changes in their circulating T-cell population: an increased frequency of $CD4^+$ T cells, a reduced frequency of $CD8^+$ T cells, and an impaired immune response to the 2009 A/H1N1 influenza vaccination. We have previously shown that increased levels of suppressor of cytokine signaling-3 (SOCS3) in obese subjects are responsible for the impaired immune response of PBMCs after Toll like receptor (TLR) stimulation. These results lead us to propose that this aberrant response in obese individuals could explain, at least in part, the complications observed in obese individuals infected with influenza A/H1N1. The aim of this work was to evaluate the production of type I interferons and pro-inflammatory and anti-inflammatory cytokines in PBMCs from obese and lean subjects with and without confirmed infection of influenza A/H1N1. The expression levels of SOCS1, SOCS3, and nuclear factor-kB were also evaluated. Materials and Methods: Cytokines were measured by real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR) and/or by ELISA in PBMCs stimulated with TLR-3 and TLR-7 ligands from either 8 obese (body mass index > 30 kg/m^2) or 4 non-obese (body mass index ≤ 25 kg/m^2) volunteers infected with influenza A/H1N1. Additionally, as control groups, 9 obese and 4 lean subjects not infected with influenza were included. The mRNA expression of both SOCS1 and SOCS3 were evaluated by qRT-PCR. Results: The obese volunteers infected with influenza A/H1N1 showed a diminished ability to produce type I interferon in response to TLR-3 ligand. Interestingly, the pro-inflammatory response was also affected in TLR-3 stimulated PBMCs. Obese influenza-free volunteers showed an increased basal expression of SOCS3, but not of SOCS1. During influenza infection, SOCS1 and SOCS3 expression was higher in the lean infected volunteers, in contrast with those who were obese infected. Conclusions: These data suggest that obesity is related to TLR-3 impairment and explain, at least in part, the inadequate immune response of obese individuals during infection with influenza A/H1N1 virus.

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CD8$^+$ T cells correlate with protection against symptomatic illness following natural pandemic influenza infection in humans

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Background: The role of T cells in mediating heterosubtypic protection against natural influenza illness in humans is uncertain. Heterosubtypic immunity in murine and non-human primate models is primarily mediated by cross-reactive cytotoxic CD8$^+$ T cells. Experimental challenge studies in humans lacking pre-existing antibodies demonstrated an inverse association for influenza-specific T-cells and symptomatic illness providing proof-of-concept for the protective capacity of T cells against influenza illness. Whether cross-reactive T cells impact clinical outcomes of natural influenza infection remains unanswered. The 2009 pandemic provided a unique natural experiment to determine whether cross-reactive cellular immunity is associated with limiting symptomatic illness following influenza infection in antibody-naive individuals. Materials and Methods: A total of 342 healthy adults (> 18 years of age) recruited between September and November 2009 after the first UK pandemic wave were followed from 2009 to 2011 to determine clinical outcomes of pH1N1 infection. Infection was defined by a four-fold rise in haemagglutination inhibition titre and/or detection of virus by RT-PCR in nasal swab samples. Among infected individuals, symptoms reported from the online surveys defined clinical outcomes associated with the influenza infection. The quality and quantity of cellular immune responses were measured using IFN-γ/IL-2 fluorescence-linked immunospot assay and multi-parameter flow cytometry. We correlated pre-existing cellular immune responses with live pH1N1 virus and conserved CD8 T-cell epitopes from virus core proteins with clinical outcomes following incident pH1N1 infection. Results: Of 51 pH1N1-seronegative individuals who developed pH1N1 infection during the study, 43 had complete clinical data and viable baseline PBMCs available. Of these 43 incident cases, symptoms and symptom scores for the clinical episode when influenza infection occurred was reliably determined in 25 individuals. Pre-existing cross-reactive T-cells to pH1N1 virus were detected in all 43 seronegative individuals with a predominance of T cells with an IFN-γ$^+$IL-2$^-$ cytokine-secreting profile. Individuals developing mild illness without fever (or cough or sore throat) had higher frequencies (P = .04) of pre-existing influenza-specific cross-reactive T cells than those with symptoms or fever. Total symptom score correlated inversely with frequencies of pre-existing cross-reactive T cells to pH1N1 virus (r = -0.39, P = .05) and conserved CD8 epitopes (r = -0.5, P = .01). Of three functional T-cell subsets, only IFN-γ$^+$IL-2$^-$ T cells were associated with limiting illness severity and viral shedding. Within this influenza-specific functional T-cell subset, CD8$^+$ cells of late-effector CD45RA$^+$CCR7$^-$ phenotype inversely correlated with symptom score (r = -0.49, P = .02) and expressed CCR5 tissue homing and CD107 degranulation markers on exposure to live virus. Conclusions: In the absence of protective antibodies, cross-reactive CD8$^+$ T cells correlate with protection against symptomatic pandemic influenza. This immune correlate of protection could guide the development and evaluation of universal T-cell–inducing influenza vaccines.

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Highly pathogenic H5N1 influenza viruses overcome a block in the replication cycle to promote productive replication in macrophages

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Background: The highly pathogenic avian (HPAI) H5N1 influenza viruses cause high morbidity and mortality in mammals. We recently identified that HPAI H5N1 viruses that are associated with the systemic spread and lethal disease in animal models productively replicate in macrophages. Unlike H1N1 influenza virus, HPAI H5N1 overcame a block early in the viral life cycle, allowing for efficient nuclear entry and subsequent viral transcription in an H5 HA-dependent manner. However, where the block occurs and the molecular mechanism(s) for the block remain unknown. Materials and Methods: RAW264.7 macrophages and Madin-Darby Canine Kidney (MDCK) epithelial cells were infected with various influenza strains, and viral localization was monitored by confocal microscopy. Viral protein production was monitored by Western blot analysis, and replication was quantified by real-time RT-PCR. Results: These studies demonstrate that the addition of the HPAI H5 HA on an H1N1 virus promotes trafficking into the nucleus with subsequent viral replication and release of infectious progeny. Surprisingly, the use of a "deselected" H5 HA (removal of the multibasic cleavage site) led to a second block downstream of viral replication and prior to release. Conclusions: The ability of HPAI H5N1 to productively replicate in macrophages may contribute to their increased pathogenesis. *In vivo* studies to determine whether productive replication of influenza viruses can be detected and whether replication in macrophages contribute to HPAI increased pathogenesis are ongoing.

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Single-cell cloning of mouse anti-HA immunoglobulins for repertoire analysis

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Background: Infection with influenza or immunization with hemagglutinin (HA) produces a diverse antibody response with many specificities, including subtype-specific neutralizing antibodies, broadly neutralizing antibodies, and non-neutralizing antibodies. However, the relative proportion of each type of antibody is not known. Materials and Methods: To quantify these components of the anti-HA response, we immunized C57BL/6 mice with H1N1pdm09 virus and boosted them with inactivated vaccine. Three days later we harvested spleens, stained with an antibody cocktail including biotinylated HA and anti-CD19, and used flow cytometry and sorting to isolate B cells specific for HA. We used PCR to amplify and sequence heavy and light chains from individual B cells. Where heavy (IgH) and light (IgL) chains were amplified from the same B cell, we subcloned the variable regions into plasmid vectors providing human heavy and light constant regions and analyzed HA binding of the resultant recombinant monoclonal antibodies (rmAb), including using a panel of HA containing point mutations to identify specific antigenic sites targeted. Results: We obtained sequences of variable regions of 54 IgH chains and 52 IgL chains. Analysis of the redundancy of germline usage allowed us to infer that the overall response to HA involves about 100 heavy chain germline sequences and about 40 light chain germline sequences; junctional diversity, somatic hypermutation, and IgH/IgL chain pairing will increase the diversity of the actual immunoglobulin repertoire above this level. Eighteen of the IgH and IgL chains were cloned from the same cell and therefore represented functional antibodies. Chimeric antibodies with these variable regions and human constant heavy or light regions were expressed in cells and the antibodies were tested for reactivity with HA. Two sets of IgH and IgL chains failed to assemble into antibodies; 15 of the remaining 16 pairs reacted with HA in

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ELISA, and 11 reacted with high affinity. Epitope mapping, using a panel of H1N1pdm09 recombinant HA point mutants, identified the binding region for 10 of these antibodies. All bound to the HA1 region of the protein and have hemagglutination inhibition (HI) and microneutralization (MN) properties and therefore may be protective in vivo. One antibody cross-reacted efficiently with other strains of virus (including H3, seasonal H1, and H7 subtypes), but this antibody failed to show HI or MN activity. Conclusions: This particular immunization regimen generated mainly (50%-90%) HA1-specific, subtype-specific antibodies that are neutralizing in vitro and may be protective in vivo. Further experiments are in progress to optimize immunization strategies that more efficiently generate broadly neutralizing antibodies.

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Evidence of cross-reactive protection against H1N1 pdm due to exposure to past H1N1 influenza viruses: preliminary findings from a prospective cohort

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Background: Evidence of cross-reactive protection against 2009 pandemic influenza A (2009 H1N1pdm) has been found among individuals born before 1957 who may have been exposed to influenza viruses genetically similar to the 2009 H1N1pdm virus. Using data from a French cohort study, designed to identify risk factors of recurrent influenza infection, we examined potential associations between hemagglutination inhibition (HI) antibody titers against past H1N1 influenza viruses and 2009 H1N1pdm and investigated an age-dependent immune response. We hereby present preliminary findings with the influenza A/1956 H1N1 strain. Materials and Methods: A total of 592 subjects were recruited to the cohort from 2008 to 2009; 534 subjects were followed until 2010. Subjects were selected using a stratified sampling method during medical visits with their general practitioner. Subjects were stratified by age (10-year age groups) and reason for medical visit (influenza-like illness [ILI] or an illness unrelated to ILI). Subjects in the ILI strata had an inclusion visit, and annual study visits were performed for all subjects. All visits included data collection, including vaccinal status (via questionnaires) and serological samples. Virological samples were collected for ILI subjects at inclusion and during ILI-related visits for all subjects. Influenza A was detected by RT-PCR; serological samples were tested for antibodies against 2009 H1N1pdm and 1956 H1N1. Infection by 2009 H1N1 pdm was defined as a positive RT-PCR sample or four-fold increase in 2009 H1N1 pdm titers. Seroprotection was defined as a pre-pandemic HI titer ≥ 1:40. This analysis relied on a total of 1575 serological samples (1132 in 406 unvaccinated and 443 in 165 vaccinated subjects) collected throughout the study. Correlations between titers were examined using Spearman's correlation. Regression models for interval-censored data were used to estimate geometric mean titers (GMTs) for HI assays and to explore potential associations; models with binary covariates estimated the multiplicative factor by which GMTs increased between the two groups, and models with continuous covariates estimated the increase in GMTs for a one-unit increase. GMT estimations were restricted to unvaccinated subjects to avoid confounding; however, associations between titers against 1956 and 2009 H1N1pdm and seasonal vaccine receipt were investigated separately. Results: Titers against 2009 H1N1pdm and 1956 H1N1 were highly correlated (r = 0.19, P < .001). Of 273 study subjects born after 1956, the correlation was highly significant (r = 0.19, P < .001) but was not significant among the 133 subjects born before 1956 (r = 0.09, P = .09). Titers against 2009 H1N1pdm were 1.13 times higher in subjects born before than in those born after 1956 (P < .001), and titers against 1956 H1N1 were 1.52 times higher (P < .001). High titers against 2009 H1N1pdm were associated with high titers against 1956 H1N1 among subjects born after 1956 (P < .001) but not among subjects born before 1956 (P = .16). Restriction of the analysis to samples

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collected before the 2009 H1N1pdm season (662 serological samples for 364 unvaccinated subjects) produced similar findings. In comparisons of vaccinated and unvaccinated subjects, titers against 1956 H1N1 were 1.39 times higher among vaccinated subjects who were born after 1956 (n = 33, P < .001); titers were not significantly different between subjects born before 1956 (n = 251, P = .146). Thirteen infected subjects were identified by RT-PCR (n = 3) or seroconversion (n = 10). None was seroprotected against 2009 H1N1 pdm, and one was seroprotected against 1956 H1N1 at baseline. Conclusion: High HI antibody titers against 1956, among subjects born before 1956, may have conferred protection against 2009 H1N1pdm infection. Recent influenza vaccination was associated with 1956 H1N1 antibody titer in subjects born after 1956, but not before. This suggests a potential vaccine-induced cross-protective response in subjects not previously exposed to H1N1 influenza viruses. These preliminary results contribute to understanding age-specific immunity to 2009 H1N1pdm within the population. Further analysis using other H1N1 antigens is planned, which will provide a comprehensive profile of pre-existing immunity in the population and may enhance knowledge of the effects of repeated exposures to H1N1 viral strains.

P1-364

Antibody landscapes: quantifying the antibody response to vaccination and infection

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Background: Interpreting antibody profiles of human sera is difficult, as they reflect a history of multiple infections and vaccinations. We exploit antigenic cartography to enable a detailed quantitative interpretation of human multi-exposure serological data by explicitly controlling for antigenic variation among viruses – the primary confounder in these data – in a method we call “antibody landscapes.” Antigenic cartography creates a quantitative visualisation of binding assay data, such that the distance between viruses in the antigenic map represents their antigenic difference. An antibody landscape extends the antigenic map with a third axis representing serological titres: high titres are shown as peaks, while antigenic regions with low antibody responses are displayed as valleys. Fitting a surface through these points creates an antibody landscape that increases the resolution of the serological values and predicts outcomes for untested strains. Materials and Methods: The method involved analysis of pre- and post-vaccination sera from 76 volunteers (18-74 years) vaccinated with A/Nanchang/933/95 and 81 volunteers (18-75 years) vaccinated with A/Sydney/5/97, which were titrated against 63 viruses isolated between 1992 and 2011 in a haemagglutination inhibition (HI) assay. Sera from 36 individuals in 6 Vietnam households in the Ha Nam cohort (5-54 years) were collected in 5 consecutive years, and HI titres against 38 viruses were determined. Results: The pre-vaccination landscape was high against older strains and lower against strains circulating after the time of serum collection. The large variability observed among individual pre-vaccination landscapes is presumably the result of diverse infection histories. Some individuals had antibody landscapes with titres below the HI assay threshold (< 10) only, while their post-vaccination responses had a range of responses. Additionally, we quantified the increase in antibody titres between the pre- and post-vaccination sera to evaluate the strength and breadth of the immune response and compared the responses to the two different vaccines. Interestingly, it was found that the landscape shape of a given individual is temporally conserved. Conclusions: Controlling for antigenic variation in serological data enables a consistent interpretation of human serological data. The intuitive high-resolution visualisations additionally showed fine-grained details

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such as the conserved shape of the antibody landscape of an individual over time, the localised and global titre changes as a result of infection and vaccination and the variation in antibody levels that exists among different members of the population.

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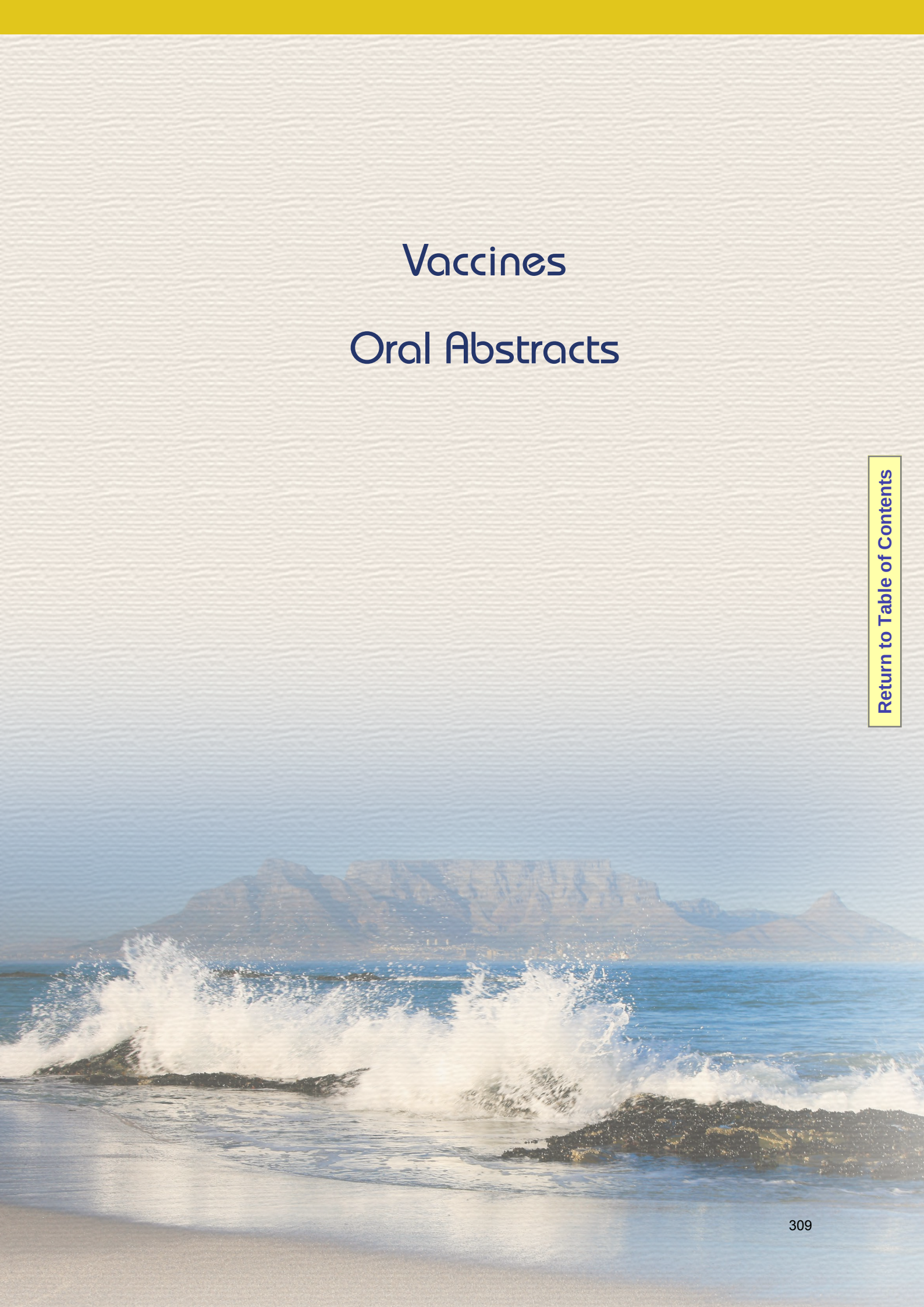
Vaccines

Oral Abstracts



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Effectiveness of seasonal trivalent influenza vaccine for preventing influenza virus illness among pregnant women: a population-based case-control study during the 2010-2011 and 2011-2012 influenza seasons

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Background: Although vaccination with trivalent inactivated influenza vaccine (TIV) is recommended for all pregnant women, no vaccine effectiveness (VE) studies of TIV in pregnant women have assessed laboratory-confirmed influenza outcomes. Materials and Methods: In our case-control study, we estimated influenza VE in preventing influenza illness among pregnant women in two metropolitan areas in the United States over two influenza seasons (2010-2011 and 2011-2012). We compared the proportion vaccinated among 100 cases, defined as women with acute respiratory illness (ARI) (with fever and cough) who were positive for influenza A or B by real-time RT-PCR, with the proportions vaccinated among 200 controls without ARI (matched by season, site, and trimester) and among 192 controls with ARI but who tested negative for influenza. VE was estimated as 100% X (1 – odds ratio) adjusted for age, race, ethnicity, and high-risk medical conditions. Analyses using influenza-negative controls were also adjusted for site, season, trimester, whether or not the illness was medically attended, and interval between illness onset and respiratory specimen collection. Results: Among influenza cases, 42% were vaccinated during the current (study) season compared with 63% and 58% vaccinated among matched ARI-negative controls and influenza-negative controls, respectively. The crude VE (95% confidence interval [CI]) of the current season vaccine against influenza A and B was 54% (CI = 26%-71%) using the ARI-negative controls and 48% (CI = 16%-68%) using the influenza-negative controls. However, receipt of the prior season's vaccine had a similar effect as receipt of the current season's vaccine, such that vaccination in either or both seasons had statistically similar adjusted VE using ARI-negative controls (VE point estimates range = 48%-76%) and influenza-negative controls (51%-76%). The likelihood of influenza virus infection was over 2-fold higher among women who received neither the current or prior season's vaccination compared with women vaccinated in either or both seasons, using matched case-controls (adjusted odds ratio [AOR] = 2.84; CI = 1.67-4.84) and influenza-negative controls (AOR = 2.36; CI = 1.39-3.98). Conclusions: In the first study of its kind, influenza vaccination was shown to reduce the risk of ARI associated with laboratory-confirmed influenza among pregnant women by over one-half, similar to VE observed among all adults during these seasons. Those not vaccinated during the current or prior season were at heightened risk of influenza illness. Our findings highlight the need for all countries with influenza vaccination programs to vaccinate pregnant women, as recommended by the World Health Organization. Supported by the Centers for Disease Control and Prevention (contract 200-2010-F-33132 to Abt Associates Inc.). The findings and conclusions in this report are those of the authors and do not necessarily represent the views of CDC, Abt Associates, Inc., or Kaiser Foundation Research Institute.

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Pandemic live attenuated influenza vaccines prime for a long lasting immune response

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Background: The pandemic threat posed by avian influenza (AI) A viruses of the H5 and H7 subtypes has led to the development and evaluation of pandemic vaccines. We generated pandemic live attenuated influenza vaccines (pLAIV) that contained the hemagglutinin (HA) and neuraminidase genes from potential pandemic viruses and the internal protein genes of the cold-adapted A/Ann Arbor/6/60 Master Donor Virus. The pLAIVs showed promising immunogenicity and efficacy in preclinical studies but were severely restricted in replication and variably immunogenic in humans. We undertook 2 studies to see if pLAIVs primed subjects for an improved antibody response to subsequent pandemic inactivated subvirion influenza vaccine (pISV). Materials and Methods: Previous recipients of pLAIV were invited to participate in studies where they would receive a single dose of 45 µg of unadjuvanted pISV and their responses were compared to those of vaccine naïve subjects. In the first study, 11, 10, and 8 previous recipients of two doses of VN04 (H5N1), HK03 (H5N1), or BC/04 (H7N3) pLAIV received a single dose of 45 µg VN04 H5N1 pISV more than 4 years later and compared to 20 naive subjects who received 1 dose and 20 naive subjects who received 2 doses of H5 pISV. In the second study, 8, 5, and 6 previous recipients of 2 doses of Neth03 (H7N7 pLAIV), 1 dose of H7N3 pLAIV or two doses of MO/06 (H2N3) pLAIV received a single dose of 45 µg H7N7 pISV approximately 18 months afterward, and were compared to 20 naive subjects who received a single dose of 45 µg of H7N7 pISV. The kinetics, magnitude and breadth of serum hemagglutination inhibition (HAI) and neutralizing antibody responses to H5N1 or H7 influenza viruses were compared among the groups. Results: Subjects who were primed with either VN04 pLAIV or HK03 pLAIV had a significantly better response to a single dose of H5N1 pISV than did naïve subjects or those who had previously received H7N3 pLAIV. In addition, subjects who had been primed with two doses of H7N7 (Eurasian lineage) pLAIV had a significantly greater response to a single dose of H7N7 pISV than did naïve subjects or those who previously received a single dose of H7N3 (North American lineage) or H2N3 pLAIV. The antibody response in VN04 pLAIV primed subjects also exceeded that following 2 doses of H5N1 pISV in naïve subjects. Recall antibody responses in primed subjects developed rapidly. Responses in primed subjects were also broadened. H5N1 pLAIV primed subjects developed antibody responses that neutralized 2 or more clades of H5N1 viruses while few subjects even in the 2 dose H5N1 pISV group developed cross-clade neutralizing antibodies. The affinity of antibodies against the HA1 domain of the H5 HA in the H5N1 pLAIV- primed groups were significantly higher than the 2 dose ISV group, and this correlated with cross-clade H5N1 neutralization. Subjects who were primed with H7N7 pLAIV, but not those who previously received H7N3 pLAIV also had a broad response that recognized H7 viruses in both the North American and Eurasian/Oceanic lineage. Conclusions: Previous exposure to pLAIV primes for vigorous responses to subsequent exposure to pISV even in the absence of detectable antibody responses to primary vaccination. The high titer and high quality, broadly cross reactive antibody response seen with H5N1 and H7N7 pLAIV priming followed by an ISV boost should prompt interest in novel vaccine schedules that combine different types of vaccines.

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Effectiveness of seasonal influenza vaccination of children in tropical developing Africa: a cluster-randomized trial

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Background: The benefit of influenza vaccine to children in tropical developing populations has not been well-studied. Additionally, because the pattern of influenza circulation and the role of children in transmission may differ markedly from those in temperate, developed settings, population-level effects of wide-spread influenza vaccination of children, including total, indirect, and overall effectiveness of vaccination, are unknown. Therefore, we initiated a multi-year project in rural Senegal to evaluate these effects after vaccination of children with seasonal trivalent inactivated influenza vaccine (TIV). Materials and Methods: Twenty villages of the Niakhar Demographic Surveillance System were randomized 1:1 for vaccination of children 6 months through 10 years of age with TIV formulated for the 2008-2009 northern hemisphere influenza season (Vaxigrip, Sanofi-Pasteur) or inactivated poliovirus vaccine (IPV) (Sanofi-Pasteur). Commercial presentations of vaccines were masked and coded before delivery to the site, and children received dosing per the manufacturer's instructions (for TIV recipients, two 0.25 mL doses for those 6-35 months, two 0.5 mL doses for those 3-8 years, and one 0.5 mL dose for those 9-10 years; for IPV recipients, equivalent numbers of doses of the 0.5 mL presentation). After immunizations were completed, surveillance for laboratory-confirmed febrile acute respiratory illness (FARI) was conducted using active, community-based (with weekly visits to households), and enhanced passive health post-based surveillance. Individuals reporting symptoms were assessed clinically, and nasal swab and throat swab specimens were collected for influenza testing using CDC's real-time reverse transcription polymerase chain reaction protocol. The primary study objective was an intention-to-treat analysis of total effectiveness of vaccination (VE_{Total}) (direct and indirect protection among vaccinees), with the cumulative incidence of laboratory-confirmed symptomatic seasonal influenza among consenting children in TIV villages compared with that in IPV villages and adjusted for village randomization. Indirect effectiveness was estimated among age-ineligible unvaccinated children and adults, and overall effectiveness was measured among the entire community. Results: Of 11,626 children eligible for study vaccinations, 5840 were from TIV villages and 5786 were from IPV villages. Starting May 26, 2009, we enrolled 3929 children in TIV villages and 3851 children in IPV villages. Median village participation rates were 73% (TIV) and 74% (IPV). During year one surveillance, beginning mid-July 2009 and ending June 2010, 1465 cases laboratory-confirmed as seasonal influenza were identified. A total of 1445 (99%) of these cases were A/H3N2, and nearly all occurred from July through November 2009 (approximately 28% of FARI cases detected during this period). Antigenic characterization of a subset of specimens indicated with high probability that all were A/Perth/16/2009 (H3N2)-like infections—a strain drifted from that included in available TIV. Twenty cases of influenza B occurred during 2010. Among age-eligible children participating in vaccinations, 812 A/H3 cases were detected, among age-eligible children not participating in vaccinations 238 were detected, and among the rest of the population 395 were detected. The average attack rate among age-eligible participating children in TIV villages was 8% while in IPV villages it was 13%. VE_{Total} was 44% (95% CI, 16% to 63%) and increased with age (25%, 54%, 64%, and 60% among children 6-35 months, 3-5 years, 6-8 years, and 9-10 years, respectively). While not statistically significant, the indirect benefit to those not eligible for vaccination was 19% (95% CI, -12% to 42%). Overall, vaccination of children was 33% effective in reducing FARI confirmed as influenza in the entire population (95% CI, 6% to 52%). Conclusions: To our knowledge, this is the largest influenza vaccine trial conducted in sub-Saharan Africa. Our cluster randomized design allowed for the measurement of total, indirect, and overall vaccine effectiveness. Although available vaccine was not matched to the drifted A/H3 strain that circulated widely in the population, significant reduction in laboratory-confirmed influenza was still measured among vaccinated children. Moreover, significant benefit was measured at the level of the entire community, including unvaccinated children too young to receive vaccine and unvaccinated adults. These data provide

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evidence that current inactivated influenza vaccines can provide benefit to developing country populations.

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Trivalent inactivated influenza vaccine immunogenicity in HIV-infected pregnant women and transplacental antibody transfer

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Background: Pregnant women, possibly more so if HIV-infected, and young infants are high-risk groups for severe influenza illness. WHO recommends pregnant women as a priority group for vaccination with seasonal inactivated influenza vaccine (IIV). Maternal vaccination may also confer protection against influenza illness to their infants < 6 months of age for whom there is no licensed influenza vaccine. IIV vaccination of pregnant women is immunogenic and safe in HIV-uninfected women, as well as increases the concentrations of transplacental HAI (hemagglutination-inhibition) antibody transfer to their foetuses. There are limited data on the immunogenicity of IIV in HIV-infected pregnant women, which suggest a lower response to the seasonal vaccine compared with HIV-uninfected pregnant women. Additional data in a larger study of monovalent pandemic H1N1 (pH1N1) vaccine found reduced immunogenicity of IIV in HIV-infected pregnant women compared with historical controls. The aim of our study was to assess the safety and immunogenicity of IIV in a large cohort of HIV-infected women vaccinated during the second/third trimester of pregnancy and the efficiency of transplacental antibody transfer to their foetuses. In a separate study, we calculated the vaccine efficacy to prevent clinical influenza-like illness and laboratory confirmed influenza in this same study cohort. Materials and Methods: We undertook a double-blind, randomized, placebo-controlled trial in Soweto, South Africa during 2011. 194 HIV-infected women were randomized to receive IIV or normal saline intramuscularly between 20-34 weeks of gestational age. IIV was composed of the following vaccine strains: H1N1 A/California/7/2009 NYMC X-179A, H3N2 A/Victoria/210/2009 NYMC X-187, and B/Brisbane/60/2008. Immune responses to each vaccine-strain, using HAI assay were measured in the women prevaccination, 1 month postvaccination and within 7 days of delivery concurrently with HAI titres being determined in their newborns. HAI titres of ≥ 1:40 were categorized as seroprotective and seroconversion was defined as at least a 4-fold increase of HAI titres 1-month postvaccination compared with prevaccination. Results: The median age of enrolled women was 27.9 years, the median HIV-1 viral load was 1067 copies/mL (IQR 61: 13923), the median CD4+ cell count was 394 cells/μl (IQR 271: 557), including 75 participants (39.5%) who had a CD4+ cell count of < 350 cells/μl. Participants delivered 188 HIV-exposed babies, of whom 87% were term and none were HIV-infected. One month postvaccination, comparing vaccinees and placebo-recipients, the proportion of women who seroconverted were 36.4% vs 2.8% to H3N2, 42.9% vs 4.2% to H1N1, 44.2% vs 15.5% to influenza-B strain (all comparisons $P < .001$). Postvaccination, the proportion of women with seroprotective HAI titres were 49.4% vs 16.9% for H3N2, 68.8% vs 23.9% for H1N1 and 71.4% vs 42.3% for influenza-B ($P < .001$ for all comparisons). Among vaccinees, HAI geometric mean titres (GMTs) increased one month post-vaccination from 17.9 to 43.0 for H3N2, 27.0 to 77.9 for H1N1 and 18.8 to 68.6 for influenza-B. Within 1 week of birth comparing newborns of IIV-recipients to placebo-recipients, the proportion of newborns with a seroprotective threshold of HAI titres were 42.9% vs 17.3% for H3N2, 58.3% vs 27.2% for H1N1 and 83.3% vs 43.2% for influenza-B ($P < .001$ for all comparisons). The geometric mean ratio of newborn to maternal HAI titres ranged from 0.62 to 1.26; being higher in the placebo group for H3N2 (1.26 vs 0.85, $P = .003$) and H1N1 (0.84 vs 0.62, $P = .04$). Conclusions: IIV vaccination of HIV-infected pregnant women induced a moderate immune response. Compared to an historical cohort of HIV-infected adults from the same setting enrolled in 2008, seroconversion rates one month post-vaccination were 36% vs 61% for H3N2, 43% vs 53% for H1N1 and 44% vs 54% for influenza-B. Influenza maternal antibodies were transferred transplacentally to the baby. New strategies for

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influenza vaccination of HIV-infected pregnant women should be explored to improve the immune response in this high risk-group.

O-862 SCHOLARSHIP Promising Investigator Scholarship

A study of the local and systemic immune responses after intranasal influenza vaccination in children

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Background: Children are the main source of the spread of influenza in the community, with WHO estimates of 20% infected annually. Annual influenza vaccination is recommended for high-risk patients and administered parenterally, but may not be the best vaccine for children. In Europe, the live attenuated influenza vaccine (LAIV) was approved for children in 2012. The palatine tonsils are rich in lymphocytes and play an important role as a reservoir of memory and effector cells. The tonsils protect the upper respiratory tract and are important in activating an immune response toward respiratory pathogens such as the influenza virus. In this study we have recruited children undergoing elective tonsillectomy and immunized them with LAIV administered as a nasal spray. The aim of the study was to investigate systemic and local immune responses by studying T- and B-cell responses after vaccination. Materials and Methods: Fifty-eight children aged 2-17 years, scheduled for elective tonsillectomy, were recruited from the Ear-Nose-Throat Clinic at Haukeland University Hospital in Bergen, Norway. Thirty-nine children were immunized with FLUENZ—a LAIV seasonal vaccine–3, 7, or 14 days prior to tonsillectomy. Thirty children received 2 doses of vaccine, 9 children received 1 dose of vaccine, and 16 children were controls. Two children withdrew after the first dose. Blood and saliva samples were collected prior to and 21 and 56 days after vaccination and at time of tonsillectomy. Mononuclear cells were separated from the blood and tonsils for use in immunological assays. The humoral immune response was evaluated using a direct antibody-secreting cell and a memory B cell ELISPOT. The cellular immune response was evaluated by IFNγ ELISPOT. CD4+ Th1 cells were evaluated by intracellular cytokine staining for INFγ, IL-2, and TNFα using multiparametric flow cytometry. The serological response was measured by hemagglutinin inhibition assay. Results: The LAIV was generally well tolerated, with few side effects. No side effects were seen in 17/39 children. Seven out of 39 children had only local reactions (stuffed/runny nose, airway symptoms), and 4 had only systemic symptoms (fever, myalgia, lethargy, headache, loss of appetite). There were no serious adverse events. We saw no significant changes in cytokine or chemokine profiles in the plasma after vaccination, corresponding with the clinical observation of low reactogenicity. Preliminary ELISPOT results showed an increased INFγ and memory B-cell response after the first vaccine dose. Flow cytometric analysis showed a significant increase in the Th1 CD4+ T-cell response after the second vaccine dose. Further research into the evaluation of immune responses in tonsils and saliva is ongoing. Conclusions: Vaccination with LAIV as a nasal spray in children is safe, is well tolerated, and induces a systemic immune response against all vaccine antigens. This vaccine may represent a good alternative for immunization of high-risk children against seasonal influenza in Europe.

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Effectiveness of 2011/12 seasonal influenza vaccines in the prevention of influenza-related hospitalization in Canadian adults: a Public Health Agency of Canada/ Canadian Institutes of Health Research (PCIRN) Serious Outcomes Surveillance Network Study

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Background: In Canada, annual influenza vaccination is recommended for adults aged 65 years and older, younger adults with medical comorbidities putting them at increased risk of severe disease, and children under 60 months of age. The goal of the Canadian influenza immunization program is prevention of influenza-associated hospitalization and death but little data is available to assess the effectiveness of seasonal influenza vaccination in the prevention of these serious outcomes. Sentinel surveillance for influenza among hospitalized adults provides a platform for assessment of influenza burden of illness and vaccine effectiveness in the prevention of hospitalization due to lab-confirmed influenza. Methods: In 2011/12, the PCIRN Serious Outcomes Surveillance Network conducted active surveillance for influenza among hospitalized adults from 1 Nov to 25 May in 40 acute care facilities in 6 provinces, encompassing ≈ 15,000 beds. A nasopharyngeal swab for influenza polymerase chain reaction (PCR) testing was obtained from all patients admitted with community-acquired pneumonia, exacerbation of chronic obstructive pulmonary disease or asthma, unexplained sepsis, any respiratory diagnosis or symptom, and, on 1 day per week, acute coronary syndrome, stroke, or other cardiac diagnoses with triage temperature ≥ 37.5°C. Cases were influenza PCR-positive; 1 or more test-negative controls matched for date, site of enrolment and age of the case (≥ 65y vs < 65y) were enrolled for calculation of vaccine effectiveness (VE). Crude VE estimates were adjusted using multivariable logistic regression with stepwise backward selection of covariates with a *P* value of < .1 in the univariate analysis. VE was estimated as (1- odds ratio of influenza in vaccinated versus unvaccinated patients) × 100. Results: In 2011/12, 514 cases and 814 controls were enrolled. 310 (60.3%) cases were ≥ 65 years (mean age, 80.5 y; range, 65-100 y); 90.9% of cases had 1 or more comorbidities. 246 (47.9%) cases and 510 (62.7%) controls had received an influenza vaccine ≥ 2 weeks prior to onset of symptoms. Vaccinated adults were more frail than unvaccinated adults prior to onset of illness (mean Frailty Index in patients ≥ 65 y 0.21 vs 0.18; *P* < .001) and were older (73.6 vs 61.4; *P* < .001). Overall, 62 (12.1%) of cases required admission to the intensive care unit, 38 (7.4%) were ventilated, and 39 (7.6%) died. Overall adjusted VE in adults was 45.8% (90% confidence interval [CI]; 30.4% to 57.8%); VE in adults ≥ 65 y adjusted for age, Frailty Index, and children 5 years or under living in the home was 56.2% (90% CI; 32.7%-71.4%). VE in adults < 65 y adjusted for age, children 5 years or younger living in the home, seniors living in the home, and current or past smoking was 37.4% (90% CI; 4.1% to 59.1%). Overall adjusted VE against Influenza A was 45.6% (90% CI; 19.9% to 63.1%): A/H3N2 VE 38.3% (90% CI; -20.4% to 68.4%); A/H1N1 VE 73.4% (90% CI; 44.6% to 87.2%). Overall adjusted VE against Influenza B was 37.2% (90% CI; 16.4% to 52.9%): B/Yamagata-like VE 35.3% (90% CI; 6.4% to 55.3%) and B/Victoria-like (vaccine strain) VE 43.2% (90% CI; 0.8% to 67.5%). Conclusion: In 2011/12, the seasonal influenza vaccines used in Canada were moderately effective in the prevention of hospitalization of adults due to lab-confirmed influenza despite co-circulation of a lineage-level mismatched B strain. The PCIRN Serious Outcomes

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Surveillance Network provides critical infrastructure for the assessment of influenza burden of disease and component-specific effectiveness of influenza vaccines in the prevention of influenza-related hospitalization. Funding: This study was funded by the Public Health Agency of Canada, the Canadian Institutes of Health Research, and GlaxoSmithKline Biologicals SA.

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Vaccine-induced anti-HA2 antibodies promote virus entry and enhance influenza A virus pathology

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Background: During the 2009 influenza (pH1N1) pandemic several reports suggested prior vaccination with the 2008-2009 seasonal inactivated influenza vaccine increased severity of clinical disease following infection with pH1N1 influenza virus in humans. The mechanism of Vaccine associated enhanced respiratory disease (VAERD) has not been fully understood. Methods: We developed a swine model to evaluate mismatched influenza vaccination associated enhanced respiratory disease (VAERD) following pH1N1 infection. Apart from following clinical diseases and symptoms in vaccinated pigs following pH1N1 challenge, polyclonal antibody repertoire generated following vaccination against was various antigenic domains in pH1N1 hemagglutinin was analyzed by surface plasmon resonance–based real-time kinetics assays. Epitopes recognized by vaccine-induced serum antibodies were mapped using phage display and impact of these antibodies on pH1N1 virus entry and pathology was investigated in various virus-cell interaction functional assays. Results: Vaccinating pigs with whole inactivated H1N2 virus vaccine (WIV-H1N2) enhanced pneumonia and disease following pH1N1 infection compared with nonvaccinated challenged animals. We elucidated the quality and specificity of antibody response elicited by the heterologous WIV-H1N2 vaccine in different assays. WIV-H1N2–induced HI antibodies against H1N2 strain but not pH1N1. WIV-H1N2 immune sera contained high titers of cross-reactive anti-pH1N1 antibodies that bound exclusively to HA2 domain of virus hemagglutinin that mapped to aa 46-91 residues of pH1N1-HA2 sequence. The cross-reactive anti-HA2 antibodies enhanced infection of MDCK cells with pH1N1 by promoting virus fusion activity. The enhanced fusion activity correlated with lung pathology for individual pigs. Conclusions: This is the first study that suggests a major role for fusion-enhancing anti-HA2 antibodies in VAERD, especially in absence of receptor-blocking antibodies. These findings should be carefully considered in the development of future universal influenza vaccines that are designed to elicit exclusively HA2 stem antibodies in the absence of anti-HA1 specific antibody response.

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Synthetic generation of Influenza vaccine viruses for rapid response to pandemics

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Background: During the 2009 H1N1 influenza pandemic, vaccines became available in large quantities only after human infections peaked. To accelerate vaccine availability for future pandemics, we developed a synthetic approach that very rapidly generates vaccine viruses from sequence data. This technology addresses 3 major technical barriers to more rapid and reliable influenza outbreak and pandemic responses: the speed of synthesizing DNA cassettes to drive production of influenza RNA genome segments, the accuracy of rapid gene synthesis, and the yield of hemagglutinin (HA) from vaccine viruses. Materials and Methods: Beginning with HA and neuraminidase (NA) gene sequences, oligonucleotides are designed and chemically synthesized. Through an entirely enzymatic, cell-free process, the oligonucleotides are assembled into HA and NA DNA segments, corrected for errors, and combined with key regulatory elements required for virus rescue by reverse genetics. The synthetic HA and NA gene cassettes are mixed with plasmid DNAs encoding viral backbone genes and transfected into an MDCK cell line qualified for vaccine manufacture. After 48 to 72 hours, influenza viruses are recovered, amplified in MDCK cells, and then rapidly analyzed for HA and NA sequence identity, growth in culture and HA production. Results: We have demonstrated that an enzymatic system of gene assembly with error correction lowers error rates sufficiently to allow the HA and NA gene cassettes to be used to rescue influenza vaccine viruses with pre-specified HA and NA sequences and backbones with high fidelity. The reliability of influenza virus rescue on a vaccine manufacturing cell line and yields of HA are increased through the use of improved vaccine backbones. Use of the system to generate a diversity of synthetic influenza viruses demonstrates the system's robustness. This process was implemented to launch an emergency vaccine seed virus development effort in response to the outbreak of human infections with avian H7N9 influenza in China that was first reported on March 31, 2013. On April 1, we employed our synthetic approach to make influenza vaccine viruses using downloaded coding region nucleotide sequences of A/Shanghai/2/2013 as the strain-specific starting materials rather than viruses or nucleic acids shipped from the site of the outbreak. A potential vaccine virus was recovered 5 days later. A number of A/H7N9 vaccine viruses have been rescued and characterized thus far in this effort, containing different backbones and 5' and 3' untranslated regions. Preliminary analyses indicate that viruses with our proprietary backbones produce higher HA yields than viruses made with the conventional PR8 backbone. Conclusion: Generation of synthetic vaccine viruses, together with more efficient vaccine release assays, could accelerate responses to influenza pandemics through a system of instantaneous electronic data exchange followed by real time, geographically dispersed vaccine production.

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Structure and accessibility of HA trimers on intact 2009 H1N1 pandemic influenza virus to stem region-specific neutralizing antibodies

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Background: The influenza virus infects millions of people worldwide on an annual basis. The major surface glycoprotein hemagglutinin is the major antigen to which neutralizing antibodies are elicited. Although vaccines and drugs are available, the virus continues to be a major health problem due to its abilities to change the antigenic makeup of hemagglutinin, and its zoonotic nature, allowing animal viruses to infect humans to which the human population has no preexisting immunity. Thus, antigenic variation of influenza virus hemagglutinin (HA) remains the principal challenge in developing more effective vaccines. However, some antibodies target stem regions of HA and show broad neutralization of influenza viral strains and subtypes making the stem region of HA an attractive target for universal influenza vaccine efforts. However, how stem antibodies bind influenza virions and how extensive is antibody binding and distribution on intact influenza virions have been unresolved questions. Materials and Methods: Using cryo-electron tomography and subtomogram averaging methods, we determined of the 3D structures of HA trimers on intact 2009 H1N1 pandemic virus particles in the absence and presence of neutralizing antibodies. We studied HA trimer structures bound to C179, a broadly neutralizing antibody that is reactive to the conserved stem region of HA and that neutralizes viruses expressing a broad range of HA subtypes (H1, H2, H5, and H9). We deduced the locations of the molecular surfaces of HA involved in interaction with this antibody. Results: Our results show that despite the dense packing of HA trimers on the surface of the virus, and the location of the stem region close to the viral membrane, we show that ≈ 75% of HA trimers have C179 bound to the stem domain. Conclusions: Thus, in conclusion, the majority of HA trimers on intact virions are available to bind antistem antibodies that target conserved HA epitopes, suggesting that universal influenza vaccines that elicit such antibodies could be effective.

O-879 SCHOLARSHIP Promising Investigator Scholarship

Universal influenza vaccinia vaccine requires CD4$^+$ T cells for heterosubtypic protection

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Background: Current influenza vaccines are ineffective against novel viruses and the source or strain of the next outbreak of influenza is unpredictable, therefore establishing universal immunity by vaccination is fundamental to limiting the impact of influenza. It is essential to improve influenza vaccines by utilizing multiple arms of the immune system. Virus-specific CD4$^+$ and CD8$^+$ T-cell responses are able to directly kill virus infected cells and co-ordinate local innate immune responses. Importanlty, T cells are able to react against different strains and subtypes of influenza. A novel vaccine has been developed using the immunogenic live vaccinia virus as a vaccine vector, containing multivalent influenza proteins derived from H5N1, plus IL-15 as an adjuvant. Previously, this vaccine demonstrated robust sterile cross-clade protection in mice for H5 influenza viruses, and here its use has been extended to mediate heterosubtypic immunity towards more distant group 1 and group 2 influenza viruses. Materials and Methods: Heterologous protection by the vaccine was

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assessed in the BalbC and B6 mouse models. T-cell responses elicited after vaccination and following influenza virus challenge were measured by Intracellular cytokine staining (ICS) assay. The T-cell compartment was selectively depleted by monoclonal antibody treatments. Results: The vaccine is able to induce robust immunological T-cell memory, enabling the survival of mice against challenge with many different influenza viruses. The vaccine protected mice against lethal challenge by increasing survival and significantly reducing virus loads against the most recent human H7N9, seasonal H3N2, pandemic-2009 H1N1, and highly pathogenic H7N7 influenza A viruses. Influenza-specific antibody responses were detectable after vaccination but were not neutralizing against heterologous viruses, nor able to mediate protection by passive transfer. Importantly, influenza specific $CD4^+$ and $CD8^+$ T-cell responses are elicited by the vaccine, and recruited following viral challenge in the lung and periphery. Selective depletion experiments for T-cell subsets revealed an important role of $CD4^+$ T cells in heterosubtypic protection depsite low peptide MHC-II epitope sequence conservation across different influenza viruses. Memory T-cell responses were found to act in synergy with higher viral loads in mice depleted of both $CD4^+$ and $CD8^+$ T-cell responses. Conclusions: This study has illustrated the potential use of multivalent-vaccinia virus as a universal influenza vaccine. The vaccine established influenza specific $CD4^+$ and $CD8^+$ T-cell responses, which were significantly larger upon viral challenge. We have established an essential role of $CD4^+$ T cells in providing universal protection against influenza established by a vaccinia-H5N1 vaccine.

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How we can use TLR2-agonist-based adjuvants to improve protection against influenza epidemics and pandemics

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Background: The discovery of Toll-like receptors (TLRs) and the use of their agonists as adjuvants is a well-documented way of enhancing protective responses to immunization with an antigen and currently form the vanguard of a new generation of rationally designed vaccine technologies. Here, we showcase how vaccination of soluble protein antigen electrostatically bound to the TLR2-agonist-based lipopeptide adjuvant R4Pam2Cys can result in the induction of protective antiviral immunity and how this strategy can be harnessed to improve and augment the efficacy of seasonal influenza vaccines. Materials and Methods: Evaluation of immune responses induced by vaccination with antigen formulated with R4Pam2Cys was performed in wild-type (C57BL/6 & BALB/c), transgenic (CD4 & B cell deficient), and knockout mice (TLR2 deficient), which enabled us to define and identify immune mechanisms mediating vaccine efficacy. Antibody responses 30 days later were measured in standard ELISAs, HI and NA assays in addition to in vitro virus neutralization assays and passive serum transfer experiments. Proliferative T-cell responses were measured 7 (primary) and 28 (memory) days following vaccination. Qualitative and quantitative CD4+ T-cell responses were analysed for their ability to differentiate into T-follicular helper cells (TFH) and promote B cell germinal center formation whilst CD8+ T-cell responses were analysed by performing intracellular cytokine straining, in vivo killing assays and memory phenotyping by flow cytometry. The ability of vaccination to protect against homo- and heterosubtypic viral challenge on day 30 was evaluated by measuring lung viral titres in plaque forming assays. We also used a contact-dependent transmission model to assess virus transmissibility from vaccinated challenged animals to naive cohoused recipients. Results: Our results demonstrate that vaccination of antigens using R4Pam2Cys induces high levels of antigen-specific antibody as well as CD8+ cytotoxic T lymphocyte (CTL) responses characterised by rapid proliferation, multiple cytokine production, granzyme B expression, and acquirement of in vivo cytolytic ability. Moreover, using this adjuvant also promotes the differentiation of CD4+ T cells into TFH cells, drives the formation of B-cell germinal centers and augments the development of CD8+ T cell effector memory and central memory populations which can be recalled effectively to mediate pulmonary anti-viral clearing responses better than other traditional adjuvants. Our studies also demonstrate that vaccination of animals with R4Pam2Cys formulated with a dose-sparing amount of influenza split virus vaccine derived from a H1N1 subtype elicits high levels of systemic neutralising antibodies and pulmonary IgA responses against the homologous strain. In addition, vaccination with this adjuvant also induces virus-specific CTL-mediated immunity, a property not

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inherent in current seasonal vaccines. These responses protect against homosubtypic as well as heterosubtypic challenge with a H3N2 subtype and can significantly mitigate the severity of infection caused by contact- dependent transmission. The absence of cross-reactive antibodies against the heterologous strain and the fact that heterosubtypic protection can still be demonstrated in antibody-deficient animals indicates that immunity bestowed by the use of this adjuvant against a different subtype is CTL-mediated. Conclusion: These discoveries provide insights into how modification of existing seasonal vaccines to include a component that can target their delivery to TLR pathways to activate CTL responses could extend their utility and spectrum of protection to provide a level of cross-protective immunity against drift variants of viruses and at the same time reduce the impact of future pandemic strains. The induction of such responses could be especially beneficial for improving community protection particularly during the period between an outbreak and when a vaccine becomes available.

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Evaluation of influenza A and B viruses isolated and passaged in the qualified MDCK suspension cell line (MDCK33016PF) and embryonated chicken eggs

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Background: To produce influenza vaccines, candidate vaccine viruses are isolated and propagated in embryonated chicken eggs, a process little changed since the 1940s. There are, however, a growing number of problems associated with this process, such as low isolation rates and poor growth with certain viruses as well as the ongoing issue of egg adaption changes. In this study conducted by the WHO Influenza Collaborating Centers at Atlanta, United Sates (WHO CC CDC) and Melbourne, Australia (WHO CC Melbourne) in collaboration with Novartis, we compared the isolation rates and characteristics of influenza A and B viruses isolated in eggs with those isolated in a qualified MDCK suspension cell line (MDCK33016PF), developed by Novartis Vaccines and Diagnostics. Materials and Methods: Influenza-positive respiratory samples that were received from 2008 to 2012 by the WHO CC Melbourne and WHO CC CDC were inoculated into both embryonated chicken eggs and the qualified MDCK suspension cell line MDCK 33016PF in parallel. Material was passaged 4 times in eggs and twice in cells at WHO CC Melbourne, and passed up to 3 times in eggs and 3 times in the Novartis suspension cells at WHO CC CDC. Virus was detected and analysed using a variety of assays. In addition, at CDC, we were interested to see if first adapting the A(H3N2) virus to the Novartis MDCK 33016PF suspension cell line would enhance it's ability to then be propagated in eggs. A subset of isolates grown in the Novartis MDCK cell suspension were inoculated into embryonated chicken eggs and passaged up to 3 times. Results: Through 2012, a total of 758 influenza positive original clinical samples were inoculated intoMDCK 33016PF cells and eggs by the WHO CC CDC. The overall isolation rate in the MDCK33016PF cell-line was 80% and 3% in eggs. By virus type, 582 of the 758 specimens were influenza A (the majority A specimens was H3N2 subtype), and 176 influenza B. The influenza A isolation rate was 82% in MDCK 33016PF cell-line and 2% in eggs, while the influenza B isolation rate was 71% in cells and 6% in eggs. In addition, a total of 148 A(H3N2) isolates propagated in the MDCK 33016PF suspension cells were inoculated into eggs. The isolation rate was 10%. A total of 284 influenza-positive respiratory samples were inoculated in both MDCK 33016PF cells and eggs by the WHO CC Melbourne. The overall isolation rate was in the MDCK 33016PF cell-line was 84% and 40% in eggs. By virus type, 196 of the 284 influenza-positive respiratory samples were influenza A, and 88 influenza B. The influenza A isolation rate was 88% in the MDCK 33016PF cell-line and 46% in eggs, while the influenza B isolation rate was 74% in the MDCK 33016PF cell-line and 28% in eggs. Sequencing of HA genes for all types/subtypes showed that cell-isolated viruses largely retained the genetic sequence of the original clinical samples, with no adaptation changes. Such changes were common in egg-isolated viruses. In particular, A(H3N2) egg-grown isolates had a high number of changes in comparison to the original influenza positive clinical samples sequence. Egg-isolated A(H1N1)pdm and B viruses also had

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adaption changes, but not in as many as the A(H3N2) isolates. In comparison, MDCK33016PF isolates had virtually no adaptation changes when compared to the original influenza-positive respiratory sample sequence. This pattern was similar to the pattern seen with regular adherent influena-positive MDCK isolates. Conclusion: The virus isolation and characterisation results obtained by the 2 WHO CC were comparable and lead to the conclusion that the MDCK 33016PF suspension cell line is a superior vaccine substrate for isolating influenza A and B viruses compared with eggs. Viruses isolated in qualified cell lines offer an alternative to egg-grown viruses and may be important for future vaccine production, as they more closely resemble influenza viruses found in epithelial cells of the upper and lower respiratory tract in humans and have fewer adaption changes compared to embryonated hens eggs.

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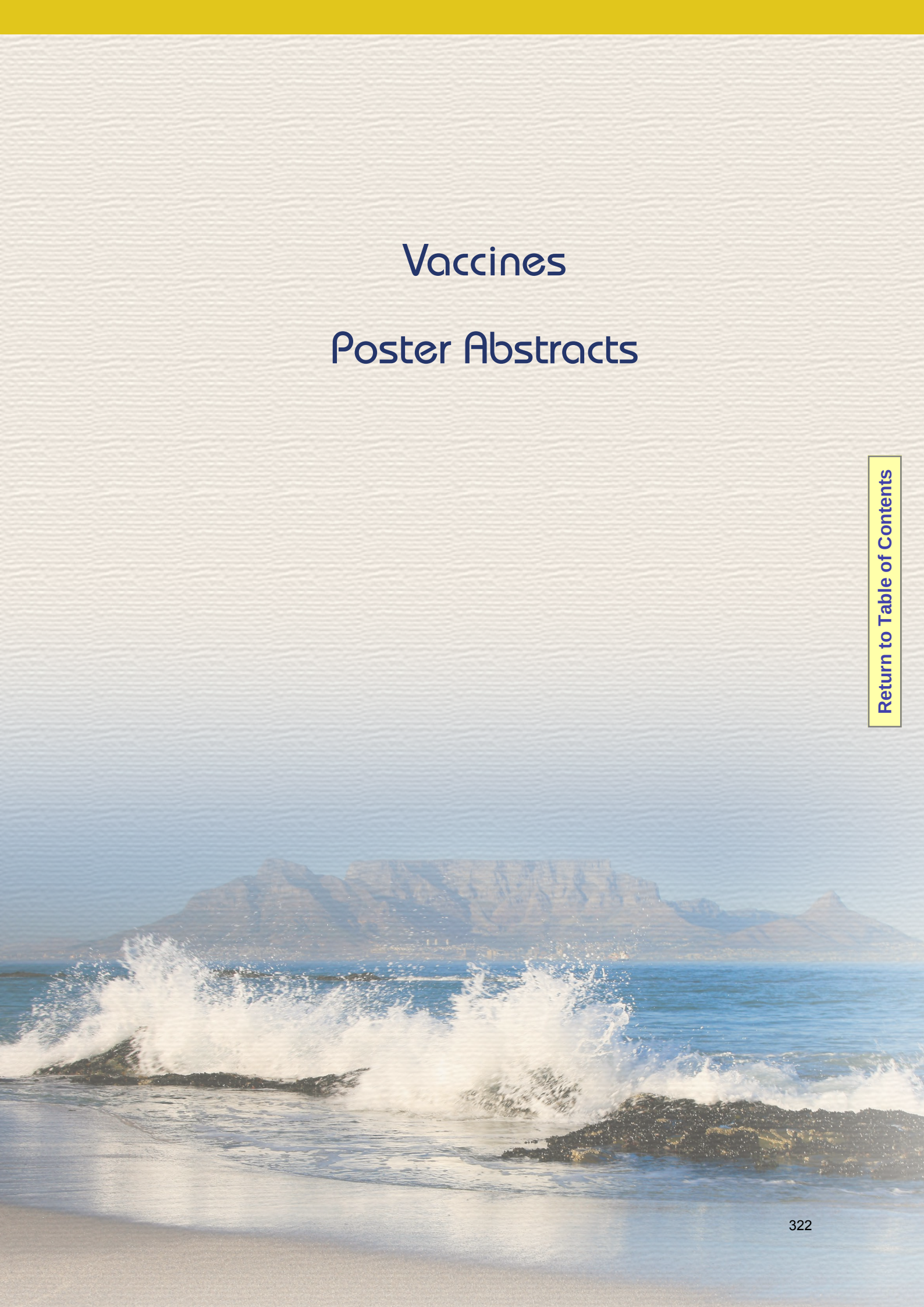
Vaccines

Poster Abstracts



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Cross-protective efficacy of vaccination with different forms of M2 vaccines and their combinations with HA vaccines against H5N1 influenza in mice

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Background: Recent outbreaks of H5N1 avian influenza virus that causes severe diseases in humans have raised concerns over an imminent influenza pandemic. As yet, there is no effective and universal vaccine that could be used to prevent and combat H5N1 infection. In this regard, development of vaccines for the prevention of H5N1 avian flu is an urgent need. Methods: In this study, we aimed to develop universal vaccine based on different forms of M2 vaccine candidates and their combinations with HA against H5N1 infection. We designed and prepared mM2e (synthetic monomer M2e peptide), M2e-MAP (tetra-branched multiple antigen peptide), fusion protein M2e-ASP-1 (1 M2e fused with ASP-1) and M2e3-ASP1 (3 M2e fused with ASP-1), DNA-M2, rAAV-M2, DNA-HA, rAAV-HA, protein HA, and inactivated virus vaccine. We tested immune responses and protective effects of these vaccine candidates, as well as effects of different aduvants. Results: Our results showed that (1) M2e-MAP, M2e-SAP-1 and M2e3-ASP-1 elicited much stronger M2e-specific antibody responses than M2e, DNA-M2 and rAAV-M2; (2) M2e-MAP and M2e3-ASP-1 provided better protection and cross-protection against viral challenges of different clades/strains of H5N1 virus and H1N1 virus than M2e-ASP-1, whereas mM2e, DNA-M2 and rAAV-M2 did not show obvious protection against viral challenge of H5N1 virus; (3) consistently, the mice vaccinated with M2e-MAP and M2e3-ASP-1 showed much lower viral load detected and less inflammation found in lung tissues; (4) cross-protective effect of M2e-based vaccines might be related to the identity of the M2e sequence between the vaccine and the H5N1 virus; (5) rAAV-HA, DNA-HA and inactivated virus vaccine provided effective protection against infection of homologous H5N1 virus, whereas these vaccine candidates and protein HA provided poor cross-protections against heterologous H5N1 viral infection; and (6) combination vaccinations with M2e-based and HA-based vaccine candidates, particularly vaccination of both M2e-MAP and inactivated virus vaccine, provided potent cross-protection against challenges with heterologous strains of H5N1 virus. Conclusion: Taken together, this study has demonstrated that both M2e-MAP and M2e3-ASP-1 can provide potent cross-protection against infections of some heterologous strains of H5N1 virus and H1N1 virus, while the strategy of combination vaccinations with both HA-based and M2e-based vaccines may be able to improve the cross-protection against heterologous H5N1 virus infection. Therefore, this study has provided the theoretical and practical basis for development of effective and universal vaccines against H5N1 influenza virus infection.

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Role of adjuvants in B-cell epitope repertoire and antibody avidity following influenza vaccination

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Background: Pandemic influenza is a persistent threat to global health. The continuing evolution of avian and swine strains contribute to unpredictability and difficulties in preparing vaccines against the next highly transmissible virus. However, efforts are under way to improve vaccine effectiveness against heterologous strains through new vaccine approaches, including novel adjuvants, prime-boost protocols, and recombinant virus-like particle (VLP) platforms. Methods: The fact that the correlates of immunity/protection remain unclear is a significant barrier to influenza vaccine research. We have developed novel technologies to delineate the immune correlates of protection during natural infection and after vaccination. Whole Genome Fragment Phage Display Libraries (GFPDL) for pandemic

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H1N1-2009 and H5N1 (Vietnam and Indonesia) strains were used to elucidate the complete antibody epitope repertoire following influenza vaccination with different vaccine modalities including, oil-in-water adjuvanted subunit vaccines, VLP, and DNA prime followed by inactivated split virus vaccine in different age populations. In addition, surface plasmon resonance (SPR) was employed to investigate the magnitude and avidity of human polyclonal antibody response against different antigenic domains within hemagglutinin following vaccination or infection. Results: DNA prime-inactivated H5N1 boost and oil-in-water adjuvanted H5N1 vaccines generated antibodies with higher neutralization capacity not only against the vaccine- homologous strains, but also against more diverse heterologous strains. GFPDL analyses of the human sera following adjuvanted vaccination revealed more diverse epitope repertoires, targeting large conformational epitopes in HA1 receptor binding domain, compared with unadjuvanted subunit H5N1 vaccination. SPR analyses revealed that oil-in-water adjuvanted vaccines and the DNA prime- followed by subunit H5N1 boost at 6 month, elicited higher serum antibody binding to oligomeric rHA1, which strongly correlated with the neutralization titers. Most importantly, the oil-in-water adjuvanted vaccines significantly enhanced antibody affinity maturation to H5N1-HA1 domain (but not HA2) as demonstrated by slower antigen-antibody complex dissociation rates in SPR, and increased resistance to 7M urea in ELISA. In the case of pH1N1 vaccination, the highest impact on antibody affinity was seen in young children. Conclusions: Thus, GFPDL and SPR identified attributes of influenza-specific antibodies, which are important for increased breadth of cross-protection against heterosubtypic strains with pandemic potential. These technologies will help in deciphering the quality of immune responses against future vaccine candidates against diverse pathogens and contribute to the design of more effective vaccines.

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TLR7 agonist imiquimod in combination with inactivated influenza vaccine induces expeditious and augmented immune responses sufficient enough to protect BALB/c mice against immediate lethal virus challenge

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Background: The potential of targeting Toll-like receptors (TLRs) to enhance vaccination efficacy had previously been explored and had shown promising effects. In this study, we investigated the possibility of using a synthetic TLR7 agonist, imiquimod, as an immune response potentiator, in combination with inactivated influenza vaccine to expedite and enhance the adaptive immune responses against influenza infection in a mouse model. Methods: Groups of female BALB/c mice were intraperitonially injected with imiquimod and inactivated monovalent A(H1N1)pdm09 vaccine, alone or in combination, at 3, 2 or 1 days prior to pdmH1N109 influenza virus challenge, or immediately before virus challenge. The mice were then inoculated with 10x mouse LD_{50} dose of pdmH1N1-Mut virus intranasally. Body weight and survival rate were monitored for 14 days postchallenge. At 1, 2, 4, and 6 days after virus challenge, 3 to 5 mice from each group were sacrificed, and blood and lung samples were collected for further studies. Results: Combination of intraperitoneal injection of imiquimod and A(H1N1)pdm09 vaccine provided an effective protection against an immediate A(H1N1)pdm2009 lethal challenge. Survival rates of 80%, 70%, and 55% were achieved respectively when the mice were challenged with 10x LD_{50} of the virus at 3, 2, or 1 day after treatment, while all control mice succumbed at day 9 post challenge. The pulmonary viral load was significantly lower at all time points for the imiquimod-vaccine combination treatment group than control group ($P < .05$). Viral specific serum IgM and IgG were detectable much earlier in these treated mice when compared with the control mice (2 days vs 6 days post challenge). Conclusions: Our findings demonstrate that this synthetic TLR7 agonist, imiquimod, in combination with inactivated influenza vaccine can expedite and augmented the adaptive immune responses sufficient enough to protect BALB/c mice against immediate lethal virus challenge.

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Broader neutralizing antibodies against H5N1 viruses using prime-boost immunization of hyperglycosylated hemagglutinin DNA and virus-like particles

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Background: Highly pathogenic avian influenza (HPAI) H5N1 viruses and their transmission capability from birds to humans have raised global concerns about a potential human pandemic. The inherent nature of antigenic changes in influenza viruses has not been sufficiently taken into account in immunogen designs for broadly protective HPAI H5N1 vaccines. Methods: We designed a hyperglycosylated HA vaccine using N-linked glycan masking on highly variable sequences in the HA1 globular head. Immunization of these hyperglycosylated HA DNA vaccines followed by a flagellin-containing virus-like particle booster in mice was conducted to evaluate neutralizing antibody responses against various clades of HPAI H5N1 viruses. Results: We introduced nine N-X-S/T motifs in five HA1 regions: 83NNT, 86NNT, 94NFT, 127NSS, 138NRT, 156NTT, 161NRS, 182NDT, and 252NAT according to sequence alignment analyses from 163 HPAI H5N1 human isolates. Although no significant differences of anti-HA total IgG titers were found with these hyperglycosylated HA compared with the wild-type control, the 83NNT and 127NSS mutants elicited significantly potent cross-clade neutralizing antibodies against HPAI H5N1 viruses. Conclusion: This finding may have value in terms of novel immunogen design for developing cross-protective H5N1 vaccines.

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Basic technological aspects of the development, safety assessment, and immunogenicity of a live cold-adapted equine influenza vaccine

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Background: For specific prophylaxis of equine influenza, the Research Institute for Biological Safety Problems developed a live vaccine based on the novel recombinant cold-adapted (*Ca)* strain A/HK/Otar/6:2/2010. The *Ca*-strain contains 2 genes (*HA, NA*) from wild-type strain A/equine/Otar/764/2007 (H3N8) and 6 genes (*PB2, PB1, PA, NP, M, NS*) from *Ca* strain A/Hong Kong/1/68/162/35 (H3N2). Materials and Methods: To optimize the cultivation of strain A/HK/Otar/6:2/2010 in chicken embryos (CE), various parameters such as the infection dose, temperature and incubation period were determined. To select the optimal stabilizing media for the vaccine virus, mono- and multicomponent media containing peptone, sucrose, trehalose, lactose, sorbitol, and glutamate in various concentrations were tested. For vaccine preparation, the virus was cultivated in CE using the optimal parameters. The oligosaccharide chitosan lactate (Sigma) was added to the viral biomass as an adjuvant (final concentration, 0.05%), then the vaccine mixture was mixed thoroughly, packaged into 1.0 mL vials, lyophilized and labeled. The safety of the vaccine was tested in 1-year-old foals and 5-year-old pregnant mares. The experimental animals were vaccinated intranasally with 10(8.2) lg EID_{50}/mL; control animals were vaccinated with PBS. The clinical state and health of the animals was evaluated over 4 weeks. To determine the immunogenicity of the vaccine, blood samples were taken from vaccinated foals at 7, 14, and 28 days post vaccination (PV) to quantify antibody accumulation using the hemagglutination inhibition (HAI) assay and an enzyme-linked immunosorbent assay (ELISA). Blood samples were taken over the same period PV to investigate cell-mediated immunity using the lymphocyte blast-transformation reaction. To determine the protectivity of the vaccine, experimental and control foals were subjected to intranasal challenge with 10(8.0) EID_{50}/mL of the wild-type virus A/equine/Otar/764/2007 (H3N8) on day 28 PV. The animals were clinically observed for 28 days postchallenge, with daily measurement of body temperature. Nasal swabs (on 1, 3, 5, 7 days postchallenge) were placed

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into tubes containing transport virus medium for titration of virus shedding in 10-day-old CE. Results: The optimal cultivation parameters for strain A/HK/Otar/6:2/2010 (H3N8) in CE were infectious dose of the virus: 100,000 EID_{50}; age of CE: 10 days; incubation temperature: 34ºC; incubation period: 48 hours. These parameters invariably led to the production of virus with infectious and HAI titers of not less than 10(8.5) lg EID_{50}/mL and 1:512, respectively. Vaccine samples stored in protective medium containing peptone (6%) and lactose (3%) were stable at 37±0.5ºC for up to 20 days; the infectivity of the virus only reduced to 10(1.59) lg EID_{50}/mL. We established that the developed vaccine was safe for foals and pregnant mares, as the clinical status of the vaccinated animals was normal during the entire period of monitoring, and pregnancy proceeded normally in vaccinated mares. Excretion of the vaccine virus in the upper respiratory tract of the vaccinated foals and pregnant mares was low; the virus was only detected on days 1 and 3 PV. In 1-year-old foals and pregnant mares vaccinated with a single dose, the antibody could not be detected using the HAI or ELISA on days 7, 14, and 28 PV. Cell-mediated immunity, as measured by the index of stimulation of peripheral blood lymphocytes, formed by day 14 PV in both foals and mares (1.73±0.26, 1.56±0.18, respectively, both $P < .05$ vs control group); however, the values at 28 day PV were not significantly higher than the respective control groups ($P > .05$). Vaccinated foals were provided with 100% protection against equine influenza with minimal virus shedding from the upper airway; virus shedding was higher and more prolonged in the control group than the vaccinated group at all periods of observation ($P < .001$ for days 1, 3, 5, and 7 postchallenge). Conclusions: We have developed the technology for manufacturing a live vaccine against equine influenza from the novel recombinant *Ca* strain A/HK/Otar/6:2/2010 which contains chitosan, allowing the preparation of a safe and immunogenic vaccine for equine influenza.

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Preclinical testing of the safety and immunogenicity of a chromatographic split-vaccine against seasonal influenza

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Background: In contrast to existing centrifuge technologies for the production of human vaccines against seasonal influenza, the RIBSP have developed effective, cheaper chromatographic split production technologies for seasonal influenza vaccines from strains recommended by the WHO. This paper presents the results of 3 experimental series of preclinical testing of the safety and immunogenicity of the chromatographic split-vaccine against influenza in laboratory animals. Materials and Methods: The chromatographic split-vaccine is a suspension of recombinant strains of the influenza virus, grown in chicken embryos, purified and concentrated by ultra/diafiltration and gel filtration, inactivated with formaldehyde and split with detergent. The experimental group were injected with the chromatographic split-vaccine, the comparison group with Fluarix vaccine (GlaxoSmithKline Biologicals, Germany), and the control group with PBS. Acute toxicity tests included identification of the clinical signs of toxicity, blood morphological and biochemical analyses, and pathomorphological analyses, including necropsy, and macroscopic and histological examinations of the viscera of mice and rats at 24 hours, 7 days, and 14 days postvaccination (PV). The allergenic properties of the vaccine were investigated using a model of delayed-type hypersensitivity in mice and immediate-type hypersensitivity in guinea pigs. The immunogenicity of the vaccine was determined on day 28 PV in mice. All studies were performed in accordance with national and international requirements and guidance on the use of laboratory animals. Results: During the acute toxicity tests, weight loss, and the clinical signs of poisoning were not observed in any mice or rats from the experimental, comparison, or control groups. All animals survived during the period of clinical observation. Morphological and biochemical analysis of peripheral blood samples revealed no significant differences between the experimental, comparison, and control groups, regardless of sex ($P > .05$). Pathologicoanatomic dissection followed by histological examination did not reveal any morphological or histological signs of disease or toxicity in mice and rats at any time point. Thus, administration of

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the chromatographic split-vaccine series at a high dose (500 times the normal human dose in terms of the mass ratio of mice, and 350 times, in terms of the body weight of rats) did not exert a negative influence on any basic physiological function, and did not induce any pathological changes in mice or rats, indicating the preparation was tolerated and safe. Laboratory symptoms of allergy were not observed in any mice in the model of delayed-type hypersensitivity. The rate of limb edema stimulation in mice from the experimental group was 29.33% for females and 12.97% for males; the corresponding rates in mice vaccinated with Fluarix were 41.68% and 19.71%, respectively; none of these values were significantly different to the control group ($P > .05$). Three-fold immunization of guinea pigs with the vaccine induced mild respiratory system changes and reactions, observed as nose scratching and sneezing; however, these signs did not develop into fatal anaphylactic shock, which demonstrates the vaccine induces a weak immediate-type allergic effect. By day 28 after a single immunization of the vaccine, the mice formed antihemagglutinating antibodies with mean geometric titers (GMT) of 197.0±5.4 to 320.0±6.1 for subtype A/H1N1, 160.0±4.5 to 242.5±5.7 for subtype A/H3N2, and 149.3±4.7 to 278.6 ± 7.7 for type B influenza virus. The GMT values of mice injected with the comparator vaccine ranged from 130.0±4.1 to 113.1±3.8, 105.6±3.6 to 149.3±4.4, and 105.6±3.6 to 121.3±4.3, respectively. Compared with the similar commercial vaccine, the chromatographic split-vaccine had a more pronounced immunogenic activity against the three influenza strains included in the vaccine. Conclusion: Preclinical studies of the developed chromatographic split-vaccine against seasonal influenza indicate that the vaccine is well tolerated, safe, and immunogenic in laboratory animals.

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Safety and immunogenicity of a monovalent inactivated whole-virion chromatographic vaccine with aluminum against influenza A (H1N1) pdm09: a randomized, blinded, dose-dependent clinical study

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Background: The pandemic influenza A/H1N1 pdm09 emerged in 2009 and led to high demand for influenza vaccines, highlighting the limited vaccine manufacturing capacity world-wide. To meet the needs of Kazakhstan for an effective vaccine against pandemic influenza, the RIBSP developed a monovalent inactivated whole-virion chromatographic vaccine with aluminum (Refluvac). This paper presents the results of a clinical phase 2 single-use study of the vaccine in healthy volunteers aged 18-60 years. Materials and Methods: The study was conducted at the clinical facilities of the Research Institute of Influenza and the Research Institute of Vaccines and Serums (Russian Federation). For the study, 80 healthy adult volunteers were randomly assigned into two groups in a ratio 1:1 for a single dose of Refluvac (3.75 or 7.5 mcg of hemagglutinin [HA]). Antibody response (seroconversion and seroprotection rates, geometric mean titer [GMT], seroconversion factors) was determined using the hemagglutination inhibition (HAI) assay. Cellular immune response was assayed using the index of stimulation of peripheral blood mononuclear cells (PBMCs) and the production of cytokines by antigen supernatant-stimulated cells. Reactogenicity and safety were assessed by monitoring adverse reactions (local and systemic reactions), physical examinations, monitoring of vital symptoms and laboratory tests (general and biochemical blood tests, Ig E, urinalysis). The study was conducted in accordance with protocol VRK-II-00-03/2010 approved by the Federal Service on Surveillance in Healthcare and Social Development of the Ministry of Health and Social Development of the Russian Federation. Results: Volunteers vaccinated with the vaccine at a dose of 3.75 mcg HA did not have any systemic reactions related to the vaccination. Weak local reactions, presenting as pain and discomfort at the injection site, were observed in 10 of the 40 (25%)

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volunteers vaccinated at a dose of 3.75 mcg HA; these reactions were not accompanied by the development of hyperemia or infiltrates, were transient (lasted no more than 2 days), and disappeared without medication. Among the volunteers vaccinated with the vaccine at a dose of 7.5 mcg HA, one medium systemic reaction was observed as an increase in temperature up to 37.8°C on the first and second days post vaccination (PV). Weak local reactions, presenting as pain and discomfort at the injection site were observed in 6 of the 39 (15.4%) volunteers vaccinated at a dose of 7.5 mcg HA; these reactions were not accompanied by the development of hyperemia or infiltrates, were transient, and disappeared without medication. Clinical and laboratory examinations of the volunteers on days 7 and 21 PV revealed that the morphological and biochemical parameters of peripheral blood, including the total level of IgE, did not significantly alter from normal physiological values (all $P > .05$). Vaccination had no adverse effect on electrocardiogram (ECG) data PV, as the ECG data for all volunteers remained similar to the baseline values. In terms of rating the immunogenic activity of the vaccine, 85.0% of the volunteers vaccinated at a dose of 3.75 mcg HA had a 4-fold seroconversion rate; the seroprotection rate was 85%, the multiplicity of growth of the antibody titer was 13.8 and the GMT was 69.0. In the group vaccinated at a dose of 7.5 mcg HA, 92.3% of the volunteers had a 4-fold seroconversion rate; the seroprotection rate was 92.3%, the multiplicity of growth of the antibody titer reached 36.0, and the GMT was 180.0. Vaccination led to the formation of a cellular immune response, which was polarized towards Th-1 production. Conclusions: This Phase II clinical study of the Refluvac vaccine at doses of 3.75 mcg or 7.5 mcg HA in healthy volunteers aged 18-60 years-old indicates the antibody can induce pronounced immunogenicity, is well tolerated, has a low reactogenicity, is safe, and leads to the formation of a cellular immune response.

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Different immunity elicited by recombinant H5N1 hemagglutinin proteins containing pauci-mannose, high-mannose, or complex type N-glycans

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Background: Highly pathogenic avian influenza H5N1 viruses can result in poultry and occasionally in human mortality. A safe and effective H5N1 vaccine is urgently needed to reduce the pandemic potential. Hemagglutinin (HA), a major envelope protein accounting for approximately 80% of spikes in influenza virus, is often used as a major antigen for subunit vaccine development. Methods: We conducted a systematic study of the immune response against influenza virus infection following immunization with recombinant HA proteins expressed in insect (Sf9) cells, insect cells that contain exogenous genes for elaborating N-linked glycans (Mimic) and mammalian cells (CHO). Results: While the antibody titers are higher with the insect cell–derived HA proteins, the neutralization and HA inhibition titers are much higher with the mammalian cell–produced HA proteins. Recombinant HA proteins containing tri- or tetra-antennary complex, terminally sialylated and asialyated-galactose type N-glycans induced better protective immunity in mice to lethal challenge. Conclusion: The results are highly relevant to issues that should be considered in the production of fragment vaccines.

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Criteria of screening of H5N1 highly pathogenic avian influenza virus as a wild-type parent for a prepandemic live attenuated reassortant influenza vaccine candidate

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Background: The H5N1 highly pathogenic avian influenza (HPAI) viruses pose a significant pandemic threat: they continue to circulate widely among wild birds and in poultry populations, and may cause severe disease and death in humans, who mostly have no immunity to them. Few amino acid substitutions might be sufficient for the virus to become airborne transmissible between mammals. The main effective antiviral prevention measure remains vaccination. Rapid evolution of H5N1 HPAI viruses requires constantly updating vaccines, but vaccine technology of the day cannot rapidly respond to the threat of newly emergent potentially pandemic influenza strains. The WHO's strategy is the creation of a bank of vaccines against potentially dangerous viruses for use in the event of emergency needs. Live attenuated cold-adapted reassortant influenza vaccines (LAIVs) are preferred, as they provide a broader and more long-lasting cross-protective immune response compared with inactivated influenza vaccines. The objective of this study was a selection of an H5N1 HPAI strain most suitable as a wild-type parent for a prepandemic reassortant LAIV candidate. The screening of prospective H5N1 HPAI viruses for an LAIV candidate was performed according to major virus properties that might determine pandemic potential: the antigenic novelty of the virus to the immune system, its virulence, and its ability to spread from person to person (transmissibility). Materials and Methods: Screening of H5N1 HPAIVs of different clades was performed to prepare the best prepandemic LAIV candidate. LAIV strains were generated by classical reassortment of cold-adapted temperature-sensitive master donor virus for Russian LAIV A/Leningrad/134/17/57 (H2N2) and PR8-based H5N1 (H5N1/PR8) candidates for inactivated vaccine (IIV) as a source of H5 HA, or by reverse genetics (rg). H5N1/PR8 IIV candidates A/Vietnam/PR8-CDC, A/Indonesia/PR8-CDC and NIBGR-23 were obtained from the CDC and WHO (corresponded wild-type strains A/Vietnam/1203/2004 clade 1, A/Indonesia/05/2005 clade 2.1 and A/turkey/Turkey/1/2005 clade 2.2). To reduce virulence, the multibasic cleavage site of H5 HA was removed. Five LAIV candidates were analyzed: two LAIV candidates were rg-constructed on the base of A/Vietnam/1203/2004 and A/Egypt/321/2007 clade 2.2, and three LAIV candidates were generated by classical reassortment. All LAIV reassortants were fully sequenced to detect spontaneous mutations. The pathogenicity of parental H5N1/PR8 strains and LAIV reassortants was evaluated by the macroscopic lessions observed on infected chicken embryos. Safety, attenuation, immunogenicity and protection of LAIV candidates were studied in ferret and mice models. Transmissibility of H5N1 parental viruses and LAIV candidates was tested in guinea pigs. Results: Four H5N1 viruses of different clades were studied as donors of HA and NA genes for LAIVs. H5N1 viruses tested were novel for the human immune system, were virulent for humans, and were isolated from autopsy samples so they may pose a pandemic threat. H5N1/PR8 viruses have been chosen for LAIV development to reduce the pathogenicity to chicken embryos and for safety lab work. The microscopic examination of infected chicken embryos demonstrated that H5N1/PR8 viruses still kept some pathogenicity despite HA modification. The most substantial macroscopic lesions were observed in embryos infected with NIBRG-23. NIBRG-23 also demonstrated the greatest degree of airborne transmissibility in a guinea pig model. This correlates with the fact that the A/turkey/Turkey/1/2005 virus possesses a Thr-156-Ala mutation in HA that some researchers (Herfst, et al, 2012) associate with the enhancement of airborne transmissibility and Glu-627-Lys mutation associated with increased virulence of H5N1 viruses in ferrets. All LAIV reassortants tested grew equally well in eggs, were attenuated in animal models, retained the antigenic properties of the parental H5N1 viruses, and were immunogenic for ferrets and mice. In contrast, NIBRG-23-based LAIV promoted broader cross-clade immune response, which is a good background for a prepandemic vaccine. Conclusions: A/turkey/Turkey/1/2005 (H5N1) is a prospective prerequisite for a prepandemic vaccine. The LAIV reassortant with modified HA of A/turkey/Turkey/1/2005 lost virulent

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determinants, it is safe, immunogenic and promotes the most broad cross-clade immunity among studied LAIV candidates in the animal model. This research was financially supported by PATH.

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Immunogenicity and protective efficacy of LAIV strains against potentially pandemic A(H2N2) influenza virus in a ferret model

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Background: Influenza A(H2N2) viruses have not circulated in the human population since 1968, so people under the age of 45 have no immunity to them and will be vulnerable to this virus if it returns to circulation. A(H2N2) influenza viruses continue to circulate among animals, maintaining an existing threat to global health should these viruses return to the human population. Therefore, generation of safe and cross-reactive H2N2 vaccine is of high priority. Materials and Methods: Classical reassortment technique was used to prepare two LAIV strains bearing HA and NA from either A/California/1/66 (H2N2) or A/Tokyo/3/67 (H2N2) wt viruses and internal genes from master donor virus (MDV) Len/17. Groups of 12 ferrets received one dose of 10^6 $TCID_{50}$ of either H2N2 LAIV or MDV Len/17, by i.n. route. Control animals received placebo (PBS). Blood was collected at -1, 14, 21 and 26 days after vaccination for HAI assay. At day 21 6 ferrets from each group were challenged with A/California/1/66 virus, and remaining 6 animals with A/Tokyo/3/67 virus at a dose of 10^6 $TCID_{50}$. Subsequently, ferrets were weighed and monitored for 5 days for clinical disease; throat swabs were taken on days 21, 23, 24, and 26. Body temperature was recorded by implanted temperature loggers. The infectious viral titers in throat swabs and nasal turbinates were determined by end-point titration in MDCK cells. In addition, histopathological evaluation of nasal turbinates was performed. Results: To generate H2N2 LAIV strains by classical reassortment an intermediate H1N1 reassortant virus bearing HA and NA genes of H1N1 wt virus and 6 internal genes of Len/17 MDV was used as a source of Len/17-specific internal genes. The resulting H2N2 vaccine reassortants A/17/California/66/395 (17/Cal/395) and A/17/Tokyo/67/326 (17/Tok/326) were compared with the MDV in aferret study. All 3 H2N2 viruses induced high homologous HI antibody titers both at 14 and 21 dpi. The 17/Cal/395 strain induced the highest homologous titers, followed by 17/Tok/326 and Len/17 respectively. In addition, the LAIVs induced high titers against the heterologous strains. The 17/Cal/395 strain induced better cross reactive antibody titers than 17/Tok/326 and Len/17 strains. Virus replication in the placebo vaccinated animals was the highest for both challenge viruses. Interestingly, 17/Cal/395 protected better than 17/Tok/326 LAIV against challenge with A/Tokyo/3/67, whereas the 17/Cal/395 and 17/Tok/326 provided equal protection after challenge with A/California/1/66. In this respect, the parental Len/17 MDV performed less well. At day 26, high virus titers were detected in the nasal turbinates of the control group animals, whereas all the LAIV vaccinated animals showed a significant reduction of virus replication either for the homologous or heterologous challenge strains. Analysis of the body temperature showed a reduction in fever for either of the challenge viruses in LAIV vaccinated groups, compared with the placebo. The temperature increase over the course of the challenge phase (area under curve) was higher in the control animals than in the vaccinated animals. Differences between the vaccines were not observed. Histopathological evaluation of nasal turbinates of control animals showed moderate damage with end score of 2.3-2.4 on a scale of 0-5. All animals vaccinated with the LAIVs showed less pathology than placebo-vaccinated ones after challenge with either of the 2 challenge strains. Between the different vaccine groups no statistical differences were observed for the end score; however, the percentage of tissue affected for the 17/Cal/395 vaccinated animals is significantly lower than for the Len/17 group when challenged with A/Tokyo/3/67. Conclusions: Three cold-adapted H2N2 LAIV strains were tested in a ferret challenge model, of which A/17/California/66/395 stimulated the best cross-reactive immune response and induced better cross-protection. These data allowed recommending A/17/California/66/395 strain for further evaluation in phase 1 clinical trial. This study was supported by WHO and PATH.

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Development of the quadrivalent live attenuated influenza vaccine (LAIV) including two reassortant influenza B viruses based on B/USSR/60/69 donor strain

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Background: Since 2001 the simultaneous circulation of 2 antigenically distinct influenza B lineages B/Victoria and B/Yamagata resulted in need to introduce both influenza B variants into seasonal influenza vaccines. For these purposes we evaluated in preclinical and clinical study the coadministration of both B lineages vaccine viruses. Materials and Methods: The reassortant B/USSR/60/69-based influenza B viruses were prepared in the Virology Department. Preclinical studies of mix inoculation with 2 reassortant influenza B viruses belonging to B/Victoria and B/Yamagata antigenic lineages were conducted in developing chicken embryos (CE) and CBA mice. Limited clinical study in 32 adult volunteers 18-56 years old was conducted to estimate a safety and immunogenicity following single intranasal immunization of the quadrivalent LAIV (qLAIV), including both vaccine strains B/Victoria and B/Yamagata compare with trivalent LAIV containing single influenza B lineage. Results: Simultaneous inoculation in CE with various equal doses of B/Victoria and B/Yamagata vaccine strains had shown that shared influenza B viruses did not interfere with each other. The intranasal introduction of single B/Victoria or B/Yamagata vaccine strain into CBA mice induced the hemagglutination-inhibition (HI) antibodies which were not cross-reacting with distinct antigen lineage, although neutralizing antibody against B/Victoria lineage were little cross-reacting against B/Yamagata. Mice immunized with 300 of 50% mouse infectious doses (MID_{50}) of both influenza B vaccine strains had demonstrated 100% lung protection against the subsequent infection with 100 MID_{50} of wild-type influenza B viruses of any antigen lineage, whereas immunization with single B lineage provide only partial protection against heterologous wild-type influenza B virus. Clinical evaluation had shown that introduction of qLAIV comprising both reassortant influenza B viruses B/Victoria and B/Yamagata was safe and areactogenic. 28 days after administration of qLAIV the geometric mean titers of HI antibodies to each of the vaccine strains were very close to those in trivalent LAIV formulation ($P > .05$). Influenza B virus RNA was detected in nasal swabs of the vaccinated using real-time PCR analysis on day 3 and day 5 after vaccination. On day 5 both M and NP genes of influenza B virus were detected in nasal swabs of 2 from 10 volunteers receiving trivalent preparation containing B/60/Wisconsin vaccine strain compare with 3 from 12 volunteers who were vaccinated with qLAIV (Fisher's exact test, $P = .594$). Conclusions: Introduction of both vaccine viruses of B/Victoria and B/Yamagata antigenic lineages had no negative impact neither on a reactogenicity of qLAIV nor on an isolation of vaccine influenza B viruses compare with trivalent preparations. Following qLAIV administration the immune response was achieved to both B/Victoria and B/Yamagata unlike trivalent LAIV. Introduction of the quadrivalent LAIV has to overcome influenza B antigen mismatching and to improve efficacy of seasonal flu vaccine.

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Gastrointestinal delivery of baculovirus-based bivalent vaccine: A broadly protective vaccine against H5N1 infection

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Background: Continuous outbreaks of highly pathogenic avian H5N1 influenza in Asia and a current situation of new H7N9 bird flu in China are increasing the threat of the next influenza pandemic. Vaccination against influenza A viruses is the first and most important step in controlling the spread of the pathogen. However, currently available influenza H5N1 vaccines induce protective immunity only

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towards vaccine-specific strains. Hence, influenza H5N1 strains already evolved and antigenically distinguished into 10 different clades and numerous subclades which hindered the selection of vaccine strain for pandemic preparedness. Therefore, it is important to develop a prepandemic vaccine that induces some degree of cross-protection against a future H5N1 pandemic strain. Several strategies have been evaluated in an effort to increase a cross-protective efficacy of the H5N1 vaccine. Our vaccine development approach involved three elements; incorporation of antigenic targets or selection of vaccine strains to achieve broad cross-reactive immunity, utilization of vector based efficient antigen delivery and route of administration of the vaccine candidates. The main objective of this study was to demonstrate the cross-protective efficacy of baculovirus displayed HAs 0f A/Indonesia/669/06 and A/Anhui/01/05 against heterologous H5N1 challenges in a mouse model. Materials and Methods: BALB/c were immunized intragastrically or subcutaneously with 200 µl containing 10^8 PFU of live or inactivated recombinant baculovirus vaccine. As a reference control, group of mice was vaccinated subcutaneously with either log 2^8 HA titer of inactive whole H5N1 virus (A/Indonesia/CDC669/2006) or 10^8 PFU of inactive bivalent-BacHA, emulsified with Montanide ISA563 adjuvant. For subcutaneous immunizations, the boost was administered on day 21 after the first immunization. For oral immunizations, the booster doses were administered on day 7 and 21 after first immunization. Results: The study results show that mice immunized orally or subcutaneously with live bivalent-BacHA vaccine significantly enhanced humoral immune responses when compared with inactivated bivalent-BacHA. In addition, oral administration of live bivalent-BacHA vaccine was able to induce significant level of antigen-specific mucosal IgA levels. Mice immunized with live bivalent-BacHA vaccine induced cross-clade neutralizing antibody titer distinct H5N1 clades (1, 2.1, 2.2, 2.3, 4, 7 and 8). Also, mice vaccinated orally or subcutaneously with live bivalent-BacHA stimulated both IFN-γ secreting TH1 cells and IL-4 secreting TH2 cells, whereas mice immunized subcutaneously with adjuvanted inactive bivalent-BacHA stimulated only IL-4 secreting TH2 cells. Cross-protective immunity study also showed that mice immunized either orally or subcutaneously with live bivalent-BacHA provided complete protection against 5MLD50 of heterologous H5N1 infections. Conclusions: Oral immunization with live bivalent-BacHA vaccine induces antigen specific humoral, mucosal and cellular immunity, and provides protection against heterologous challenge in mice. Compared with systemic vaccines, oral vaccine stimulates antigen specific mucosal immunity and also showed increased patient adherence, safety, and ease of administration. Also, baculovirus-based delivery system is advantageous over other human viral vectors due to its low cytotoxicity, lack of replication in mammalian cells and absence of preexisting immunity in humans.

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Influenza vaccination coverage and effectiveness in a cohort of young children in Bangkok, Thailand

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Background: Since 2009, Thailand has recommended influenza vaccine for several groups at high risk for severe influenza, including children aged 6-24 months. However, the number of persons for which influenza vaccination is recommended far exceeds the supply of influenza vaccine purchased by the Thai government annually. We evaluated influenza vaccination coverage during 2010-2013 and estimated the effectiveness of the 2011 Southern Hemisphere influenza vaccine in young children in Bangkok. Methods: In August 2011, we began a prospective cohort study of children aged ≤ 36 months at Queen Sirikit National Institute of Child Health. The study enrolled an equal number of children with and without an underlying medical condition. We followed each child for 2 years and contacted parents weekly to inquire about whether their child had acute respiratory illness. Ill children came to the hospital and had a combined nasal and throat swab collected and tested for influenza viruses by realtime reverse transcription polymerase chain reaction. Influenza vaccination status was

verified since enrollment and every 6 months by reviewing each child's vaccination card. Since Thailand uses Southern Hemisphere vaccine and influenza viruses circulate year-round, we defined the season from June, when vaccine was available, until May. Complete vaccination was defined as having received 2 vaccine doses in the current season that were administered ≥ 28 days apart or ≥ 1 dose in any previous season and 1 dose in the current season. Partial vaccination was defined as having received only 1 of 2 recommended vaccine doses during the current season and no vaccination in the previous season. We limited analysis to children aged ≥ 6 months at the beginning of each season. For 2011-2012 and 2012-2013 seasons, VE was estimated as 100% × (1-hazard ratio). To adjust for age at respiratory illness and underlying disease condition, we used the Cox proportional approach. Results: During 2010-2013, 1050 children aged 6-44 months were enrolled (115 during the 2010-2011 season, 308 during 2011-2012, and 627 during 2012-2013); 424 (40.4%) children had ≥ 1 medical condition considered a risk for severe influenza. Vaccine coverage (partial or complete) by season was 27.8% (32/115), 40.2% (124/308), and 32.0% (201/627), respectively. Complete vaccination was 18.3% (21), 34.4% (106), and 23.9% (150), respectively. Only in the 2011-2012 season, complete vaccination was significantly higher among children aged 24-44 months compared with those aged 6-23 months (22/37 [59.4%] vs 84/271 [31.0%]; P < .01). In 2012-2013, the complete vaccination rate was significantly higher among healthy children compared to children with medical conditions (106/373 [28.4%] vs 44/254 [17.3%]; P < .01), but this was not found in other seasons. In 2011-2012, there were 246 ARI episodes; of which 8 (3.2%) were influenza positive (age range, 9-36 months; 4.2 per 1,000 vaccinated child-weeks and 7.4 per 1000 unvaccinated child-weeks). The unadjusted and adjusted VE for complete vaccination was 44% (95% confidence interval [CI]; -154%, 88%) and 62% (CI; -63%, 91%), respectively. In 2012-2013, there were 822 ARI episodes; of which 46 (5.6%) were influenza positive (age range, 7-46 months; 3.6 per 1000 vaccinated child-weeks and 4.3 per 1000 unvaccinated child-weeks). The unadjusted and adjusted VE for complete vaccination was 28% (CI; -78%, 71%) and 37% (CI; -63%, 76%), respectively. Conclusions: Influenza vaccination rates were moderate in this cohort of young children in Bangkok; however, our data may not be representative of all Thai children. Additional efforts are needed to increase vaccination rates particularly in children aged 6-24 months or those with underlying disease. The 2010-2011 VE point estimate suggests the vaccine was moderately effective at protecting young children against influenza; however, it was much lower in 2012-2013. The VE estimates did not reach statistical significance, possibly due to the small number of influenza infections. Continued evaluation of influenza vaccination coverage and effectiveness is merited to evaluate national influenza vaccination program impact among young children who are at high risk of severe influenza.

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Identification of key amino acids in influenza matrix protein 2 ectodomain which affect vaccination effectiveness against high pathogenic influenza challenge

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Background: The ectodomain of influenza matrix protein 2 (M2e) is an ideal target for universal vaccine development due to its highly conserved property among all subtypes of influenza virus. However, it has been reported that some alternations on the domain sequence could resulted in the vaccination ineffective. Methods: In this study we investigated the relationship between the vaccine protection and the amino acid alternation on the M2e sequence to identify critical amino acids that could affect M2e vaccine efficacy. M2e tetrameric peptide of Vietnam/1194/04 H5N1 strain (P0) and 9 other peptides with amino acid changes were synthesized. Their protection against H5N1 viral challenge was investigated. Results: Our results showed that the protection would be completely abolished when amino acid at position 10, 14, and 16 changed respectively from Pro to Leu, Glu to Gly, and Glu to Gly. However, single amino acid altered at these positions would not affect the protection against the viral challenge. Increased protection was observed when amino acid at position 20 changed from Ser to Asn. Antibody binding assay also supported the survival result, as there was a direct relationship between the binding ability of vaccine-induced antibodies and the protection against lethal viral challenge. Different secondary structures of these peptides were shown by 3D

prediction, which might affect the induction of antibodies. Conclusion: Our study has demonstrated some critical amino acids in M2e sequence which should be considered in development of universal vaccine based on M2e.

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Cross-protective immunity of a new trivalent MVAtor-H5HA vaccine candidate in mice and guinea pigs against various H5N1 influenza clades

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Background: Highly pathogenic H5N1 influenza strains have a potential for causing a pandemic disease that remains a serious public health concern. Moreover, influenza strains frequently evolve into new types by random mutation leading to diverse antigenic determinants, thus posing serious challenges to the control of influenza infection by currently existing vaccines. To overcome the limitations of individual strain-specific antigens the concept of universal vaccines covering a broad spectrum of strains is in the focus of vaccine developers around the globe. EPIC Bio, the joint venture of Emergent BioSolutions and Temasek Life Sciences Laboratory, developed a new trivalent MVAtor™ (modified vaccinia virus Ankara vector) vaccine candidate expressing 3 different H5HA antigens from the strains A/Vietnam/1203/04 (clade 1), A/Indonesia /669/06 (clade 2.1.3) and A/Anhui/02/2006 (clade 2.3.4). These H5HA antigens were selected for broad cross-protection against different clades by covering all major amino acid variations of neutralizing epitopes of H5HA in H5N1 strains. Therefore, the new trivalent vaccine candidate is intended to provide cross-clade protection against various H5N1 influenza strains. The aim of the reported study was to assess the immunogenicity of the trivalent MVAtor-H5HA vaccine candidate in mice and guinea pigs. Materials and Methods: BALB/c mice were immunized intramuscularly twice with 8×10^7 TCID50/animal on days 0 and 28 with trivalent MVAtor-H5HA or MVAtor-LacZ control. Serum of mice was collected at days 27, 47, and 63 and analysed by hemagglutination inhibition (HAI) assay and micro-neutralization assay. Mice were challenged at day 49 with 10 MLD50 of homologous clade 1.0 (A/Vietnam/1203/04) or heterologous clade 7.0 (A/Shanxi/2/06) H5N1 influenza virus. Immune response, survival, weight loss, and viral load in lungs were assessed. Additionally, immune sera in guinea pigs were created by immunization with trivalent MVAtor-H5HA with 8×10^7 TCID50/animal on days 0, 28, and 49, and sera for analysis in HAI and micro-neutralization assay were collected on day 63. Results: Immunogenicity and protective efficacy of a recombinant, trivalent MVAtor-H5HA vaccine candidate expressing three different H5HA influenza virus hemagglutinin antigens was evaluated in mouse and guinea pig models. After 2 immunizations, BALB/c mice vaccinated with trivalent MVAtor-H5HA showed a robust immune response evident by both HAI titers and serum micro-neutralization titers. Importantly, the trivalent MVAtor-H5HA vaccine candidate protected 100% of mice in a challenge study against 10 MLD50 of homologous clade 1.0 (A/Vietnam/1203/04) and heterologous clade 7.0 (A/chicken/ Shanxi/2/06) H5N1 strains. Moreover, sera from vaccinated guinea pigs showed strong HAI response and effective neutralization against the three homologous H5N1 strains (A/Vietnam /1203/04, A/Indonesia/669/06 and A/Anhui/02/2006), as well as 14 different heterologous H5N1 strains, including strains from clade 0, 4, 7 and 9. Conclusions: The newly developed trivalent MVAtor-H5HA vaccine candidate induced high levels of cross-clade neutralizing antibodies in mice. In addition, the vaccine candidate provided cross-clade protection against homologous and heterologous H5N1 viral infection without any clinical symptoms. Furthermore, vaccination of guinea pigs with the trivalent MVAtor-H5HA resulted in high HAI titers and micro-neutralization titers against 3 homologous and 14 heterologous H5N1 strains, indicating extensive cross-reactivity. These promising findings indicate the feasibility of the development of a prepandemic vaccine candidate that provides broad protection against various influenza H5N1 influenza strains by combination of carefully selected divergent H5HA antigens in a single vaccine.

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Viral vectored vaccines against influenza

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Background: Recombinant, replication-deficient viral vectored vaccines are among the leading candidates in the fields of HIV, malaria, and tuberculosis vaccine development. Clinical trials have demonstrated that they are safe and highly immunogenic in people of all ages. They are capable of inducing both humoral and T-cell–mediated immune responses against multiple antigens, and can prime novel immune responses as well as boosting existing memory responses. This talk will discuss progress in both preclinical and clinical development of viral-vectored vaccines against influenza, including approaches that are being taken to induce broadly cross-reactive T-cell responses to conserved internal antigens of influenza, as well as the use of haemagglutinin (HA) stem as an antigen. Materials and Methods: Viral vectored vaccines (both Modified Vaccinia virus Ankara and a simian adenovirus) expressing nucleoprotein and matrix protein 1 have been tested in a series of clinical studies of healthy volunteers aged between 18 and 85 years, including coadministration of trivalent inactivated vaccine (TIV) in one study. Immune responses to the vaccine were measured by interferon gamma ELISpot assay and HI titre. In preclinical studies, the effect of priming immune responses to HA stem prior to booster immunization with a series of vaccines has been assessed. Results: The viral-vectored vaccines were safe and immunogenic in all trials. T cell memory to NP and M1 was boosted, and coadministration of MVA-NP+M1 and TIV resulted in increased HI titres to TIV. Priming of influenza-naive animals with HA-stem resulted in changes in humoral responses to subsequent HA vaccination. Conclusions: These studies illustrate the potential of viral-vectors to produce a new generation of influenza vaccines that can induce broadly protective immunity to influenza in people of all ages. Combined with new improvements in vector design, this approach has much to offer.

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Efficacy of MVAtor-based trivalent vaccine candidate against heterologous pandemic influenza viruses

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Background: Immunogenicity and efficacy of a MVAtor™-based (modified vaccinia virus Ankara-based) vaccine candidate, expressing 3 influenza virus antigens, the nucleoprotein (NP), matrix 1 (M1) protein, and extracellular domain of the matrix 2 (M2) protein, were evaluated in a mouse model. BALB/c mice were immunized intramuscularly twice before challenge with live influenza virus pH1N1 (A/Mx/4108/09). Immune response, survival, weight loss, and viral load in respiratory tissues were assessed. Materials and Methods: BALB/c mice were immunized intramuscularly twice with 8×10^7 TCID50/animal on days 0 and 22 with trivalent MVAtor (NP/M1/M2) or MVAtor control. Serum of mice was collected at days 21, 39, and 56 and analysed by hemagglutination inhibition (HAI) assay and micro-neutralization assay and ELISA. Mice were challenged with $20 \times LD_{50}$ (20 times the 50% lethal dose) of influenza virus pandemic H1N1 (pH1N1) (A/Mx/4108/09) virus on day 42, ie, during maximal immune response. Survival, weight loss, and viral load in lungs and nares were assessed. Results: Using ELISA, robust anti-NP and anti-M2 immune responses were quantified. All vaccinated animals survived the challenge with 20 LD_{50} of the pH1N1 virus, compared to 13% survival in the negative control group (MVAtor control). After maximal weight loss on day 3, vaccinated animals recovered their weight to baseline values; no weight recovery was observed in control animals. A 10-100–fold reduction in viral loads in nasal turbinates was observed in the vaccinated groups on day 4

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postchallenge. No reduction in viral loads in lungs was observed in vaccinated groups on day 4 postchallenge. Conclusion: A trivalent MVAtor-based vaccine candidate containing conserved influenza antigens has potential to be protective against pH1N1 pandemic influenza viruses.

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An updated review on the effectiveness of the Brazilian influenza vaccination policy

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Background: Since 1999, Brazil has undertaken annual influenza vaccine campaigns, free of charge, targeting the elderly population, health professionals, and immune-deficient patients. In 2010 new groups were included: children from 6 months to 2 years of age, pregnant women, and indigenous peoples. Campaigns have been considered largely successful. Uptake among the elderly in the first decade of the initiative reached the 70% target in all but 1 year. Recently, the target was increased to 80%, and again it has been met. In 2010, the campaign against the pandemic A/H1N1 2009 virus vaccinated over 89 million people, almost 50% of the Brazilian population. Despite the success in vaccinating the target groups little is known about the effectiveness of the campaigns in reducing influenza related morbidity and mortality. We conducted, in 2010, the first systematic review of the literature on order to estimate the effectiveness of the initiative. Now, we present an updated review, including publications until May 2013. Methods: The keywords influenza, vaccine, Brazil, and effectiveness were used to search the following databases: Medline, PubMed, the Latin American Scielo, and Lilacs, the Brazilian BVS, and also Google Scholar. In this phase, there was no restriction in publication dates. In the second phase, we applied the inclusion and exclusion criteria. The inclusion criterion was any publication addressing the influenza vaccine campaigns conducted in Brazil since 1999. The exclusion criteria were: articles published before 1999, clinical trials of influenza vaccines conducted in Brazil, evaluation of influenza vaccination in occupational settings outside of the Brazilian public policy, outbreak investigations, papers on laboratory methods for influenza diagnosis, and review articles. Results: 657 publications matched our search keywords. 61 of them remained in the review after the application of inclusion and exclusion criteria. They were classified into 5 categories: vaccine coverage surveys, adverse events studies, effectiveness studies using an ecologic design, effectiveness studies using an individualized design, and studies on the seasonality of influenza in Brazil. Conclusions: Vaccine coverage is high, though not so high as informed by the Information System of the National Immunization Program. Ecologic studies on mortality and hospital admissions due to influenza related causes present conflicting results: some observed a reduction in mortality and hospital admissions, some saw no changes, and some pointed to an increase in influenza related mortality and hospital admissions after the campaigns were introduced. Methodological issues such as the causes of death or hospitalization considered to be influenza related, the different time frames analyzed, the different geographic regions considered, might account for the opposing results. At least some of the different outcomes might be related to the different seasonal pattern of influenza in equatorial Northern Brazilian States and the subtropical South and Southeast. Influenza peaks apparently occur before winter months in North and Northeastern Brazil, and also before the scheduled dates of the vaccine campaigns. Only 2 studies presented estimates of vaccine effectiveness, 1 among transplant patients and the other among the elderly. Brazilian epidemiologists still owe society more and better studies on the effectiveness of the influenza vaccine campaigns.

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Immunogenicity of MF59®-adjuvanted influenza vaccine in subjects ≥ 60 years old: results from a meta-analysis

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Background: An increased risk for influenza-related complications is demonstrable in adults over 60 years and many countries recommend routine vaccination at that age. Older adults exhibit reduced immune responses to conventional influenza vaccines. To enhance antibody responses to influenza immunization in this age group, an adjuvanted subunit vaccine containing the oil-in-water emulsion adjuvant, MF59® (Novartis Vaccines and Diagnostics), was developed and licensed in Europe in 1997. We reviewed evidence for the immunogenicity of the MF59-adjuvanted vaccine in subjects ≥ 60 years-old. Materials and methods: The 17 randomized, observer blind, actively controlled clinical studies included in this meta-analysis were similar in design and part of an integrated clinical program performed for registration of an MF59-adjuvanted trivalent influenza vaccine (ATIV) in Europe. All subjects, ≥ 60 years old who would normally be candidates for routine seasonal influenza vaccination, received one dose of ATIV (Fluad®, Novartis Vaccines and Diagnostics; N = 2371) or nonadjuvanted subunit (TIV) vaccine (N = 1948). Subjects with immunodeficiency or severe acute diseases, and those with contraindications to influenza vaccination (eg, egg allergy) were excluded. All subjects were ambulatory except for some patients in 2 studies conducted in extended care facilities. Serum hemagglutination inhibition (HI) titers were determined at pre- and postvaccination periods (at 28 and 180 days after vaccination). The immunogenicity variables assessed included: geometric mean antibody titers (GMTs) and percentages of subjects with HI titers ≥ 40. For the meta-analysis, the Dersimonian/Laird random effects model was used. Statistical heterogeneity was assessed using the Higgins I^2 test. Meta-regression was also used to analyze the extent to which statistical heterogeneity between results of multiple studies was related to preimmunization titre and vaccine groups. All clinical trials were approved by corresponding local ethics committees. All subjects provided written informed consent. Results: Recipients of seasonal MF59-adjuvanted and of nonadjuvanted comparator influenza vaccines showed no significant differences for age, gender, weight, height, body mass index and history of previous influenza vaccination at study entry. The pooled analysis of immunogenicity showed that the mean percentage of subjects with HI titer ≥ 40 at day 28 postvaccination ranged from 95.9% to 98.4% in the ATIV group and from 90.8% to 96.5% in the TIV group, depending on influenza vaccine virus strain. At 6 months postvaccination, mean percentages of subjects with HI titer ≥ 40 ranged from 88.8% to 97.0% and from 88.7% to 96.8% in ATIV and TIV groups respectively, fulfilling the CHMP criterion of > 60%. The MF59-adjuvanted influenza vaccine was more immunogenic than conventional influenza vaccines in subjects ≥ 60 years old; the pooled adjuvanted/comparator GMT ratios at day 28 for subtype A antigens (A/H3N2 and A/H1N1) were 1.34 (95% CI 1.16, 1.53) and 1.17 (95% CI 1.05, 1.30) respectively, and for the B antigen 1.28 (95% CI 1.20, 1.36). We also analyzed the pooled ATIV/TIV GMT ratios at day 28 for different age groups: for the 60–64 age group (data available from three trials only; ATIV N = 63 and TIV N = 181) the ATIV/TIV GMT ratio for the A(H3N2) antigen was 2.21 (95% CI -0.3, 4.7), for A/H1N1 1.34 (95% CI 0.4, 2.2) and for the B antigen 1.26 (95% CI 0.9, 1.56); for the ≥ 65 age group (data available from 17 trials; ATIV N=2278 and TIV N = 1746) the ATIV/TIV GMT ratios were 1.23 (95% CI 1.13, 1.34), 1.41 (95% CI 1.01, 1.26) and 1.30 (95% CI 1.22, 1.38) for the A (A/H3N2 and A/H1N1) and B antigens, respectively. In all instances the MF59-adjuvanted influenza vaccine was more immunogenic than the non-adjuvanted conventional vaccines with no statistically significant differences between age groups (*P*-values for differences between age groups for the A/H3N2 = .16, A/H1N1 = .37, and B antigens = .44). Conclusions: The MF59-adjuvanted influenza vaccine was more immunogenic in subjects ≥ 60 years of age than conventional non-adjuvanted influenza vaccines. Efficacy studies to assess the protective effect of the MF59-adjuvanted influenza vaccine against infection by different strains, pneumonia, hospitalization, and death in this age group are needed.

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Indirect effectiveness of vaccinating school children against influenza

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Background: Children and adolescents play an important role in maintaining the transmission of influenza. Moreover, with the occurrence of influenza in children, there are important socioeconomic consequences to families affected by the disease, related to absenteeism, spending on health services and medications. Some evidence suggests that vaccinating schoolchildren might reduce transmission to their unvaccinated household contacts. This study evaluates the direct and indirect effectiveness of influenza vaccination in school-age children and in their unvaccinated household contacts. It was conducted in 2009, in São Paulo, Brazil. Methods: A randomized double-blind community trial with a six-month follow-up was undertaken to assess the direct and indirect effectiveness of the Southern hemisphere trivalent vaccine. The experimental group received the influenza vaccine and the control group the conjugated meningococcal and varicella vaccines. After vaccination, the volunteers and their unvaccinated household contacts were followed for 6 months, by telephone calls, in order to identify cases of acute respiratory infection (ARI). When an ARI case was identified, the field teams visited the household to collect biological samples for the diagnosis of influenza with real-time PCR (RT-PCR). At the end of the study, we described the occurrence of ARI and laboratory-confirmed influenza (LCI). Results: 1742 schoolchildren enrolled in 10 schools (1021 in the experimental and 721 in the control group) and 5406 household contacts (3184 contacts of the experimental group and 2222 of the control group) participated in the study. 632 ARI cases were detected, of which 103 had a positive result for influenza. Influenza cases were classified as A/H1N1 pdm 2009 (55 cases) or “seasonal influenza” (47 cases). Incidence density of “seasonal influenza” was 0.5 per 1000 person/week for the experimental group and 0.54 for the control group. Incidence density was 0.11 per 1,000 person/week among the household contacts of the experimental group and 0.28 among the contacts of the control group. The effectiveness of the vaccine in protecting infection with seasonal viruses was 61% (95% CI 5.8; 84.7) for household contacts, and 23.3% (95% CI 66.3%;-74.9%) among the schoolchildren. No serious adverse events were observed. Conclusions: The vaccination of schoolchildren with influenza vaccine protected, significantly, their household contacts against seasonal influenza, but the effectiveness among the vaccinated children could not be demonstrated. A high proportion of losses among the schoolchildren reduced the power of the analysis. The occurrence of the 2009 pandemic has probably also contributed to this result, once the pandemic virus circulated simultaneously to other seasonal subtypes and the 2009 seasonal vaccine did not protect against it.

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Single replication influenza vaccine M2SR elicits long-lasting, cross-protective immunity

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Background: Influenza vaccines have remained virtually unchanged for decades. The most commonly used vaccine is the trivalent inactivated vaccine (TIV). Despite annual updating to match the circulating influenza strains, the effectiveness of TIV is only 60%. A vaccine with greater efficacy that also provides heterosubtypic protection would be considered a major transformation in the influenza vaccine market. Our M2SR (single replication) vaccine candidate, an influenza virus that does not

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express M2 protein, has achieved these characteristics in animal models and against multiple influenza subtypes (H3N2, H1N, and H5N1). Flow cytometric monitoring of infection dynamics has shown that M2SR protein production kinetics, unlike FluMist, is similar to wild-type virus. However, M2SR does not produce progeny virions; ie, does not shed virus, due to the deletion of M2. Materials and Methods: M2SR viruses—Viruses were generated by standard influenza rescue techniques. H5N1 and H1N1 M2SR viruses encode the hemagglutinin (HA) and neuraminidase (NA) from A/Vietnam/1203/2004 (VN1203, H5N1) and A/California/07/2009 (H1N1), respectively. Mice—Mice were intranasally inoculated with 1 or 2 doses of H5N1 M2SR or H1N1 M2SR (10^6 $TCID_{50}$/mouse) or VN1203 HA protein (1.5 µg/mouse) administered intramuscularly or mock-immunized. Sera were collected on days 7, 14, 21 postinoculation. Mice were challenged with a lethal dose of VN1203 ($20MLD_{50}$) virus 20 weeks later and evaluated for weight loss, clinical symptoms, and survival. Mice organs were harvested on day 4 post-challenge for virus titer determination. Ferrets—Ferrets immunized with one or two doses of H5N1 M2SR or H1N1 M2SR (10^7 $TCID_{50}$/ferret) were challenged 8 weeks later with a lethal dose of VN1203 (10^4 pfu) and observed for weight loss, clinical symptoms and survival. Virus titer was determined in lungs, nasal turbinates, and brain harvested on days 3 and 6 postchallenge and in nasal washes collected on days 1, 3, 5, 7, 9, and 14. Immune assays—HA-specific antibody responses were analyzed by ELISA or hemagglutination inhibition (HI) assays. Influenza-specific cell-mediated responses in bronchoalveaolar lavage or medial lymph nodes were analyzed by flow cytometry for cell surface markers and intracellular cytokines. Ferret peripheral blood mononuclear cells were analyzed for interferon gamma secretion by ELISA. Natural killer (NK) cell activation by serum antibodies via antibody-dependent cell-mediated cytotoxicity (ADCC) was measured by flow cytometry. Results: Mice—H5N1 M2SR vaccinated mice induced high levels of IgG and IgA in both serum and lung wash. These mice also displayed high HI titers whereas the H1N1 M2SR, VN1203 HA protein and mock-immunized groups did not display any HI activity. All of the M2SR vaccinated mice survived lethal VN1203 challenge whereas none of the mock-immunized and only 40% of the VN1203 HA protein group survived. The H5N1 M2SR vaccinated mice did not have any detectable challenge virus in any organ indicating sterile immunity. In contrast, virus was recovered from multiple organs in the mock-immunized mice (systemic infection). Ferrets—The H5N1 and H1N1 M2SR ferrets vaccinated with two doses were 100% protected from lethal H5N1 challenge showing no clinical symptoms or weight loss. The single-dose vaccine groups (H5N1 M2SR and H1N1 M2SR) displayed ≈ 5% weight loss and minor clinical symptoms but recovered with 67% in each group surviving the lethal VN1203 challenge. Challenge virus levels in nasal washes of vaccinated ferrets were lower than in mock-immunized ferrets. H5N1 M2SR elicited HI titers against VN1203 whereas H1N1 M2SR did not. Mechanism of cross-protection—HA specific antibodies activated mice NK cells in the presence of HA proteins from either H5N1 or H1N1 viruses. This suggests that H1N1 M2SR virus induced cross-reactive antibodies that mediate ADCC capable of recognizing VN1203 influenza virus. Cell-mediated responses also contribute to M2SR cross-protection. Conclusions: A single dose of M2SR vaccine induced long-lasting cross-protective immunity against lethal H5N1 challenge in mice and ferrets (supported by NIAID Contract # HHSN272201000031I). Preliminary results suggest that multiple cross-reactive immune responses contribute to the breadth of protective immunity afforded by M2SR against different influenza virus subtypes.

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Influenza vaccine effectiveness among southern California and Illinois residents in 2012-13

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Background: For the 2012-13 influenza season, the US Centers for Disease Control and Prevention (CDC) estimated interim adjusted vaccine effectiveness (VE) to be 56% for overall influenza, 47% for influenza A(H3N2), and 67% for influenza B. They found similar VE estimates across age groups, except for lower VE in the > 65 year age group. VE fluctuates every year depending on the match of the influenza vaccine strains to circulating strains. It may also vary depending on the type of vaccine used (trivalent influenza vaccine (TIV) versus live, attenuated influenza vaccine (LAIV), and the age

and health of the population. Consequently, it is important to assess VE to evaluate the overall benefit of the vaccine, identify differences between vaccine type, and identify populations that may be at greater risk of infection even after vaccination. Materials and Methods: VE for the 2012-13 influenza season was assessed by the Naval Health Research Center (NHRC) using influenza-like illness (ILI) surveillance cases from DoD dependent and other civilian populations living in California and Illinois between December 2012 and March 2013. 1090 ILI cases were identified during the study period among four study populations. Among these cases, 935 with known vaccination status were included in this study. Influenza cases were laboratory confirmed by PCR assay and prior vaccine status was collected by chart review. Participants were considered vaccinated if they were more than 14 and less than 180 days postvaccination. Descriptive statistics, including frequencies and chi-square tests, were run to identify differences between cases and controls. Crude and adjusted VE were calculated using logistic regression and taking 1- OR. Characteristics with P <. 1 were assessed in the multivariate model and backwards selection (removing variables with P > .05) was used to create the adjusted model. Results: Among the 935 ILI cases in this study, 347 (37%) were vaccinated and 371 (40%) were influenza positive. Vaccination was higher among influenza negative cases (47%) compared with influenza positive cases (22%). The study population was young, with 71% under 25 years old, and had slightly more women (56%). Age group, hospitalization, symptom duration before seeking care, study population and vaccination status were all significantly different between those who were influenza positive and those who were influenza negative. Sex and vaccine type were not significantly different between the 2 groups. Adjusted VE was 64% (CI: 50, 73) for all types of influenza, 73% (CI: 59, 82) for influenza A(H3N2), and 48% (CI: 21, 66) for influenza B. Type of influenza vaccine was available for 86% of vaccinated participants. The adjusted VE was 68% (CI: 53, 78) for TIV and 65% (CI: 19, 85) for LAIV. The adjusted, age stratified VE estimates were 64% (CI: 23, 81) for 0-4 years, 64% (CI: 42, 78) for 5-24 years, 66% (CI: 10, 87) for 25-49 years, 51% (CI: -55, 84) for 50-64 years, and 81% (CI: 34, 95) for > 64 years. Conclusions: The results of our study indicate that influenza vaccine reduced overall influenza infection by approximately two-thirds. VE was lower, although not significantly different, against influenza B compared with influenza A(H3N2). Lower influenza B VE could be the result of 2 cocirculating influenza B strains for this season, of which only 1 was included in the vaccine. Our study showed similar VE for TIV and LAIV and did not show evidence of reduction in the elderly. In contrast to the CDC's interim estimates, our study had higher VE for influenza A(H3N2) compared with influenza B which may reflect regional differences in circulating strains. VE estimates for overall influenza in our study population were also slightly higher than those calculated by the CDC. Some of these differences could be explained by variances in the study populations.

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Construction pre-pandemic vaccine strains of H5N1 avian influenza isolated in China since 2006

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Background: Avian influenza viruses do not efficiently infect human for the viral genetic determinants and host factors. The first human case caused by highly pathogenic avian influenza H5N1 cross barrier was reported in Hong Kong in 1997. Since then, HPAI H5N1 viruses continue to evolve at both genetic and antigenic levels. HA gene diversity made H5N1 virus classification update periodically. 12 new H5N1 clades have presented in recent years. At antigenic level, H5N1 virus showed intraclade and interclade cross reactivity. Despite the cross reactivity of certain H5N1 viruses, it became difficult to predict whether a vaccine strain will protect against a strain of a different clade (or even sometimes of the same clade). If an H5N1 strain becomes readily transmissible between humans, it likely will results in an influenza pandemic. In recent years, plasmid-based reverse genetics has been applied to the rational development of influenza virus high growth reassortants. 6:2 reassortants were created in which the HA and NA segments were derived from a variety of wild-type viruses, including H1N1, H3N2, H5N1, and H5N3 human and avian viruses with the remaining six segments from A/PR8/34.Materials and Methods: Cells, Virus and Sera—H5N1 human isolate A/Xinjiang/1/2006(XJ), A/Guangxi/1/2009(GX), A/Hubei/1/2010(HB), A/Guangdong/1/2011 (GD) virus, anti-XJ ferret serum,

anti-GX ferret serum, anti-HB ferret serum and anti GDSZ ferret serum were provided by the national influenza center, IVDC. Plasmid DNA: The plasmid containing the PR8 internal genes and pCIpolI vector were gift from US CDC. Isolation of viral RNA, reverse transcription, PCR amplification and sequencing. The RNeasy Kit (Qiagen) was used to extract vRNA. For RT-PCR, the One-Step RT-PCR kit (Qiagen) was used according to the protocol provided. Mutagenesis and cloning: PCR was used to amplify the HA1 and HA2 genes of A/Xinjiang/1/2006, A/Guangxi/1/2009, A/Hubei/1/2010,A/Shenzhen/1/2011 to remove the multibasic amino acids located at the cleavage site. HA1 and HA2 genes were digested with SapI to form the blunt end, ligased with the pCIpolI vector. The NA gene was amplified by PCR, digested with SapI and ligased into the pCIpolI vector. The 6 internal genes of A/PR/8/34 have already ligased into the pCIpolI vector. Plasmids were sequenced and the sequences were compared with those generated from the wild-type virus. Virus rescue: The modified HA and NA of XJ, GX,HB,GDSZ plamids and the 6 internal genes of PR8 (1ug of each) were transfected to 293T cell. Cells were scratched after 72 h and inoculate eggs. Trypsin dependence study: Virus were examined by plaque assay in chicken embryo fibroblast cells. Confluent cell monolayers were infected with 10-fold dilutions of virus. Cells were covered with an overlay MEM containing 0.8% agar and 1 ug/ml TPCK-trypsin. After 3 days, the cells were stained with crystal violet. Chicken embryo lethality test: Ten-day-old chicken embryos were inoculated in the allantoic sac with 0.1 ml of log10 dilution of each virus with known infectious titers. After 48 hours, the embryo viability was recorded. Antigenic characterization by HI assay: Antigenic characterization of H5N1 viruses was performed using reference ferret antisera in a hemagglutination inhibition (HI) assay with turkey erythrocytes as described. Results: In this study, 4 recombinant viruses were rescued based on reverse genetics. Reassortants were created in which the HA and NA segments were derived from A/Xinjiang/1/2006, A/Guangxi/1/2009, A/Hubei/1/2010, A/Guangdong /1/2011 and the remaining 6 segments from A/PR/8/34. HA genes were modified to remove the multibasic amino acids in the cleavage site. All reassortant viruses showed high yield, avirulent and remained the antigenicity of parental virus respectively. Conclusions: This study suggests that four recombinant viruses might serve as candidate vaccine strains for the prevention the pre-pandemic H5N1 influenza viruses.

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Preclinical studies of live cold-adapted influenza prepandemic vaccines

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Background: Recent emergence of a new H7N9 virus and threat of transmission of avian viruses to human elevated the need for research and development of live attenuated cold-adapted influenza vaccines against newly appeared influenza viruses. The following paper describes the preclinical studies of new H1N1, H7N3, H5N2, H2N2 live attenuated influenza vaccine (LAIV) candidates in mice. The objectives of this study were: 1. Demonstration that cold-adapted (ca) reassortant influenza A vaccine candidates are indistinguishable from the parental A/Leningrad/134/17/57 (H2N2) master donor strain (MDS) virus with regards to replication efficiency in upper and lower respiratory tract of mice. 2. Demonstration of safety of LAIV candidates in toxicology studies in rodents. 3. Demonstration of immunogenicity of LAIV candidates at different doses in mice. 3. Demonstration of protective efficacy of cold-adapted (ca) reassortant influenza A vaccine candidates in mice against a homologous wild-type virus challenge. Materials and Methods: Virus replication kinetic and neuroinvasion potential. Female Balb/c mice, 6-8 weeks of age were inoculated with 100 MID_{50} of A/17/California/2009/38 (H1N1), A/17/mallard/Netherlands/00/95 (H7N3), A/17/Vietnam/1203/04 (H5N1), A/17/turkey/Turkey/05/133 (H5N2), A/17/California/66/395 (H2N2), A/17/Tokyo/67/326 (H2N2) and A/Leningrad/134/17/57 (H2N2) MDS as well as wild-type parent viruses and the viral loads were measured in respiratory and brain tissues collected at 3 and 6 days postinfection (dpi). Mice were inoculated intranasally with 50µl volume. Tissue homogenates were prepared using a disruptor and clarified supernatants titrated on eggs at permissive temperature to determine infectious virus concentration. Immunogenicity and Protection Studies. To assess the immunogenicity of LAIV candidates, groups of animals were inoculated with 1000 and 100 MID_{50} of each virus intranasally, after collecting a pre-immunization blood sample. A second blood sample was collected at 28 dpi and

on the same day animals received a second intranasal inoculation of the same virus that was used for priming at 0 dpi. A third blood sample was collected on dpi 56. To assess protection, all animals were infected on dpi 42 with 100 MID_{50} of wild-type parent virus by intranasal route. Four animals from each group were euthanized on 45 dpi and respiratory and systemic organs harvested for virus titration. HAI antibody titers were determined for individual serum samples collected on days 0, 28, and 42. Sera were tested for reactivity against homologous influenza viruses. Toxicology Studies. Evaluation of the acute toxicity of LAIV strains was performed after intraperitoneal administration of viruses in mice and intradermal administration in guinea pigs after single dose administration of preparation. The control animals received sterile saline. During the course of the study all animals were observed daily for clinical signs of toxicity, behavior, food and water uptake, weight change. After 7 days the animals (mice) were sacrificed and organs (brain, heart, liver, kidney, and spleen) were collected for histopathology study. Results: It was shown that H1, H5, H7 and H2 LAIV were indistinguishable from parental H2N2 MDS in terms of replication in lungs and noses of mice. All tested strains showed no neuroivasive capacity and failed to infect brains. Single dose administration of LAIV strains in volume maximal for injection did not cause death of animals, change of behavior or significant weight loss. The candidate vaccine preparations were well tolerated. The dynamics of weight change in vaccinated animals was similar to that of the control group that received placebo. LAIV candidates were immunogenic, elicited antibody response confirmed by HI and ELISA assays. Vaccinated mice were completely protected against homologous wild-type challenge strains. Conclusions: Live attenuated cold-adapted (ca) influenza vaccines are an effective means for the control of influenza, most likely due to their ability to induce both humoral and cellular immune responses. In our study, we confirm that new LAIV candidates are safe, immunogenic and confer protection in mice. Based on the results of pre-clinical studies the permits for phase 1 clinical studies were obtained from the Russian Federation Ministry of Health. The phase 1 clinical studies of LAIV are ongoing. The study was supported by PATH.

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Influenza vaccine effectiveness in preventing medically-attended influenza A (H3N2) and the effect of prior season vaccination on estimates: results from the US Flu VE Network, 2012-13

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Background: In the United States, annual seasonal influenza vaccination is recommended for all persons ≥ 6 months of age. Historically, there have been inconsistent findings regarding the effect of annual vaccination on estimates of vaccine protection. During 2012-13, the A(H3N2) vaccine component (A/Victoria/361/2011) differed from the 2011-12 A(H3N2) vaccine component (A/Perth/16/2009). We compared the effect of vaccination during the previous season ("prior vaccination") on the vaccine effectiveness (VE) of the current season vaccine ("current vaccination") against A(H3N2) infection associated medically attended acute respiratory infection (MAARI) as measured by the US Flu VE network during 2012-13. Methods: We enrolled adults and children seeking outpatient care for ARI at participating health care facilities in Michigan, Pennsylvania, Texas, Washington and Wisconsin; only persons aged ≥ 9 years of age were included in this analysis. Persons were eligible if they had an acute respiratory illness with cough of ≤ 7 days duration. After consent, participants completed an enrollment interview and a combined nasal and throat swab was collected and tested for influenza, including subtype, using real-time reverse transcription polymerase chain reaction (RT-PCR) with CDC primers and probes. Applying the test-negative approach, influenza positive enrollees were "cases" and influenza negative enrollees were "controls". Participants who received at least 1 dose of any seasonal influenza vaccine ≥ 14 days prior to illness onset were considered vaccinated. Vaccine effectiveness was estimated as 100 × (1 – adjusted odds

ratio) in logistic regression models adjusted for age group, race/ethnicity, study site, days from illness onset to enrollment, and self-rated health status. Effect modification of prior year vaccination was assessed using an interaction term. Additional VE analyses assessed vaccine exposure as a 4 level variable that included all combinations over two seasons: current only, current and prior, prior only, or no vaccination either year (referent). Results: During 2012-13, we enrolled 4608 patients aged ≥ 9 years; 947 had A (H3N2) infection (cases), and 2973 were controls. Overall, 47% were vaccinated (27% received current and prior vaccination, 20% current only) and 53% were unvaccinated in 2012-13 (7% received prior vaccination and 46% were not vaccinated either season). The adjusted VE against A (H3N2) was 45% (95% CI, 36 to 53) when prior vaccination was not included in the model. Among persons aged 9-17, 18-49, 50-64, and ≥ 65 years, the adjusted VE against A(H3N2) was 27% (-9, 51), 40% (23, 53), 54% (34, 67), and 23% (-25, 53), respectively. Significant effect modification from prior vaccination was present (*P* = .0008). Influenza A(H3N2) infection was detected in 17% of patients who received current season vaccine only, 22% who received vaccine in both seasons, 21% who were vaccinated in prior season only, and 29% who were unvaccinated in both seasons. In the model that evaluated all combinations of vaccine exposure over two seasons, adjusted VE was 49% (37, 60) for current season vaccination only; 37% (23, 49) for current and prior season vaccination; and 38% (14, 55) for prior season vaccination only (referent group: unvaccinated in both seasons). Among persons aged 9-17, 18-49, 50-64, and ≥ 65 years, respectively, the adjusted VE in the two season vaccination model was: 67% (34, 84), 44% (23, 59), 55% (28, 71), 31% (-41, 67) for current vaccination only; 7% (-49, 42), 41% (18, 57), 57% (33, 72), 28% (-29, 60) for current and prior vaccination; and 58% (7, 81), 34% (-5, 59), 30% (-45, 66), 27% (-80, 70) for prior vaccination only. Conclusions: Compared with persons who were unvaccinated in both years, vaccination in 2011-12 and/or 2012-13 was associated with a reduced risk of medically attended influenza A(H3N2) infection. Our results suggest that current year vaccine effectiveness was modified by prior year vaccination, when the A(H3N2) vaccine components were different. However, we cannot exclude unmeasured confounding in this association. Further studies are required to understand the interplay of virologic and host factors that affect measured VE.

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Effectiveness of influenza vaccine for preventing laboratory-confirmed influenza hospitalizations in older adults

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Background: Current evidence supporting the effectiveness of influenza vaccine in preventing hospitalizations in older adults is sparse. Methods: During 5 influenza seasons spanning 2006-2009 and 2010-2012, adults aged ≥ 50 years hospitalized with acute respiratory symptoms were prospectively enrolled. The 2009-2010 pandemic influenza season was not included due to vaccine being unavailable during most of the season. Nose and throat samples were collected from participants and tested for influenza virus by reverse-transcriptase polymerase chain reaction. Verification of vaccination status was performed by contacting both primary care and non-traditional vaccine providers. Vaccine effectiveness was estimated by comparing vaccination status between influenza positive cases and influenza negative controls using logistic regression models with LASSO penalty on all covariates. LASSO type methods have been developed recently for high-dimensional data analysis when the number of parameters to be included in the model is large relative to the number of cases available. The penalty functions are chosen so that the model can yield zero estimates when the parameter values are close to zero and hence can perform the variable selection procedure. Covariates included in the model were age, gender, race, smoking status, insurance, week of enrollment, use of supplemental oxygen at home, immune suppression (cancer or immunosuppressive medications), kidney or liver disease, asplenia, pulmonary disease, cardiovascular disease, and diabetes. Confidence intervals were constructed using 1000 bootstrap samples. Results: Overall, 967 of 1656 (58%) eligible patients were enrolled; 932 (96%) had adequate respiratory samples and known influenza vaccination status. Of these, 247 (27%) had either only self-reported vaccination or verified vaccination within 14 days of symptom onset and 21 (2%) had missing data on covariates and hence were excluded leaving 664 participants in the final

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analyses. The proportion of influenza positive patients was 7%, 19%, 4%, 16%, and 10% during the five successive seasons. The proportion of vaccinated patients was 62%, 60%, 72%, 70%, and 70% during the same five seasons. Overall, 29 of 68 (43%) influenza positive cases were vaccinated compared with 423 of 596 (71%) influenza negative controls. Patients included in the analyses had a median age of 67.6 years, 41% were male, 25% were black, 97% had insurance, 24% were smokers, and the median time of admission was 10 weeks into influenza season. The median duration of hospitalization was 3 days, 10% were admitted to intensive care units and 1.1% died in the hospital. Adjusted vaccine effectiveness for the 5 influenza seasons combined was 69.6% (95% Confidence Interval 40.0%, 80.7%). Adjusted vaccine effectiveness was 68.4% (28.0%, 85.3%) for adults <65 years and 64.9%% (11.4%, 83.3%) for adults ≥ 65 years of age. Inclusion of those with self-reported vaccination yielded an adjusted vaccine effectiveness of 67.8% (33.4%, 78.5%) for all ages, 67.1% (27.4%, 84.8%) for adults < 65 years, and 58.6% (3.1%, 82.1%) for adults ≥ 65 years. Conclusion: Influenza vaccine prevents a significant number of acute respiratory hospitalizations in older adults including those aged ≥65 years.

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Safety and immunogenicity of a quadrivalent inactivated influenza vaccine (QIV) containing two influenza A and two influenza B strains among healthy children/adolescents, adults, and elderly subjects in phase III clinical studies

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Background: For more than a decade, strains from two distinct influenza B virus lineages (B/Victoria and B/Yamagata) have cocirculated worldwide. Selecting which influenza B-lineage strain to include in the annual trivalent influenza vaccines (TIVs) that will match with circulating strains during the season has been a challenge to public health authorities and often resulted in a TIV with a B-strain mismatch. In view of the current epidemiology of influenza B in which both lineages cocirculate, quadrivalent influenza vaccines have been developed that incorporate a strain from each of the two influenza B lineages. Materials and Methods: Two phase 3 clinical trials were conducted to evaluate the safety and immunogenicity of a quadrivalent inactivated influenza vaccine (QIV; Sanofi Pasteur) in children/adolescents 9 to 17 years of age (N = 385), adults 18 to 60 years (N = 2488), and elderly above 60 years (N = 785). In each study, healthy subjects were randomized to receive either (i) QIV (containing a A/H1N1, a A/H3N2, a B/Victoria and a, B/Yamagata strain), or (ii) a licensed TIV (Sanofi Pasteur's Vaxigrip® containing a A/H3N2, a A/H1N1 and a B/Victoria strain) or in one study (iii) AltB-TIV containing an influenza strain from alternate B-lineage (a A/H3N2, a A/H1N1 and a B/Yamagata strain). Injection-site and systemic reactions, other adverse events (AEs), and serious AEs (SAEs) were monitored post-vaccination. Humoral antibody responses to study immunogenicity of different vaccine formulations were evaluated using standard Hemagglutination Inhibition assay and influenza virus seroneutralization assays. Results: For 3653 subjects enrolled in these 2 studies, 3093 subjects received QIV, 337 subjects received TIV, and subjects received 223 AltB-TIV. The overall safety profile of QIV was similar to the TIVs. The most frequently reported adverse reaction following vaccination with was injection site pain. Most reactions occurred within the first 3 days following vaccination and resolved spontaneously within 1 to 3 days after the onset. Reactions were mild. Overall, adverse reactions were generally less frequent in elderly than in younger adults and children/adolescents. Other AEs and SAEs were reported at similar rates across age groups. Serum antibodies measured by HAI assay after vaccination with QIV show induction of robust immune responses against A/H1N1, A/H3N2, and both B strain lineages which either met or exceeded the CHMP immunogenicity criteria defined for adults and elderly population. In general, higher immune responses against all four strains were observed after QIV vaccination in children/adolescents when compared with responses observed in adults age group. QIV demonstrated superior immunogenicity when compared to TIV containing the alternate B strain. Thus QIV can provide superior coverage against all circulating influenza B strains. In addition, results of these studies provided evidence that the addition of the second B strain did not interfere with the generation of immune responses against other two influenza A or influenza B strains included in the vaccine. Same trends in immune

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responses were also observed when serum samples were tested using the Seroneutralization (SN) assay. Conclusions: Sanofi Pasteur's candidate QIV is a potential alternative to currently available licensed TIV and offers simultaneous protection against two influenza A strains and strains from both influenza B lineages. These studies were funded by Sanofi Pasteur.

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Adjuvanticity of mOMV for recombinant avian influenza virus antigen in the vaccination of mouse and chicken

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Background: A recombinant H5N2 (rH5N2) virus was generated to be used as vaccine antigen towards avian influenza viruses H5N1 and H9N2. To increase efficacy of the rH5N2 vaccine against avian influenza virus infections in animals, a new particulate adjuvant called mOMV (modified outer membrane vesicle) was incorporated, and its potential adjuvant effects were evaluated in the animal models. mOMVs are derived from an *E. coli* K-12 *msbB*/*pagP* mutant possessing less-endotoxic penta-acylated lipid A moiety of LPS. Materials and Methods: The mOMV-adjuvanted rH5N2 vaccine was prepared by mixing with recombinant H5N2 whole virus antigen (rH5N2) generated by reverse genetics with combination of H5N1-HA gene and H9N2-NA gene in the PR-8 (H1N1) backbone. Mice and chickens were vaccinated by prime-booster regime with the rH5N2 vaccine dose (1.75 μg of HA equivalent) plus mOMV (10 μg) or aluminum hydroxide (alum) adjuvant. The vaccinated animals were challenged with lethal doses of H5N1 or H9N2 viruses, and body weight change and survival rates were monitored daily for 15 days post infection.Results: Both mOMV- and alum-adjuvanted rH5N2 vaccine groups showed high survival rate and low level of body weight change compared with non-adjuvanted control groups. Interestingly, the mOMV-adjuvanted group showed better results in the cross-reactivity of antibodies (in vitro) and cross-protection (in vivo) against heterologous virus (H5N1 or H9N2) challenge. Conclusions: Our results suggest that mOMV could be used as vaccine adjuvant for development of cheap and effective veterinary vaccines controlling avian influenza A virus transmissions.

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Knowledge, attitudes, and practices about influenza vaccination among pregnant women in Thailand

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Background: In 2009, Thailand expanded recommendations for influenza vaccination to include pregnant women as a high-priority group for vaccination. Vaccine uptake among Thai pregnant women is likely low compared with other high-risk groups, but perceived barriers to vaccination have not been evaluated. This study examined knowledge, attitudes and practices related to influenza vaccination among Thai pregnant women. Materials and Methods: During Dec 2012-Feb 2013, a cross-sectional survey was conducted among Thai pregnant women aged > 15 years attending

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antenatal clinics at government hospitals. We selected a convenience sample of eight of 77 provinces. In each province, we selected the provincial hospital and a convenience sample of three geographically disparate district hospitals. Hospitals were visited on antenatal clinic days until the sample size was reached. The sample size was determined using an outcome frequency (vaccination or willingness to be vaccinated) of 50%, a precision of 3% and 95% confidence level. The allocation of the total sample size for each region was proportional to the regional live births. The number of pregnant women surveyed was equally distributed by hospital in each province. Self-administered questionnaires were used to ascertain demographics, perceived risk and severity of influenza as well as perceived benefits of and barriers to influenza vaccination, in conjunction with the health belief model. In bivariate analysis and logistic regression, we evaluated predictors of (1) influenza vaccination by comparing responses of women who had and had not received influenza vaccine during the current pregnancy, and (2) willingness to receiving influenza vaccine by comparing responses of women who said they were willing versus not willing to receive vaccine. In the model, we controlled for factors that were significant in the bivariate analysis, gestational age and economic status. Results: At 32 hospitals, 973 pregnant women were eligible to participate; 967 (99%) completed the survey. The median age among survey respondents was 25 years (IQR 20-30 years), the median gestational age at the time of the survey was 26 weeks (IQR 18-35 weeks), and 929 (96%) and 599 (62%) reported having heard about influenza and influenza vaccine, respectively. During the current pregnancy, 174 (18%) women had received a health care provider recommendation for influenza vaccination and 25 (3%) had received influenza vaccine (28% in the first trimester, 44% in the second, and 28% in the third). The median gestational age at vaccination was 21 weeks (IQR 19-35 weeks). Of the 599 respondents who had heard about influenza vaccine, the vaccine was perceived as safe by 299 (50%), beneficial to the woman by 396 (66%), beneficial to the baby by 377 (63%), and as an effective influenza prevention method by 354 (59%). The most commonly reported barriers to vaccination were paying for the vaccine (38%), difficulty traveling to get vaccinated (16%) and lack of vaccine at the hospital (13%). Fifty-eight percent of the pregnant women who were not vaccinated expressed a willingness to get vaccine. In bivariate analysis, factors associated with influenza vaccination during the current pregnancy included any prior receipt of influenza vaccine (OR 8; 95% CI 3-25),receipt of influenza vaccine during a previous pregnancy (OR 14; 95% CI 6-33), willingness to pay for vaccine (OR 5; 95% CI 1-21), and health care provider recommendation for vaccination during pregnancy (OR 22; 95% CI 7-66). Only health care provider recommendation for vaccination remained significant in multivariate analysis (OR 11; 95% CI 3-43.). When the outcome was changed to willingness to get influenza vaccine, hearing or reading a recommendation that pregnant women should receive the influenza vaccine was the only significant factor in the multivariate analysis (OR 6; 95% CI 2-17). Conclusions: Over half of Thai pregnant women were willing to get an influenza vaccine; however, influenza vaccine uptake was low. Subsidizing vaccine and encouraging health care providers of pregnant women to support influenza vaccination recommendations may be effective strategies to improve influenza vaccine uptake in Thai pregnant women.

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Recombinant adenovirus encoding hemagglutinin (HA) and conserved matrix protein M for induction of broad protection against influenza in mice

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Background: Influenza vaccines aimed at inducing antibodies (Abs) against viral surface hemagglutinin (HA) and neuraminidase (NA) provide sterile immunity to infection with the same subtypes. Vaccines targeting viral conserved determinants shared by influenza A virus (IAV) subtypes offer heterosubtypic immunity (HSI), a broad protection against different IAV subtypes. We proposed

that vaccines targeting both HA and conserved ectodomain of matrix protein 2 (M2e) could concurrently induce sterile immunity to virus sharing the same HA and broader protection against other influenza A virus subtypes. Materials and Methods: We generated recombinant adenovirus (rAd) vector encoding both HA of H5 virus and M2e (rAdH5/M2e) and evaluated the induction of cross-protection upon intranasal immunization with the rAd vector-based vaccine candidate. Mice were intranasally immunized with the vaccine candidates and 4 weeks later challenged with homotypic H5 or heterosubtypic H1 virus. Results: We found that single intranasal immunization of mice with rAdH5/M2e induced significant HA and M2e specific Ab responses along with protection against homotypic and heterosubtypic challenge. The protection is greater as compared to that induced by adenovirus vector encoding either HA (rAdH5) or M2e (rAdM2e). While protection against homotypic H5 virus is mediated by virus neutralizing (VN) Abs, the cross-protection is associated with Abs directed to conserved stalk HA and M2e that seem to have synergistic effect. Accordingly, adoptive transfer of immune sera induced by rAdH5/M2e provided greater protection against heterosubtypic challenge as compared to that mediated by sera of mice immunized with rAdH5 or rAdM2e. Conclusions: The results underscore the usefulness of rAd vector encoding both H5 and M2e as a universal vaccine against pre-pandemic H5N1 influenza and outbreaks caused by newly emerging subtypes.

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Physical and antigenic stability of spray freeze-dried influenza vaccine for pulmonary delivery

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Background: One of the advantages of dry influenza vaccines over conventional liquid flu vaccines is that they can be used for alternative routes of administration. The pulmonary route improves compliance level by eliminating drawbacks like pain and needle fear, the chance for needle stick injuries and the need for trained health care workers to administer the injection. Moreover, pulmonary vaccination can potentially induce local immune response besides a systemic immune response. Spray freeze-drying is an excellent technique to produce vaccine powders that are suitable for pulmonary delivery. It would be preferable if could be possible to store these powders at room temperature without change in physical powder and biochemical properties to ensure reproducible lung deposition and immune response, respectively. Therefore, the aim of this study was to evaluate these aspects of storage stability of spray freeze-dried vaccine powders. Materials and Method: Whole inactivated influenza virus was spray freeze dried using inulin, dextran and mixture of dextran/trehalose as stabilizer. We evaluated the physical powder and immunogenic stability of the vaccine that was stored for three months at various temperatures. Results: The formulated powder vaccines were evaluated for its antigenic stability by. The physical stability of the powders were confirmed by Scanning electron microscopy, size distribution and specific surface area measurements indicated that the physical powder properties did not change during storage for three months at 30°C. Furthermore, TEM, SDS-PAGE and the haemagglutination titers indicated that also the biochemical properties of the flu vaccine did not change during storage under these conditions. Furthermore, *in vivo* experiments confirmed that the immunogenic properties of the vaccine were preserved during storage for three months at 30 °C. Conclusion: The physical powder and immunogenic properties of the flu vaccine spray freeze dried in the presence of inulin, dextran and mixture of dextran/trehalose were maintained during storage for three months at 30°C. Hence, it is expected that vaccine powders prepared with either of these sugars can be safely stored at room temperature for extended periods without loss of their potency after pulmonary administration.

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Immunogenicity and safety of an *EB66* cell culture-based H5N1 candidate influenza vaccine: a randomized controlled study

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Background: New technologies to manufacture influenza vaccines, including cell culture-based methods, offer advantages over traditional methods with embryonated eggs, allowing for large-scale production to cover high demands for vaccines in case of pandemics. For influenza antigens that are poorly immunogenic, like H5N1, the use of adjuvants has been shown to improve the immune response to vaccination. We assessed the safety and immunogenicity of GlaxoSmithKline Vaccines' inactivated *EB66* cell culture-based A/Indonesia/5/2005 split virus H5N1candidate influenza vaccine (CC-PAN-H5N1) with or without the adjuvant AS03. Materials and Methods: In this phase I, observer-blind, randomized, controlled study (NCT01236040) with staggered enrolment in the US, healthy adults aged 18–49 years were enrolled in 2 waves and randomized 1:1:1:1 into 4 groups per wave to receive 2 vaccine doses (at days 0 and 21). In wave 1, subjects received either (1) $AS03_A$-adjuvanted CC-PAN-H5N1 containing 3.75 µg A/Indonesia/5/2005 hemagglutinin (HA) (CC-PAN-H5N1-3.75 µg+$AS03_A$), (2) nonadjuvanted CC-PAN-H5N1 containing 15 µg HA (CC-PAN-H5N1-15 µg), (3) nonadjuvanted egg-derived Q-PAN-H5N1 containing 15 µg HA (Q-PAN-H5N1-15 µg), or (4) saline placebo. In wave 2, subjects received either (1) CC-PAN-H5N1-3.75 µg+$AS03_A$, (2) CC-PAN-H5N1-1.9 µg+$AS03_B$, (3) Q-PAN-H5N1-3.75 µg+$AS03_A$, or (4) saline placebo. The AS03 adjuvant effect (co-primary objective) was evaluated in wave 1 based on the Center for Biologics Evaluation and Research (CBER) criteria and considered beneficial if, at day 42 (21 days post-dose 2), the lower limit (LL) of the 95% confidence interval (CI) for the A/Indonesia/5/2005 HA inhibition (HI) antibody geometric mean titer (GMT) ratio (CC-PAN-H5N1-3.75 µg+$AS03_A$ over CC-PAN-H5N1-15 µg) was > 1.0, and if the LL of the 95% CI for the difference in seroconversion rates (SCRs) (CC-PAN-H5N1-3.75 µg+$AS03_A$ minus CC-PAN-H5N1-15 µg) was > 0. Antigen-sparing effect of the adjuvanted CC-PAN-H5N1 formulation (co-primary objective) was assessed in wave 1 on day 42 according to CBER immunogenicity criteria for A/Indonesia/5/2005 HI SCRs and seroprotection rates (SPRs) (LL of 95% CI for SCR ≥ 40%, for SPR ≥ 70%). HI responses to the drift variant H5N1 virus (A/Vietnam/1194/2004) and long-term persistence up to Month 12 were assessed on the entire study population (according-to-protocol cohorts for immunogenicity). Solicited symptoms were recorded for 7 days after each vaccine dose. Serious adverse events (SAEs) were recorded until Month 12. Results: 521 subjects were included in the total vaccinated cohort (50 in CC-PAN-H5N1-3.75 µg+$AS03_A$, wave 1), 507 in the day 42 according-to-protocol cohort for immunogenicity (48 in CC-PAN-H5N1-3.75 µg+$AS03_A$, wave 1) and 504 in the Month 12 according-to-protocol cohort for immunogenicity. The criteria for confirmation of the adjuvant effect of AS03 in CC-PAN-H5N1 were met; the LL of the 95% CI for the HI GMT ratio at day 42 was 17.51 (> 1.0) and for the SCR difference was 56.37 (> 0). The HI antibody response to the homologous A/Indonesia/5/2005 virus induced by CC-PAN-H5N1-3.75 µg+$AS03_A$ exceeded CBER criteria, with a day 42 SCR of 97.9% (95% CI: 88.7-99.9) and SPR of 100% (95% CI: 92.5-100). The HI antibody response to the drift variant A/Vietnam/1194/2004 at day 42 induced by CC-PAN-H5N1-3.75 µg+$AS03_A$ also exceeded CBER criteria (LL for SCR: 58.9%, for SPR: 71.1%). HI antibody responses against the homologous and drift variant viruses elicited by CC-PAN-H5N1-3.75 µg+$AS03_A$ declined over time but remained above baseline until Month 12. Pain was reported more frequently for the adjuvanted than the nonadjuvanted formulations of both the cell-culture and egg-derived vaccines. 13 nonfatal SAEs were reported throughout the study; none were assessed as vaccine-related. Conclusions: Antigen sparing properties of the AS03-adjuvanted *EB66* cell culture-based H5N1 candidate vaccine were demonstrated: 21 days after a 2-dose vaccination schedule, the vaccine elicited homologous HI antibody responses that exceeded CBER criteria and were superior to those elicited by the nonadjuvanted formulation. The vaccine also induced cross-reactive HI antibodies against a drift variant. This study demonstrated for the first time the feasibility of producing an immunogenic inactivated split virus influenza vaccine with an acceptable safety profile using the novel *EB66* cell line. Funding: GlaxoSmithKline Biologicals SA and BARDA.

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Full inactivation of human influenza virus by high hydrostatic pressure preserves virus structure and membrane fusion while conferring protection to mice against infection

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Background: Seasonal influenza virus causes significant morbidity and mortality worldwide and prevention is considered to be the most effective method of reducing the socio-economic burden of influenza. The currently available human vaccines are usually subunit vaccines, trivalent, containing 2 influenza A and 1 influenza B subtypes. Whole virus vaccine formulation is described as more immunogenic in a naive population and may be needed in a pandemic situation to elicit an adequate immune response. Whole inactivated vaccines (WIV) preserving fusogenic activity are more protective then nonfusogenic WIV. In the current study we present a WIV based in a high hydrostatic pressure (HHP) inactivation as a possible candidate of vaccine model. Material and Methods: In this work, we show the inactivation, the structural alterations and glycoproteins activity of human influenza virus X-31(H3N2) subjected to HHP (289.6 MPa). We also investigated the potential as a vaccine model using 2 routes of administration in BALB/c mice: intranasal and intramuscular (vaccination was performed in 2 doses with 15 days of interval and challenge was performed fifteen days after the second dose). To verify structural changes, we used spectroscopic measurements, light scattering, and electron microscopy. Inactivation was confirmed by $TCID_{50}$ and RT-PCR. To investigate glycoproteins activity, we performed hemagglutination assay and evaluation of neuraminidase activity by fluorescence. Fusogenic activity was assayed by confocal microscopy. Immune responses were evaluated by ELISA assay for serum specific influenza IgG1, IgG2a, and IgA. Clinical signs were tested by monitoring weight change. Pulmonary physiological conditions were analyzed by positron emission tomography (PET) using labeled fluorinated glucose. Results: Structural changes detected by spectroscopy and light scattering show that these alterations are slight and essentially reversible when pressure is removed. Electron microscopy data show pore formation on viral envelope, but general morphology was well preserved and small variations were seen in the particle structure. The activity of hemagglutinin (HA) in the process of binding and fusion was affected in a time dependent-manner, but neuraminidase (NA) activity was not affected. Infectious activities were abolished after three hours of pressurization and mice were protected from infection after being vaccinated, with strong Igs response. Preliminary results suggest that vaccination could prevent pulmonary physiological impairment as visualized by glucose uptake. Conclusions: Our results indicate that HPP is an efficient tool to inactivate entire virus to be used in vaccines. This kind of inactivation produces an antigen with many of the chemical and physical properties of the intact viral particles, which is essential for satisfactory immune response. A vaccine based on pressurized virus is a simple, fast, and low cost model that could offer an important alternative of large production vaccines. Despite our promising results, further investigations are necessary to evaluate the protection level, reactogenicity and immunological response of a based HPP WIV.

P1-402 G FELLOWSHIP GATES Fellowship

Inactivation of infectivity of avian influenza viruses using BPL, formalin, and ether for preparation of candidate vaccines

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Background: Inactivation of virus infectivity is an essential step for preparation of inactivated vaccines and for use of virus antigens as reagents for diagnosis of avian influenza (AI). The available information on sensitivity or resistance of AI viruses to different inactivating agents is inadequate and

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ambiguous. A rational choice of inactivating agents and inactivation conditions is crucial in preparation of inactivated vaccines. Beta-propiolactone (BPL), formalin, and ether have been used for preparation of inactivated vaccines. BPL is an alkylating agent which modifies the structure of nucleic acids inducing nicks in DNA, cross-linking between DNA and proteins as well as between the DNA strands in the double helix. The mechanism by which viral inactivation is achieved by aldehydes is through the alkylation of amino and sulphydrilic groups of proteins and purine bases. Formalin cross-links the viral proteins inhibiting the viral replication. Ether treatment of influenza virus causes disruption of the viral envelope to smaller fragments. In the current scenario of emerging AI viruses, the present study was undertaken to probe the structure, function and infectivity of highly pathogenic avian influenza (HPAI) and low pathogenic avian influenza (LPAI) viruses, using BPL, formalin and ether. Materials and Methods: A total of 9 AI viruses comprising 4 subtypes were included in the study. BPL, formalin and ether were used as inactivating agents. Viruses were treated with 0.05% and 0.1% BPL for 16 hours at 4°C; 0.02%, 0.04%, and 0.1% of formalin at 3 different incubation time points and with ether. The confirmation of virus inactivation was performed by 2 serial passages of inactivated viruses in 10-day-old embryonated chicken eggs. Results: 0.1% BPL, 0.04%, and 0.1% formalin eliminated infectivity of AI viruses at all incubation time periods. The elimination of infectivity of few HPAI viruses using only ether may not be adequate. Treatment with all inactivating agents retained hemagglutinating titers of viruses. Inactivated viruses reacted with the immune sera raised against homologous antigens indicating that inactivated viruses retained their immunogenicity. Conclusions: 0.1% BPL, 0.04%, and 0.1% formalin can be used for inactivation of infectivity of AI viruses. However, confirmation of virus inactivation after each inactivation treatment is necessary. This data might be useful for inactivation of AI viruses and is an important step for preparation of inactivated vaccines and reagents.

P1-403

Immunogenicity and safety of influenza vaccine given concomitantly with 13-valent pneumococcal conjugate vaccine or 23-valent polysaccharide pneumococcal vaccine in adults aged ≥ 65 years

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Background: 13-valent pneumococcal conjugate vaccine (PCV13) and 23-valent polysaccharide pneumococcal vaccine (PPV23) are being just introduced in the elderly in Korea. Although it is common to administer PCV13/PPV23 and trivalent inactivated influenza vaccine (IIV3) concomitantly for convenience, there is no direct comparison study that evaluates the immunogenicity and safety of IIV3 given concomitantly with 13-valent pneumococcal conjugate vaccine (PCV13) or 23-valent polysaccharide pneumococcal vaccine (PPV23) in the elderly. Materials and Method: Prior to the beginning of the 2012-2013 influenza season, healthy volunteers ≥ 65 years were randomized to 1:1 to receive IIV3 with PCV13 or PPV23. Serum hemagglutination-inhibiting (HI) antibodies for IIV3 strains were measured at the time of vaccination and then at 1 month after vaccination. At the time of vaccination, each patient received a clinical diary to monitor for any local or systemic reactions to the vaccines for 7 days to evaluate the safety. Results: Of 224 subjects recruited, 110 (98.2%) of PPV23 with IIV3 and 110 (98.2%) of PCV13 with IIV3 volunteers were finally followed up. At 1 month after vaccination, both vaccine groups satisfied the Committee for Proprietary Medicinal Products (CPMP) criteria. Seroprotection rate, seroconversion rate and GMT fold were not significantly different between 2 groups. The most common local and systemic reactions were local pain at the pneumococcal injection site and muscle aches. For most local and systemic reactions, the severity was reported to be mild. The occurrences of each reaction were similar between two vaccine groups without statistical significance. Conclusions: Concomitant vaccination IIV3 with PCV13 or PPV23 demonstrates acceptable immunogenicity and safety.

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Rapid changes in serum cytokines and chemokines in response to inactivated influenza vaccination

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Background: Among the barriers to influenza vaccination is fear of side effects. Although the phenomenon of myalgias and malaise following vaccination is well described, the biological basis of these events is poorly understood. We examined serum cytokine kinetics in response to inactivated trivalent influenza vaccine (TIV) in attempt to define the relationship between markers of inflammation and TIV-related side effects. *Materials and Methods:* Healthy, nonpregnant adults aged 18-50 years were eligible for enrollment. Two small cohorts of 10 subjects each received the 2011-2012 US FDA-licensed TIV. Cohort 1 had blood drawn at baseline and then 3, 7, 24, and 48 hours and 14 days later. Cohort 2 had blood drawn at baseline and 16 and 40 hours, and 14 days after vaccination. Subjects were questioned about local and systemic reactions to TIV. Sera were tested for a panel of 15 cytokines and chemokines as well as antibody titers. Results: 89%-100% of subjects had a sero-response to 1 or more of the influenza strains in the vaccine. Cytokine and chemokine responses were evident as early as 3-4 hours after immunization. Although serum cytokine responses varied by analyte, IFN-γ, IP-10, and IL-8 had distinctive temporal patterns. Significant increases in IFN-γ occurred by 7 hours and peaked between 16 and 24 hours before returning to baseline by 44 hours postvaccination. Similarly, IP-10 also increased significantly by 7 hours, with a peak at 24 hours, declining at 44 hours, with a return to baseline by day 14. IL-8 levels were decreased compared with baseline at 44 hours postvaccination and remained low at day 14. Fifteen of 20 subjects reported at least 1 adverse event after vaccination, the majority mild. One subject, however, who reported moderate myalgias and injection site pain after vaccination displayed an amplified cytokine response profile that included robust IL-6, IL-2, IL-8, IP-10, MCP-1, TNF-α, TARC, and MCP-4 responses between 3 and 24 hours after vaccination. Conclusions: Despite a modest sample size, we identified distinct serum cytokine patterns to TIV which began as early as 3 hours postimmunization. We also observed an outlier response that coincided with moderate side effects to the vaccine. These data suggest that early cytokine/chemokine responses may provide additional insight into vaccine responses.

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Results of phase I study of KD-295 (novel influenza vaccine by cell culture)

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Background: Highly pathogenic avian influenza H5N1 viruses have the potential to become pandemic agent, thus representing a threat for humans. Although vaccination is one of the effective tools to defend against a pandemic threat, manufacture of traditional influenza vaccines that use egg-based technology could be restricted by the supply problem of chicken eggs in pandemic situation. Therefore, development of cell culture-derived vaccines, which has advantages of shorter lead-in times and greater flexibility of production, is desired. For these reasons, we developed AS03-adjuvanted cell culture-based influenza vaccine (KD-295), and conducted phase 1 study. Materials and Methods: KD-295 investigational vaccine, contains purified and inactivated HA protein from influenza virus of A/Indonesia/5/2005(H5N1)/PR8-IBCDC-RG2 strain cultured using duck embryonic stem cell-derived cell line (EB66® 1) proprietary to Valneva) is the main component, which is mixed with AS03 adjuvant upon administration. AS03 is an o/w emulsion containing DL-α-tocopherol and squalene. Content of DL-α-tocopherol per 0.5 mL of KD-295 is 11.86 mg for AS03A, and 5.93 mg for

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AS03B. Healthy male adult volunteers (20-40 years old) were the target population, and adverse events, clinical laboratory test results, HI antibody titers, and neutralization antibody titers after KD-295 administration (0.5 mL) intramuscularly twice at 21-day interval were evaluated 21 days after the second administration. Three different doses of KD-295 [L group 1.9µgHA+AS03B, M group: 3.8µgHA+AS03A, and H group: 7.5µgHA+AS03A] were administered to 20 subjects in each group (total of 60 subjects). The L group received half the volume of M group. Results: During the course of the study, no death, serious adverse events or any adverse events that led to discontinuation of the investigational drug administration were reported. In total, 54 subjects (90.0%) reported 202 adverse events; all of which were well tolerated. Injection site pain was the most frequently reported adverse event. There was no dose dependency in the incidence, time-to-onset, and duration of the adverse events. HI antibody (horse blood cells) met the criteria in the Japanese guidelines for a prototype vaccine (seroconversion rate: > 40%, rate of increase in geometric mean antibody titer: > 2.5, seroprotection rate: > 70%) for all groups. Neutralizing antibody titers made notable rise in all groups. Cross immunity of neutralization antibody against heterologous virus strains of the same H5N1 subtype (Vietnam, Qinghai, and Anhui strains) were detected. Conclusion: AS03-adjuvanted cell culture based influenza vaccine named KD-295 (1.9µgHA+AS03B, 3.8µgHA+AS03A, and 7.5µgHA+AS03A), administered as 2 doses, was highly immunogenic, and induced cross immunity, with a clinically acceptable safety profile. 1) EB66® cell line exclusively licensed from Valneva (created from the merger between Intercell AG and Vivalis SA) by GSK Biologicals S.A. for the field of Influenza and sublicensed to Kaketsuken.

P1-406 SCHOLARSHIP International Research Scholarship

Uptake and effectiveness of a trivalent inactivated influenza vaccine in urban and rural Kenya, 2010-2012

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Background: In recent years, surveillance has demonstrated a high burden of influenza throughout Africa. However, influenza vaccine is rarely used on most of the continent. Little is known about the effectiveness of the vaccine in Africa, where HIV, malnutrition, malaria, and other comorbidities are prevalent. Materials and Methods: The Kenya Medical Research Institute/Centers for Disease Control-Kenya offered free influenza vaccine to children in 2 resource-poor communities in Kenya—Kibera, an informal settlement in Nairobi, and Lwak, a rural community in western Kenya—with a population of ≈ 50,000. We conducted a case-control study to evaluate the effectiveness of trivalent inactivated influenza vaccine during 3 years. During July-September of 2010, April-June of 2011, and March-May 2012, we offered free vaccine (southern hemisphere composition) to children aged 6 months-10 years who were residents of 3 sites in each of the 2 communities. We selected the vaccination period based on the availability of the vaccine, which varied each year, and surveillance data showing influenza peaks in both communities during June-August. Vaccination was voluntary and required parental consent; children < 9 years old were offered 2 doses of vaccine if they had not been previously vaccinated. We collected naso- and oro-pharyngeal swabs from children ≤ 10 years old who presented to the free medical clinic in each of the 2 communities with severe acute respiratory illness (SARI) or influenza-like illness (ILI). Swabs were tested for influenza A and B by real-time reverse-transcription polymerase chain reaction (rt RT-PCR); specimens positive for influenza A were subtyped by RT-PCR. Influenza-positive specimens were cultured, and isolates were sent to CDC-Atlanta for antigenic characterization using a panel of postvaccination ferret antisera. We compared the odds of vaccination among case-patients who tested positive for influenza to the odds of vaccination among controls who tested negative for influenza. Up to 4 controls were enrolled for each case-patient, matched on age (6-23 months; 2-5 years; and 5-10 years), date of sample collection (+/- 14 days from the date the case-patient sample was collected), syndrome (SARI vs ILI), and site (Kibera vs Lwak). We excluded 52 cases and controls who were partially vaccinated. We used a conditional logistic regression model that controlled for interval from symptom onset to sample

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collection. For each year, our evaluation period was 9 months, starting 2 weeks after the final dose was administered. We estimated vaccine effectiveness (VE) as [100 x (1 - adjusted odds ratio)]. Results: Of the approximately 10,000 eligible children in the 2 sites, 30%, 36%, and 38% were fully vaccinated in 2010, 2011, and 2012, respectively; 11%, 12%, and 13% were partially vaccinated. During the three 9-month follow-up periods, there were 144, 77, and 102 cases of influenza, respectively, among children aged 6 months-10 years. The median age of cases was 4.8 years and 149 (46%) were female. In the first 2 years, influenza A predominated (67% of cases in 2010-2011 and in 2011-2012), and influenza A comprised 50% of influenza cases in 2012-2013. In 2010-2011 and 2011-2012, most influenza A cases were influenza A(H1N1)pdm09, while in 2012-2013, influenza A(H3N2) predominated. We included 244 cases and 608 test-negative controls in our evaluation. VE among fully vaccinated children was 44% for the entire study period (95% confidence interval (CI) = 20%-61%). Season-specific VE was 51% (95% CI = 16%-71%) during 2010-2011, 48% (95% CI = -16%-77%) during 2011-2012, and 26% (95% CI =-29%-60%) during 2012-2013. We cultured 48/144 specimens in 2010-2011 and 29/77 specimens in 2011-2012; all isolates were considered well-matched to vaccine strains. Strain information for 2012-2013 is not yet available. Conclusions: During 3 years in an urban and rural community in Kenya, over one-third of eligible children were fully vaccinated through a campaign offering free trivalent inactivated influenza vaccine. Among children aged 6 months-10 years, fully vaccinated children were ≈ 40% less likely to have an influenza-associated medically attended respiratory illness overall. VE appeared lower in 2012-13; pending antigenic characterization data may help explain this finding.

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The overview of government-funded influenza vaccination program during influenza season 2011-2012

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Background: Influenza vaccination is publicly known as the most effective method for preventing influenza infection. To protect the public's health, the Taiwan Department of Health started to launch influenza vaccination programs since 1998, focusing on high-risk groups. In the government-funded influenza vaccination program for influenza season 2011-2012, trivalent inactivated influenza vaccines (TIV) were used in 6 high-risk groups, including the elders aged more than 65 years, children aged 6 months through 6 years and elementary school students from grade 1 through 4, residents and staff in nursing homes and other long-term care facilities, healthcare and public health personnel, poultry or livestock farmers, and animal health inspectors, and people with catastrophic illness. Materials and Methods: The influenza vaccine uptake rates for each high-risk group were obtained via the Influenza Vaccine Information System (IVIS). Results: During the influenza season 2011-2012, 2,582,859 doses of influenza vaccines were inoculated. The coverage rates for each group were as follows: the elders aged more than 65 years: 40.2%; preschool children aged above 6 months with at least 1 dose: 31.9%, with complete vaccination: 28.7%, with partial vaccination: 7.2%; elementary school students from grade 1 through 4: 72.2%; high-risk groups above fifth grades in elementary schools and aged less than 65 years: 41.2%. Overall, the coverage rates for each high-risk group increased significantly comparing with the 2010-2011 influenza season. The coverage rate increased by 4% for adult groups, by 2.7% for elementary school children, and by 6.2% for infants and toddlers aged less than 6 years. Conclusions: The coverage rate increased the most for infants and toddlers comparing with those for other groups, but was still lower than the rates in the past, leaving room for improvement.

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Progress update on the Global Action Plan for Influenza Vaccines (GAP)

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Background: The Global Action Plan for Influenza Vaccines (GAP) aims at ensuring rapid and equitable access to vaccines in the event of an influenza pandemic. The three major pillars of the GAP are: 1) increase of evidence-based seasonal influenza vaccine use, 2) increase of global pandemic production capacity and 3) development of more effective influenza vaccines using new technologies. Increase of evidence based seasonal vaccine uptake: Main activities include increasing knowledge about influenza burden of disease, vaccine performance and vaccine cost-effectiveness for different target groups in various geographical settings. In addition, this objective aims that all countries have a policy by 2016 to vaccinate at least 1 group of at-risk population, as indicated in the 2012 WHO position paper on vaccines against influenza, that recommended five priority risk groups for seasonal influenza vaccination, pregnant women being the highest priority. Increase production capacity: The target is to increase by 2016 global vaccine production capacity to cover within 6 months from transfer of master seeds to industry, 70% of the world population with a pandemic influenza vaccine that provides adequate protection. Fourteen manufacturers from low- and middle-income countries have received grants and technology transfer support, resulting in a capacity today of 280M doses. This is included in the 2011 global seasonal capacity of 1.4B doses in 2011. Assuming a conversion factor of 3, this translates into a pandemic vaccine capacity of 4.2B doses. The sustainability of these facilities now needs to be improved through the development of various business models. Developing more effective vaccines using new technologies: The target is to license or have in an advanced clinical development, by 2016, more effective new vaccines. Activities include promoting research on new correlates of protection, prototype pandemic seed strains and other new technologies. Following a January 2013 meeting in Hong Kong discussing progress and knowledge gaps, an influenza vaccine research agenda is being developed to address: universal vaccines, vaccines for viruses with pandemic potential, vaccine safety and regulatory issues, production strategies, adjuvants, delivery methods, new approaches and clinical trials in risk groups. In addition to the above priority activities, WHO has conducted a series of influenza vaccine workshops together with the US Health and Human Services Department (HHS). These workshops have addressed gaps that might delay the implementation of the GAP in developing countries. Issues considered include selection of production technology, regulatory strengthening, workforce training, disease burden and cost-effectiveness data for decision-making, business planning for sustainability and communication challenges with media, healthcare workers and the public. WHO functions as the GAP secretariat, supported by an Advisory Group with international expertise in public health, vaccinology, epidemiology, influenza virology, modeling, and immunization. This group periodically reviews activities, provides recommendations on implementation, identifies key priorities and revises and updates the plan as science, technology and preparedness progresses. The plan further aims to create an informal network of partnership offices as a means for international agencies to share what they are doing and what GAP priorities they may be able to assume. Currently 2 US agencies have heavily contributed: HHS/BARDA and the CDC. Additional GAP partnership offices are being considered, initially in China and Japan. Conclusion and next steps: Since 2006, GAP has been a catalyst for significant expansion in global influenza vaccine manufacturing capacity. It is anticipated that enough production capacity could be in place by 2016, but concerted efforts are needed thereto. Evidence-based policy and communication strategies need to be implemented. Sustainability is a challenge for influenza manufacturers in developing countries. Research and development for new vaccines should continue and include use of antigen-sparing technologies (such as live-attenuated vaccines) and high yield strains.

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Effectiveness of seasonal influenza vaccine in preventing laboratory confirmed influenza in a primary heath care setting in South Africa, 2010-2012

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Background: Sentinel influenza surveillance systems provide platforms for the estimation of influenza vaccine effectiveness . South Africa has long-standing outpatient sentinel surveillance for influenza, the Viral Watch Programme (VWP), which was started in 1984 to describe influenza epidemiology in South Africa and to provide influenza strains for global vaccine strain selection. The objective of this study is to describe the vaccine coverage amongst amongst individuals of with influenza-like illness presenting to the VWP sentinel sites in South Africa from 2010 through 2012 and to estimate influenza vaccine effectiveness (VE) annually amongst these individuals. Methods: Patients countrywide presenting with an influenza-like illness (ILI) to participating centres of the South African VWP between 1 January 2010 to 31 December 2012 were eligible for inclusion. ILI was defined as: Patients of all age groups, with an acute respiratory illness with a measured temperature of ≥ 38˚C, or a history of fever, and cough, with onset within the past 7 days. The influenza season was defined as at least 2 consequtive weekly influenza detection rates of ≥ 10%, and only specimens collected during the season were included for the VE analysis. Pateints who met the ILI case definition, presented within 7 days of onset, with a known influenza vaccine history, and who were 6 months or older were included. Patients who presented at points of entry into South Africa were excluded. Specimens were tested using multiplex reverse transcription real-time polymerase chain reaction (RT-PCR) assays for influenza A and B. Influenza A positive specimens were further subtyped. Patients testing positive for any influenza were considered a case, and those testing negative a control. Logistic regression was used for the analysis and VE estimates were adjusted for age and underlying medical conditions. VE was calculated as 1-odds ratio (OR) for influenza in vaccinated and unvaccinated patients. Patients were considered to be vaccinated if they had received influenza vaccine 2 weeks or more prior to onste of symptoms. Results: Between 2010 and 2012, 7385 patients with ILI were enrolled (range, 1945-3131 per annum). Of theses 3884 (53%) were included in the VE analysis. The influenza seasons occurred from week 23 (week starting 7 June) to week 40 (week starting 4 October) in 2010; week 18 (week starting 2 May) to week 29 (week starting 18 July) with a second smaller peak from week 33 (week starting 15 August) to week 41 (week starting 10 October) in 2011; and week 23 (week starting 4 June) to week 41 (week starting 8 October) in 2012. Influenza was detected in 587/1157(50.7%) in 2010; 731/1688(43.3%)in 2011 and 558/1039(53.7%) in 2012. The dominant subtype differed by year with influenza B accounting for 298/587(50.8%) in 2010, influenza A(H1N1pdm09) for 565/731(77.3%) in 2011, and influenza A(H3N2) for 337/558(60.4%) in 2012. Annual influenza vaccine coverage in patients included was low in both cases and controls (1.7% and 4.9% in 2010; 1.9% and 5% in 2011; 1.3% and 2.9% in 2012 for cases and controls respectively) with coverage highest in the ≥ 45 year age group (3.3% and 12.46% in 2010; 3.5% and 7.9% in 2011; 3.1% and 2.0% in 2012 for cases and controls respectively) and lowest in the < 20 year age group (1.2% and 2.0% in 2010; 2.2% and 1.9% in 2011; 1.1% and 1.4% in 2012 for cases and controls respectively). VE estimates adjusted for age and underlying condition for 2010-2012 were: 58.23% (95% CI: 7.11%-81.22%); 58.70% (95% CI: 22.28%-78.05%); 49.78% (95% CI: -39.70%-84.94%). Conclusion: Our results show that influenza vaccine was effective in 2010 and 2011. In 2012 the molecular characterisation of the circulating influenza A(H3N2) showed genetic drift, and a new H3N2 vaccine strain was selected for inclusion in the Southern hemisphere vaccine for 2013. The VWP is a useful platform for assessing annual influenza vaccine effectiveness, but low vaccine coverage limits statistical power to generate precise estimates of vaccine effectiveness or evaluate effectiveness in different subgroups.

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Computationally optimized broadly reactive antigens (COBRA): developing broadly reactive vaccines against emerging and seasonal influenza

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Background: Pandemic outbreaks of influenza are caused by the emergence of a pathogenic and transmissible virus to which the human population is immunologically naive. Recent outbreaks of highly pathogenic avian influenza (HPAI) of the H5N1 subtype are of particular concern because of the high mortality rate (60% case fatality rate) and novel subtype. In this study, we have engineered an influenza virus-like particle (VLP) that contains a synthetic, consensus-based HA molecule based upon a new methodology, computationally optimized broadly reactive antigen (COBRA). Methods: Three COBRA H5N1 HA proteins have been engineered based upon 1) human clade 2 H5N1 sequences, 2) human and avian clade 2 sequences, and 3) all H5N1 influenza sequences. COBRA vaccines were generated for H1N1 based upon seasonal and novel H1N1 sequences. Each HA retained the ability to bind the appropriate receptors, as well as mediate particle fusion. Noninfectious recombinant VLP vaccines using the COBRA HA molecules from were generated from a mammalian expression system. COBRA VLP vaccines were administered to mice, ferrets, and cynomolgus macaques and the humoral immune responses were compared with those induced by VLPs containing an HA derived from a primary viral isolate or a mixture of primary isolates. Results: Using a single vaccination at 0.6ug HA dose with an adjuvant, all animals vaccinated with COBRA clade 2 HA H5N1 VLPs had protective levels of HAI antibodies to a representative isolate from each subclade of clade 2, but lower titers against other clades. The addition of avian sequences from other clades expanded breadth of HAI antibodies to the divergent clades 1, 4, and 7. Furthermore, all vaccinated animals were completely protected from challenge with the highly pathogenic clade H5N1 viruses. Little damage was observed in the lungs of COBRA VLP vaccinated animals as detected by H&E staining and little virus could be detected by immunohistochemistry staining of lung tissues or by viral plaque assay of lung homogenates. Similar results were observed using an H1N1 HA COBRA VLP vaccine. Conclusion: This is the first report describing the use of a H5N1 VLP vaccine containing a synthetic HA antigen. The results show that the COBRA HA H5N1 VLPs elicit broad humoral immunity and is an effective influenza vaccine against HPAI virus in multiple animal models.

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Immunogenicity and safety of a heterologous booster dose of $AS03_B$-adjuvanted H5N1 influenza vaccine in children 6 through 35 months of age

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Background: H5N1 avian influenza represents a pandemic risk to humans if the continuous genetic reassortment of virus subtypes leads to an increased transmission to humans while retaining virulence. The rapid production and world-wide distribution of strain-specific influenza vaccines are

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crucial for pandemic prevention and control. Infants and children play a potentially important role in H5N1 transmission during pandemics. Cross-immunogenicity could be an essential feature of H5N1 vaccines during pandemics through the use of booster vaccination with strains drifted from the primary vaccine strain. In this study, we evaluated the safety and immunogenicity of an $AS03_B$-adjuvanted H5N1 influenza candidate vaccine (H5N1, GlaxoSmithKline Vaccines) following a heterologous booster dose in children 6-35 months of age. Materials and Methods: In this phase 2, nonrandomized, open-label multicenter study (NCT01323946) in Australia and Singapore, all subjects were primed with 2 doses of A/Indonesia/05/2005 (H5N1-In) vaccine on days (D) 0 and 21 and received 1 booster dose of A/turkey/Turkey/01/2005 (H5N1-Tu) vaccine at D182. Both vaccines contained 1.9 µg of hemagglutinin antigen adjuvanted with $AS03_B$ (5.93mg of α-tocopherol, half the standard dose). The study assessed whether the hemagglutination inhibition (HI) responses to H5N1-Tu 10 days following booster vaccination (D192) met the EU Committee for Medicinal Products for Human Use (CHMP) immunogenicity licensure criteria: point estimates for seroconversion rates (SCRs) > 40%, for seroprotection rates (SPRs) > 70% and for mean geometric increases (MGIs, compared with D0) > 2.5. HI and neutralizing antibody responses were evaluated at D0, D42, D182, D192, and D364. Solicited local and general adverse events (AEs) were evaluated for 7 days following each dose. Unsolicited AEs were evaluated for 21 days following each dose and during days 0-84 overall. Medically attended AEs (MAEs), potential immune-mediated diseases (pIMDs) and serious AEs (SAEs) were assessed throughout the study. Results: Of the 113 vaccinated subjects, 86 were included in the month 6 according-to-protocol (ATP) cohort for immunogenicity and 100 in the month 12 ATP cohort for immunogenicity. At D192, the HI immune response to the H5N1-Tu booster dose met all CHMP criteria: point estimates were 100% for SCR and SPR, and 357.7 for MGI (geometric mean titer [GMT] = 2026.2). At D192, postbooster SCR and booster factor (BF) were 98.8% and 22.7, respectively. HI antibodies against H5N1-Tu persisted up to D364: SCR was 96.0%, SPR was 100% and MGI was 14.9. Postbooster SCR was 96.0% and BF was 14.9 (GMT = 1266.8). For H5N1-In, at D42 SCR and SPR were 100% and MGI was 215.7 (GMT = 1078.6). At D192 and D364, SCR and SPR were 100%, and MGIs were 357.7 and 208.7, respectively (GMTs were 1787.6 and 1043.3, respectively). Neutralizing antibody responses were comparable with HI antibody responses. During the 7-day postvaccination periods, for 91.1% of subjects at least 1 general symptom was reported, and for 62.5% at least 1 local symptom was reported. Increased reactogenicity following each additional dose, particularly comparing dose 1 to dose 3, was noted. Solicited or unsolicited local symptoms 7 days after each dose were reported for 32.1%, 34.2%, and 51.9% of subjects, following dose 1, 2, and 3, respectively. Fever was reported for 12.5%, 32.4%, and 50.0% of subjects and grade 3 fever for 1.8%, 5.4%, and 10.2% of subjects, after dose 1, 2, and 3, respectively. During the 21 days following each dose, at least 1 unsolicited AE was reported for 63.7% of subjects. MAEs were reported for 60.2% of subjects throughout the study. From D0 up to D364, 13 SAEs (none fatal or vaccine-related) and no pIMDs were reported. Conclusions: The heterologous booster dose of H5N1-Tu given 6 months following 2-dose primary vaccination with H5N1-In elicited a robust immune response that met all CHMP criteria and persisted to D364. An increase in reactogenicity following each additional dose of study vaccine, particularly comparing dose 1 to dose 3, was noted. Overall, no safety concerns were identified. Funding: GlaxoSmithKline Biologicals SA.

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$AS03_B$-adjuvanted H5N1 vaccination in children 3 to 17 years of age: an open, controlled evaluation of safety and immunogenicity following primary and heterologous booster vaccination

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Background: The development of safe and effective vaccines is key in case of an H5N1 influenza pandemic. Heterologous prime-boost vaccination schedules could simulate vaccination scenarios through using booster pandemic-specific strains differing from strains used in primary vaccination.

This study assessed the superiority of immune responses to an H5N1 vaccine containing 1.9µg A/turkey/Turkey/01/2005 hemagglutinin (HA) antigen and AS03B adjuvant (5.83mg tocopherol) (H5N1-Tu) in subjects primed with 2 doses of AS03B-adjuvanted H5N1 vaccine containing 1.9 µg A/Indonesia/05/2005 HA antigen (H5N1-In) versus H5N1-unprimed subjects. Materials and Methods: In this phase III, randomized, open, active-controlled study (NCT01379937), subjects 3-17 years of age from the Philippines were randomized (3:3:2:2) to receive: (1) 2 H5N1-In doses (days (D) 0 and 21) followed by either 1 H5N1-Tu dose (D182) and 1 hepatitis A vaccine (HAV) dose (D364) (H5_H5) or 2 HAV doses (D182, D364) (H5_HAV), or (2) a first HAV dose (D0) followed by either 1 H5N1-Tu dose (D182) and a second HAV dose (D364) (HAV_H5) or a second HAV dose (D182) (HAV_HAV). Blood samples were collected on D0, D42, D182, D192 (except for H5_HAV and HAV_HAV) and D364 (except for HAV_ HAV). Superiority of the booster response to H5N1 vaccine was shown if at D192 the lower limit of the 95% confidence interval (CI) for the hemagglutination inhibition (HI) geometric mean titer (GMT) ratio for subjects primed with 2 doses of H5N1 vaccine versus H5N1-unprimed subjects was above 1. HI antibody responses were evaluated against H5N1-In and H5N1-Tu in terms of GMTs, seroprotection rates (SPRs), seroconversion rates (SCRs, only for H5N1-In), and post-booster SCRs and booster factors (BFs, compared to D182), only for H5N1-Tu. Neutralizing antibodies were evaluated in terms of GMTs, vaccine response rates (VRRs) and booster VRRs. Solicited local and general symptoms 7 days after each dose and unsolicited adverse events (AEs) 21 days after each dose and during D0–84 were assessed. Medically-attended adverse events (MAEs), potential immune-mediated diseases (pIMDs) and serious adverse events (SAEs) were collected throughout the study. Results: Of the 520 vaccinated subjects, 464 were included in the month 6 and 501 in the month 12 according-to-protocol (ATP) cohort for immunogenicity. The HI antibody response against H5N1-Tu was superior in the H5_H5 versus HAV_H5 group: the lower limit of the 95% CI for GMT ratio at D192 was 7.30. At D192, GMTs were 737.6 and 24.7; SPRs were 100% and 33.3%; post-booster SCRs were 100% and 32.1%; and BFs against H5N1-Tu were 18.6 and 4.1, for H5_H5 and HAV_H5 groups, respectively. At D364, GMTs were 215.1 and 18.4; SPRs were 99.3% and 20.0%; post-booster SCRs were 69.5% and 18.0%; and BFs against H5N1-Tu were 5.6 and 3.0 in H5_H5 and HAV_H5 groups, respectively. At D192, GMTs, SPRs and SCRs against H5N1-In were 674.1 and 7.6, 100% and 3.6%, and 100% and 3.6% in H5_H5 and HAV_H5, respectively. The neutralizing antibody response was comparable to the HI antibody response. For children < 6 years of age, the results suggested an increase in fever frequency following the second and particularly the third H5N1 vaccine dose. From D0 to D182, at least 1 MAE was reported for 36.9% of subjects in the pooled H5N1 groups (H5_H5, H5_HAV) and for 30.8% of subjects in the pooled control groups (HAV_H5, HAV_HAV). By the D364 visit, at least 1 MAE was reported for 41.7% (H5_H5), 53.8% (H5_HAV) and 39.4% of subjects (HAV_H5 and HAV_HAV). No fatal or vaccination-related SAEs or pIMDs were reported throughout the study. Conclusions: This study demonstrated the superiority of the immune response and the existence of an anamnestic immune response against H5N1-Tu in children 3-17 years of age previously primed with 2 doses of H5N1-In versus those unprimed with H5N1. No safety concerns were identified throughout the study except for the increased incidence of fever in children < 6 years of age. Funding: GlaxoSmithKline Biologicals SA.

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Characteristics of vaccine failures in a randomized placebo-controlled trial of inactivated influenza vaccine in children

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Background: Influenza vaccine is efficacious in preventing influenza infections in school-age children. While infections in vaccinated persons, referred to as vaccine failures, are known to occur in vaccines with imperfect efficacy, few studies have investigated whether trivalent inactivated influenza vaccine (TIV) can reduce seriousness of illness or the degree or duration of viral shedding in vaccine failures

in children. In a large randomized controlled trial in children aged 6-17 y, TIV had moderate to high efficacy in preventing confirmed influenza B virus infections (Cowling B, et al. *Clin Infect Dis.* 2012). In further analysis of data from that trial, here, we examined the effect of TIV on patterns in viral shedding and illness associated with confirmed influenza B virus infections in children. Materials and Methods: 796 children 6-17y of age were randomly allocated to receive 1 dose of TIV (0.5 mL VAXIGRIP; Sanofi Pasteur) or saline placebo from August 2009 through February 2010. One of the vaccine strains was a B/Brisbane/60/2008-like (Victoria-lineage) virus. After vaccination, subjects and their households were intensively monitored for 9-12 m for acute respiratory illnesses and nose and throat swabs were collected for testing by quantitative RT-PCR. Viral loads were analyzed using a log-linear mixed-effects model that allows for repeated measures for each subject and inclusion of vaccination status and day from symptom onset as covariates. Time to alleviation of respiratory symptoms (cough, sore throat, or runny nose), systemic symptoms (chills, headache, fever, or muscle pain), and fever was compared between TIV and placebo groups using accelerated failure time models. Results: Of 796 randomized children, 470 (TIV) and 312 children (placebo) completed follow-up. Among these 782 children, we collected 1471 nose and throat swabs and were able to identify 60 episodes of PCR-confirmed influenza. Due to small numbers of PCR-confirmed influenza A(H1N1)pdm09, A(H3N2), and B(Yamagata-lineage) infections, the present analyses focused on the 32 episodes of confirmed influenza B(Victoria-lineage) infections. These episodes occurred a median of 127 days (range, 48-251 days) following vaccination. The circulating influenza B viruses remained antigenically close to the vaccine strain based on phylogenetic analyses of their HA gene. Overall, 20 (63%) of the PCR-confirmed influenza B virus infections could be matched with a URTI episode. While TIV showed 66% (95% CI: 33%-83%) efficacy in preventing PCR-confirmed influenza B, the proportion of PCR-confirmed infections that could be matched with illness episodes did not differ by vaccination status (P = .83). Vaccine failures had lower postvaccination HAI titers compared to the other TIV recipients (P = .04) while the difference in prevaccination titer (P = .31) and geometric HAI titer rise following vaccination (P = .10) were not statistically significant. Viral shedding was observed to decline following a log-linear trend and was projected to cease at around day 10 with no significant difference between the TIV group (ie, vaccine failures) compared to the placebo group (P = .78). Patterns in illness were similar, and there was no significant difference between confirmed infections in the TIV versus placebo recipients in time to alleviation of respiratory symptoms (P = .21), systemic symptoms (P = .44), or fever (P = .89). Conclusions: While TIV was efficacious in preventing influenza B virus infection, in this randomized placebo-controlled trial we did not find any evidence that TIV additionally reduced the duration of illness, or the degree or duration of influenza B virus shedding in PCR-confirmed cases. Most importantly, low antibody response to TIV was observed among vaccine failures. A low level of post-vaccination HAI antibody titer in vaccine failures may therefore explain why those subjects were not protected against infection, and why there was no apparent effect on the degree and duration of illness and viral shedding.

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Seasonal influenza vaccine uptake in Kenya in 2012: does father involvement make a difference?

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Background: Paternal involvement in health decision-making for children has been associated with positive child health outcomes, including increased rates of vaccination (Rammohan A, et al. *BMC Public Health*. 2012;12:336); however, the role that fathers play in the health of children in tropical Africa remains underexplored. Seasonal influenza vaccine is not routinely offered by Kenya's Expanded Programme on Immunization, and therefore, not readily available in Kenya. In 2010, KEMRI/CDC launched a 3-year observational seasonal influenza vaccine effectiveness study in 2 population-based infectious disease surveillance (PBIDS) sites: Kibera, an informal settlement in Nairobi, and Lwak, a rural community in Nyanza Province, Western Kenya. Free seasonal influenza vaccine was offered to children, 6 months to 10 years of age, who were enrolled in PBIDS in the 2

sites. We assessed the extent of fathers' involvement in seasonal influenza vaccine uptake in these sites. Materials and Methods: Annual vaccination campaigns were conducted from designated health-facilities in both sites. In 2012, nurses at health facilities administered standardized questionnaires to the parents or caretakers of vaccinated children during the campaign from March-May. We also sampled approximately 500 households at each site and conducted structured interviews of male healthcare decision-makers/caretakers from July-August. Households were stratified into 3 categories: fully vaccinated against influenza (all children in the household recieved the recommended number of doses of influenza vaccine), partially vaccinated (1 or more of the children in the household received a dose of vaccine, but not all children received all needed doses), and not vaccinated against influenza (no children in the household received vaccine). Multivariate ordinal logistic regression analysis was performed using Proc GENMOD in SAS to adjust for clustering of responses within households to evaluate associations between independent variables measuring paternal and maternal age and educational variables, paternal and maternal attitudes towards the importance of seasonal influenza vaccine, and paternal engagement in vaccine decision-making with a dependent variable of household vaccination status. Results: We provided 27,602 doses of vaccine to 17,613 children in the 2 communities. Of the 1074 sampled households, 456 (42.5%) were fully vaccinated against influenza, 405 (37.7%) were partially vaccinated, and 213 (19.8%) were not vaccinated. Over 90% of children were brought by their mothers to the vaccination campaign. Households in which both fathers and mothers were away from home for a duration > 3 months at the time of vaccination were less likely to vaccinate their children compared with households in which both parents were in the home during the vaccination campaign (aOR = 0.29; 95% CI 0.09-0.88; *P* = .03). However, households where either the father (aOR= 0.81; 95% CI 0.62-1.06; *P* = .12) or the mother (aOR= 1.60; 95% CI 0.68-3.74; *P* = .28) was away during the vaccination campaign were more likely not to have their children vaccinated than households where both parents were present. Households with fathers who believed that influenza vaccines are important (aOR = 5.10; 95% CI 1.63-15.97; *P* = .01); mothers who believed that influenza vaccines are important (aOR = 11.98; 95% CI 3.97-36.14; *P* < .001); and where both parents believed that influenza vaccines are important (aOR = 8.35; 95% CI 3.16-22.06; *P* < .001) were each more likely to have their children vaccinated when compared with households where both parents did not believe that the vaccines were important. Finally, households where fathers joined in the decision to have children vaccinated were more likely to vaccinate their children than households where the father did not join the vaccination decision (aOR = 3.01; 95% CI 1.62-5.62; *P* < .001). Conclusion: Mothers are highly visible parental caretakers at the time of vaccination, as 90% of vaccinated children were brought by their mothers for influenza vaccination in Kenya. However, both father's and mother's belief in the importance of influenza vaccine, and fathers' involvement in the decision to vaccinate children, were significantly associated with vaccination against seasonal influenza in Kenya. Despite the visibility of mothers at the time of a child's vaccination, the fathers' role in vaccine decision-making should not be overlooked. Targeting fathers in sensitization activities during future campaigns may help to increase vaccination coverage rates.

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Seasonal influenza vaccine coverage by risk group in Thailand, 2010-2012

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Background: The World Health Organization (WHO) currently prioritizes seasonal influenza vaccinations for populations who are at the highest risk for serious complications from influenza, as well as health care personnel. In Thailand, universal health care covers influenza vaccine for persons with a high-risk condition and elderly persons (> 65 years), and the Ministry of Public Health covers

vaccine for health care personnel. In 2009, the Advisory Committee on Immunization Practice of Thailand expanded recommendations for influenza vaccination to the 5 WHO recommended target groups [pregnant women, health care personnel, children (6 months to 2 years), elderly, and persons with a high-risk condition], as well as obese persons and persons who are mentally disabled. We assessed vaccination coverage by age group and pregnancy status, and overall vaccine wastage, in Thailand from 2010 to 2012. Materials and Methods: From 2010 to 2012, the National Health Security Office (NHSO) purchased seasonal influenza vaccine for the universal health care program for persons with a high-risk condition and elderly persons; the Department of Disease Control (DDC) purchased vaccine for health care personnel. Vaccine was distributed to provinces and providers were supposed to vaccinate these 3 target groups first. The total number of vaccine doses purchased was obtained from NHSO and DDC. NHSO maintains records of all individuals who receive publicly purchased seasonal influenza vaccines. These data were analyzed to determine the number of individuals who received the influenza vaccine by risk group. Census data for registered nationals obtained from the Thailand National Statistics Office were used to identify the number of children aged 6 months to 2 years and elderly persons. The number of live births was used as a proxy for the number of pregnant women (Bureau of Policy and Strategy, Ministry of Public Health, Thailand). Vaccine coverage was defined as the proportion of individuals in each category who received the vaccine. Denominator data for other risk groups is being sought. Vaccine wastage was defined as the proportion of vaccines purchased by the public sector that were not used. Results: From 2010 to 2012, 2.37, 2.52, and 3.29 million doses were purchased, respectively; the number of influenza vaccines publicly available increased 39% from 2010 to 2012. Vaccines administered by year were 2,170,813 (91.6%), 2,259,614 (89.8%), and 2,976,836 (90.4%); vaccine wastage was 8.4% in 2010, 10.2% in 2011, and 9.6% in 2012. Over the 3 years combined, 3,974,848 (54%) doses were administered to nonelderly persons with a high-risk condition; 1,721,490 (23%) to elderly persons, 1,426,686 (19%) to health care personnel; 78,885 (1.1%) to obese persons, 82,570 (1.1%) to young children; 26,481 (0.4%) to persons who were mentally disabled; 17,787 (0.2%) to pregnant women, and 78,651 (1.1%) to persons whose risk group was unknown. The average vaccine coverage across all 3 years was 11.8% in elderly persons, 1.5% in young children, and < 1% in pregnant women. Compared with elderly men, elderly women had higher vaccine coverage (13% vs 11%, $P < .01$). No gender differences in coverage among young children was observed (male: 1.5%, female: 1.4%; $P > .01$). Vaccine coverage for elderly was higher outside of Bangkok (12% vs 5.6%, $P < .01$) but was comparable among young children (1.4% vs 1.7%, $P < 0.01$). Conclusion: In Thailand, the availability of seasonal influenza vaccine for use in the public sector increased 39% from 2010 to 2012; however, vaccine wastage also increased during this time. Vaccine coverage was highest in elderly persons, but still remained low at < 12%. These findings underestimate vaccine coverage since private and other public sector sales and use were not included. Results may reflect low rates of vaccine acceptance, insufficient capacity of immunization clinics, and shortcomings in vaccine management in Thailand. Annual monitoring of the vaccination campaign is warranted to assess progress and insure high coverage in the recommended risk groups. Future studies should validate the immunization data sources and seek to identify potential barriers to receiving influenza vaccine in Thais, as well as logistical considerations which may increase vaccine wastage.

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Safety, infectivity, and immunogenicity of H2N2, H5N1, H6N1, H7N3, and H9N2 live attenuated influenza vaccines

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Background: Hemagglutinin (HA) and neuraminidase (NA) genes of avian influenza viruses of pandemic potential (H2N2, H5N1, H6N1, H7N3, and H9N2) have been used to prepare investigational pandemic live attenuated influenza vaccines (pLAIV) based on the cold adapted A/Ann Arbor/6/60 donor strain. Clinical trial results in seronegative adults have been reported individually but have not been collectively summarized. Materials and Methods: Open-label inpatient clinical trials of 2 doses of $10^{6.7}$ to $10^{7.5}$ median tissue culture infectious doses ($TCID_{50}$) of H2N2 (Ann Arbor/1960),

H5N1 (Hong Kong/2003 and Vietnam/2004), H6N1 (teal/Hong Kong/1997), H7N3 (chicken/British Columbia/2004) and H9N2 (chicken/Hong Kong/1997) pLAIV were conducted in seronegative adults. A total of 173 adults (18-49 years of age) were vaccinated; in individual trials 52%-68% of subjects were male. Vaccine virus replication was measured by testing nasal wash specimens using quantitative viral culture or sensitive real-time reverse transcriptase polymerase chain reaction (rRT-PCR) assays. Serum antibody responses were assessed using hemagglutination-inhibition (HAI), microneutralization, HA-specific IgG- or IgA-enzyme-linked immunosorbent (ELISA) assays. Nasal antibody responses were assessed using HA-specific IgA ELISA. For each pLAIV studied, the percentage of subjects with a 4-fold rise in antibody titer after any dose was summarized. Results: Vaccine shedding following dose 1 was modest and variable. The percentage of subjects with vaccine virus detected in nasal specimens by culture was 0% (Vietnam/2004 H5N1 and H6N1), 5% (H9N2), 6% (Hong Kong/2003 H5N1), 19% (H2N2), and 24% (H7N3). Use of rRT-PCR did not substantially increase the proportion of subjects with detectable shedding beyond day 1. The percentage of subjects with a 4-fold rise in serum or nasal antibody titer differed among different vaccines, as did the assay that detected the greatest proportion of seroresponders. Overall seroresponse rates measured using HAI and microneutralization assays were similar; slightly more seroresponders were detected using HAI compared with microneutralization. Serum HAI response rates were modest for some vaccines (0% for A/Hong Kong/2003 H5N1, 5% for H6N1, and 12% for H2N2). Higher rates of 62% and 92% were observed for the H7N3 and H9N2, respectively. Response rates using serum IgG and IgA ELISA assays also varied for the different vaccines, and there was generally concordance between the 2 assays. For vaccines that had a low HAI seroresponse rate (H5N1, H6N1, and H2N2), the IgG and IgA ELISA detected substantially higher seroresponse rates. For example, the A/Vietnam/2004 H5N1 pLAIV, which had a 10% HAI seroresponse rate, had a 52% IgA ELISA seroresponse rate. Similarly, the H6N1 pLAIV had a 5% HAI seroresponse rate but a 27% IgG ELISA seroresponse rate. For all pLAIV tested, a smaller proportion of subjects developed nasal IgA responses compared with serum IgA responses. The percentage of subjects with a seroresponse in any assay was 100% (H9N2), 81% (H7N3), 62% (Vietnam/2004 H5N1), 36% (H6N1), 24% (Hong Kong/2003 H5N1), and 24% (H2N2). No effect of age or gender on immune response was noted. Conclusions: The proportion of seronegative subjects with detectable pLAIV replication was modest. Although rates for the H7N3 pLAIV (24%) and H2N2 pLAIV (19%) were comparable with the 17% rate for seasonal LAIV in adults of mixed baseline serostatus, other pLAIV containing avian HA/NA genes were less infectious in humans than seasonal LAIV containing human HA/NA genes. Serum antibody responses to pLAIV were modest. The seroresponse rate in any assay ranged from 24% to 100% for pLAIVs formulated at a potency of at least 10^7 $TCID_{50}$. HAI response rates ranged from 0% to 92%. By comparison, the serum HAI response rate of seronegative adults was approximately 12% to 14% to human influenza A strains in trivalent seasonal LAIV and 15% to monovalent H1N1 2009pdm LAIV. Thus, the modest serum antibody response rates measured for pLAIV are consistent with experience with seasonal LAIV. However, even in the absence of measurable shedding and/or immune responses, immunological priming might still occur. Sponsored by MedImmune and the NIAID Division of Intramural Research, NIH.

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Potential impact of prior vaccination on live attenuated influenza vaccine efficacy

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Background: A recently published observational study of mostly inactivated vaccine recipients raised the question of whether influenza vaccine effectiveness is lower among subjects vaccinated against influenza in the prior season, perhaps due to suboptimal immune responses when sequential vaccines are closely related antigenically. The aim of this study was to present data from randomized controlled trials describing the efficacy of live attenuated influenza vaccine (LAIV) by vaccine type/subtype (A/H1N1, A/H3N2 or B) in 2 consecutive years, and to compare vaccine efficacy in year 2 depending upon whether the vaccine strains differ or not from those contained in year 1. Material

and Methods: All 2-year randomized clinical trials of LAIV versus placebo were reviewed. Vaccine efficacy against laboratory confirmed cases of influenza due to antigenically similar strains in year 1 and 2 was assessed in subjects vaccinated with 2 doses of LAIV in year 1 and one dose of LAIV in year 2 versus subjects who received placebo in year 1 and year 2. A log binomial model was used to estimate vaccine efficacy for each study, as well as consolidated estimates adjusting for study variation. Consolidated vaccine efficacy was not estimated when heterogeneity of vaccine efficacy between studies was significant. Results: Four published prospective double-blind randomized clinical trials comparing the efficacy of live attenuated influenza vaccine (LAIV) versus placebo during 2 consecutive years were identified. These studies enrolled previously unvaccinated children aged 6 to 83 months. A total of 4145 children were assessed in year 2: 2497 LAIV recipients and 1648 placebo controls. A/H1N1 strains contained in the vaccine were the same in 3 studies. Consolidated vaccine efficacy (95% confidence interval) was 89% (79%, 94%) in year 1 and 93% (77%, 98%) in year 2. In the study in which A/H1N1 strains differed in years 1 and 2, no case of laboratory confirmed A/H1N1 was identified in year 2. A/H3N2 strains contained in the vaccine in years 1 and 2 were the same in one study. Vaccine efficacy was 96% (89%, 99%) in year 1 and 100% (NA, 100%) in year 2. Among the 3 studies in which A/H3N2 vaccine strains differed in years 1 and 2, vaccine efficacy was 73% (56%, 83%) in year 1 and 88% (82%, 92%) in year 2. B strains contained in the vaccine in years 1 and 2 were the same in 2 studies. Vaccine efficacy was 89% (73%, 95%) in year 1 and 100% (NA, 100%) in year 2. Vaccine efficacy in year 2 was significantly different between the 2 studies in which B strains differed in years 1 and 2: 82% (55%, 92%) versus −102% (−998%; 63%); therefore no consolidated efficacy was calculated. Conclusions: LAIV consolidated efficacy estimates in year 2 were consistently greater than 80%, regardless of whether vaccine strains in year 1 and 2 were the same or different. These data suggest that LAIV efficacy in children does not decline with revaccination. Sponsored by MedImmune.

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Sublingual priming and heterologous intramuscular boosting of immune responses with whole inactivated virus vaccine influenza

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Background: Vaccination is the best approach to contain the spread of influenza virus during pandemics. However, production of vaccine for a newly emerging strain can be very time consuming. If a potentially pandemic strain is known, as is the case for H5N1, vaccine can be produced and stockpiled. While the stockpiled vaccine might not completely match with the final H5N1 pandemic strain it has been shown to be still useful for priming of an H5N1-specific immune response. We hypothesized that priming could be effectively achieved by sublingual vaccination, and would improve the response to intramuscular (IM) boosting with a drifted strain. In this study, this approach of sublingual priming and IM boosting with heterologous flu vaccines was evaluated. Materials and Methods: Freeze-dried whole inactivated virus (WIV) influenza vaccine prepared from strain NIBRG-14 was reconstituted in water and used for immunization of mice via the sublingual route. After 56 days, the immune response was boosted by IM injection of NIBRG-23. After another 28 days, antibodies in lung and nasal washes were evaluated along with the systemic immune response. Mice immunized once with NIBRG-23 by an IM injection at day 56 were used as control. Results: A single IM immunization of mice with NIBRG-23 did induce serum IgG but hemagglutination inhibition (HAI) titers were below the detection limit. If mice were primed by sublingual immunization with NIBRG-14 prior to IM immunization with NIBRG-23, NIBRG-23-specific antibody titers were significantly enhanced and HAI titers were readily detectable. Both the antibody response and the HAI titers induced by 2 vaccine doses, ie, sublingual prime and intramuscular boost were found to be significantly higher as compared to those induced by one intramuscular vaccine dose (NIBRG-23). Moreover, the sublingual prime/IM heterologous boost regime resulted in induction of influenza-specific IgA in nose and lungs. Conclusions: In conclusion, the sublingual immunization with a stockpiled vaccine strain can effectively prime the immune system for a subsequent IM immunization

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with a drifted influenza strain. This immunization regime resulted in increased serum antibody titers and the induction of IgA in the upper airways, the port of virus entry. Hence, this approach of vaccination may help to face the vaccine shortage during pandemic outbreaks.

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Pressure-inactivated avian influenza virus: implications for vaccine development

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Background: Influenza virus poses a serious global health threat, particularly in light of newly emerging strains, such as the avian virus H5N1 and H7N9. Vaccination remains the primary method for prevention of acquiring influenza or to avoid developing serious complications related to the disease. Vaccination based on virus-inactivated split vaccines and on chemically inactivated whole virus have some important drawbacks, including changes in immunogenic properties of the virus. To induce a greater mucosal immune response, vaccines by intranasal administration are highly desired as the intranasal vaccination strategy can not only prevent disease, but can also block infection at its primary site. Hydrostatic pressure has been used as a potential method for viral inactivation and vaccine production. Materials and Methods: An egg-grown inactivated H3N8 whole virus vaccine was prepared using hydrostatic pressure as inactivating agent. The residual infectivity of the pressurized virus samples was assayed for 3 sequential serial passages in embryonated hens eggs and MDCK cells monolayer. The hemagglutinin and neuraminidase activity was assayed by hemagglutination and sialidase assay. Visualization of pressurized and control viruses were performed in a transmitted electron microscope. To evaluate the immunogenic capacity of inactivated particles, we used 6-week-old female Balb/c mice. The animals were immunized intranasally at weekly intervals, with 3 doses of 100 µg/mL pressurized virus (1 $TCID_{50}$, 265HAU/50 µL). On day 21, mice were challenged in intranasal route with 400 µg/mL (512 HAU/50 µL) of wild-type H3N8 virus. After vaccination and infection, IgG2a, IgG1 and IgA were measured in sera, feces, and nasal wash using an ELISA. To further investigate the ability of the pressure-inactivated virus in stimulating the immune system we evaluated the production of cytokines (IL-2, IL-4, IL-6, IL-10, IFN-γ, and TNF-α) in the supernatants of splenocytes and BAL. We also performed lung histopathology analysis by light microscope. Results: In this study we show that hydrostatic pressure is able to inactivate the H3N8 avian influenza virus, while still maintaining hemagglutination and neuraminidase functionality. Challenged vaccinated-animals showed no disease signs (*ruffled fur,* lethargy, weight loss, and huddling). In the same way, these animals showed less Evans blue leakage and cell counts in bronchoalveolar lavage when compared to challenge non vaccinated group. We found that the whole inactivated particles were capable to generate neutralizing antibody response in serum, also been found IgA in nasal mucosa and feces. After vaccination and challenge we found Th1/Th2 cytokines secretion with prevalence of IFN-γ. Vaccinated and further-challenged mice showed no lung damage. Conclusions: Our results showed that the animals present a satisfactory immune response after vaccination and are protected against challenge. These data may pave the way into the development of a novel pressure-based vaccine against influenza virus.

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Safety and immunogenicity of a vero cell culture-derived whole-virus H5N1 influenza vaccine in a pediatric population

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Background: The development of vaccines against H5N1 influenza viruses is a cornerstone of pandemic preparedness. A nonadjuvanted, Vero cell culture-derived whole-virus H5N1 vaccine has been shown to be safe and immunogenic in multiple clinical studies in adult and elderly populations, and to effectively induce neuraminidase-inhibiting (NAi) antibodies in adults. As children are at increased risk of severe disease attributable to influenza, and are particularly vulnerable to infection with novel influenza viruses, the safety and immunogenicity of this vaccine was further investigated in a pediatric population aged 6 months to 17 years. Antibody responses to both hemagglutinin (HA) and neuraminidase (NA) were assessed, and the induction of immunological memory and the ability of a heterologous booster immunization to induce cross-clade antibody responses were evaluated. Materials and methods: Between December 21, 2009 and May 24, 2012, a phase 1/2 partially blinded study was conducted at 20 study centers in Finland, Spain, Singapore, and Australia. Participants were sequentially enrolled after initial review of safety data in each successive age cohort (ie, 9-17 years, 3-8 years, and 6-35 months) by an independent Data Monitoring Committee. Adolescents aged 9-17 years received 2 priming immunizations, 21 days apart, with the H5N1 vaccine containing 7.5 µg HA antigen of clade 1 A/Vietnam/1203/2004. Children and infants aged 3-8 years and 6-35 months were randomized 1:1 to receive 2 immunizations with a 7.5 µg or 3.75 µg HA dose of A/Vietnam/1203/2004 strain H5N1 vaccine. A subset of participants received a booster immunization with a clade 2.1 A/Indonesia/05/2005 strain H5N1 vaccine approximately 1 year later. Antibody responses were assessed by virus neutralization (MN) and single radial hemolysis (SRH) assay. An enzyme-linked lectin assay was used to assess NAi antibodies. Vaccine safety was assessed throughout the study. Results: The H5N1 vaccine was safe and well-tolerated in all age groups. Adverse reactions were transient and predominantly mild in severity. No vaccine-related serious AEs were reported. Two immunizations with the 7.5 µg dose of A/Vietnam vaccine induced MN titers ≥ 1:20 against the A/Vietnam strain in 85.4%, 72.9%, and 68.8% of participants aged 9-17 years, 3-8 years and 6-35 months, respectively. After the booster vaccination with the 7.5 µg dose of A/Indonesia H5N1 vaccine, the corresponding rates of MN titers ≥ 1:20 against the A/Vietnam and A/Indonesia strains were ≥ 93.1%, ≥ 94.7%, and 100.0%. The MN data were broadly supported by the SRH data. N1-specific NAi antibodies were induced in 99.7%, 91.8%, and 90.3% of participants in the 9-17 years, 3-8 years and 6-35 months age groups, respectively, after two immunizations with the 7.5 µg A/Vietnam vaccine. After the A/Indonesia booster vaccination, 100% of participants in all age groups had N1-specific NAi antibodies. Conclusions: A nonadjuvanted, Vero cell culture-derived whole-virus H5N1 influenza vaccine is safe and well-tolerated in healthy infants, children and adolescents aged 6 months to 17 years. Functional antibody responses to H5N1 HA and NA were effectively induced after two immunizations with the H5N1 influenza vaccine, which primed for robust cross-clade neutralizing antibody responses as well as NAi responses after boosting with a heterologous H5N1 vaccine approximately one year after priming. These data support use of the H5N1 influenza vaccine in both pre-pandemic and pandemic vaccination programs for the highly vulnerable pediatric population.

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Evaluation of different formulations of an H9N2 vaccine with or without AS03 adjuvant in adults aged 18-64 years

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Background: Influenza A virus subtype H9N2 is a low pathogenic avian influenza virus with limited incidence and severity in humans that has the potential to become pandemic. Development of vaccines against influenza caused by potential pandemic subtypes is a key strategy for pandemic preparedness. Antigen-sparing through adjuvantation allows for a faster and broader pandemic vaccination response. We evaluated the immunogenicity, reactogenicity and safety of different formulations of monovalent split-virus influenza A/chicken/Hong Kong/G9/1997 NIBRG-91 (H9N2) vaccine (produced by GlaxoSmithKline, Quebec, Canada) with or without adjuvant system 03 (AS03) in adults 18 to 64 years of age. Materials and Methods: In this phase I/II observer-blind, randomized, placebo-controlled study in the United States and Canada (NCT01659086), adults were randomized (1:1:1:1:1:1:1:1:1:1:4) to receive 2 doses (on days [D] 0 and 21) of either different H9N2 hemagglutinin (HA) antigen formulations or saline placebo. H9N2 vaccine regimens administered were: 1.9µg HA adjuvanted with $AS03_A$ (1.9µgHA+$AS03_A$) or $AS03_B$ (1.9µgHA+$AS03_B$), 3.75µg HA adjuvanted with $AS03_A$ (3.75µgHA+$AS03_A$) or $AS03_B$ (3.75µgHA+$AS03_B$) or 15µg HA H9N2 plain antigen (15µgHA). AS03 contains α-tocopherol ($AS03_A$: 11.86mg; $AS03_B$: 5.93mg) and squalene as an oil-in-water emulsion. Hemagglutination inhibition (HI), seroprotection rates (SPRs), seroconversion rates (SCRs), and mean geometric increase (MGI) were evaluated on D21 and D42 according to the US Center for Biologics Evaluation and Research (CBER; lower 95% confidence interval [CI] for SCR ≥ 40%, for SPR ≥ 70%) and the EU Committee for Medicinal Products for Human Use (CHMP; point estimate for SCR > 40%, for SPR >70%, for MGI > 2.5) immunogenicity criteria. Adjuvant benefit was demonstrated if the lower limit of the 95%CI of the HI geometric mean titre (GMT) ratio of the adjuvanted formulations over plain antigen was > 1. Solicited local and general adverse events (AEs) were evaluated for 7 days and unsolicited AEs for 21 days after each dose. Medically attended adverse events (MAEs), potential immune-mediated diseases (pIMDs), and serious adverse events (SAEs) were collected throughout the study. Results are reported here up to 21 days postdose 2 (D42); this ongoing study includes a booster vaccination at D182 with different H9N2 vaccine regimens. Results: Of 420 vaccinated subjects, 404 were included in the according-to-protocol cohort for immunogenicity (ATP-I) D21 and 383 in the ATP-I D42. At D21 and D42, the immune response to adjuvanted H9N2 vaccines met all CHMP and CBER immunogenicity criteria. At D21, SCRs ranged from 91.1% to 96.3%, SPRs from 96.4% to 100%, and MGIs from 24.19 to 43.95 in the adjuvanted groups. At D42, SCRs ranged from 98.1% to 98.3%, SPRs were 100%, and MGIs ranged from 82.79 to 124.36 in the adjuvanted groups. The immune response to H9N2 HA plain antigen also met all CHMP and CBER immunogenicity criteria at D21 and D42. An adjuvant effect was demonstrated for 1.9µgHA+$AS03_A$, 3.75µgHA+$AS03_A$ and 3.75µgHA+$AS03_B$ at D21 and all adjuvanted formulations at D42. Pain was reported more frequently in the adjuvanted groups than in the 15µg HA or placebo groups. No increase in AE frequency was observed after dose 2. Up to D42, MAEs were reported by 4.9% (1.9µgHA+$AS03_A$) to 14.4% (placebo) of subjects. Up to D42, 3 SAEs (none fatal or vaccine-related) and no pIMDs were reported. Conclusions: The HI immune response to $AS03_A$- or $AS03_B$-adjuvanted H9N2 vaccine containing 1.9µg or 3.75µg HA antigen met all CBER and CHMP immunogenicity criteria 21 days after dose 1 and 2. Although adjuvant benefit was demonstrated at D42 for all adjuvanted groups, establishing antigen sparing, the plain antigen formulation also met immunogenicity criteria at D21 and D42. The different H9N2 vaccine formulations were generally well tolerated and no safety concerns were identified up to D42. Funding: GlaxoSmithKline Biologicals SA and BARDA.

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Influenza symptoms and impact on quality of life in elderly adults: effect of AS03-adjuvanted or –non-adjuvanted inactivated trivalent seasonal influenza vaccines

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Background: The burden of seasonal influenza in the elderly is high, and vaccination against seasonal influenza is recommended routinely for the elderly in many countries. However, it is well recognised that the immune response to vaccination is lower in the older adults compared with younger adults, with a corresponding decrease in vaccine effectiveness. In the geriatric setting, patient-reported outcomes (PROs) are particularly relevant, since the most important characteristic of an improved vaccine may include reduction of symptom severity rather than prevention of infection. Although clinical trials of influenza vaccines usually focus on prevention of illness, independent of severity, reduction in disease severity may be a more realistic and clinically significant goal. The effects of disease symptoms on an individual are best measured by PROs, which have become recognised as important endpoints in clinical trials in many therapeutic areas and validated by the US Food and Drug Administration (FDA). The aim of this study was to evaluate PROs in subjects aged ≥ 65 years receiving two different vaccines. Materials and Methods: This was a phase III, randomised, observer-blinded study (NCT00753272) of the AS03-adjuvanted inactivated trivalent split-virion influenza vaccine candidate (AS03-TIV) versus a non-adjuvanted commercially available trivalent influenza vaccine (TIV) in elderly patients aged ≥ 65 years. A new instrument, the Influenza Intensity and Impact Questionnaire (FluiiQ™), previously developed to meet FDA PRO standards, was used to compute symptom scores (systemic, respiratory, total) and life-impact scores (activities, emotions, relationships). The minimal important difference in influenza severity between vaccines for the post hoc analysis was > 7%. Vaccine efficacy of AS03-TIV relative to TIV in moderate to severe influenza (hospitalisation, complication or most severe one-third of episodes based on the AUC for systemic symptom score) was calculated post hoc. The main exploratory analyses were conducted on influenza cases confirmed by detection of virus or its RNA in the per-protocol cohort (n = 280 AS03-TIV, n = 315 TIV). Results: More than 80% of study subjects provided calculable FluiiQ™ data indicating that, in this population, PRO assessment is feasible and acceptable to study subjects. Patients vaccinated with AS03-TIV had lower mean systemic symptoms, total symptoms and impact on activities compared with those given TIV. Mean respiratory symptoms, impact on emotions and impact on relationships were similar between vaccines. Influenza tended to be less severe with AS03-TIV, but the minimal important difference was reached only for the impact on activities score (mean difference: 9.0%). Relative vaccine efficacy in severe influenza was 29.38% (95% confidence interval: 7.60–46.02). Conclusions: This exploratory analysis suggested that vaccination with AS03-TIV

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reduced the impact of influenza on symptoms and daily activities as well as in prevention of moderate to severe illness. A key strength of the study was the use of the validated FluiiQ™ questionnaire to study the impact of influenza vaccines on PROs and disease severity. Assessment of PROs characterizing a patient's experience of illness may assist with making influenza vaccine efficacy trials more clinically relevant. Funding: GlaxoSmithKline Biologicals SA.

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Development of candidate vaccine virus derived from A/Anhui/1/2013(H7N9) strain

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Background: On March 2013, the first case of human infection with avian influenza A/H7N9 virus in China was reported. Since there was a threat of pandemic, we have commenced to develop candidate vaccine viruses (CVVs) in April, along with other WHO Collaborating Centers. For this purpose, we have created 6:2 genetic reassortant viruses between A/Anhui/1/2013(H7N9) and high growth master virus A/PR/8/34 (H1N1) using reverse genetics technique. The viruses are in the evaluating process of their suitability for the practical use in vaccine production. Materials and Methods: A/Anhui/1/2013 (H7N9), one of egg-isolated strains, was kindly provided from China CDC. We have created the reassortant viruses using the 12 plasmid-based reverse genetics technique (kindly provided from Dr Yoshi Kawaoka, University of Wisconsin). In brief, the cDNA of HA and NA genes of A/Anhui/1/2013 was prepared by reverse transcription, cloned into the pPolI plasmid vector and was utilized to transfect LLC-MK2 cells together with another 10 plasmids encoding genes of PR8. The transfectants of LLC-MK2 cells were harvested at 48 hours posttransfection and inoculated into 10-days old specific-pathogen–free (SPF) embryonated chicken eggs to amplify the rescued viruses. The growth ability in eggs, genetic and antigenic properties of the obtained viruses have been characterized. Results: Direct sequencing on RT-PCR products of HA and NA genes amplified from the original virus showed that A/Anhui/1/2013 HA gene has a mixture of sequences encoding different amino acids at positions 123, 125, or 149 (H7 numbering). Genetic cloning and sequence analysis of HA cDNA revealed that three types of HA with 123N/125A/149N (identical with GISAID database EPI439507), 123N/125T/149N, and 123D/125A/149D were identified. These amino acid positions locate around the receptor-binding pocket of HA and a new N-linked oligosaccharide attachment site was created at position 123N by the amino acid 125T substitution. Nucleotide sequence of NA cDNA was concordant with that shown in the GISAID database (EPI439509). Using these HA and NA cDNAs, we have rescued three reassortant viruses, NIIDRG-10, NIIDRG-10.1 and NIIDRG-10.2, with the respective amino acid residues of HA, 123N/125A/149N, 123N/125T/149N, and 123D/125A/149D. After propagation in eggs, NIIDRG-10 showed 128 HA titer, while NIIDRG-10.1 and 10.2 showed higher HA titers of 1024-2048. NIIDRG-10.1 showed genetic stability of HA and NA genes during 4 passages of the viruses in eggs. These results suggested that the amino acid sequences 123N/125T/149N and 123D/125A/149D contributed to the growth ability of the virus in eggs. HI test using ferret antisera against A/Anhui/1/2013 revealed similar antigenic properties among these CVVs. The suitability of these CVV for the practical use in vaccine production, such as HA protein yields, immunogenicity, and pathogenicity, is under examination. Conclusion: During the pandemic and interpandemic phases, the high growth ability and high HA yields are desirable properties for CVV because such CVV would shorten a period of vaccine production. In this study, we developed several CVV derived from the original virus specimens and examined the characteristics of all the created viruses. As a result, we identified NIIDRG-10.1 with a possible advantage in the vaccine production because of their high HA titers and the genetic stability of HA gene. Field isolates frequently contain genetic variants arisen by viral evolution. As long as the original virus isolates would be quasi-species, to examine the properties of all the obtained viral clones is important for the establishment of the preferable CVV. We are grateful to Drs Y Kawaoka (Univ. of Tokyo) and T Odagiri (NIID) for providing antisera used for antigenic characterization of our CVVs.

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Influenza vaccine-induced immune responses in children in rural India

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Background: The effectiveness of influenza vaccines is dependent not only on vaccine strain match with circulating strain, but also on induction of neutralizing antibodies. Little is known about vaccine-induced immune responses in children in developing countries where nutritional and other environmental factors may result in altered responses. We report results of antibody responses in subsets of children (vaccine naive) enrolled in a house randomized, double blinded, active controlled, phase IV clinical trial of trivalent inactivated influenza vaccine (TIV) given to children 6 months through 10 years of age. Methods: Children from ages of 6 months to 10 years were enrolled from three villages under the Comprehensive Rural Health Services Project, located in the Haryana state of North India to receive one (9-10 y) or two (6 mo-8 y) doses of either inactivated TIV (2009-2010 northern hemisphere; Sanofi) or inactivated polio vaccine (IPV; Sanofi). A subset of children (3-6 y of age) were enrolled for an immunogenicity study, and informed consent was taken prior to blood sample collection on day 0, and at week 4 each after either 1 or 2 doses for TIV or IPV. Antibody response to vaccine homologous antigens which included seasonal A/Brisbane/59/2007(H1N1)-like, A/Brisbane/10/2007(H3N2)-like and B/Brisbane/60/2008-like viruses, as well to A/California/7/2009pdm09was detected by means of hemagglutination inhibition assay (HI) using turkey red blood cells with ether treatment of the influenza B antigen. Rise in antibody titers to > 4-fold above baseline was considered seroconversion and a titer > 1:40 was considered seroprotection. Geometric mean titers (GMT) and 95% confidence intervals (CIs) were calculated. The vaccine allocation was unblinded after all laboratory results were generated. Results: Of 161 children from whom blood samples were collected, 76 received TIV (mean age 4.7±0.9 y) and 85 received IPV (mean age 4.5±0.8 y). Of the 76 TIV recipients, 41 received 1 dose and 35 received 2 doses of TIV. The overall seroconversion (≥ 4 fold) subsequent to 1 or 2 doses of TIV ranged from 71%-79% with minimal titers (1%-2%) for IPV group for each of the 3 antigens. There was a significant rise in GMT titers postvaccination in TIV (4-9 fold for all 3 antigens) vs IPV group ($P < .05$). The proportion of subjects with HI titer of 1:40 or greater (sero-protected) after full vaccination were higher for influenza A (79% for H1N1 and 91% for H3), when compared with Influenza B (71%). Overall, 56.6% of TIV recipients were seroprotected and 51.3% seroconverted to all 3 antigens compared with1.3% and 0% in IPV ($P \leq .05$). Analysis of antibody response to A/H1N1pdm09 revealed 6% (10/164) had HI titers at the baseline, whereas additional 24% (41/164) children revealed titers (1:20-1:640) after 4-8 weeks, likely reflecting natural infection during the peak of pandemic infection in Delhi area in November-December 2009. As expected, no difference was observed between antibody responses to A/H1N1pdm09 in TIV or IPV vaccinated group. Conclusion: Influenza vaccination in children between ages of 3-6 years induced strong antibody responses to all 3 viruses contained in 2009-2010 influenza vaccine in the rural community of India. Vaccine clinical data will be analyzed to assess overall effectiveness of influenza vaccination. These studies were supported in part by cooperative agreements U01 IP000177 from the Centers for Disease Control and Prevention, Atlanta, USA.

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Comparisons of circulating influenza viruses in Kenya with WHO-recommended vaccine strains, 2007-2012

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Background: There are limited data on influenza viruses from tropical Africa to inform WHO global vaccine strain recommendations. Such data might also inform an influenza vaccination strategy in Kenya, where despite the presence of 2 licensed influenza vaccines, only 30,000 influenza vaccine doses are currently sold annually in a country of 40 million people. We present data on the influenza viruses detected in Kenya from 2007 through 2012 and compare these viruses with vaccine virus strains. Materials and Methods: Nasopharyngeal/Oropharyngeal (NP/OP) specimens were systematically obtained from patients meeting a respiratory illness case definition who presented to12 national influenza sentinel and population-based surveillance sites from May 2007 to April 2012. These specimens were tested at the KEMRI/CDC laboratories (Nairobi, Kenya) by rtRT-PCR for influenza A and B, and influenza A-positive specimens were subtyped. Influenza viruses were isolated from a convenience subset of the influenza-positive specimens selected from all regions of the country. Isolates were sent to the WHO Collaborating Center at CDC (Atlanta, Georgia, United States), where antigenic characterization was carried out by hemagglutination inhibition (HI) assay. An influenza virus was considered antigenically related to a vaccine or reference strain if its HI titer was equal to or within a 4-fold difference to the vaccine/reference strain. Isolates obtained from specimens collected during the Northern Hemisphere (NH) influenza season (November through April) were compared with NH vaccine formulations while isolates obtained from specimens collected during the Southern Hemisphere (SH) influenza season (May through October) were compared with Southern Hemisphere vaccine formulations for the same period. Results: During 2007-2012, 51,068 NP/OP specimens were tested for influenza viruses, of which 5155 (10.1%) were positive for influenza A, 1475 (2.9%) for influenza B, and 143 (0.3%) for both influenza A and B. Among 6630 influenza-positive specimens, 3203 (48.3 %) were collected during the NH season and 3427 (51.7 %) during the SH season. Antigenic characterization was carried out on 739 isolates; 420 isolates from specimens collected during the SH season and 319 from specimens collected during the NH season. 329/420 (78.3%) of the SH season isolates and 285/319 (89.3%) of the NH season isolates were antigenically related to the corresponding vaccine strains. A total of 543 influenza A isolates were characterized; 108 A(H1N1), 201 A(H1N1)pdm09, and 234 A(H3N2). Forty (37%) of the A(H1N1) isolates, all collected from November 2008 to October 2009, were antigenically related to the vaccine strain A/Brisbane/59/2007(H1N1)-like virus; 193 (96%) of the A(H1N1)pdm09 isolates were similar to A/California/7/2009(H1N1)-like virus. Of 234 A(H3N2) isolates characterized, 198 (84.6%) were antigenically related to the corresponding vaccine strains. These 198 isolates included 2/16 (13%) collected between May 2007 and April 2008 that were antigenically related to A/Wisconsin/67/2005(H3N2)-like virus; 78/79 (98.7%) collected between May 2008 and April 2009 that were antigenically related to A/Brisbane/10/2007(H3N2)-like virus; 0/19 (0%) samples collected between May 2009 and April 2010 that were antigenically related to A/Brisbane/10/2007(H3N2)-like virus; and 118/120 (98.3%) samples collected between May 2010 and April 2012 were antigenically related to A/Perth/16/2009(H3N2)-like virus. Of the196 Influenza B isolates, 183/196 (93.4%) were similar to the corresponding vaccine strain. Of the influenza B viruses collected after April, 2008 183/185 (99%) were antigenically related to the corresponding vaccine strains. Conclusions: Of the 739 influenza isolates obtained through the systematic surveillance in Kenya, slightly over half were collected during the time period corresponding to SH influenza seasons. Over three-quarters of the Kenyan influenza isolates tested were antigenically related to corresponding WHO vaccine strains. As influenza viruses circulate for an average of 10 months per year in Kenya, both during the SH and the NH seasons, an influenza vaccination strategy in Kenya may benefit from using either the NH or the SH formulation. Additional work is currently underway to determine the added costs and benefits of using both vaccine formulations over a single formulation.

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Role of the quaternary structure of human secretory-IgA in nasal mucus on neutralization activity to influenza A virus

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Background: Secretory IgA (S-IgA) antibodies, the major contributor to humoral mucosal immunity, are polymeric immunoglobulins in many external secretions. A large number of studies show that the protective immunity, which is induced by influenza virus infection, is mainly corresponding to S-IgA and IgG antibodies in respiratory tract. Notably, preexisting S-IgA antibodies can provide immediate immunity via their unique capability to eliminate a pathogen before it even pass the mucosal barrier and enter the human body. Polymeric S-IgA antibodies neutralize influenza viruses more effectively than monomeric IgA or IgG in vitro and experimental animal model. The polymeric nature of S-IgA antibodies also explains why S-IgA antibodies crossreact with variant influenza viruses to a greater extent than serum IgG antibodies. However, little is known of the quaternary structures and neutralizing potencies of the various forms of polymeric S-IgA antibodies on viral infection in human upper respiratory tract. It is thus unclear whether polymeric S-IgA antibodies provide more effective protection against influenza virus than monomeric IgA or IgG antibodies in human. In the present study, the quaternary structures and neutralizing potencies of nasal antibodies were examined using nasal wash samples from healthy adults after intranasal administration of inactivated influenza vaccines. Materials and Methods: Nasal wash samples were obtained from 5 healthy adult volunteers, who showed relatively high serum neutralizing antibodies responses against A/Victoria/210/2009 (H3N2; Victoria) virus after receiving 2 intranasal vaccinations with inactivated A/Victoria/210/2009 (H3N2)-like virus whole virion vaccines and were administrated 3 additional intranasal vaccinations (total 5 vaccinations). Then, the nasal wash samples containing various size immunoglobulins were separated by gel filtration chromatography followed by examination of neutralization activity against influenza virus. The quaternary structures of the nasal IgA antibodies induced by intranasal inactivated influenza vaccines were also determined using biochemical techniques, high-mass MALDI-TOF MS and high speed AFM. Results: It was found that human nasal IgA antibodies were composed of at least 5 forms of quaternary structures, monomer, dimer, trimer, tetramer, and larger polymer than tetramer. In particular, the high-speed AFM observation clearly revealed that trimeric or tetrameric IgA antibody had 6 or 8 antigen-binding sites, respectively, which show large-scale conformational change asynchronously and capture the HA antigens in solutions. Among these antibodies, polymeric over dimeric IgA antibodies have a relative higher neutralizing potency to influenza virus. Conclusions: In conclusion, intranasal administration of inactivated influenza virus whole virion vaccines resulted in induced various molecular forms of neutralizing antibodies in human nasal mucus. Within these antibodies, polymeric over dimeric IgA antibodies have the highest neutralizing potency with their characteristic quaternary structures such as flower-shaped complexes, and might play pivotal roles in protection of viral infection on human upper respiratory tract.

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Effectiveness of seasonal influenza vaccination of children against H1N1 (2009) influenza during the first wave of the pandemic in Senegal

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Background: Studies in Canada and Hong Kong have reported higher rates of 2009 pandemic H1N1 (pH1N1) infection after receipt of seasonal trivalent inactivated influenza vaccine (TIV). An "infection-blocking" hypothesis, whereby TIV protects against seasonal influenza infection that might otherwise confer cross-protection against pH1N1 has been described. Another hypothesis is that TIV induces cross-reactive, but not cross-protective, antibodies that result in antibody-dependent enhancement of infection. We conducted a large-scale cluster-randomized trial of TIV in rural Senegal to evaluate the effects of wide-spread influenza vaccination of children. In 2009, A/H3N2 influenza circulated extensively. Subsequently, in early 2010, the first wave of pH1N1 occurred in Senegal and allowed us to explore this issue. Materials and Methods: In May 2009, 20 villages of the Niakhar Demographic Surveillance System were randomized 1:1 for vaccination of children 6 months through 10 years of age with TIV formulated for the 2008-2009 northern hemisphere influenza season (Vaxigrip, Sanofi-Pasteur) or inactivated poliovirus vaccine (IPV) (Sanofi-Pasteur). Commercial presentations of vaccines were masked and coded before delivery to the site, and children received dosing per the manufacturer's instructions. Immunizations were given over a 6-week period beginning May 26, and thereafter, surveillance for laboratory-confirmed febrile acute respiratory illness was conducted using active, community-based (with weekly visits to households), and enhanced passive health post-based surveillance. Individuals reporting symptoms were assessed clinically, and nasal swab and throat swab specimens were collected for influenza testing using CDC's real-time reverse transcription polymerase chain reaction protocol. The primary objective, reported separately, was total effectiveness of vaccination (VE_{Total}) against seasonal influenza. A posthoc analysis estimated VE_{Total} against pH1N1, as 1 minus the relative risk of laboratory-confirmed symptomatic pandemic influenza among enrolled children in TIV villages (measured as cumulative incidence) compared with that in IPV villages, adjusting for village-randomization. Results: Of 11,626 children age-eligible for study vaccinations, 5840 resided in TIV villages and 5786 in IPV villages. Starting May 26, 3929 were enrolled for vaccination in TIV villages and 3851 in IPV villages. Median village participation rates were 73% (TIV) and 74% (IPV). Through November 2009, A/H3N2 (A/Perth) circulated extensively in the population. Subsequently, pH1N1 circulated from January through April 2010. A total of 321 cases laboratory-confirmed as pH1N1 were identified among participants. The average attack rate of pH1N1 among these children in TIV villages was 5% while in IPV villages it was 3%, for a VE_{Total} against pH1N1 of -44% (95% CI, -166% to 21%). VE_{Total} also exhibited negative but statistically nonsignificant effectiveness in all age groups (-24%, -29%, -90%, and -89% among children 6-35 months, 3-5 years, 6-8 years, and 9-10 years of age, respectively). Attack rates of pH1N1 in IPV villages were 3.7%, 3.1%, 2.5%, and 3.6% among children 6-35 months, 3-5 years, 6-8 years, and 9-10 years, respectively, but in the second half of 2009, attack rates of symptomatic seasonal A/H3N2 influenza in these same control villages had been 22%, 16%, 10%, and 7%, respectively. In a subanalysis among the 6973 enrolled children who had not been identified as having had recent symptomatic A/H3N2 infection in 2009, VE_{Total} against pH1N1 remained negative (-43%; 95% CI, -83 to -12%). Conclusions: In this large randomized trial in Africa, we measured an increased risk of pandemic influenza after receipt of seasonal vaccine as compared with after receipt of placebo (negative VE). Although this negative VE was not significant in the full analysis, it was significant among those without clinical seasonal influenza A/H3N2 identified during the previous season. Relatively high attack rates of previous seasonal influenza were measured, but we only evaluated symptomatic seasonal cases. Thus, this subanalysis of total effectiveness against pH1N1 may still have included many who may have had cross-protection against pH1N1 from recent undetected seasonal infection. Thus, infection-blocking by seasonal influenza infection could have caused lower pH1N1 rates in the

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control arm. Our data, like others', also point to the need for further studies of the immune response to natural influenza infection and influenza vaccines.

P2-511

Effectiveness of 2012/13 seasonal influenza vaccines in the prevention of influenza-related hospitalization in Canadian adults: a Public Health Agency of Canada/ Canadian Institutes of Health Research (PCIRN) Serious Outcomes Surveillance Network Study

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Background: Ongoing assessment of effectiveness of influenza vaccines is critical to inform public health decision making regarding publicly funded immunization programs. The goal of the Canadian influenza immunization program is prevention of influenza-associated hospitalization and death, but little data is available to assess the effectiveness of seasonal influenza vaccination in the prevention of serious outcomes. The PCIRN Serious Outcomes Surveillance Network provides critical infrastructure for the assessment of overall and strain-specific influenza vaccine effectiveness against hospitalization in Canadian adults. Methods: In 2012/13, the PCIRN Serious Outcomes Surveillance Network conducted active surveillance for influenza among hospitalized adults from 15 Nov to 30 April in 45 acute care facilities in 7 provinces, encompassing ≈ 18,000 beds. A nasopharyngeal swab for influenza polymerase chain reaction (PCR) testing was obtained from all patients admitted with community-acquired pneumonia, exacerbation of chronic obstructive pulmonary disease or asthma, unexplained sepsis, any respiratory diagnosis or symptom, and, on 1 day per week, acute coronary syndrome, stroke, or other cardiac diagnoses with triage temperature ≥ 37.5°C. Cases were PCR-positive for influenza; up to 2 test-negative controls matched for date, site of enrolment and age of the case (≥ 65 y vs < 65 y) were enrolled for calculation of vaccine effectiveness (VE). Interim unadjusted, unmatched VE was estimated as (1-odds ratio of influenza in vaccinated versus unvaccinated patients) × 100 for cases and controls enrolled up to April 25, 2013. Crude VE estimates will be adjusted using multivariable logistic regression with stepwise backward selection of covariates with a *P* value of < .1 in the univariate analysis. Results: 1503 cases and 2117 controls were enrolled; 1026 (68%) of cases were ≥ 65 years (mean age, 80.8 y; range, 65-102 y); 736 (49%) were > 75 y. 827 (55%) cases and 1368 (64.6%) controls had received an influenza vaccine. Overall unadjusted, unmatched VE in adults was 33.0% (90% confidence interval [CI]: 25.0% to 40.2%); VE in adults ≥ 65 y was 29.1% (90% CI: 17.9% to 38.7%); VE in adults < 65 y was 48.3% (90% CI: 36.6% to 57.8%). Conclusion: In 2012/13, interim estimates to April 25 suggest that the seasonal influenza vaccines used in Canada had suboptimal effectiveness in the prevention of hospitalization of adults due to lab-

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confirmed influenza. VE was higher in those < 65 y than in those ≥ 65 y. Funding: This study was funded by the Public Health Agency of Canada, the Canadian Institutes of Health Research, and GlaxoSmithKline Biologicals SA.

P2-512

The comparative effectiveness of adjuvanted and unadjuvanted trivalent inactivated influenza vaccine in the elderly

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Background: Influenza has a high mortality and a high rate of catastrophic disability in older adults. Vaccine effectiveness decreases with increasing age and immunosenescence. Enhanced vaccines are needed to provide adequate protection in the elderly. Methods: We evaluated the comparative effectiveness of Fluad® an MF59 adjuvanted and unadjuvanted influenza vaccine in the elderly through a test negative case control study. Cases had laboratory confirmed influenza. All eligible elderly (65+) tested for influenza in the winters of 2011-12 and 2012-13 were included. Logistic regression was used to calculate the odds ratio of testing positive for influenza. Adjuvanted vaccine effectiveness was compared with unadjuvanted. Results: A total of 1175 eligible participants were enrolled (454 cases). Over half (658) were in a long term care facility and almost half (549) were 85 years of age or older. Analysis was completed separately for those in long term care (LTC) and community (non-LTC). In 2011-12 in a variety of multivariate analyses, ATIV was significantly protective at around 60% and TIV was ineffective. In 2012-13, ATIV and TIV showed similar VE in non-LTC (VE: 54%, 24%-75% and 57%, 14%-76%, respectively). In non-LTC cases, ATIV showed marginally significant protection against hospitalization (OR: 0.44, 0.19-1.02). TIV did not. In LTC, ATIV showed significant protection against hospitalization (OR: 0.14, 0.05, 0.36) compared with no vaccination. Conclusion: The MF59 adjuvanted vaccine appears to provide improved protection against influenza in the elderly, particularly against hospitalization.

P2-513

Effectiveness of seasonal influenza vaccinations against laboratory confirmed influenza-associated infections among Singapore military personnel in 2010-2013

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Background: Limited information is available about seasonal influenza vaccine effectiveness (VE) in tropical communities. Virus subtype–specific VE was determined for all military service personnel in the recruit camp and 3 other nonrecruit camps from 1 June 2009 to 30 June 2012. Methods: Consenting servicemen underwent nasal washes, which were tested with RT-PCR and subtyped. The test-positive case and test-negative control design was used to estimate the vaccine efficacy (VE). To estimate the overall effect of the program on new recruits, we used an ecological time series approach. Results: A total of 7016 consultations were collected. The crude estimates for the VE against influenza A(H1N1)pdm09 and influenza B were 86% (95% CI 82%-89%) and 84% (95% CI 785-88%), respectively. VE against influenza A(H3N2) was markedly lower (VE 33%, 95% CI 4%-57%). An estimated 70% (RR = 0.30; 95% CI 0.11-0.84), 39% (RR = 0.61;0.25-1.43) and 75% (RR = 0.25; 95% CI 0.11-0.50) reduction in the risk of influenza A(H1N1)pdm09, influenza A(H3N2) and

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influenza B infections, respectively, in the recruit camp during the postvaccination period compared with during the prevaccination period was observed. Conclusions: Overall, the blanket influenza vaccine program has had a moderate to high degree of protection against influenza A(H1N1)pdm09 and influenza B, but not against influenza A(H3N2). Blanket influenza vaccination is recommended for all military personnel.

P2-514

Efficacy of a plant-derived influenza H5 virus-like particle vaccine against heterologous influenza virus challenge in mice

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Background: Avian influenza virus (H5N1) has been a continuing problem in the poultry industry for many years, and is currently a public health threat due to its high mortality rate when the virus crosses the species barrier into people. This study compared H5-virus like particle (VLP) and recombinant H5 (rH5) hemagglutinin vaccines against heterologous challenge in mice with influenza A/Vietnam/1203/2004 (H5N1), influenza A/California/04/2009 (H1N1pdm), or influenza A/Japan/305/1957 (H2N2) virus. Methods: Groups of 6-week old female BALB/c mice were vaccinated by the intramuscular route. Placebos received sterile saline by the same route. Blood was collected on days 0 (prevaccination) and 21 (prechallenge). All mice received intranasal challenge with 1× LD_{90} of H5N1, 3× LD_{50} of H1N1pdm, or 3× LD_{50} of H2N2 on day 21. Five mice were sacrificed on days 0, 3, and 6 postchallenge for lung lavage. Following challenge, mice were observed for morbidity and weight loss through day 21. Kaplan-Meier survival curves were compared by the Log-rank test followed by pairwise analysis using the Gehan-Breslow-Wilcoxon test in Prism 5.0f (GraphPad Software Inc.). Mean body weights were analyzed by 1-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. Evaluation of serological responses following vaccination included measurement of virus-specific antibodies by hemagglutination inhibition (HI) assay and virus neutralization (VN) of influenza virus on MDCK cells. Cellular immunity was evaluated by quantitation of cytokines and chemokines using a multiplex ELISA on lung lavage samples collected post-challenge. The results from serological assays and cellular responses were evaluated by ANOVA. Results: The H5-VLP vaccine provided complete protection from challenge with the H5N1 virus and also provided protection from weight loss following challenge. However, no significant differences in mortality or weight loss were observed between vaccine and placebo groups following challenge with the H1N1 virus. The H5-VLP vaccine did provide 70% protection from challenge with the H2N2 virus. Whereas, the same dose of rH5 vaccine did not provide significant protection against homologous or heterologous challenge. In addition, virus titers from lung lavage collected after challenge showed that the H5-VLP vaccinated group had a significant reduction in virus on days 3 and 6 postchallenge with H5N1, and also showed a significant reduction in virus titer on days 3 and 6 postchallenge with H2N2. Treatment with both H5-VLP and rH5 vaccines showed reduction in virus titer on day 3 postchallenge with H1N1, but only the H5-VLP showed a reduction in virus on day 6. Modest HI titers against H5N1 were observed for both H5-VLP and rH5 vaccinated groups, and against the H2N2 by the H5-VLP vaccinated group. However, the titers were not statistically significantly. Modest VN titers against H5N1 were also observed for both H5-VLP and rH5 vaccinated groups, and against the H2N2 by the H5-VLP vaccinated group. Although, the VN titers from the H5-VLP vaccinated group were significant. The most striking observations on cytokine and chemokine levels in lung lavage following challenge include a decrease in IL-4 levels following vaccination with the H5-VLP for all three challenge viruses at all times tested; and an increase in TNFα levels following vaccination with the H5-VLP for all viruses at all times tested. The results for IL-12p70 and MIP-1α levels were also of interest. IL-12p70 levels decreased following vaccination with the H5-VLP for all viruses at all times tested. However, significant changes were only observed after H1N1 or H2N2 challenge. Conclusions: The H5-VLP vaccine provided complete protection from challenge with the H5N1 virus and also provided significant protection from weight loss following challenge. In addition, the H5-VLP vaccine provided 70% protection from challenge with the H2N2 virus. Modest HI and VN titers were observed following vaccination with the H5-VLP, although, only the VN titers were

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statistically significant. These data suggest that cellular immunity plays an important role in providing protection from challenge following vaccination. Supported by Contract HHSN272201000039I from the Respiratory Diseases Branch, NIAID, NIH.

P2-515

Current and previous season vaccination have similar effectiveness for preventing medically attended influenza A (H3N2) and B: results from a community cohort over 8 seasons

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Background: Influenza vaccination is recommended annually for all persons aged ≥ 6 months in the United States. Recent single-season studies suggest that vaccination in prior years may reduce the effectiveness of current season vaccination. Marshfield Clinic has conducted annual studies of influenza vaccine effectiveness (VE) using the test-negative case-control design since 2004-05. We used enrollment and vaccination data from multiple seasons to assess the independent and combined effects of current and prior season vaccinations for prevention of medically attended influenza A (H3N2) and B.
Methods: Patients ≥ 9 years old with medically attended acute respiratory illness (MAARI) were actively recruited during 8 influenza seasons: 2004-05 through 2012-13. Enrollments during the 2009-10 pandemic were excluded. RT-PCR (or culture during 2004-05 and 2005-06) was performed on respiratory swab specimens for detection of influenza and type/subtype. Dates of influenza vaccinations were determined using a validated immunization registry. VE was calculated as 100 × (1 – adjusted odds ratio) in logistic regression models that adjusted for age (years), high-risk medical conditions (with 4 dichotomous variables: cardiovascular, diabetes, pulmonary, other), interval (days) from onset to sample collection, season, gender, and MAARI during the prior influenza season. We modeled exposure variables for vaccination in the current season, vaccination 1 season prior, and an interaction term. VE was estimated for all combinations (4 categories) of vaccine exposure in the current and prior season; individuals who were unvaccinated in both seasons served as the referent group. Separate analyses were performed for H3N2 and B. H3N2 analyses excluded the 2008-09 season when no cases of H3N2 were identified. Results: There were 7315 analyzable enrollments for persons aged ≥ 9 years during the 8 seasons; 1053 (14%) had influenza A (H3N2), 647 (9%) had influenza B, 3 (< 1%) had both H3N2 and B infection, and 5612 were negative. The mean (±SD) age was 42.0 (±21.3) years, 31.2 (±19.9) years, and 40.6 (±21.6) years, respectively for H3N2 cases, B cases, and influenza negative controls. Overall, 35% received both current and prior season influenza vaccine; 10% were vaccinated in the current season only; 9% were vaccinated 1 season prior; and 46% were not vaccinated in either current or prior seasons. The interaction term for current and prior season vaccination status was significant in separate models for H3N2 (P = .05) and for B (P < .0001). For H3N2, the VE for current year vaccination was -7% (95% CI: -45, 21) among individuals who had received influenza vaccine in the prior season and 28% (95% CI: 6, 46) among those who had not. For type B, the VE for current year vaccination was -33% (95% CI: -107, 15) among individuals who had received influenza vaccine in the prior season and 66% (95% CI: 51, 77) among those who had not. Similar differences were observed when the models excluded individuals ≥ 50 years old who were more likely to be vaccinated repeatedly. When VE against H3N2 was estimated for each of the 4 categories of vaccination exposure, the level of protection was similar (VE point estimates range = 30% to 34%) for those vaccinated in the current season only, prior season only, or in both seasons (compared to those not vaccinated in either season). VE against influenza B was 52% to 66% for individuals vaccinated in one or both seasons. The 4 category vaccine exposure VE estimates were similar when individuals ≥ 50 years old were excluded. Conclusions: Current season vaccination and prior season vaccination provided similar but low levels of protection against medically attended H3N2 infection in a community cohort over multiple seasons. The same effect of prior season vaccination was observed for protection against influenza B, but VE estimates were higher. These effects are consistent with residual protection from prior season vaccination and/or

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vaccine interference. Further studies should examine the immunologic effects of repeated annual influenza vaccination for a variety of populations, influenza viruses, and seasons.

P2-516

Vaccine effectiveness did not vary by influenza B lineage in the 2012-13 season: preliminary results from the US Flu VE Network

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Background: Two different influenza B lineages have circulated in humans since the 1980s. During the 2012-13 season, both B lineages circulated widely in the United States but only the B/Yamagata lineage was included in the licensed trivalent vaccine. We report preliminary results from the US Flu VE Network on vaccine effectiveness for preventing medically attended influenza B caused by vaccine lineage (Yamagata) and alternate lineage (Victoria) strains during the 2012-13 season. Methods: We enrolled adults and children seeking outpatient care for acute respiratory illness at participating health care facilities in Michigan, Pennsylvania, Texas, Washington, and Wisconsin. Adults and children ≥ 6 months old were eligible if they had an acute cough illness ≤ 7 days duration. After consent, participants completed an enrollment interview and a combined nasal and throat swab was obtained (nasal swab only for children < 2 years old). Samples were tested for influenza, including subtype and B lineage, using real-time reverse transcription polymerase chain reaction (RT-PCR) with CDC primers and probes. Vaccination status was determined from electronic registries or medical records at 4 sites, and by self-report at 1 site. Participants ≥ 9 years old were considered vaccinated if they received at least 1 dose of any seasonal influenza vaccine ≥ 14 days prior to illness onset; younger participants were considered fully vaccinated if they had received both recommended doses. Vaccine effectiveness was calculated for each B lineage as 100 × (1 – adjusted odds ratio) in a logistic regression model that included age group, study site, race/ethnicity, self-rated health status, and interval (days) from onset to swab collection. Test-negative controls served as the comparison group, and influenza A cases were excluded from the analysis. Results: The preliminary analysis included 283 cases of B/Victoria, 538 cases of B/Yamagata, and 3801 test-negative controls. The median age (IQR) in each group was 12 years (6 to 35), 14 years (7 to 44), and 31 years (11 to 51), respectively. The median interval from illness onset to swab was 3 days for each group. Overall adjusted VE against influenza B (any lineage) was 63% (95% CI, 55 to 69). VE against the vaccine lineage (B/Yamagata) was 64% (95% CI, 56 to 71). VE against the alternate lineage (B/Victoria) was 60% (95% CI, 47 to 70). Among adults 18-49 years old, VE was 52% (95% CI, 26 to 69) and 58% (95% CI, 26 to 77), respectively, for the vaccine lineage and alternate lineage. Similarly, VE among adults 50-64 years old was 78% (95% CI, 61 to 88) and 79% (95% CI, 39 to 93) for vaccine and alternate lineage illness. VE in adults ≥ 65 years old was higher for the alternate lineage (73%, 95% CI, 23 to 91) compared with the vaccine lineage (39%, 95% CI, -53 to 75), but confidence intervals overlapped. In children < 9 years old (fully immunized), VE was 69% (95% CI, 51 to 80) and 51% (95% CI, 19 to 70), respectively, for vaccine and alternate lineage illness. In children and adolescents 9-17 years old, VE was 56% (95% CI, 30 to 73) for vaccine lineage and 38% (95% CI, -17 to 67) for alternate lineage. Conclusions: The 2012-13 trivalent vaccine provided moderate and similar levels of protection against medically attended illness caused by the vaccine lineage (B/Victoria) and the alternate lineage (B/Yamagata). Further research is needed to confirm these findings and assess potential mechanisms for cross-lineage protection.

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P2-517

Thai physician perspectives on a seasonal influenza immunization program for pregnant women

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Background: Pregnant women are at increased risk for influenza-related morbidity and death. In Thailand, seasonal influenza vaccination has been recommended for pregnant women since 2009, but acceptance of influenza vaccination among this group remains suboptimal. Information from other countries indicates that physician recommendation for influenza vaccination increases influenza vaccine acceptance and uptake among pregnant women. Therefore, we conducted a nation-wide cross-sectional survey of Thai physicians who provide antenatal care to determine their knowledge, attitudes, and practices (KAP) related to influenza vaccination of pregnant women. Material and Methods: During January-April 2013 (prior to the 2013 vaccination campaign), a self-administered questionnaire on KAP related to influenza vaccination for pregnant women was distributed via postal services to all public and private hospitals with an antenatal care clinic (ANC) in Thailand. At each hospital, 1 physician responsible for the ANC was selected to complete the survey with priority given to selection of obstetricians/gynecologists before selection of general practitioners. We used logistic regression to compare factors between physicians offering and not offering influenza vaccine to pregnant women. Results: Of 1134 hospitals invited to participate in the survey, between January and April 2013, 595 (53%) submitted surveys with complete responses [481 of 889 (54%) public hospitals and 114 of 245 (47%) private hospitals]. Of the 595 respondents, 327 (55%) were male, 294 (49%) were obstetricians/gynecologists, and 254 (43%) were general practitioners. The mean age was 38 years (range, 23-77 years), and the average time in practice was 13 years (range, 1-49 years). The majority of respondents believed that influenza causes a substantial disease burden (70%) and was associated with increased risk of severe complications in pregnant women compared with the general public (85%). The majority of respondent also believed that influenza vaccine was safe for pregnant women (69%) and the fetus/baby (65%), was an effective influenza prevention measure for pregnant women (61%), and should be given to pregnant women in Thailand (74%). However, only 24% of respondents had ever offered influenza vaccines to pregnant women. Among the physicians who did not offer influenza vaccine, the most commonly cited barriers were lack of the vaccine at their hospitals or clinics (51%), lack of insurance coverage for influenza vaccination (49%), refusal of vaccination by pregnant women (45%), and being unaware of the Ministry of Public Health (MOPH)'s recommendation for influenza vaccination (44%). Furthermore, 62% of participants thought current influenza vaccination recommendations by MOPH were unclear or confusing. In bivariate analysis, factors positively associated with offering influenza vaccine to pregnant women included positive perceptions of the vaccine on its safety for the fetus/baby (OR 4.8, 95% CI 1.1-20.6), its effectiveness in preventing disease in pregnant women (OR 3.6, 95% CI 1.5-8.4), its effectiveness to prevent disease in infants (OR 3.2, 95% CI 1.4-7.0), its ability to reduce maternal mortality (OR 2.3, 95% CI 1.2-4.8), specialty of physicians (obstetricians/gynecologists, OR 2.2, 95% CI 1.5-3.3) and clinician gender (male, OR 1.5, 95% CI 1.0-2.2). Only specialty of the physicians (obstetricians/gynecologist) remained significantly associated with offering vaccine in the multivariate analysis after controlling for those factors significantly associated in the bivariate analysis (OR 2.4, 95% CI 1.2-4.9). Conclusion: To improve influenza vaccination coverage among pregnant women, strategies are needed to increase vaccine availability and free vaccine services in ANCs in Thailand. In addition, efforts to increase healthcare provider awareness of Ministry of Public Health recommendations for influenza vaccination are warranted and additional targeted outreach to physicians providing care to pregnant women should be considered.

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P2-518 G FELLOWSHIP GATES Fellowship

The genetic match between vaccine strains and circulating seasonal influenza A viruses in Vietnam, 2001-2009

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Background: Vietnam is currently developing domestic capability to manufacture influenza vaccines but information on the genetic and antigenic characteristics of locally circulating seasonal influenza viruses is limited. In order to assess the relevance of WHO recommended vaccine strains to the situation in Vietnam, we analyzed the genetic relatedness of the hemagglutinin (HA) gene of seasonal influenza A viruses circulating in Vietnam from 2001-2009 to WHO recommended vaccine strains over the same period. Methods and Principal Findings: We sequenced the HA gene of 32 H1N1 and 44 H3N2 seasonal influenza A isolates from laboratory-based sentinel surveillance sites in Hanoi from 2001-2005, and from a national influenza surveillance system from 2005-2009. H1 and H3 HA phylogenetic trees rooted to vaccine strains A/Beijing/295/1995 (H1N1) and A/Moscow/10/1999 (H3N2), respectively, were constructed with contemporary HA sequences of isolates from neighboring countries. We found some genetic differences between seasonal influenza H3N2 viruses and 3 WHO influenza vaccine strains recommended for use in the northern and southern hemispheres for the 2001 to 2004 and 2007-2008 seasons, and close genetic identity of circulating H3N2 strains with the recommended WHO southern hemisphere vaccine strains for 2004 and 2009 seasons. The genetic similarity of circulating H1N1 strains with the WHO recommended vaccine strains are described for the study period, 2001-2009. Conclusions: The HA gene of seasonal influenza virus strains in Vietnam (especially influenza A/H3N2) showed varying degrees of genetic identity compared with those of the northern or southern hemisphere vaccine strains recommended by WHO. The close relatedness of the HA of Vietnamese strains and contemporary strains from nearby countries indicate a good genetic match of circulating strains during study period. Greater representation of virus isolates from South East Asia in the vaccine strain selection process is desirable of influenza vaccine development in Vietnam.

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P2-519

Evaluation of heterosubtypic cross-protection by active infection and TIV-vaccination of seasonal influenza virus in ferret models

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Background: Since 1997, highly pathogenic avian influenza (HPAI) H5N1 viruses circulated among domestic poultry and wild birds in Asia, Africa, and Europe and believed to have undergone genetic evolution. Moreover, World Health Organization (WHO) reported a mortality rate of 59% out of the 630 human cases of HPAI H5N1 infections worldwide from 2003 to June 2013. There is, therefore, a growing concern that HPAI H5N1 viruses might cause the next pandemic. Here, we investigated the long term heterosubtypic cross-protection by natural infection, or immunization with trivalent human seasonal inactivated vaccine (TIV) followed by fatal HPAI A/VN/1204/04 (H5N1) virus challenge in ferret models. Materials and Methods: Groups (n = 6) of ferrets were inoculated intranasally with human seasonal A/H1N1 (A/California/07/2009, group A) or A/H3N2 (A/Perth/16/2009, group B) virus for imitating natural infection, and additional ferrets were immunized with seasonal TIV (group C)

three times with 2-week interval. Three months after infection or last immunization, antibody responses were measured by hemagglutination inhibition (HI) and virus neutralization (VN) assays. After serological assays, group A, B, C and nontreated ferrets (group D) were challenged with lethal dose of HPAI A/Vietnam/1204/04(H5N1) virus, and evaluated the viral shedding from nasal washes, tissue samples and their mortality. Result: Serological assays revealed that the sera from groups A, B, and C could elicit moderate titers against homologous viruses at 4 weeks after natural infection or TIV immunization; cross reactivity against the HPAI H5N1 virus was not detected. However, these serologic titers appeared to have receded after 12 weeks posttreatments (infection or immunization). Following viral challenge with HPAI H5N1 virus, the ferrets from group A and B survived but those from group C and D all died. Group A and B exhibited significantly attenuated viral loads in nasal washes, lung, brain, and spleen and showed recovery within 9 days postinfection. Notably, group A showed the most reduction of HPAI H5N1 virus replication and attenuated clinical signs. In contrast, the ferrets in groups C and D demonstrated high fever with substantial weight loss accompanied by high replication of the challenge virus at the upper respiratory tract and tissues samples. Therefore immunization with the human seasonal TIV could not protect ferrets from fatal HPAI H5N1 infection. Conclusion: Taken together, our findings revealed that natural infection with human seasonal strains are more effective than TIV immunization against fatal HPAI H5N1 infection. Data also suggest that natural virus exposure might provide a long-term protective impact against the A/Vietnam/1204/04(H5N1) challenge virus. Thus, the inactivated human seasonal influenza vaccine might not protect the vaccinated host, while healthy individuals who were previously infected with seasonal influenza A viruses might be more protected from the lethal HPAI H5N1 virus infection.

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Protection of cynomolgus monkeys from H5N1 HPAI virus challenge by recombinant influenza vaccine based on vaccinia virus vector

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Background: H5N1 highly pathogenic avian influenza (HPAI) viruses have caused epidemics in bird populations, and human infections transmitted from infected birds have continuously been reported in several countries. H5N1 HPAI viruses have to be considered a potentially serious pandemic threat. Therefore, the development of safe and effective vaccines is urgently required. Vaccinia virus LC16m8 strain is one of the most attenuated strains of vaccinia virus and has been authorized as a smallpox vaccine in 1975 in Japan, as its safety and long-term immunity have been proven. In this study, we investigated efficacy of recombinant LC16m8 expressing HPAI virus hemagglutinin against challenge with H5N1 HPAI virus in cynomolgus monkeys. Materials and Methods: We generated recombinant vaccinia virus LC16m8 strain which expresses hemagglutinin (HA) protein of H5N1 HPAI virus by homologous recombination (rVV-H5 Flu; clade 2.2 and clade 2.3.4). Female cynomolgus monkeys were injected intradermally with the rVV-H5 Flu in their upper arms using a bifurcated needle under anesthesia. The monkeys immunized with recombinant LC16 m8 which contains only promoter sequence (rVV-Empty) was used as a control group. The monkeys were intranasally infected with the H5N1 HPAI virus [A/whooper swan/Hokkaido/1/2008 (H5N1), clade 2.3.2.1] 1 month or 1 year after the vaccination. Results: We measured anti-H5N1virus antibody in serum of the monkeys by ELISA. In the rVV-H5 Flu vaccinated group, H5N1 virus antigen-specific IgG was detected from 2 weeks after the vaccination and gradually increased until 4months later. The high level of anti-H5N1 virus IgG was kept even 1 year later. We carried out the intranasal infection of the monkeys with H5N1 HPAI virus 1 month after the vaccination. The vaccinated group recovered earlier from influenza symptoms, including high fever than the control group. Infectious H5N1 virus in swab samples from nose, mouth, trachea, and bronchus was also rapidly eliminated in the vaccinated group. At 7 days after the infection, we carried out the autopsy. No infectious H5N1 virus was detected in all lobes of the lung of vaccinated monkeys, while H5N1 virus was detected in several lobes of the lung of control monkeys. The vaccinated group prevented the development of severe

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pneumonia. Furthermore, other groups of the vaccinated monkeys were intranasally infected with the H5N1 HPAI virus (clade 2.3.2.1) 1 year later. Both rVV-H5 Flu (clade 2.2)- and rVV-H5 Flu (clade 2.3.4)-vaccinated groups rapidly eliminated infectious H5N1 HPAI virus in the swab samples and alleviated the influenza symptoms such as high fever and severe pneumonia. Conclusions: Single immunization with rVV-H5 Flu vaccine protected cynomolgus monkeys from the challenge with H5N1 HPAI virus. These results suggest that vaccinia virus LC16m8-based attenuated recombinant influenza vaccine (rVV-H5 Flu) is one of promising H5N1 vaccine candidates.

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The mutation in PA determines the enhanced growth properties of candidate vaccine viruses

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Background: Since the first outbreak in humans in 1997, highly pathogenic avian influenza (HPAI) H5N1 viruses have continuously threatened human population worldwide. Due to pandemic potential of H5N1 viruses, candidate vaccine viruses (CVVs) have been developed and stockpiled for urgent use. However, most CVVs meet the challenge of low yields. Hence, we studied high-yielding molecular determinants of H5N1 CVVs. Materials and Methods: H5N1 CVVs were generated by plasmid-based reverse genetics using HA and NA genes of the A/Chicken/Korea/IS/2006 (HPAI H5N1, clade 2.2.1) virus and 6 internal genes of the A/Puerto Rico/8/34 (PR8) human vaccine donor. These CVVs were then serially passaged 15 times in fertile chicken eggs, and their plaque phenotypes and growth properties were investigated in eggs and cultured cells. In the subsequent sequence analysis, we identified several amino acid mutations. After introduced in new CVVs, these mutations were further analyzed for their impacts in enhanced viral growths. Results: After serial adaptations in eggs, we found that one CVV obtained an increased plaque phenotype. This CVV also exhibited enhanced growths in both eggs and tested cells. Sequence analysis revealed one amino acid mutation in PA gene. In a subsequent luciferase mini-replicon assay, we found that the PA mutation resulted in elevated polymerase complex activity. Further analysis also confirmed growth enhancements of the PA mutated CVV in both eggs and cells. Conclusions: Considered all, we suggest that this PA mutation is a high yielding genetic determinant for both classical egg-grown and cell-based influenza A vaccine backbone.

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Vaccine effectiveness against laboratory-confirmed influenza in those hospitalised with severe acute respiratory illnesses in New Zealand: 2012 to 2013

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Background: The Southern Hemisphere Influenza and Vaccine Effectiveness Research and Surveillance (SHIVERS) Project commenced in 2012 and is a 5-year multi-centre research collaboration between the Institute of Environmental Science and Research, Auckland District Health

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Board (ADHB), Counties Manukau District Health Board (CMDHB), University of Otago, University of Auckland, WHO Collaborating Centre at St. Jude Children's Hospital and the US Centers for Disease Control and Prevention. One aim of SHIVERS is to estimate the annual vaccine effectiveness (VE) of seasonal trivalent inactivated influenza vaccine (TIV) against hospitalised laboratory-confirmed influenza in the Auckland population admitted with a febrile respiratory illness to the four major public hospitals in Central and South Auckland. These data, from this southern temperate country, will allow VE assessment of vaccines against circulating influenza viruses at a time when influenza activity is low in northern temperate countries. Materials and Methods: Since May 2012, enhanced, active, year-round, population-based surveillance has been established for all cases of severe acute respiratory infections (SARIs) admitted to hospitals in the central, south and west of the Auckland region (population: 838,000) covering the ADHB and the CMDHB. A case test-negative study was undertaken during the period 1 May 2012 to 14 February 2013 (ended prior to the start of influenza vaccination for the 2013 season). A nasopharyngeal swab sample was collected from all persons who met the SARI case definition (acute respiratory illness with fever and cough and onset within 7 days of hospitalisation) and was tested by real-time reverse transcription polymerase chain reaction for influenza viruses. Cases were defined as persons with SARI who tested positive and controls as persons with SARI who tested negative for influenza. Basic data on demographics, medical history, a frailty measurement based on long-term oxygen use, time of onset and sampling were gathered from a case report form and hospital electronic records. Influenza vaccination history was assessed by patient recall and health provider records. VE was estimated by comparing vaccination coverage between cases and controls in adjusted logistic regression models. We used propensity scores based on a range of covariates (history of chronic disease, obesity, smoking status, frailty, pregnancy) that could be associated with vaccination to match cases and controls. Results: Of 5501 patients screened, a total of 1299 SARI patients were included in this analysis, of whom 290 (29%) were influenza-positive. Of the 1009 influenza-negative cases, 416 (41%) were vaccinated; of the 290 influenza-positive cases, 104 (36%) were vaccinated. The cases were more likely than the controls to be younger (P = .03), of Pacific ethnicity (P = .04) and less frail (P = .02). The vaccine formulation used in New Zealand in 2012 included a virus that matched the circulating 2009 H1N1 virus, but was less well matched for influenza B and the H3N2 subtype. Propensity-adjusted VE for hospitalisation for laboratory-confirmed influenza was 27% (95% CI 2% to 45%); VE was 27% (0% to 47%) for influenza A, 29% (–22% to 58%) for influenza B, 50% (16% to 71%) for H1N1 and 6% (–46% to 40%) for H3N2. Conclusions: Overall VE against influenza-associated SARI was low but statistically significant and comparable with other international studies. VE against matched pandemic H1N1 strains was moderately high but was not significant against less well matched Influenza B and H3N2. These are the first VE data from SHIVERS. As part of the broader project, community-based surveillance will commence starting in 2013, enabling measurements of VE for the prevention of influenza illness in primary care settings.

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Mismatch between vaccine strains and circulating influenza B viruses in different regions of Brazil: 2001-2013

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Background: Vaccination against influenza plays a pivotal role in averting severe disease and reducing morbidity, mortality, and the socioeconomic costs associated with primary and secondary

infections. Since the vaccine comprises only one influenza B lineage, a successful intervention relies on agreement between circulating viruses and vaccine-selected strains. We investigated influenza B viruses from different Brazilian regions, and their match with vaccine-strains, during the period 2000 - 2013. Materials and Methods: This study included 202 viral sequences, distributed as follows: north (n = 7), northeast (n = 26), southeast (n = 81), and south (n = 88). South (Rio Grande do Sul, Parana, Santa Catarina), southeast (Minas Gerais, Espirito Santo, Rio de Janeiro), and northeast (Bahia, Alagoas, Sergipe) samples were sequenced at the WHO/National Influenza Center, Rio de Janeiro, as described elsewhere (Motta FC, et al. *J Clin Virol* 2006;36:208). Samples from other states were downloaded from the GISAID database. Nucleotide alignment was carried out using Muscle, and ML trees were reconstructed using HKY nucleotide substitution model (gamma distribution, n = 5), with 1000 bootstrap replicates (Mega 5.2). Results: Victoria and Yamagata strains have co-circulated in Brazil since 2005. In previous years, only one strain was identified. Key mismatches between the Southern Hemisphere vaccine and the most prevalent circulating viruses were identified in 2005 (n = 4/6), 2008 (n = 32/49), 2010 (n = 23/32), and 2013 (n = 20/23). In this context, Brazilian regions were distinctly affected. In 2008, most circulating viruses in the South of Brazil were identified as Victoria-like (n = 23/28), in contrast with the northeast (n = 3/6) and the southeast (n = 6/15) regions. In 2010, whereas northeast viruses were essentially Victoria-like (n = 4/5), in accordance with B/Brisbane/60/2008, Yamagata-like viruses were more frequently found in the southeast (n = 11/16) and south (n = 11/11). In 2012, Yamagata-like viruses were only identified in the northeast (n = 4/9). Finally, Victoria-like (n = 20/23) viruses have been predominant in 2013, in disagreement with the recommended strain—B/Wisconsin/1/2010. Conclusions: Our findings corroborate the international literature on the poor concordance between vaccine and circulating influenza B strains, in specific influenza seasons. Inclusion of both influenza B lineages in vaccine composition would avoid challenging predictions and their respective public health consequences. Funding: Carlos Chagas Filho Foundation for Research endowment for Rio de Janeiro, process no. 111.802/2012; Science and Technology Department, Ministry of Health and National Counsel of Technological and Scientific Development, Brazil.

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Influenza antigenic variant detection using quantitative PCR based approximate ligation assays

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Background: Identification of influenza antigenic variants is the key to a successful influenza vaccination program for both pandemic preparedness and seasonal influenza prevention and control. Immunological tests, such as hemagglutinin inhibition (HI) assay and microneutralization (MN) assay, are usually utilized to identify antigenic variants among the circulating strains. These empirical methods to determine influenza virus antigenic properties are time-consuming, mid-throughput, and require live viruses. Materials and Methods: A novel quantitative PCR-based antigenic characterization method based on proximity ligation assays was developed and validated in this study. Results: This method is able to detect an influenza virus with less than 100 TCID50, using homologous polyclonal sera. It can not only differentiate different subtypes of influenza A viruses but also effectively identified antigenic drift events of H3N2 seasonal influenza viruses, and the results are consistent with those from the routine serological assays, including both HI and MN assays, used in influenza surveillance. This method effectively quantified antigenic distances among the wild type strains, A/Johannesburge/33/94, A/Nanchang/933/94, and A/Sydney/05/97, and their corresponding mutants with single, double, or triple mutations, which caused antigenic changes among these viruses, as confirmed by both HI and MN assays. This novel method was applied in 100 clinical samples from 2012-2013 influenza season, which were confirmed to be H3N2 influenza positive. Both the antisera against A/Victoria/361/2011(H3N2) and the antisera against A/Perth/16/2009(H3N2) showed significant cross activities with 97 of 100 samples, whereas the antisera against A/Brisbane/10/2007(H3N2) only with 43 of these 100 samples. The mean Ct values were 6.71

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(standard deviation, 2.15), 7.66 (3.66), and 3.84 (4.73), respectively, and these titers among 3 sera groups were significantly different through pairwise paired t-test (P < .001). Conclusions: The quantitative PCR based approximate ligation assays developed in this study is sensitive and robust in antigenic variant detection, has the potential to be applied in a large scale platform in influenza surveillance using directly clinical samples.

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Using vaccinated cases only to evaluate influenza vaccine effectiveness against death following admission to the hospital in the tropics

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Background: Influenza infection is known to increase the risk of mortality, particularly in the elderly. This burden in tropical countries has been shown using modeling studies to be substantial and similar to that experienced in temperate regions. In contrast with temperate climates, influenza occurs all year round in tropical regions, with two major peaks observed in April to July and November to January corresponding with the winter epidemics occurring in the Northern and Southern Hemisphere, respectively. There remains a lack of population-level studies to evaluate the impact of influenza vaccination on mortality in tropical regions. We describe a novel cohort study method using only vaccinated individuals to verify the effectiveness of influenza vaccine on preventing death following emergency admission to a large acute hospital in tropical Singapore. Materials and Methods: Since the 2009 H1N1 pandemic, the influenza vaccine strain has remained the same until the 2012 season in the Northern Hemisphere. Therefore, we extracted all emergency admission episodes from 1 January 2010 to 31 August 2012, which is defined as the observation period, to Tan Tock Seng Hospital (TTSH)—an acute hospital of the National Healthcare Group (NHG) serving the central region of Singapore. Influenza vaccination records from the same period were also extracted from the medical database of NHG, which includes other hospitals and primary care clinics. Using Singapore's unique identity card number, we populated the influenza vaccination records into the admission database. Only cases with a positive vaccination history were used for subsequent analysis. We then included information on whether a patient died following admission to the hospital using data from hospital records. We defined influenza vaccine protection duration as 15 days after the date of influenza vaccination to 365 days after the date of influenza vaccination, ie, approximately just less than 12 months. A multivariable logistic model was developed to assess the odds of death following admission to the hospital within the protective period compared with that outside the protective period. Results: From 1 January 2010 to 31 August 2012, there were 136,338 emergency admissions from 84,404 individuals to TTSH; 5636 (7%) had a history of having received influenza vaccine, and there were 564 deaths in this cohort following admission. The median ages of the patients who died within the protective period and outside the protective period were 70 and 71 years, respectively, with an interquartile range of 60 to 80 years. Univariate analysis showed statistically significant differences in sex and ethnicity ($P < .01$). A total of 237 patients (4.3%) admitted died during the protective period compared with 332 (14.6%) patients outside the protection period ($P < .01$). After adjustment for possible confounders of age, sex, ethnicity, as well as month and year of admission, the odds of death following hospital admission during the protective period was 0.25 (95% CI, 0.20 to 0.30) compared with that outside the protective period. Conclusions: We have described and demonstrated a novel cohort study method using vaccinated cases only to assess the effectiveness of influenza vaccine in preventing death following hospital admission. Influenza vaccine was found to be about 75% effective at preventing death if admission took place within the defined protective period when compared with those admitted more than 12 months after receipt of their influenza vaccination. Using only vaccinated cases negates the problems of healthy user bias commonly associated with other observational methods used to measure vaccine effectiveness. Increasing influenza vaccination coverage in tropical regions could provide substantial clinical benefits in terms of preventing mortality following hospitalization.

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A novel approach using vaccinated cases only to evaluate influenza vaccine effectiveness against all-cause hospital admissions in the tropics

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Background: Despite misconceptions, the burden of influenza in tropical regions is substantial and not dissimilar to that experienced in temperate regions. In contrast with temperate climates, influenza occurs all year round in tropical regions, with two major peaks observed in April to July and November to January corresponding with the winter epidemics occurring in the Northern and Southern Hemispheres, respectively. Public health advocacy for influenza vaccination in the tropics is severely limited by a lack of evidence from real-life population-level influenza vaccine-effectiveness studies. This paucity in tropical regions is primarily due to the combined lack of public health surveillance systems with the capacity to monitor vaccine impact as well as low uptake of influenza vaccine, although it is recommended in national guidelines. We describe a novel method using the self-controlled case series (SCCS) normally used to verify adverse events following immunization to evaluate the effectiveness of influenza vaccine on all-cause hospital emergency admissions in an acute hospital in Singapore, a tropical city state. Materials and Methods: Since the 2009 H1N1 pandemic, the influenza vaccine strain has remained the same until the 2012 season in the Northern Hemisphere. Therefore, we extracted all emergency admission episodes from 1 January 2010 to 31 August 2012, which is defined as the observation period, to Tan Tock Seng Hospital (TTSH)—an acute hospital of the National Healthcare Group (NHG) serving the central region of Singapore. Influenza vaccination records from the same period were also extracted using the medical database of the NHG, which includes other hospitals and polyclinics. Using Singapore's unique identity card number, we populated the influenza vaccination records into the admission database. Only cases with one positive vaccination history were used for subsequent analysis. The observation period was divided into three periods: pre-protection period, defined as the period from 1 January 2010 to 15 days after influenza vaccination; protection period, defined as 15 days after the date of influenza vaccination to 365 days after the date of influenza vaccination; post-protection period, defined as 365 days after influenza vaccination to the end of the observation period (31 August 2012). A subject-specific Poisson regression model with the log link adjusted for month and year was applied to ascertain the incidence risk ratio (IRR) of emergency admission during the protection period compared with the baseline (pre-protection and post-protection). Results: From 1 January 2010 to 31 August 2012, there were 136,338 emergency admissions from 84,404 individuals to TTSH. Of these, 4313 had a history of having received one influenza vaccine only. The mean age of the cohort was 65 years, and there were slightly more males (60%). After adjustment for month and year, the IRR during the protection period was 0.89 (95% CI, 0.85 to 0.93) compared with the baseline risk of hospital admission. Conclusions: We have described and demonstrated a novel approach using the SCCS to estimate vaccine effectiveness using only vaccinated cases, which negates the problems of healthy user bias commonly associated with other observational methods to measure vaccine effectiveness. The SCCS also adjusts for known and unknown fixed confounders, such as sex and genetics. The year-round circulation of influenza and influenza vaccination makes the SCCS uniquely suitable for evaluating the impact of influenza vaccine in tropical regions. Influenza vaccine is 11% effective at protecting against all-cause emergency admissions in tropical Singapore. Increasing influenza vaccination coverage in tropical regions could provide substantial public health benefits in terms of relieving hospital admissions in acute hospitals.

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Influenza vaccine effectiveness, by age group and strain, at preventing laboratory-confirmed influenza admissions in adults 18 years of age or older targeted for influenza vaccination during the 2012-2013 influenza season: a test-negative study

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Background: Influenza epidemics—associated with excess morbidity, hospitalizations and deaths—occur annually. Influenza vaccination is considered the most effective strategy for preventing influenza and is recommended for individuals at high risk of serious influenza-related complications. As a consequence of antigenic drift, influenza vaccine effectiveness varies season by season. Objective reappraisal of this evidence is possible by using sensitive and specific real-time reverse transcription–polymerase chain reaction (RT-PCR) and the execution of test-negative studies, their major strengths being comparability of cases and controls and laboratory-confirmed outcomes. In addition, RT-PCR allows definition of a more timely and focused picture of the burden of disease due to different influenza strains. Based on these developments, our aim was to estimate influenza vaccine effectiveness (IVE) at preventing influenza-confirmed admissions by strain and age group. Materials and Methods: We conducted a prospective, hospital-based, test-negative case-control study to estimate IVE at preventing influenza-confirmed hospitalizations in patients 18 years of age and older targeted for influenza vaccination. The study was performed in five health districts in Valencia, Spain, which provide hospital care to 1,266,899 inhabitants. We invited all emergency hospitalized patients with a predefined set of medical conditions to participate in the study. IVE was estimated for subjects 18 years of age or older pertaining to target groups for vaccination and for the period defined by two or more admissions due to influenza in at least two consecutive weeks. Influenza season began on week one in 2013 and ended on week 15 in 2013. Subjects who tested positive for influenza were considered cases, and those who tested negative for influenza were considered controls. IVE is estimated as (1-0R)*100. We provide overall crude and adjusted estimates by influenza strain and by age group (18 to 69 y and 70 y or older) and by age group and strain. Results: Overall, 1034 admitted patients met all inclusion criteria. We identified 172 admissions with laboratory-confirmed influenza. We observed two influenza waves, the first due to influenza B Yamagata and the second due to influenza A(H1N1)pdm09. The predominant circulating influenza virus strains in Valencia were B Yamagata (n = 108 cases; 63%), H1N1pdm09 (n = 54; 31%), H3H2 (n = 5; 3%), and B Victoria and B nontypable (n = 5; 3%). Influenza B Yamagata was identified in all age groups, whereas A(H1N1)pmd09 was less predominant in the 75- to 84-year age group and nearly absent in the 85 years or older age group. Overall, non-adjusted influenza vaccine effectiveness was 37% (13% to 55%). For all age groups considered together, IVE at preventing admissions related to B Yamagata was 45% (17% to 63%) and related to H1N1pdm09 was 27% (–31% to 59%). After adjustment, only IVE against B Yamagata was significant (45%, 11% to 66%), while IVE against H1N1pdm09-related admissions was −5% (–98% to 44%). For those 18 to 69 years of age, adjusted IVE was 26%. It was 56% against influenza B Yamagata and 16% against H1N1 pdm09. In all estimates in this age group, the lower limit of the confidence intervals was negative. For those 70 years of age or older, adjusted IVE was 38% (2% to 61%). It was 45% (3% to 69%) against B Yamagata, and −25% against A(H1N1)pdm09. Conclusions: Influenza vaccine prevented half the admissions due to influenza B Yamagata and a third of the admissions due to overall influenza. IVE effectiveness against A(H1N1)pdm09 was from low to nonexistent, in agreement with the recently described drift of circulating H1N1pdm09 strains compared with vaccine strains. The overall IVE age

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effect could thus be explained by the predominance of H1N1pdm09 in younger age groups. Our results are limited because of the small numbers. This clearly upholds the convenience of networking and data pooling between sites applying similar protocols. This study was funded by a grant from Sanofi Pasteur. Valencia Hospital Network for the study of Influenza & RVD is a member of the Global Influenza Hospital-based Surveillance Network.

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Variability in the immunogenicity of inactivated seasonal influenza vaccine in children due to age and recent previous influenza vaccination

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Background: Annual receipt of trivalent inactivated influenza (TIV) vaccination is recommended for school-age children in some countries. However, there is little data on the variability of the immunogenicity of influenza vaccination in children and how this is affected by their age and recent influenza vaccination history. Materials and Methods: We used data on children in a Hong Kong community-based study who were randomized to receive TIV before the 2009-2010 influenza season. Antibody titers against seasonal and pandemic A(H1N1), seasonal A(H3N2), and two B influenza viruses (B/Brisbane and B/Florida) were measured by hemagglutination inhibition immediately before and 1 month after vaccination (Cowling et al. *Clin Infect Dis*. 2012). Multivariate regression models were fitted in a Bayesian framework to characterize the distribution of changes in antibody titers following vaccination and update previous findings by considering the correlation between virus strains (Ng et al. *Pediatr Infect Dis J*. 2013). Results: In 452 subjects, statistically significant rises in the geometric means of all antibody titers were observed, with those against the virus strains included in the TIV rising by geometric means of 7.95 to 13.36; those against pandemic A(H1N1) and B/Florida rose by 1.47 and 4.21, respectively. Geometric standard deviations were between 3.76 and 8.41 around the geometric means, with pandemic A(H1N1) showing the least variability in rises. The most closely correlated titer increases were those for the two influenza B viruses, while increases in pandemic A(H1N1) titers were unrelated to any other titer. Being vaccinated in either of the two previous years significantly reduced the increase in seasonal A(H1N1) and A(H3N2) antibody titers, while among children not vaccinated in the previous 2 years, those aged > 9 years experienced significantly higher increases in the influenza B titers than those aged 6-8 years. Conclusions: Increases in antibody titers following vaccination can vary depending on age and vaccination history. Results from our study suggest that humoral antibody response to TIV may be lower in children receiving repeated vaccination, but receipt of TIV induced seroprotection in most subjects.

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Supplementation of oil-based inactivated H9N2 vaccine with M2e antigen enhances resistance against heterologous H9N2 avian influenza virus infection

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Background: Avian influenza virus (AIV) subtype H9N2 has become established in multiple avian species across large geographical areas, including Asia, the Middle East, and Africa, causing serious economic losses in the poultry industry. To control H9N2 infections in poultry, an oil-based inactivated H9N2 vaccine was used in the chicken industry to prevent H9N2 infection in several Asian countries.

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Previous studies have demonstrated that H9N2 viruses underwent antigenic drift and thus could evade vaccine protection. Therefore, development of broadly cross-protective vaccine is required to improve vaccine efficacy against newly emerged viruses. Recent studies have shown that supplementation with M2e antigen potently provides cross-protective immunity against heterologous influenza virus infection. In this study, we investigated the efficacy of an inactivated H9N2 oil-emulsion vaccine mixed with M2e proteins expressed in *Escherichia coli* against a vaccine escape mutant H9N2 virus. Materials and Methods: AIV H9N2 M2e antigen was expressed in *E. coli* and was used as a supplement to inactivated H9N2 oil-emulsion vaccine. Specific pathogen-free chickens received a single injection of inactivated H9N2 oil-emulsion vaccines with (iH9-M2e) or without (iH9) M2e supplementation. At 3 weeks post-vaccination, hemagglutination inhibition (HI) tests and enzyme-linked immunosorbent assays were performed to determine serological immune responses. A challenge study using a vaccine escape H9N2 variant was performed to evaluate the efficacy of M2e supplementation. Results: Significantly increased levels of HI titers were induced both in iH9- and in iH9-M2e–vaccinated chickens. The difference in the HI titers between these groups was not statistically significant. Even after a single immunization, the M2e-specific antibody titers in the iH9-M2e–vaccinated group were approximately 100-fold higher than those in the iH9-vaccinated group, which indicates that the recombinant M2e antigen used in this study significantly enhanced M2e-specific antibodies without interfering with the induction of HI antibodies when added to oil-based inactivated H9N2 vaccine. M2e-supplemented vaccination significantly reduced challenge virus shedding in the oropharynx, both at 3 and 5 day post-challenge, while no significant reduction in challenge virus shedding was observed in the non-supplemented vaccination group. Conclusions: Our results suggest that supplementation of inactivated H9N2 vaccine with prokaryote-expressed M2e antigen improved inactivated H9N2 virus vaccine efficacy and achieved cross-protection against heterologous antigenic variant. Supplementation with M2e protein could be an attractive vaccine strategy to better control AIVs in poultry species.

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Influenza-specific cross-reactive CD8+ T-cell responses induced in HLA-A2.1/H-2D^{d} transgenic mice by an H7N3-inactivated virus

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Background: The ideal influenza vaccine should induce broad cross-reactive B- and T-cell responses and thus provide protection against heterologous viruses. Several studies have demonstrated that the majority of virus-specific memory T cells, particularly CD8+ T lymphocytes, are directed against relatively conserved viral proteins and that the magnitude and breadth of virus-specific T cells contribute to heterosubtypic immunity. Therefore, efforts to ameliorate the induction and recall of virus-specific T cells by influenza vaccination would help limit viral spread and severity of disease. Vaccination using whole inactivated influenza virus remains the primary method of prevention, especially when considering pre-pandemic vaccines. In particular, vaccine preparations based on reassortant viruses containing the six internal gene segments of A/PR/8/34 virus and the hemagglutinin (HA) and neuraminidase genes from potentially pandemic virus strains could provide both humoral and cellular immune responses. Here, we used HLA-A2.1/H-2D^{d} transgenic mice to examine the CD8+ T-cell responses elicited by a non-replicating H7N3 virus and the cross-reactivity with A/PR/8/34 virus. Materials and Methods: HLA-A2.1/H-2D^{d} mice were immunized intraperitoneally with 500 HAU of either live or heat-inactivated A/Turkey/Italy/214845/02 (HI-H7N3) virus. Viral inactivation was performed at 56°C for 30 minutes. To evaluate recall responses, anesthetized mice were infected intranasally with sublethal doses of the A/PR/8/34 virus. Fourteen peptides that bind HLA-A2.1 molecule and are conserved among diverse influenza subtypes were used in an IFN-γ ELISPOT assay to investigate the CD8+ T-cell response in the spleen of mice. The D^{b}-restricted peptide epitope NP$_{366}$ from influenza nucleoprotein (NP) was included in the assays. Results: We selected a panel of the immunogenic and HLA-A2–restricted influenza-specific peptides, consisting of 2 NP epitopes, 10 polymerases epitopes (4 PA, 4 PB1, 2 PB2), and 2 matrix (M1) epitopes. Following

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intraperitoneal injection with live H7N3 virus, most of the peptides tested were able to stimulate IFN-γ secretion from mouse splenocytes. Strong CD8+ T-cell responses to the immunodominant $M1_{58}$ (HLA-A2-restricted) and NP_{366} (D^b-restricted) epitopes were observed in all mice. In contrast with live viral infection, HI-H7N3 administration elicited CD8+ T-cell responses that were restricted to epitopes $M1_{58}$ and NP_{366}. Reproducible T-cell responses were also observed after stimulation with the overlapping peptide $M1_{59}$. Then, we sought to determine whether cross-reactive recall responses to the subdominant epitopes may be affected in HI-H7N3–vaccinated mice following heterologous influenza virus infection. One month after HI-H7N3 vaccination, mice were infected with A/PR/8/34 virus. The frequencies of T cells specific for the immunodominant epitopes were higher than those of unprimed mice. However, CD8+ T-cell responses specific for subdominant epitopes, including the PA_{225} epitope, were similar to those observed in primary infections. This suggests the lack of priming by non-replicating flu vaccines for these antigens. Finally, we investigated whether pre-existing immunity to a heterologous influenza virus affected the T-cell responses elicited by HI-H7N3 virus. H7N3 vaccine administration exclusively boosted the CD8+ T cells specific for immunodominant epitopes. Similar results were obtained in A/PR/8/34-preimmune mice vaccinated with heat-inactivated X31 virus. In both cases, the frequencies of T cells specific for subdominant epitopes were not increased. Conclusions: Our data indicate that inactivated whole-virus vaccines elicit CD8+ T-cell responses exclusively toward dominant epitopes of the most abundant internal proteins in both naive and preimmune mice. In humans, conventional PR8-based inactivated vaccines would be able to recall cross-reactive immune responses to M and NP proteins and thus play an important role in the case of mismatches between strains recommended for pre-pandemic vaccine formulation and field isolates. However, the paucity of conserved immunodominant epitopes and potential emergence of antigenic variants from animal reservoirs suggests that further investigation of the cross-reactivity of M and NP proteins from human and avian viruses in the context of different HLA class I and class II molecules is needed. It would help to identify and develop alternative pre-pandemic vaccines that are able to elicit the appropriate immune response.

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Main factor of the low antigen content of inactivated H5N1 influenza vaccines—decreased stability of the H5 hemagglutinin protein

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Background: Since 1997, highly pathogenic avian influenza viruses (HPAIVs) of the H5N1 subtype have been circulating in wild and domestic birds and periodically infecting humans; thus, they pose a pandemic threat. The major influenza virus surface antigen hemagglutinin (HA) is the target for the virus-specific antibodies and, therefore, it is the main component of anti-influenza vaccines. The conventional inactivated vaccines made from H5N1 HPAIV (eg, NIBRG14 vaccine strain) were shown to reveal decreased HA yields compared with the seasonal preparations. The HA of HPAIV differs from that of human influenza viruses by the increased threshold pH of the HA conformational change (pH of fusion or activation), which is related to decreased HA conformational stability. We showed earlier that decreasing the threshold pH of the HA activation by site-directed mutagenesis leads to increased virus stability toward a low pH and high temperature, which in turn increases virus infectivity for mammals and the immunogenicity of live influenza vaccines. The aim of this study was to investigate whether the increased pH threshold of H5 HA activation is related to the decreased HA antigen content of whole virion inactivated influenza vaccines. Materials and Methods: The single mutation HA_2 (K58I), known to decrease the threshold pH of fusion for the avian HA, was introduced in the HA of HPAI isolate A/chicken/Kurgan/5/05(H5N1), and two 6:2 reassortants differing by a single K58I substitution were constructed by reverse genetics in the A/PR/8/34 background. The pH threshold of virus HA activation was measured in a hemolysis test. Virus stability was estimated on the basis of low pH, high temperature, and freeze-thawing stress. The HA conformational stability was assessed in ELISA by binding efficiency to the IIF4 monoclonal antibody, which is specific to low pH HA conformation and by resistance to trypsin proteolysis in Western blotting. Viruses were propagated in chicken embryos or MDCK cells, inactivated with 0.015% formaldehyde for 24 h at

32°C and purified by ultracentrifugation in 20%-60% sucrose density gradient. The total protein content was measured by Lowry protein assay. The quantity of immunocompetent HA antigen in the whole virus inactivated preparations was determined by SRID assay. Results: The reassortant containing the modified HA was characterized by a 0.3 pH unit lower threshold of the HA activation. The conformational change in the mutant virus HA detected by the binding of IIF4 antibodies occurred at 0.3 pH units lower than that of the original strain. The mutant virus demonstrated increased stability toward a low pH and elevated temperature when compared with the original non-mutated strain. The HA protein of the mutant virus was more resistant to the freeze-thawing stress, being less damaged, while the HA protein of the original strain appeared as rare and shortened spikes when analyzed by electron microscopy. Whole virus inactivated preparation made from the original unstable virus contained more HAs in the low pH conformation, which were more sensitive to digestion by trypsin than an analogous preparation made from a more stable mutant strain. The relative HA content in an inactivated whole virus formulation prepared from the mutated virus was 1.8 times higher than in the preparation made from the original strain. Conclusions: The obtained data demonstrate that decreasing the threshold pH of the HA activation of the H5N1 virus by introducing HA_2(K58I) mutation increased its HA conformational stability, resulting in a higher content of immunocompetent HA in the experimental whole virus inactivated formulation. This research was supported by the Russian Foundation for Basic Research grant 12-04-31397.

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Influenza vaccine effectiveness in the United States military during the 2012-2013 influenza season

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Background: Influenza is one of the most commonly encountered infectious diseases in the US military, especially among young, non-immune personnel. Thus, assessment of influenza vaccine effectiveness (VE) is routinely performed on a year-to-year basis among active component service members (SMs). We estimated influenza VE among all active component, non-recruit SMs during the period 1 September 2012 to 30 April 2013. Materials and Methods: Assessment of VE was performed by a case-control approach in which cases were individuals with positive laboratory tests for influenza. The Defense Medical Surveillance System (DMSS) was used to identify Health Level 7 (eg, laboratory data) in the DMSS, which was used to identify influenza-like illnesses (ILIs) that were laboratory confirmed by a rapid influenza test, reverse transcriptase polymerase–chain reaction (RT-PCR), or culture. Controls were of two kinds: (1) SMs who tested negative by RT-PCR or culture ("internal controls") or (2) SMs who experienced health care encounters for musculoskeletal conditions (without respiratory diagnoses, "external controls") and who were matched to cases by sex, age, date of diagnosis (+/- 3 days), and treatment facility. Vaccination status was determined by review of immunization records documented in the DMSS. Individuals were considered vaccinated if their ILI diagnosis occurred more than 14 days after influenza vaccination. Crude odds ratios were calculated, and logistic or conditional logistic regression was used to calculate adjusted odds ratios. Analyses were stratified by influenza type (A or B), subtype (H1, H3 or B), and vaccine type (trivalent inactive vaccine [TIV] and live attenuated influenza vaccine [LAIV]). Results: Preliminary analyses showed moderate protective levels (~ 34%-35%) depending on the vaccine type. TIV and LAIV appeared to confer similar levels of protection in all preliminary analyses. VE for influenza A (subtype H3) or subtype B resulted in non-statistically significant findings, most probably due in part to limited numbers of laboratory-confirmed influenza infections during the study period. Conclusions: Influenza continues to be a significant military-relevant disease, and annual vaccination continues to be mandated; however, moderate levels of VE are documented. Additional factors such as repeated immunization, circulating influenza strains, and health status may play a role in variable degrees of VE in the military. Updated estimates and the potential role of these other factors for all active component SMs will be examined in more detail and presented in final form.

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The study of heterosubtypic antibody responses against influenza A viruses elicited by seasonal vaccination using a pseudotype neutralisation assay

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Background: The study of heterosubtypic antibody responses directed against influenza A haemagglutinins in human populations is an important facet of pandemic preparedness. The evaluation of the ability of vaccines to increase heterosubtypic antibody responses to confer broad protection against different influenza subtypes is one approach to this. Classic serological assays, such as haemagglutination inhibition and microneutralisation, have demonstrated low sensitivity for the detection of cross-neutralising antibodies, especially those directed against epitopes in the haemagglutinin HA2 stalk region. For this reason there is a need for new assay formulations that are able to detect and quantify these heterosubtypic antibody responses. Influenza pseudotypes represent safe tools to study the neutralising antibody response since they are replication-defective viruses and they harbour on their envelope only the haemagglutinin that is the major target of this response. Materials and Methods: We have generated a panel of group 2 influenza A pseudotypes (H3 A/Udorn/307/1972, H4 A/duck/Czechoslovakia/1956, H7 A/chicken/Italy/1082/1999, H10 A/chicken/Germany/N49, H14 A/mallard/Astrakhan/263/1982, H15 A/shearwater/West Australia/2576/1979) and we have used them as surrogate antigens in neutralisation assays to study the presence and magnitude of heterosubtypic neutralising antibody responses in human sera collected before and after the 2007-2008 seasonal influenza vaccination. Results: In the human sera tested, neutralising antibody responses are detected against not only human influenza viruses, but also against influenza pseudotypes harbouring avian haemagglutinins belonging to group 2 viruses. After seasonal vaccination, the pseudotype neutralisation assays detect variation in the neutralising antibody titres against avian influenza pseudotypes. Conclusions: The increased sensitivity of the pseudotype neutralisation assay performed using a panel of influenza A pseudotypes permits the detection of heterosubtypic antibody responses before and after seasonal influenza vaccination. This has implications for the development of pandemic preparedness plans at the population level.

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Host genetic factors as determinants of immunologic and adverse responses on influenza vaccination in humans

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Background: Seasonal influenza epidemics cause a great burden of illnesses, hospitalizations, and deaths worldwide. Although influenza vaccination has generally been regarded as safe and effective in preventing influenza infection, some people do develop poor immune responses or occasional serious adverse events on receiving the vaccination. Little is known about how host genetic determinants are affecting responses to influenza vaccination in humans. Materials and Methods: We used a genetic association study with a candidate gene approach based on a randomized placebo-controlled trial on influenza vaccination to examine the role of host genetic variation on immune

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response and adverse reaction to influenza vaccine in humans. In the trial, 915 children aged 6-15 years were randomized to receive either an inactivated trivalent seasonal influenza vaccine (TIV) (Vaxigrip, Sanofi Pasteur) or placebo in phases from 2009 to 2010. Vaccine response was defined by a post-vaccination antibody titer of 1:40 or ≥ 4-fold rise in all TIV components. An adverse vaccine responder was defined by an aggregated symptom score ≥ 2 on day 1 post-vaccination, based on 10 symptoms each on a scale of 0 (absent), 1 (mild), 2 (moderate, or 3 (severe), thus having at least 2 mild or 1 moderate symptoms. All participants kept a daily symptom dairy. Whole blood samples from 535 participants receiving TIV were collected for genetic analysis in this study. DNA was extracted and genotyped for single nucleotide polymorphisms for IL-1B-511G>A (rs16944), IL-6-5843A/G (rs1818879), IL-8-251T/A (rs4073), IL-10-082A/G (rs1800896), -819T/C (rs1800871), -592A/C (rs1800872), MBL-2-5232G>A (rs1800451), 221C/G (rs7096206), -34C>T (rs5030737), -550G>C (rs11003125), MxA-88G/T (rs2071430), OSA1-347A/G (rs2660), RIG1 G/C (rs9695310), TLR3-1377T/G (rs3755290), -7G/T (rs3775296), TLR4 G/A (rs5030718), Asp299Gly (rs4986790), TLR7 Gln11Leu (rs179008), 1817G/T (rs5741880), TLR8-129G/C (rs3764879), Met1Val (rs3764880), and (rs11003131)G/T. Logistic regression models were used to evaluate the relationship of polymorphisms with various outcomes and to compute the ORs and 95% confidence interval (CIs) in relation to vaccination response and adverse vaccination reaction. The heterozygous and homozygous variant genotypes were analyzed both as a nominal and an ordinal variable as consisting, respectively, of one and two variant alleles and compared with the wild-type homozygous genotype. The heterozygous genotype was also grouped with either of the two homozygous genotypes to analyze in a dominant or recessive model. Two-sided *P* values are reported and $P \leq .05$ was considered to indicate statistical significance. Results: Among 535 subjects receiving TIV, 295 were classified as vaccine responders. Polymorphisms IL-6 rs1818879 G mutation in an ordinal model (OR = 1.56, CI = 1.054-2.31), AG (OR = 1.667, CI = 1.109-2.504), and combined AG/GG (OR = 1.637, CI = 1.093-2.454) in a dominant model were associated with increased odds of response. TLR7 rs5741880 GT (OR = 0.161, CI = 0.046-0.566), combined GT/TT (OR = 0.371, CI = 0.159-0.866) in a dominant model, and TLR3 rs3755290 GG (OR = 0.572, CI = 0.335-0.976) in a recessive model compared with GT/TT were associated with lower odds of response. No serious vaccine response, including anaphylaxis or shock, was reported by any recipient. With a symptom score ≥ 2, 26.1% were classified as adverse responders for TIV. IL-6 rs1818879 AG (OR = 1.833, CI = 1.14-2.945) and combined AG/GG (OR = 1.778, CI = 1.108-2.854) were associated with a higher risk, while CCL1 rs2282691 AT (OR = 0.578, CI = 0.347-0.963) was associated with a lower risk of adverse response. All these effects of polymorphisms in relation to vaccination response are compatible with the current understanding regarding the role played by those genes in either the pathogenesis or immunological response to influenza infection. Conclusions: Our findings suggest the potential role of host genetic variation and identified genetic determinants that affect the immunological and adverse responses to seasonal influenza vaccination in humans. These findings may help to explain the great variability in protection achieved by influenza vaccination.

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Immunogenicity and reactogenicity of a quadrivalent influenza vaccine administered intramuscularly to children 6 to 35 months of age in 2012-2013: day 56 results of a randomised, double-blind, controlled, multi-centre, multi-country, clinical trial (NCT01711736)

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Background: Influenza has a high attack rate in infants and young children. Vaccines containing both lineages of influenza B (Yamagata and Victoria), in addition to H3N2 and H1N1 antigens, may improve the protection of this vulnerable population. Materials and Methods: Children 6 to 35 months

of age in stable health were randomised (1:1) to receive 0.5 mL of a candidate quadrivalent influenza vaccine (QIV) or a trivalent control vaccine (TIV) by the intramuscular route. The QIV contained 15 µg haemagglutinin (HA) from 4 strains: A/California/7/2009(H1N1)pdm09, A/Victoria/361/2011(H3N2), B/Brisbane/60/2008 (Victoria lineage) and B/Hubei-Wujiagang/158/09 (Yamagata lineage) in a thimerosal-free formulation. The TIV contained the same H1N1, H3N2 and B Yamagata components. Vaccine unprimed participants (VUPs) (no prior receipt of seasonal influenza vaccine in the past or only one dose for the first time in 2011-2012) received vaccines on days 0 and 28, whereas vaccine primed participants (VPs) received one dose (day 0). Blood samples for haemagglutinin agglutination (HI) antibodies were collected on days 0 and 28 from VPs and on days 0 and 56 from VUPs. Solicited injection site and general adverse events (AEs) were collected by parents/guardians on diary cards on days 0-6 after each vaccination; unsolicited AEs will be collected until day 180. The primary objectives were to assess QIV immunogenicity based on the Center for Biologics Evaluation and Research seroconversion rate (SCR) acceptance criterion for each strain 28 days after completion of dosing and solicited AEs on days 0-6. Results: A total of 601 children (TIV, 302; QIV, 299) were enrolled and randomised at 8 sites in three countries (Canada, Dominican Republic, Honduras); 96% (580/601) completed the day 56 visit of the study. The mean ages were 18.2 months (SD 8.17) and 18.1 months (SD 8.34) for the QIV and TIV groups respectively. Only 13 (4.35%) and 15 (4.97%) children were considered VPs, respectively. The primary immunogenicity objective was met, as the lower limit (LL) of the two-sided 95% confidence interval (CI) for SCR in QIV recipients ranged from 66.6% to 81.3%, which was ≥ 40% against all 4 strains; the SCR point estimates ranged from 72.2% to 85.9%. The immunogenic superiority of the B/Victoria strain present in QIV (in terms of geometric mean titre and SCR) compared with TIV was concluded, as the LL of the two-sided 95% CI of the GMT ratio (QIV/TIV) (6.28; 95% CI 5.32, 7.41) was greater than 1.5, and the LL of the two-sided 95% CI for the difference in SCR (QIV – TIV, 64.19%; 95% CI 57.65, 69.95) was greater than 10%. At least one symptom (solicited and unsolicited) was reported for 63.5% and 67.9% of subjects in the QIV and TIV groups, respectively, during the 7 days after vaccination. Injection-site pain was the most frequently reported solicited injection-site AE (QIV, 32.6%; TIV, 30.6%) with Grade 3 pain in 7 (2.4%) and 3 (1.0%) participants, respectively. Irritability/fussiness was the most frequently reported solicited general AE (40.7% and 41.6% of subjects in the QIV and TIV groups, respectively). Grade 3 irritability/fussiness was reported in 5.2% and 4.7% of QIV and TIV recipients, respectively. Grade 3 fever after QIV and TIV was reported in 14.5% and 14.5% of recipients after the first dose and in 10.3% and 9.1% of children after the second dose, respectively. Grade 3 or higher fever (≥ 39.0°C) occurred in 7.9% and 4.0% of children. In an exploratory analysis, the relative risk of fever in QIV compared with TIV recipients during a 4-day follow-up period was 1.12 (95% CI 0.76, 1.64). No withdrawals related to vaccination have occurred up to day 56; follow-up to 6 months is ongoing. Conclusion: A candidate QIV containing B Yamagata and B Victoria lineages is immunogenic in VUPs, with a similar reactogenicity profile to TIV. Funding: GlaxoSmithKline Biologicals SA.

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Modeling the long-term public health impact of quadrivalent influenza vaccination in the US population

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Background: Vaccination is widely recognized as the most efficient intervention to prevent influenza infection. Current trivalent influenza vaccines (TIVs) contain two influenza A and one influenza B strains. Yearly co-circulation of two genetically distinct B lineages and difficulty in predicting which B lineage will predominate during the next influenza season results in frequent mismatches. New quadrivalent influenza vaccines (QIVs) containing a strain from each B lineage should address these issues, but their impact still needs to be estimated. Our aim was to estimate the relative public health benefit of using QIV versus TIV for routine influenza vaccination in the US. Materials and Methods: We developed a dynamic compartmental model able to take into account potential interactions between influenza A subtypes (H1N1 and H3N2) and influenza B lineages (Victoria and Yamagata) in

terms of natural or vaccine-induced immunity and cross-protection. The model is able to qualitatively assess the long-term impact of vaccination on influenza circulation by taking into account changes in vaccine composition. This age-structured model realistically accounts for population dynamics, for vaccine efficacy per strain, and for the weekly ramp-up of vaccination coverage during the influenza season. We calibrated the model using US demography and epidemiological data for the last 10 years and made projections on the next 10 years. Our reference scenario for TIV assumes a yearly switch of B lineage in its composition. Sensitivity analyses were performed on the basic reproduction rate R_0 ($1.5 < R_0 < 2.5$), natural and vaccine-induced cross-protection between B strains, duration of acquired immunity, vaccine efficacy, and frequency of vaccine mismatch due to B lineage. Results: Assuming a cross-protection of 20%, the model predicts that switching to QIV would result in an average 47% reduction of incident cases due to B-lineage strains over the next 10 years. Varying cross-protection from 0% to 40% results in B-case reductions of 60% and 26%, respectively. Conclusions: Our model predicts that replacement of TIVs with QIVs will substantially reduce the incidence of influenza B cases regardless of the level of existing cross-protection between lineages.

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Error-prone PCR based mutagenesis to generate influenza egg high yield strains without subsequent adaptation

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Background: Influenza viruses cause both occasional pandemic and seasonal epidemic, presenting continuous challenges to public health. Vaccination is the primary option to counteract and reduce the impacts of influenza outbreaks. A high yield vaccine seed strain is required for vaccine manufacturing, thus, the key to a successful influenza vaccine program. However, it is not uncommon that the recommended vaccine strains by the World Health Organization have poor yield in chicken embryonic eggs (abbreviated as eggs). A poor yield strain will be required to be engineered into a high yield strain, and this process can be time consuming and sometimes will significantly delay vaccination production. Conventional methods to increase vaccine yields include egg adaption method and site directed mutagenesis method. The egg adaptation method is to select a high-yield variant by growing a vaccine candidate in eggs, usually, with multiple passages. The site mutagenesis is based on empirical experience to mutate the hypothetical markers to increase yields in eggs. Both methods are very time consuming and usually require months to generate a desired strain. Materials and Methods: In this study, we developed a novel error-prone PCR-based mutagenesis approach for rapidly generating high-yield influenza vaccine strains. Results: This method allows us to generate vaccine candidates within 7 to 10 days, compared with months by other conventional methods. This method was further validated in generating high yields of 2009 H1N1 pandemic virus with A/California/4/2009(H1N1) as the template. A total of 43 mutants were rescued, and 6 had at least 4-fold higher HA titers than the pandemic wild-type–based reassortant (pdmR) when being grown in eggs. The growth kinetics in MDCK cells further demonstrated that these 6 mutants replicated more efficient than pdmR. The HA peak titer of these viruses in MDCK cells reached to a range from 128 to 512 units whereas that for pdmR was only 32. The peak $TCID_{50}$ of these mutants had 0.4-1.0 log10 $TCID_{50}$ higher than that for pdmR. Antigenic analyses demonstrated that these mutants grown in both eggs and cells did not have significant altered antigenicity. Conclusions: Thus, this novel approach presented in this study allows us to rapidly generate egg or cell based high yield influenza vaccine strains and can be used in vaccine strain preparation for influenza prevention and control.

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Efficacy of inactivated influenza vaccine in HIV-infected pregnant women: a randomized, double-blind, placebo-controlled trial

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Background: HIV infection and pregnancy may act synergistically to predispose women to severe influenza illness. The WHO recommends that pregnant women be prioritized for inactivated influenza vaccination (IIV) receipt. In Bangladesh, IIV of pregnant women in the third trimester of pregnancy reduced the rate of respiratory illness with fever in mothers by 36% (95% CI: 4 to 57) up until 6 months postpartum. In African HIV-infected predominantly non-pregnant adults, the efficacy of IIV against laboratory-confirmed influenza (LCI) illness was 76% (95% CI: 9 to 96). We determined that IIV is only modestly immunogenic in HIV-infected pregnant women. However, no published data on the efficacy of IIV in HIV-infected pregnant women exist. An aim of our study was to evaluate the efficacy of IIV against LCI and protocol-defined influenza-like illness (ILI) in HIV-infected women vaccinated during the 2nd/3rd trimester of pregnancy. Materials and Methods: We undertook a double-blind, randomized, placebo-controlled trial in Soweto, South Africa, in 2011. We randomized 194 HIV-infected women between 20 and 34 weeks of gestational age to receive either trivalent IIV or normal saline placebo intramuscularly. Trivalent IIV contained the recommended Southern Hemisphere vaccine strains for the 2011 influenza season: H1N1 A/California, H3N2 A/Victoria and B/Brisbane. Participants were followed-up until 6 months postpartum for clinical and laboratory-confirmed endpoints. The primary objective of the study was to assess the safety and immunogenicity of IIV in HIV-infected pregnant women, while secondary objectives were to assess vaccine efficacy (VE) against ILI and LCI. A clinical diagnosis of ILI was standardized as the presence of fever and at least one of three symptom groups of < 7 days duration. Participants were screened weekly for the presence of acute respiratory illness or hospitalization for acute cardio-pulmonary illness. When ILI was confirmed by a study physician, oropharyngeal and nasopharyngeal swab specimens were collected for influenza virus testing by real-time reverse transcriptase–polymerase chain reaction. Specimens were tested for influenza A and influenza B viruses; influenza A virus isolates were subtyped for pandemic H1N1 (pH1N1) and H3N2, and influenza B isolates were tested for Yamagata and Victoria lineages. Results: At enrolment the median age of the participants (n = 194) was 27.9 years, the median HIV-1 viral load was 1067 copies/ml (IQR 61: 13,923), and the median CD4+ cell count was 394 cells/µl (IQR 271: 557). There was at least one physician-attended illness visit in 149 (76.8%) participants, with 253 and 270 physician-attended illness visits in IIV recipients and placebo recipients, respectively. Eighty-one (16%) physician-attended illness visits fulfilled the ILI criteria. Trivalent IIV was not associated with a reduction in the first episode of ILI (VE: 10.9%; 95% CI: –40.2 to 43.4). The dominant strain of influenza virus identified was pH1N1: 7 (43.8%) cases in the control group and 6 (85.7%) in the vaccines; H3N2 was identified in 5 women (4 placebo recipients and 1 IIV recipient). Influenza B was identified in 4 cases (4 placebo recipients and 0 IIV recipients). Of the positive specimens for influenza B virus, one was of Victoria lineage and three were of Yamagata lineage. LCI illnesses were identified in 7/99 (7.1%) IIV recipients and in 16/95 (16.8%) placebo recipients. Trivalent IIV was associated with a reduction in first episode of LCI illness (VE: 58%; 95% CI: 2.5 to 81.9; *P* = .035). After exclusion of the non-vaccine influenza B Yamagata cases from the analysis, there was positive but non-significant VE against LCI (VE: 48.3%; 95% CI: –23.9 to 78.4). Conclusions: HIV-infected pregnant women had a high influenza attack rate during the study. IIV of HIV-infected pregnant women appeared to prevent LCI illness, even though it induced only modest immune responses measured by hemagglutination-inhibition assay. Based on the attack rate in the placebo group (16.8%), our study had an 80% power to detect at least a 78% reduction in LCI disease; hence, the data from our study need to be corroborated by further studies.

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Plant-made virus-like particle vaccines for pandemic influenza: advantages in speed, delivery to antigen-presenting cells, and induction of cellular memory

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Background: Plant-made virus-like particles (VLPs) have several potential advantages as influenza vaccine candidates, in terms of both speed of production and immunogenicity. The ongoing threat of avian influenza strains with pandemic potential (H5N1, H7N9, and others) strongly supports the detailed evaluation of these candidates. Three aspects of our recent work with Medicago's candidate H5- and H7-VLP vaccines are presented: 1) speed test for H7-VLP production, 2) delivery of H5-VLP vaccine to regional lymph nodes and association with antigen-presenting cells (APCs), and 3) the induction of balanced humoral and cellular responses. Materials and Methods: Speed test—Deposited H7N9 sequence data were used to produce HA consensus constructs for transfection of routine production lots of 4-week-old *Nicotinaia benthamiania* plants. VLP–immune cell interactions—An H5-eGFP fusion construct (H5 external - eGFP internal) was generated to track VLP movement within the immune system *in vivo* and to assess VLP–immune cell interactions *in vitro*. Balanced humoral and cellular responses—Multiparameter flow cytometry was applied to PBMCs, collected from human volunteers 6 months after administration of 2 doses of an H5-VLP vaccine, to analyze CD4 and CD8 memory for homo- and heterosubtypic strains. Results: Speed test—H7 consensus constructs were transfected into *N. benthamiania* within 1 week of deposition of H7 sequence data. Candidate H7-VLPs were physico-chemically characterized, and the first bench-scale lot was administered to 6-week-old female BALB/c mice 19 days after the DNA gene was received at Medicago. Immunogenicity after a first dose of 3 µg IM was excellent and further improved with a second dose 21 days later. VLP tracking—The H5-eGFP VLPs were observed to be slightly larger than H5-VLPs (150 vs 120 nM) but were otherwise identical by all physicochemical measures, except that the eGFP-bearing VLPs were strongly fluorescent. The H5-eGFP VLPs were also fully immunogenic in 6-week-old BALB/c mice after IM injection. Mixed with mouse splenocytes, the H5-eGFP VLPs preferentially associated with monocytes, dendritic cells, neutrophils, and (to a lesser extent) NK cells. After IM injection in the hind limb, H5-eGFP VLPs moved to the regional (inguinal) lymph nodes within 10 minutes, where, again, they were found to be associated preferentially with APCs and neutrophils. Mixed with human PBMCs, the H5-eGFP VLPs rapidly associated with all immune cell subsets, including T and B lymphocytes. The mean fluorescence intensity was highest in APCs and neutrophils. Balanced humoral and cellular immunity—When wild-type HA was used as the target antigen, adults (age range, 18-59 years) mounted excellent humoral responses to 2 doses of H5-VLP candidate vaccines (5-20 µg with alum IM) or a single dose of non-adjuvanted H1 VLPs (5-28 µg), which readily met all 3 CHMP criteria for licensure. A companion poster (Tarbet B, et al) details the cross-reactivity of these antibodies in a mouse model. At 6 months after immunization, strong multi-functional CD4 and CD8 memory T-cell responses were readily detected in almost all of the subjects. These responses were significantly greater than those seen following trivalent influenza vaccination (H1-VLPs vs Fluzone™) when cross-reactivity was evaluated (eg, H1-VLP vaccination elicited a strong response to H5 antigen). Conclusions: In aggregate, these data suggest that plant-made VLP vaccines address several of the problems with current commercial influenza vaccines, particularly with regard to speed of manufacturing in the event of a pandemic or a severe seasonal mismatch and the initiation of balanced and cross-reactive humoral and cellular responses. The *in vitro* and *in vivo* tracking data of these plant-made VLPs suggest that selective uptake by APCs and rapid transit to regional lymph nodes likely contribute to their powerful immunogenicity. Some aspects of this work were supported by a CIHR-Industry grant to BJW and NL.

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Cross-reactivity of serological responses after H7N1 vaccination to influenza H7N9 viruses

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Background: The recent zoonotic outbreak of influenza H7N9 in China has highlighted the importance of pandemic preparedness. We developed a cell-based candidate pandemic H7 split virus vaccine based on a highly pathogenic A/Chicken/Italy/13474/99 (H7N1) virus, which was tested previously in preclinical studies and in a human phase 1 clinical trial. In this study we evaluated the ability of the H7N1 vaccine to elicit cross-reactive serological responses to A/Anhui/1/2013 and A/Shanghai/1/2013 (H7N9) viruses. Materials and Methods: We previously conducted a phase 1 clinical trial of an aluminium adjuvanted H7N1 vaccine and collected biobank samples. The H7N1 vaccine was poorly immunogenic, and sera were chosen from 10 volunteers who had neutralisation antibody to the homologous vaccine strain (A/Chicken/Italy/5074/99 (H7N1)). Pre- and post-vaccination human sera were tested for their ability to cross-react with H7 viruses by haemagglutination inhibition (HI) and microneutralisation (MN) assays using A/Anhui/1/2013 (H7N9) virus (or HI using virus-like particles), A/Mallard/Netherlands/12/2000 (H7N7) virus and the homologous vaccine virus A/Chicken/Italy/5074/99 (H7N1). The antibody response was also evaluated by ELISA using haemagglutinin from the A/Anhui/1/2013 (H7N9) and A/Shanghai/1/2013 (H7N9) viruses expressed in the baculovirus expression system. Results: The vaccine induced low MN serum antibody titres to the homologous vaccine strain (MN titres 7-160). Low cross-reactive MN titres were detected in the post-vaccination sera of 3 subjects to the H7N9 virus (MN titres 7, 14 and 14) and 2 volunteers to H7N7 (MN titre 7). When sera were preadsorbed with horse red blood cells and tested in the modified HI assay using H7N9 virus-like particles, we found that 13 of 60 volunteers had HI titres of 16 to 32. We further investigated post-vaccination sera from all subjects with MN titres to the homologous vaccine virus and detected an IgG response to both A/Anhui/1/2013 and A/Shanghai H7 HAs by ELISA. Importantly, we previously showed in preclinical studies that the protective efficacy of this vaccine to homologous challenge may not be directly correlated with the pre-challenge levels of serum antibodies. In ongoing studies, we will examine the ability of lymphocytes collected during the trial to recognise H7N9 viruses. Conclusions: We have shown using non-traditional assays that human vaccinees immunised with a candidate H7N1 vaccine show some cross-reactivity to H7N9 viruses isolated during the Chinese outbreak.

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A genetic survey of vaccine recommendations and circulating influenza A(H3N2) strains during the period from 1999 to 2012 in Brazil

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Background: Influenza vaccination is the most important public health measure to prevent severe cases and death due to influenza infection. However, influenza viruses are constantly evolving,

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requiring the continuous reformulation of the vaccines. Few studies in Brazil have evaluated the evolution of influenza viruses and compatibility between circulating viruses and vaccine recommendations. The subtype A(H3N2) is known as the fastest evolving and the dominant subtype in most seasonal influenza epidemics since 1968, when it was introduced in the human population. Our goal in this study was to check the genetic compatibility among Brazilian samples and the chosen A(H3N2) vaccine component by year, throughout 1999 to 2012. Materials and Methods: Samples were obtained in the scope of the Brazilian influenza surveillance network and were subtyped by real-time RT-PCR. A representative number of these samples was characterized by Sanger sequencing. A total of 237 sequences of the HA1 portion of the hemagglutinin (HA) gene—collected in the northeast, southeast, and south Brazilian regions and from different regions of the world contained in the GenBank and GISAID databases—were evaluated and sequences of vaccine strains were recommended throughout this period. We used MEGA v5.10 to infer nucleotide and amino acid distances between sequences and annual vaccine prototypes, and we used the PhyML program to carry out the phylogenetic reconstructions with the maximum likelihood algorithm. Based on these results, we assessed the identity of Brazilian sequences and compared the effectiveness of the influenza vaccination in three regions of Brazil within hypothetical alternative scenarios defined by different timings of vaccine delivery as well as Southern or Northern Hemisphere compositions. Scores were based on the temporal overlap between vaccine-induced protection and circulating strains. Results: Based on our analysis, strains circulating in 1999 and 2003 were significantly different from those present in the vaccines. We found that, during 2010 to 2012, the A/Perth/09-like and A/Victoria/09-like strains co-existed in distinct regions of Brazil, despite the composition of the vaccine being A/Perth/09 throughout the whole period. Most amino acid mutations associated with branches that gave rise to positively selected variants were located in HA epitope sites, with significant statistical support (aLRT > 0.90). Using phylogenetic analysis we identified 5 of 13 seasons when community-circulating samples were related to their respective vaccine prototype: 2001, 2002, 2005, 2009, and 2010. Based on the Brazilian vaccination schedule, the better matches were obtained using the southern vaccine formulation for all three regions included in this study. Conclusions: During the period 2010-2012, phylogenetic differentiation was statistically supported, and the mutations focused in the HA epitope regions could explain the reduced efficacy of the vaccine during this period. We made two public health–relevant conclusions from our analyses: first, the strains in the influenza vaccine generally arrived after their circulation had been replaced by others. This disappointing outcome might be partially responsible for the low efficacy of the vaccine. Second, our data did not confirm the previous results from Mello et al. In the northeast region, we found that it was mostly irrelevant whether the Northern or Southern Hemisphere recommendation was used. Nevertheless, in the south and southeast region, the current choice of the Southern Hemisphere recommendation is indeed superior. In addition, studies of viral evolution can help elucidate which influenza viruses are circulating globally, which is useful in the selection of future vaccine candidates.

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An adaptable pseudovirus assay enables safe, robust measurements of influenza-neutralizing antibody activity and accurately predicts protection in a challenge study utilizing highly pathogenic H5N1 virus

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Background: Protection from influenza virus A&B infections is mediated by neutralizing antibodies (nAb). Accurate and sensitive assessments of nAb targeting the hemagglutinin (HA) protein are critical to characterizing protective immune responses. The HA inhibition assay (HAI) is considered the gold standard for assessing nAb titers that correlate with immune protection yet it suffers from limitations in reproducibility and sensitivity and may present biohazard risks to laboratory personnel. We have developed a versatile, biosafe pseudovirion assay (PSVA) with enhanced sensitivity and reproducibility that accurately measures HA nAb activity. Materials and Methods: HEK293 cells are cotransfected with an envelope-defective HIV-1 genomic vector containing a luciferase reporter gene along with influenza HA and NA expression vectors and an airway serine protease that mediates HA

processing. Pseudovirions produced by transfected cells are used to inoculate HEK293 target cells and infectivity is assessed by measuring luciferase activity (relative light units [RLU]). HA nAb activity is evaluated over a broad range of nAb concentrations. The HA pseudovirus assay was used to identify H5 HA immunogens that elicit broad neutralizing activity against highly pathogenic H5N1 viruses. Libraries of chimeric HA sequences were created using in vitro homologous DNA recombination of wild-type H5 HA sequences derived from 3 clades. Thirty HA variants were selected for mouse immunizations, and sera was analyzed against 10 pseudoviruses containing HAs representing 4 different H5N1 clades (clade 0, 1, 2, and 7). The variants stimulating the most broadly neutralizing responses, 9281 and 9282, plus the parental HAs were evaluated for protection against a lethal H5N1 challenge in mice. Mice were immunized with Viet04, Indo05, 9281, 9282, or an irrelevant DNA, and then challenged intranasally with highly pathogenic A/Vietnam/1203/2004. Results: Pseudovirions representing > 75 HAs (H1-H11, B) and > 60 NAs (N1-4, 6-8, B) were evaluated for infectivity and susceptibility to vaccine sera and monoclonal antibodies. Pseudovirus infectivity (RLU) spanned > 7 $\log_{10}$. Based on > 10,000 assays, nAb titers of HA immunized animals ranged from < 1:10 to > 1:1,000,000. PSVA nAb titers were 10-100× higher than HAI or microneutralization assay (MN) titers. PSVA reproducibility was ≤ 2.5-fold and correlated well with HAI and MN neutralization within the measurable range of the HAI and MN assays. In the study of multiple H5 immunogens, PSVA cross-neutralization titers correlated with vaccine efficacy after challenge while HAI and MN titers did not. Two HA chimeric variants (9281 and 9282) induced neutralizing antibody responses to a broader range of viruses compared to the wild-type H5 HA proteins. Sera from 9281- and 9282-immunized mice exhibited moderate to strong nAb activity against most pseudoviruses, while the sera nAb activity from wild-type immunogens exhibited subclade specificity. 9281 sera exhibited strong neutralizing activity against clade 2.3.2 HA, while none of the wild-type immunogens elicited activity against this strain. The nAb of these sera were also evaluated in standard HAI and MN assays against 3 viruses. Mice immunized with negative controls succumbed to challenge between 5-8 days postinfection. Mice immunized with the Viet04 or variant 9282 survived challenge, and 90% were protected by immunization with variant 9281. Mice immunized with variant 9282 displayed fewer signs of disease after infection, including less weight loss and shorter duration of disease symptoms, even compared with the Viet04 group, which was perfectly matched to the challenge virus HA. These data indicate that antibody breadth as determined in the HA pseudovirus assay can be a superior predictor of vaccine efficacy. Conclusions: An accurate, sensitive, versatile, and biosafe pseudovirion assay has been developed to characterize influenza HA nAb responses. Assay accuracy was assessed against conventional HAI and MN assays. A large, diverse library of influenza HA genes enables rapid and comprehensive assessments of nAb breadth and potency. This PSVA provides improved performance over HAI and MN assays and flexibility that enables evaluation of HA infectivity and nAb activity, as well as the characterization of naturally occurring and genetically engineered HA variants, including those of highly pathogenic viruses.

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P2-548 SCHOLARSHIP Promising Investigator Scholarship

A universal influenza virus vaccine based on the stalk domain of the hemagglutinin

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Background: Influenza virus infections remain a significant cause of morbidity and mortality worldwide. Current vaccines show good efficacy against antigenically matched viruses, but fail to protect against drifted and pandemic strains since they do not induce broadly neutralizing antibodies. Due to the rapid antigenic drift of influenza viruses, these vaccines have to be reformulated, generated, and administered through a cumbersome and expensive process every year. The membrane-proximal stalk domain of the viral hemagglutinin exhibits a high degree of both sequence and structural conservation across influenza virus subtypes. Furthermore, antibodies directed against this region typically show broad neutralizing activity. Materials and Methods: We therefore hypothesize that a

vaccine strategy that stimulates a robust immune response towards this region of the hemagglutinin could provide universal influenza virus protection. Such a vaccine would thus abolish the need for annual vaccine reformulation and further enhance our pandemic preparedness. We developed a universal influenza virus vaccine based on the conserved stalk domain of group 1 and group 2 hemagglutinins. By sequential vaccination of mice with these chimeric hemagglutinin constructs we were able to boost broadly neutralizing antibody titers against conserved epitopes in the hemagglutinin stalk domain. Results: Mice vaccinated with our constructs were protected from morbidity and mortality induced by infection with a panel of heterologous and heterosubtypic influenza A viruses. Further, we used passive transfer and $CD8^+$ T-cell depletion experiments to show that the observed protection is solely antibody mediated. It is of note that our vaccination regimen protected animals from a stringent H7N1 challenge and also induced high titers of antibodies that reacted strongly with the H7 HA protein derived from the H7N9 strain A/Shanghai/1/13. These findings are of high importance in the light of the emergence of the novel H7N9 strain in China. Conclusions: The present data suggest that this vaccine strategy could be successfully developed in humans to provide broad influenza virus protection and enhance our pandemic preparedness. A universal influenza virus vaccine, which—similar to the ones developed for polio and measles viruses—requires a single or only a few immunizations, would represent a major advance towards the control of influenza worldwide.

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Live virus and vector-based influenza vaccines for induction of antibodies specific for stalk domain of hemagglutinin

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Background: Influenza A virus (IAV) causes an acute respiratory disease that affects millions of people annually. The virus undergoes mutations in the two outer membrane glycoproteins—hemagglutinin (HA) and neuraminidase (NA)—that lead to antigenic changes (antigenic shift and antigenic drift). Vaccines against influenza need to be updated every year because they fail to induce cross-protection against infection with drifted and shifted viruses. Attempts to develop universal vaccines that provide broad protection have been made. Those vaccine candidates include live virus and vector-based vaccines that could induce antibodies (Abs) specific for virus conserved stalk domain of HA and hence broad protection. Materials and Methods: We evaluated in mice the induction of Abs specific for conserved stalk domain of HA by intranasal immunization with live attenuated influenza virus, replication-deficient adenovirus encoding influenza HA or formaldehyde inactivated virus. The role for such Abs in cross-protection was studied by adoptive transfer of immune sera into naïve mice and subsequent challenge with heterosubtypic virus strains. Results: We found that live attenuated influenza virus or replication-deficient adenovirus encoding influenza HA induced significant levels of Abs against conserved stalk domain of HA as compared with that induced by inactivated vaccine. When passively transferred to naive mice, sera derived from mice immunized with a live virus or vector protected the recipient mice against homotypic or heterosubtypic virus challenge, whereas sera from mice immunized with inactivated virus did not. In search for the cause, we found that inactivation of the viruses by formaldehyde significantly reduced the binding of conserved stalk as compared to that of globular head HA to specific monoclonal Abs, suggesting that formaldehyde cross-linking significantly altered conformational epitopes of stalk HA. Conclusions: The results indicate that intact viral conserved stalk HA expressed by live virus or vector-based vaccines is important for induction of stalk-recognizing Abs. Such Abs could play an important role in cross-protection against infection with different influenza virus subtypes. The findings implicate strategies in the development of universal influenza vaccines.

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Biosafety and biosecurity in a biocontainment laboratory working with highly pathogenic avian influenza (HPAI)

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The critical aspects of biosafety, biosecurity, and biocontainment have gained increased awareness for governments around the globe that wish to pursue high-risk microbiological life sciences research. In recent months, there has been acute focus for HPAI laboratories to optimize standards according to current oversight frameworks in order to increase the safety of laboratory workers and decrease the risk of accidental release of a biological agent. Our purpose is to highlight how a CEIRS/WHO collaborating center in the US manages biosafety and biosecurity for HPAI, a high-risk pathogen known for its global public and animal health threat. We implement four levels of controls that focus on personal protective equipment, work place practices, administrative, and facility engineering. Taken together, these controls maximize the safety of the laboratory worker, allow for efficient progress of research in animals, and minimize opportunities for accidental exposure or release. For successful implementation, it requires the cooperation of the scientific investigators, the Environmental Health and Safety office, and the animal facility veterinarians and their technicians. With full support from all departments, we have created a system that is consistent and compliant with all regulations, and developed an excellent "culture of safety" among the research staff.

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Utilizing endogenous microRNA to confer molecular biocontainment to gain-of-function influenza viruses: towards risk mitigation

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Background: Influenza A virus (IAV) is primarily a seasonal pathogen with the potential to cause catastrophic pandemics. Zoonotic influenza viruses have the capacity to jump species and successfully establish respiratory infections in humans. Because of the potential introduction into and sustained circulation of zoonotic influenza viruses among populations of immunologically naive, susceptible hosts, studies examining pathogenesis and transmissibility are necessary to develop risk assessments. Avian H5N1 and H7N9 are two influenza virus strains that are currently of significant concern to public health. Although natural H5N1 transmission is restricted to avian species, gain-of-function studies have elucidated the minimal amino acid changes that are sufficient to permit aerosol transmission between mammals. This finding, although informative to pandemic preparedness plans, generated concern for the threats associated with transmissible H5N1 and began a public dialogue regarding the risks and benefits of this type of research. Endogenous miRNAs were explored as a means to confer intrinsic molecular biocontainment to influenza virus and to provide an added level of risk mitigation to studies assessing pathogenicity and transmissibility of zoonotic influenza viruses with pandemic potential. Materials and Methods: The H3 and H5 genomic sequences encoded by A/Wyoming/3/2003 (H3N2) and A/Vietnam/1203/2004 (H5N1), respectively, were genetically modified

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to 1) introduce duplicated 5′ packaging sequences and 2) insert scrambled noncoding RNA or 4 fully complementary miR192 target sites. Target sequences for miR192 were selected because this miRNA is expressed in lung tissues of mice and humans but is not expressed in lung tissue of ferrets, which is the classic animal model used for influenza virus transmission studies. The effect of species-specific RNA interference on influenza virus replication, pathogenesis, and transmission was examined in vitro and in vivo. Results: The in vitro replication of wild-type and control H3N2 or H5N1 influenza viruses encoding scrambled noncoding RNA sequences in the 5′ packaging sequences of HA were similar in canine kidney and human lung epithelial cells. The replication of miR192-targeted influenza viruses was comparable to that of wild-type and control viruses in canine kidney cells; however, the replication of miR192-targeted viruses was attenuated in human lung epithelial cells. Northern blot and Western blot analyses revealed a correlation between the level of endogenous miRNA expression and the degree of knockdown of H3 or H5 expression in human lung epithelial cells or in infected mouse lungs. Consistent with miR192 expression and the in vitro replication data, miR192-targeted H5N1 influenza virus did not induce morbidity or mortality following intranasal infection of mice. Conversely, wild-type and control virus infection resulted in significant morbidity, and all animals succumbed to infection. However, in ferrets, which do not express miRNA192 in lungs, wild-type, control, and miR192-targeted H3N2 influenza viruses replicated and transmitted among ferrets with comparable efficiencies. Conclusions: Incorporation of species-specific miRNA target sites in the influenza virus HA results in unperturbed replication and transmissibility in desired species, while eliminating pathogenicity in others. This strategy introduces a new biosafety approach for the molecular biocontainment of pathogens that can be added to present risk-minimizing strategies.

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Limited airborne transmission of influenza A/H7N9 virus between ferrets

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Background: At the end of March 2013, the World Health Organization was notified by the Chinese authorities of three human cases of infection with a novel avian-origin influenza A/H7N9 virus. As of June 2013, 132 A/H7N9 laboratory-confirmed cases have been reported in six different provinces of China, including 37 deaths. The A/H7N9 viruses harbor genetic traits that have been associated with human adaptation of avian viruses and increased transmission among mammals. However, no sustained human-to-human transmission of A/H7N9 viruses has been reported to date. Apart from two confirmed cases that might have arisen from family clusters and for which human-to-human transmission cannot be ruled out, human cases of A/H7N9 infection were epidemiologically unrelated and identified in seven different areas of China. Here, we assessed the airborne transmissibility of a human isolate, A/Anhui/1/2013 (AN1), in the ferret model. Materials and Methods: Four ferrets were inoculated intranasally with 10^6 $TCID_{50}$ of AN1 virus. One day after inoculation, one naive recipient ferret was placed opposite each donor ferret. Nose and throat swabs were collected on 1, 3, 5, and 7 dpi for donor ferrets and on 1, 3, 5, 7, and 9 dpe for the recipient ferrets. A positive nose swab sample from a recipient ferret was used for the second transmission experiment. Sanger sequencing was used to determine the consensus genome sequence of the four airborne-transmitted AN1 viruses isolated from recipient ferrets. Next-generation sequencing was performed with respiratory samples of the donor/recipient pairs for which airborne transmission was observed and with the original virus stock. Wild-type virus and viruses with mutations of interest (N123D and N149D) were assessed for their binding properties using a modified turkey red blood cell assay, pH-dependent HA-mediated cell fusion, and their stability. Results: Virus shedding from the donor ferrets was detected from 1 dpi onwards, with infectious virus titers up to $10^{6.5}$ $TCID_{50}$/mL. AN1 virus was transmitted to 3 of 4 recipient ferrets. Sanger sequencing showed that two substitutions, N123D and N149D in HA, were consistently found in all three airborne-transmitted viruses. Virus recovered from a recipient ferret was used to inoculate four additional donor ferrets. One day later, these animals were paired with 4

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recipient ferrets in transmission cages of which one became infected. Substitutions N123D and N149D in HA and M523I in PB1 were found consistently in the two subsequent transmission experiments. Residues N123 and N149 are adjacent to the receptor binding site but do not interact directly with α2,3- and α2,6-linked sialic acids. Binding to α2,3-linked sialic acids of $AN1_{N123D}$, $AN1_{N149D}$, and $AN1_{N123D,N149D}$ viruses was increased slightly by 2- to 4-fold compared with AN1 virus. Neither the single N123D and N149D substitutions nor the double N123D + N149D substitution affected the threshold pH for HA-mediated membrane fusion compared with the wild-type AN1 HA. Fusion for AN1 HA occurred at pH 5.6, which is higher than reported previously for human viruses. Substitution N123D and the double substitution N123D + N149D decreased the temperature stability compared with AN1. Conclusions: Here we report that airborne transmission of AN1, as previously reported for A/Shanghai/2/2013, can occur between ferrets. Keeping in mind that quantitating transmission in our current experimental model is difficult and that any transmission may not be directly extrapolated to transmission between humans, these data suggest that AN1 transmission is more efficient than that for avian influenza viruses (which are not airborne transmitted in ferrets) but less robust—with fewer animals becoming infected and less and delayed virus shedding—as compared with seasonal and pandemic A/H1N1 virus transmission. Influenza viruses carrying human adaptation markers can arise in poultry. Fortunately, additional determinants beyond human receptor preference, such as the proposed increase in HA stability, are needed for avian influenza viruses to transmit efficiently in mammals. Increased understanding of the mechanisms and molecular determinants that facilitate crossing the species barrier and airborne transmission of avian influenza viruses between mammals is urgently needed.

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Transmission bottlenecks in the ferret model differ between respiratory droplet and contact transmission routes

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Background: The ferret is a favored model for influenza transmission. It is believed that viruses capable of sustained transmission between humans also transmit efficiently between ferrets housed in adjacent cages, termed the respiratory droplet (RD) route. Other viruses (eg, some swine influenza strains) infect ferrets but only transmit to animals in the same cage. The significance of this direct contact transmission route for the epidemiology of human influenza is not clear. To better understand the dynamics of virus transmission in the two ferret models, we attempted to estimate the number of influenza viruses that are transferred from one animal to the next at the point of transmission. Materials and Methods: We used reverse genetics to engineer a synonymous nucleotide change as a tag into the genome of a recombinant influenza A pH1N1 2009 virus. We showed that the tag did not affect virus replication in vitro in MDCK cells or in highly differentiated cultures of human airway epithelium. We then mixed the tagged virus with the wild-type recombinant untagged strain at a known ratio and infected donor ferrets. Sentinel animals were exposed at days 1-2 after infection by direct contact (cohousing) or RD (adjacent cages) routes, and the ratio of genotypes detected in their nasal wash virus was quantified by pyrosequencing for the tag. Results: Using pyrosequencing, we were able to show that the ratio of the two genotypes remained constant for the duration of viral shedding from the nose of the donor animals, suggesting no fitness consequence of the tag in vivo. In all cases of RD transmission, we saw purifying transmission of only one of the two genotypes. The data suggest that the number of particles that initiate infection in RD sentinel animals is very low, less than 5. In contrast, where transmission occurred by contact between cohoused animals, we found evidence for a less stringent bottleneck, whereby the minority variant was transmitted in addition to the majority genotype. This mixed population of virus was sustained through at least one further contact transmission event in a chain of transmission. Bearing this in mind, we tested the transmission of a mixture of oseltamivir-resistant and -sensitive virus by both routes. This pair of viruses were based on a 2010 isolate from the United Kingdom and was reverse genetically engineered so they

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only differed at H275Y in the NA gene, a mutation known to confer oseltamivir resistance. In human airway cultures, the drug-sensitive wild-type virus outcompeted the resistant virus within 24 hours. In vivo during transmission events, we found evidence that drug-resistant variants could be transmitted under contact transmission conditions but were outcompeted by wild-type drug-sensitive virus during RD transmission. Conclusions: The number of influenza viruses that cross into the new host at transmission was greater when transmission was by the direct contact route than when it was through the air. These data have implications for understanding how transmission impacts on influenza evolution, as well as leading to a better characterization of the ferret model of transmission and how it relates to circulation of the virus in humans.

O-868

Efficacy of surgical face masks in reducing influenza virus dissemination in human exhaled breath

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Background: The use of surgical face masks is one of the most widely recognized nonpharmaceutical interventions used during epidemics of respiratory infections. However, little is known about the efficacy of face masks in preventing the dissemination of respiratory viruses in exhaled breath to the environment as a source control. The objective of our study was to measure influenza virus concentration in the exhaled breath of outpatients with influenza virus infection in Hong Kong and to estimate the effectiveness of surgical face masks in reducing influenza virus dissemination. Materials and Methods: Patients with acute respiratory illness (ARI) were recruited in an outpatient clinic in January-April 2013. A rapid diagnostic test (Sofia influenza A+B fluorescent immunoassay analyzer; Quidel, San Diego, California) was used to identify patients with influenza virus infection. Those with positive rapid test results were subjected to exhaled breath collection for 30 minutes. Each patient was randomly assigned to either wear a surgical face mask (Lite One procedure mask; Kimberly-Clark, Roswell, Georgia) during collection or not, and each patient was invited to stay for a second exhaled breath combination of the alternate type. Influenza virus in exhaled breath was captured by a Gesundheit-II (G-II), a device specifically designed to collect human exhaled breath and reported to be 85% efficient for collecting particles > 50 nm in diameter and comparable to the SKC BioSampler for detection and recovery of influenza A/PR/8/34 H1N1 by RT-PCR and culture (McDevitt et al., 2013 Aerosol Sci Tech; Milton et al., 2013 PLoS Pathog). The G-II device collected exhaled breath particles in two size fractions, ≥ 5 μm (coarse) and < 5 μm (fine). After completion of exhaled breath sampling, patients were followed up for a further 10 days to measure course of illness, mask-wearing behavior, and transmission to other household members. Nose swabs, throat swabs, and exhaled breath samples were tested by RT-PCR, and quantitative viral culture results are pending. Results: A total of 78 participants were recruited and tested using the rapid test. Fifteen (19%) were influenza positive (A:12, B:3) by the rapid test. Of these 15 positive patients, the median age was 35 years (range, 12-65) and 27% were male. Twenty exhaled breath samples were collected from the 15 participants, including 11 samples with a surgical mask and 9 samples without a surgical mask. Influenza A virus infection was confirmed by RT-PCR in nasal swabs (geometric mean viral load, 6.99 $\log_{10}$ copies/mL) in 8 patients, whereas no patients had confirmed influenza B. Seven of 8 of these confirmed cases were A(H1N1)pdm09. Influenza A virus was detected in 2 of 5 patients (4.45, 3.49 $\log_{10}$ copies/mL) in the fine fraction of the exhaled breath sample without wearing a surgical mask compared with 2 of 5 (3.22, 3.00 $\log_{10}$ copies/mL) with a mask. Influenza A virus was detected in 2 of 5 (3.31, 2.14 $\log_{10}$ copies/mL) in the coarse fraction of the exhaled breath sample without wearing mask compared with 0

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of 5 samples with a surgical mask. There was a high correlation (r = 0.82) between viral load in nasal vs throat swabs but a moderate correlation in viral load in nasal swabs vs coarse (r = 0.47) or fine (r = 0.52) particles. Three of 8 patients (38%) with PCR-confirmed influenza A returned symptom diaries, and one patient with undetectable virus in exhaled breath reported symptomatic illness in a household member during the follow-up period. Conclusions: In our preliminary analysis of exhaled breath in the first 15 patients, we were underpowered to identify a significant difference between virus concentrations in exhaled breath with and without surgical masks, although the point estimates were consistent with the effect previously reported in a study in the United States (Milton et al., 2013 PLoS Pathog). Further patient recruitment during the coming summer influenza season should increase the power to identify differences and may allow us to identify differences in viral loads for patients infected in the winter epidemics compared with summer epidemics of influenza in Hong Kong.

O-869 SCHOLARSHIP Promising Investigator Scholarship

Household transmission of influenza in Nicaragua

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Background: Few data exist on the epidemiology and transmission of influenza in tropical, poor countries. Materials and Methods: The Nicaraguan Household Transmission of Influenza Study is a case-based ascertainment study of influenza transmission in Managua, Nicaragua. Index influenza patients who are positive for influenza by rapid test within 48 hours of illness onset and are the only person in their household who has had influenza-like symptoms in the previous 2 weeks were identified and enrolled. The index case's household is then visited by study personnel that day, and all household members are invited to participate in the study. At enrollment, study staff record detailed information about clinical history, socioeconomic status, and individual and household risk factors and collect a respiratory sample. Study staff then visit the household every other day to collect clinical information and a respiratory sample for a total of five visits (≈ 12 days). A blood sample is collected at enrollment and 30-45 days later to assess influenza infection and nutritional status. Results: To date, we have enrolled 124 participants from 21 households. Study acceptance among household contacts has been consistently high, and 94.2% of the potential participants approached gave consent for participation in the study. Participants ranged in age from 0 to 65 years old, with a mean age of 14 years. Three (14.3%) households had a dirt floor and 6 (28.6%) households had an inside water tap. Nine (42.9%) households had 1 or more smokers. Thirty-five percent of participants had exposure to household animals, including 13.4% to chickens, 8.9% to ducks, and 12.9% to pigs. A total of 459 respiratory samples have been collected during household visits. The average number of respiratory samples for participants who had completed the initial 12 day follow-up period to date was 4.5. Seventy-four of 124 participants contributed an enrollment and end of study blood sample. Of those that had not contributed a blood sample, 47 were recently enrolled and so had yet to reach the end of the study, 1 was under the age of 6 months, and 2 were lost to follow-up. Of the 16 index patients with RT-PCR results up to date, 15 tested positive for influenza by RT-PCR. Of the 71 household contacts of influenza-positive individuals that had completed the initial 12 day period of study participation, 12 were RT-PCR-positive for influenza virus during the 12-day follow-up period, yielding a secondary attack rate of 16.9%. Of the 12 RT-PCR-positive contacts, 10 had fever and/or respiratory symptoms. Conclusions: This study will provide important information about the transmission of influenza in a tropical urban area.

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Transmission and Infection Control

Poster Abstracts

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GATES Fellowship

Availability, consistency and evidence-base of policies and guidelines on the use of mask and respirator to protect hospital health care workers: a global analysis

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Background: Currently there is an ongoing debate and limited evidence on the use of masks and respirators for the prevention of respiratory infections in healthcare workers (HCWs). This study aimed to examine available policies and guidelines around the use of masks and respirators in HCWs and to describe areas of consistency between guidelines, as well as gaps in the recommendations, with reference to the WHO and the CDC guidelines. Materials and Methods: Policies and guidelines related to mask and respirator use for the prevention of influenza, SARS, and TB were examined. Guidelines from the World Health Organization (WHO), the Center for Disease Control and Prevention (CDC), 3 high-income countries, and 6 low/middle-income countries were selected. Results: Uniform recommendations are made by the WHO and the CDC in regards to protecting HCWs against seasonal influenza (a mask for low risk situations and a respirator for high-risk situations) and TB (use of a respirator). However, for pandemic influenza and SARS, the WHO recommends mask use in low risk and respirators in high-risk situations, whereas, the CDC recommends respirators in both low- and high-risk situations. Among the 9 countries reviewed, there are variations in the recommendations for all 3 diseases. While, some countries align with the WHO recommendations, others align with those made by the CDC. The choice of respirator and the level of filtering ability vary amongst the guidelines and the different diseases. Lastly, none of the policies discuss reuse, extended use or the use of cloth masks. Conclusion: Currently, there are significant variations in the policies and recommendations around mask and respirator use for protection against influenza, SARS, and TB. These differences may reflect the scarcity of level-one evidence available to inform policy development. The lack of any guidelines on the use of cloth masks, despite widespread use in many low and middle-income countries, remains a policy gap. Health organizations and countries should jointly evaluate the available evidence, prioritize research to inform evidence gaps, and develop consistent policy on masks and respirator use in the health care setting.

P1-417

GATES Fellowship

Use of cloth masks in the practice of infection control—evidence and policy gaps

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Background: Cloth masks are commonly used in low- and middle-income countries. Currently there is a lack of evidence on the efficacy of cloth masks. It is generally believed that the primary purpose of cloth masks is to prevent spread of infections from the wearer. However, historical evidence shows that they have previously been used to protect health care workers (HCWs) from respiratory infections. Most studies on cloth masks were conducted in the first half of the 20th century. After the development of disposable surgical masks in the 1960s, very few studies have been conducted subsequently, and to date there have been no RCT of cloth masks. The few available studies on cloth masks are either descriptive or in vitro. The aim of this review was to examine the evidence on the efficacy of cloth masks and discuss the use of cloth masks as a mode of protection from infections in HCWs. We also reviewed the various approaches implemented to try and improve the effectiveness

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of cloth masks, for example, type of fabric, masks design and face fit. Materials and Methods: We searched available studies related to the use of cloth masks via MEDLINE and EMBASE with the help of key words. After reviewing the titles and abstracts, only studies that examined the use of cloth masks (in a controlled, community, laboratory or hospital setting) were selected for this review. Initially 32 studies fulfilled the selection criteria. After reading the full article, 19 studies were selected for this review. Results: The available literature suggests that cloth masks may provide some protection and reduce exposure to respiratory aerosols. The following factors were highlighted in studies in regard to the filtration capacity of a cloth mask: (1) Closeness of the gauze/cloth threads: The filtration capacity improved when the number of threads increased in the gauze and the mesh became finer, compared with coarse gauze with lower thread counts; (2) Number of gauze/cloth layers: The number of layers was found to be directly proportional to the filtration capacity in most of the laboratory studies; and (3) Type of gauze/cloth: Certain types of cloth provided better protection than others, eg, fine muslin (loosely woven cotton fabric) was better than gauze. In the studies reviewed, the filtration effectiveness significantly improved with increasing number of layers in the mask. The design of a mask was also important and some designs were more effective than others, particularly those with a tight seal around the face. Conclusions: Despite common use, recommendations on cloth masks to protect against respiratory virus transmission are currently not made in any publicly available guidelines. Research is required to test the clinical efficacy of cloth masks. Other future research questions should include filtration efficacy, length of use, methods of decontamination and fit testing. The use of cloth masks should be addressed in policy documents to inform best practice in low- and middle-income countries.

P1-418

Impact of live poultry market closure in the control of human infection with avian influenza A(H7N9) virus

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Background: A novel influenza A(H7N9) virus has emerged in China, causing serious disease in more than 125 individuals. The virus appears to be predominantly spread by interspecies zoonotic transmission. Live poultry markets in the provincial capitals of Shanghai, Hangzhou, and Nanjing, where the majority of cases have occurred, were swiftly closed as a precautionary public health measure. Our objective was to assess the effectiveness of market closure in reducing the risk of human infection with influenza A(H7N9) virus. Materials and Methods: We used data on the illness onset dates and geographical locations of all laboratory-confirmed A(H7N9) cases that were officially announced by 28 April 2013. We constructed a statistical model to explain the patterns in incident cases reported in each city based on the assumption of a constant force of infection prior to closure and a different constant force of infection after closure. Within the model, we were also able to estimate the distribution of delays between infection and illness onset (ie, the incubation period). We fitted the model using Markov chain Monte Carlo. Results: In total, 68 of 125 cases occurred in Shanghai, Hanghzhou, and Nanjing and surrounding areas. Closure of the live poultry markets reduced the risk of human infections by 92% in each of the three cities. Given that live poultry markets were the predominant source of H7N9 exposure, we estimated the mean incubation period to be 2.4 days (95% CI, 1.1-4.6 days). Conclusions: Our findings demonstrated that closure of live poultry markets in the three provincial capitals substantially reduced the risk of laboratory-confirmed human infection with avian influenza A(H7N9) virus. Incidence of cases fell dramatically within 2-3 days of closure, indicating (1) the primary role of live poultry markets and live poultry exposure in driving the

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risk of influenza A(H7N9) virus infection in urban areas and (2) the relatively short incubation period. If the H7N9 epizootic/epidemic continues, live poultry market closure should be immediately implemented and sustained in all affected and at-risk areas. In the longer term, evidence-based discussions and deliberations about the role of central slaughtering of all live poultry should be renewed.

P1-419

Detonation nanodiamond materials as the sorbents for influenza human and bird viruses

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Background: One of the ways to prevent the transmission of an infection is to create an effective barrier for the micropathogens. Viruses, being in majority cases nano-sized objects, are rather hard-to-catch particles even for the modern sorbents . This work continues the serial investigations on the search of the effective sorbent for viruses via nano materials of a different origin. The Detonation Nanodiamond Materials and their derivatives were chosen due to their low toxicity, reasonable cost, and the fact that they are have been already studied by the scientists for different potential application in the medicine. We have investigated along with the base Detonation Nano Diamonds (DND), the modified detonation nanodiamond materials, such as graphitized, animated, and chlorinated DND. Materials and Methods: Viruses: pandemic strains A/California/07/09, A/South Caroline/02/2010 (H1N1)pdm09, reference strains A/Wisconsin/67/05 (H3N2), B/Florida/04/06, A/duck/Primorie/ 2621/01 (H5N2). The viruses were grown in chicken embryonic eggs or tissue culture cell MDCK, concentrated by ultracentrifugation, then diluted in physiological solution (PS). The hemagglutination test has been used as criteria of virus interaction with sorbents in solutions. The fragments of cDNA were obtained in PCR reaction from influenza A and B virus. DND were given by company SINTA (Minsk, Belarus). Animated, chlorinated, graphitized DND materials were investigated by electron microscopy and infrared spectroscopy. The interaction between viruses and adsorbents was investigated by the method described in (VT Ivanova, MV Ivanova, BV Spitsyn, et al. Interaction of nanodiamonds materials with influenza viruses. Journal of Physics: Conference Series 345 (2012) 012019 doi:10.1088/1742-6596/345/1/012019). Results: The DND materials can interact with biological objects due to atoms O,S.N,H presented on surface (A. M. Schrand, S. A Hens, O. A. Shenderova, Nanodiamonds particles: properties and perspectives for bioapplications, Critical reviews in solid and material sciences 34, 1-2, 18-74 [2009]) and due to DND ability to collect into clusters. Each type of the examined DNDs interacted with influenza viruses and cDNA. The hemagglutination titers of virus solutions decreased up to ≤ 500 times for concentrated viruses diluted in PS and up to ≤ 8 times for allantoic viruses. The efficiency of viral sorption depended on the sorbent concentration, different impurities (presence of different proteins, like in allantoic viruses) and the type of modification of DND. The sorption does not noticeably changes in temperature range 4º-36º C. The intensive sorption takes place during first 15 minutes of contact with sorbents. The desorption of viruses from DND into PS within 48 hours was not observed. Complex DND + virus after immunization of the rats resulted the antibodies to virus in titer 80 in HI test. Conclusion: It was established that nanodiamond materials can be used as the sorbents for influenza human and bird viruses for decontamination of these viruses from water solutions and for preparation of new diagnostic systems. In case the solution is decontaminated by proteins and other substances of bigger size (compared with viruses), the preliminary purification is recommended. The mix of different types of DND can be used to make the sorption ability more universal. Our investigations were supported by the Center for Disease Control and Prevention, Atlanta, United States, CoAg: U51POOO527-02, the grant of President Russia Federation YC -3937.2013.3.

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Modes of transmission of influenza A and B viruses in households in Hong Kong and Bangkok

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Background: Influenza A and B viruses spread between humans through contact, large respiratory droplets, and small particle droplet nuclei (aerosols). However, the relative importance of these three modes of transmission has not been established. Materials and Methods: Large randomized controlled trials were conducted in Hong Kong and Bangkok during 2008-2011 to investigate the efficacy of enhancing hand hygiene and wearing surgical face masks in reducing transmission of influenza in households. In each study, index patients who reported at least two symptoms of respiratory illness and lived with at least two other household members of whom none had reported upper respiratory tract infections in the preceding 14 days were enrolled from local outpatient clinics. Rapid diagnostic tests were used to determine influenza virus infection, and index patients with a positive rapid test result and their household contacts were randomly allocated into 1 of 3 study groups (control with health education only, control plus hand hygiene, or control plus hand hygiene plus surgical face masks) and intensively followed up for approximately 7 days. Daily symptoms were reported by all participants, and nasal and throat swab specimens were collected from each participant for laboratory confirmation of influenza A and B virus infection by reverse transcriptase polymerase chain reaction (RT-PCR). Using data collected in this study, we constructed a mathematical model that accounted for the alternative modes of transmission and used it to infer the relative importance of alternative modes of transmission assuming that the risk of fever plus cough varied depending on the mode of transmission. Analyses were stratified by influenza type. Results: There were 275 and 507 households in Hong Kong and Bangkok, respectively, with an index patient with confirmed influenza A and 104 and 113 with confirmed influenza B, respectively. Average household sizes in Hong Kong and Bangkok were 3.0 and 2.5, respectively. The risks of confirmed secondary infection were 10% and 19% overall in Hong Kong and Bangkok and were similar by influenza type. When we plotted the cumulative incidence of confirmed influenza infections reporting fever plus cough among household contacts, we identified an increase in intervention groups that was particularly apparent in the households in Bangkok. These changes are consistent with our hypothesis that hand hygiene and face mask interventions would reduce contact and large droplet (but not aerosol) transmission, increasing the contribution of aerosol transmission in the intervention arms, which consequently led to an increased proportion with fever plus cough among the confirmed influenza virus infections in household contacts in the intervention arms. Under an example scenario in which randomization to the hand hygiene intervention reduced contact transmission by 50% and randomization to face masks plus hand hygiene reduced both contact and droplet transmission by 50%, we estimated that (1) for influenza A, aerosol transmission contributed to 51% and 42% of household transmission events in Hong Kong and Bangkok, respectively, in the absence of interventions and (2) for influenza B, aerosol transmission contributed to 37% and 26% of household transmission events in Hong Kong and Bangkok, respectively, in the absence of interventions. Conclusions: Our model indicated that aerosol transmission is an important mode of transmission for influenza A and B viruses in households. This implies that measures to reduce transmission by contact or large droplets may not be sufficient to control influenza virus transmission in households. The importance of aerosols for transmission of influenza B appears slightly lower than its importance for influenza A transmission. If aerosol transmission is less important for influenza B, interventions against contact transmission such as hand hygiene may be more effective against influenza B than influenza A. Our findings were derived from households in tropical and subtropical settings, but the proposed framework can be used to assess transmission modes in other climates and settings.

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Driving factors of influenza transmission in the Netherlands

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Background: Influenza epidemics in temperate regions show a characteristic seasonal pattern with peak incidence occurring in winter. Despite decades of research, the mechanisms that drive influenza seasonality in temperate regions remain poorly understood. Previous research showed that low absolute humidity and school holiday can both affect influenza transmission. During an epidemic, transmission is additionally strongly influenced by the depletion of susceptibles. Materials and Methods: To assess how much variability in influenza transmission intensity is due to each of these driving factors, we analyzed a time series of weekly visits to the general practitioner related to influenza-like illness in the Netherlands spanning the period 1970-2011. In our method we first transform this time series into weekly effective reproduction numbers, defined as the number of secondary infections produced by a typical infective individual. The reproduction number is used as measure of the transmission intensity of influenza, which we then decompose it into biological relevant variables. This decomposition is translated into a regression equation while retaining the biological interpretation of the variables. A test against simulated data showed that our method is able to identify factors that influence transmission. We then quantify how the reproduction number is affected by each driving factor. Results: A model that included the depletion of susceptibles, between season effects, and absolute humidity could explain 60% of the variance in influenza reproduction numbers. We found a clear ranking of importance of driving factors: most variation (30%) is explained by the depletion of susceptibles during the season, between-season effects explained 27%, and 3% of the variation was explained by absolute humidity. Christmas school holidays did not have a statistically significant effect on influenza transmission. A permutation test where we correlated reproduction numbers against randomized weather data showed that there was no confounding by season in the effect of absolute humidity. Conclusions: The percentage of variance of the influenza reproduction number that is explained by absolute humidity is relatively small (3%).The regular seasonal fluctuations of absolute humidity, combined with the low value of the influenza reproduction number (hovering around unity), helps to explain why influenza epidemics in summer are unlikely (the influenza reproduction number is lower than one) and why likelihood of influenza epidemics increases during autumn and winter (the influenza reproduction number is above one).The large part of the variance in the influenza reproduction numbers explained by the depletion of susceptibles confirms that once an epidemic is going, it is strongly governed by internal transmission dynamics, as should be expected for immunizing infections such as influenza.

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Impact of international and internal travel restrictions on the spread of human influenza: a systematic review

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Background: Travel restrictions have been recommended by the World Health Organization (WHO) for the rapid containment of pandemic influenza. The effectiveness of these interventions has been assessed by different authors mainly through mathematical modelling scenarios. An evaluation of evidence currently available in the scientific and grey literature is essential to inform public health policy regarding the control of influenza. The primary aim of this study was to conduct a systematic review on the effectiveness of internal and international travel restrictions for the rapid containment of human influenza. Materials and Methods: This study was conducted according to the PRISMA statement. The protocol was registered with PROSPERO prior to executing our literature search strategy. Health care databases (PubMed, Medline, EMBASE, CINAHL, and WHO Medical Global Index) and sources of grey literature were searched by two researchers. We screened all records against the protocol eligibility criteria by title, abstract, and full text. Data extraction and risk of bias assessments were undertaken in duplicate using a piloted template. We developed and applied a novel risk of bias tool for mathematical modelling studies. Results were synthesised narratively according to a framework by the Economic and Social Research Council. Results: Eighteen mathematical modelling studies, one time-series analysis, one systematic review, and two literature reviews met the protocol eligibility criteria for inclusion. The risk of bias varied across studies. The main biases we observed were assumptions made regarding the transmissibility of strains of influenza virus (R_0), susceptibility of populations, and seasonality. The impact of travel restrictions in the magnitude of influenza epidemics (eg, attack rate [AR]) was minimal. The maximum AR reduction did not exceed 0.1% when 95% internal travel restrictions (domestic land and air travel) were implemented for up to four weeks. Limited impact was observed in delaying the spread and the peak of influenza epidemics. Internal travel restrictions achieved a delay in the epidemic peak of only one week, which increased to up to three weeks when combined with national border controls. International travel restrictions were associated with negligible or limited impact (< 2%) on AR across the included studies. Delays up to two weeks to reach the epidemic peak were estimated for restrictions up to 90% and R_0 = 1.7. Air travel restrictions of 80% and 90% only delayed spread between 3 and 14 days, respectively when R_0 = 1.7. Near total bans (ie, 99.9% air travel restrictions across all infected countries) produced an estimated delay of between 3 and 4 months in epidemic peak. However, this was not deemed feasible by most authors due to the potential impact on global travel and trade. Selective restriction of travel by specific age groups did not reduce the spread of influenza. However, targeted air travel restrictions on specific routes were more effective than general air travel restrictions by 19% in reducing the number of infected travellers. Travel restrictions were most effective when implemented within two weeks of the first case of an epidemic. However, the impact of travel restrictions was reduced when interventions were implemented more than 45 days after the notification of influenza epidemics and if influenza strains involved were of high transmissibility (R_0 > 1.9). Additionally, models suggested that the effectiveness of international travel restrictions were more limited when the geographical source of influenza epidemics were large urban hubs with a high population density and extensive links to the global travel network. Conclusions: Internal and international travel restrictions will have limited impact on the rapid containment of human influenza. The maximum delay observed in spread of influenza epidemics was four months for international interventions. However, the extent of travel restrictions required to achieve this estimate would be unfeasible due to the negative economic and social impact caused. Our systematic review indicated that travel restrictions are not effective for the containment of human influenza.

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The impact of neuraminidase inhibitors on influenza transmission: a systematic review and meta-analysis

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Background: Treatment of individuals infected with influenza and prophylaxis of their contacts with antiviral drugs are considered important to reduce transmissibility and spread. These measures are currently advocated by the World Health Organization (WHO) for rapid containment. However, systematic reviews have considered only randomised controlled trials evaluating the efficacy of

neuraminidase inhibitors for prophylaxis mainly against seasonal influenza. An evaluation of all available evidence from experimental and observational studies on seasonal, pandemic, and avian influenza is essential to inform public health policy. The primary aim of this study was to conduct a systematic review and meta-analysis on the effectiveness of neuraminidase inhibitors for preventing community transmission of influenza. Materials and Methods: This study was conducted according to the PRISMA statement, and the protocol was registered with PROSPERO prior to executing the literature search strategy. Two researchers searched health care databases (MEDLINE, EMBASE, CINAHL, Cochrane Library, PubMed, and WHO Medical Global Index) and sources of grey literature. All retrieved records were screened against the protocol eligibility criteria. Data extraction and risk of bias assessments were undertaken in duplicate using a piloted template. Results were synthesised narratively. Meta-analyses of pooled odds ratios (including 95% CIs), including tests for heterogeneity, publication bias, and sensitivity analyses, were conducted. Results: Nine randomised controlled trials, eight observational studies, and seven systematic reviews met the protocol eligibility criteria. Risk of bias varied across studies. The main sources of bias were assumptions made regarding sample size calculations, variation in vaccination status of study participants, susceptibility of populations, and influenza type. Narrative synthesis showed that oseltamivir and zanamivir provided protective efficacy of 58% to 84% for households and 67% to 89% for individuals following postexposure prophylaxis (PEP) with or without treatment of the index patient. Larger effects were observed when medication was commenced within 24 to 48 hours of symptom onset. The typical reduction in secondary attack rate associated with treatment of the index patient alone was 40%. However, this result is based on few studies. Short-term pre-exposure prophylaxis of individuals was found to be of uncertain benefit. Long-term prophylaxis (≥ 28 days) showed strong evidence of individual protection, which ranged between 74% and 87%. Meta-analyses of individual protection showed that the pooled odds of laboratory-confirmed influenza was statistically significantly lower compared with placebo or no therapy following PEP with oseltamivir (n = 8 studies; odds ratio [OR] = 0.11; 95% CI = 0.06 to 0.20; $p < 0.001$; $I^2 = 58.7\%$) and zanamivir (n = 4 studies; OR = 0.23; 95% CI = 0.16 to 0.35; $p < 0.001$; $I^2 = 0.0\%$). Sensitivity analyses of the oseltamivir data demonstrated pooled estimates comparable to the primary analysis from experimental studies of seasonal influenza (n = 4 studies; OR = 0.15; 95% CI = 0.07 to 0.33; $p < 0.001$; $I^2 = 37.3\%$) and observational studies of 2009 pandemic influenza A/H1N1 (n = 4 studies; OR = 0.09; 95% CI = 0.04 to 0.19; $p < 0.001$; $I^2 = 61.7\%$). Meta-analyses of household protection showed the pooled odds of laboratory-confirmed influenza was statistically significantly lower compared with placebo or no therapy following PEP with oseltamivir (n = 2 studies; OR = 0.23; 95% CI = 0.09 to 0.59; $p = 0.002$; $I^2 = 39.0\%$) and zanamivir (n = 2 studies; OR = 0.18; 95% CI = 0.11 to 0.31; $p < 0.001$; $I^2 = 0.0\%$). No evidence of publication bias was present in our meta-analyses. We found no evidence that neuraminidase inhibitors are effective in reducing wider community transmission as envisaged by the WHO rapid containment strategy. Conclusion: The evidence base indicates that oseltamivir and zanamivir are effective for PEP of individuals and households irrespective of treatment of the index patient and long-term pre-exposure prophylaxis. Sources of heterogeneity are present among the included studies, which may limit the generalizability of our findings. There are no data that support an effect on community transmission. This study was commissioned by the Global Influenza Programme, World Health Organization. The University of Nottingham Health Protection and Influenza Research Group (JSN-V-T, CRB, HEO, GNO) is an official WHO Collaborating Centre for pandemic influenza and research. It receives limited funding from WHO in support of specific activities.

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P1-424 G FELLOWSHIP GATES Fellowship

The role of community-acquired influenza patients and health care workers in transmitting influenza infections in tertiary hospitals in Bangladesh

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Background: The potential for novel influenza viruses to spread in hospital wards is a global concern, especially in resource-poor settings like Bangladesh, where inadequate infection control practices and limited personal and respiratory hygiene practices provide opportunities for airborne and droplet transmission of influenza viruses. Hospital transmission of influenza infection can cause serious health consequences, including fatal outcomes among patients with coinfections. Admitted patients, health care workers, and visitors have been previously reported as sources of influenza in hospitals worldwide. A study in Bangladesh reported influenza as the most commonly identified virus associated with hospital-acquired viral respiratory illness, but the source of the influenza virus infection was not investigated. Identifying the most common sources could help to develop effective infection control interventions to reduce influenza transmission in hospitals. The objective of this analysis was to identify clusters of influenza infection in Bangladeshi hospital wards using existing surveillance systems to investigate likely sources of influenza infection. Materials and Methods: We analyzed data collected from four adult and two pediatric medicine wards in two tertiary hospitals during June 2008-April 2011. Surveillance physicians in these wards identified patients admitted with fever and cough or sore throat with onset in the last 7 days as part of community-acquired influenza surveillance, as well as patients with new onset of cough, fever, rhinorrhea, or difficulty breathing ≥72 hours posthospital admission as part of hospital-acquired influenza surveillance. The physicians collected nasopharyngeal and throat swabs from patients who met these case definitions. Health care workers in these wards were asked to self-report new onset of respiratory illnesses to surveillance physicians who also collected nasopharyngeal and throat swabs from them. Swab specimens were tested for evidence of influenza RNA using real-time reverse transcriptase polymerase chain reaction; specimens with evidence of influenza A RNA were subtyped. We defined a cluster of influenza infection as identification of more than one person with the same influenza type and subtype occurring on the same ward within 7 days of one another. Clusters consisting of only patients with community-acquired infections were excluded from analysis because they do not represent hospital transmission events. Results: We identified 81 laboratory-confirmed influenza cases in the 6 study wards: 45 patients with community-acquired influenza, 34 hospital-acquired influenza infections, and two physicians with influenza. The median duration of hospitalization prior to onset of influenza among hospitalized patients was 6 days (IQR: 4-7). We identified 8 clusters, all occurring during May and/or June, involving 19 cases. In 4 clusters, hospitalized patients developed new-onset influenza within a week of hospitalization of community-acquired influenza patients. In another cluster, two physicians developed influenza infections that were followed within a week by a new influenza infection in a hospitalized patient. Three clusters consisted only of patients with hospital-acquired influenza. Twenty-three (68%) of 34 patients with hospital-acquired influenza were not associated with clusters. Four clusters were associated with influenza A/H3, two with influenza B, and one each with influenza A/H1 and influenza A(H1N1)pdm09. Four clusters occurred in pediatric wards, three in female medicine wards, and one occurred in a male medicine ward. Eighteen of 19 clustered cases recovered, and one left against medical advice. Conclusions: The majority of patients who acquired influenza infection in our study hospitals were not associated with concurrent hospitalizations of patients with community acquired-illness. Our surveillance for health care worker illnesses was not very sensitive because they were requested to self-report; therefore, there may be health care worker illness associated with these transmission events that we did not capture. Given the context of Bangladeshi hospitals where family caregivers and visitors outnumber patients, these community members could be the most likely source of influenza infection in hospital wards and a potential target for preventative interventions.

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Hand hygiene and risk of influenza virus infections in the community: a systematic review and meta-analysis

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Background: Community-based prevention strategies for seasonal and pandemic influenza are essential to minimize their potential threat to public health. Hand hygiene intervention is an appealing NPI because it is straightforward and can be applied in both developed and developing regions at low cost. For influenza virus transmission, the relative importance of small droplet (aerosol), large droplet, and contact transmission is controversial, although the potential for efficacy of hand hygiene implicitly requires that direct or indirect contact is an important mode of transmission. Because small droplet transmission is enhanced by low or very high humidity, it has been hypothesized that in temperate zones with a cool and dry winter, influenza transmission is predominantly by aerosol, whereas in tropical zones with a warm and humid environment, the virus is more commonly transmitted by the contact route. If this hypothesis is correct, the effectiveness of hand hygiene interventions would be expected to vary by latitude, ambient temperature, and humidity. This systematic review and meta-analysis aimed to evaluate the hand hygiene intervention in preventing influenza virus transmission in a community and to investigate the possible effects of latitude, temperature, and humidity on the efficacy of hand hygiene in controlling influenza virus transmission. Materials and Methods: We searched for relevant studies in PubMed, MEDLINE, and EMBASE. We included any RCT comparing the effect of hand hygiene interventions with no intervention in reducing influenza virus transmission in community settings, in which study participants or cluster units in a population were assigned prospectively into intervention and control groups using random allocation. The primary outcome was the relative reduction in the number of influenza virus infections confirmed by reverse transcriptase polymerase chain reaction (RT-PCR), virus culture, or rapid antigen test in the hand hygiene intervention group compared with the control group. Results: We identified 936 articles in the initial database search, of which 46 were retrieved based on their title and abstract content and 10 were, finally, included for meta-analysis. For studies conducted in developed countries, hand hygiene was associated with a statistically significant 22% relative risk reduction for laboratory-confirmed influenza outcome in the pooled analysis (RR = 0.78; 95% CI = 0.64 to 0.96; I^2 = 0%; p = 0.02). There were only two studies conducted in less developed countries, and the efficacy of hand hygiene was not statistically significant in the pooled analysis. In a meta-regression model, we found that a 10 percentage point rise in relative humidity was not associated with a statistically significant change in the efficacy of hand hygiene (RRR = 0.68; 95% CI = 0.41 to 1.14; p = 0.14) in developed countries, but the direction of the estimate was consistent with the hypothesis stated above. Conclusions: Hand hygiene interventions are an effective low-cost intervention to reduce the risk of influenza virus transmission. We did not identify a statistically significant effect of ambient humidity on the efficacy of hand hygiene interventions in controlling influenza transmission in developed countries.

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Transmission of influenza infection within households or educational facilities (elementary schools and preschools) and assessment of virus titers over time by TCID50 assay in children treated with NA inhibitors

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Background: An influenza epidemic in a community is greatly affected by household or school transmission. It is important to clarify the dynamics of influenza transmission in a population and take precautions to control the spread of infection. The most basic strategy to prevent influenza exposure is patient isolation. In Japan, influenza-infected schoolchildren are required to stay at home and to be absent from educational facilities until 5 days after onset of symptoms, and 2 days after fever has disappeared. We conducted epidemiologic studies and evaluated the timing of disappearance of the virus to validate the isolation period. Materials and Methods: A total of 2398 patients infected with influenza A from 1931 families were analyzed to estimate family infection rate. Epidemic status of 615 affected children aged 7-12 years in 195 classrooms of elementary schools and 143 affected children aged 0-6 years in 42 classrooms of preschools was investigated using data collected during the 12 previous seasons in 2001 and 2002. Moreover, we identified 55 patients aged 3-12 years as positive for influenza A by rapid diagnostic test within the first 48 h of fever onset during the 2012-13 season. The patients were randomly assigned to oseltamivir (n = 16), zanamivir (n = 12), peramivir (n = 16), or

laninamivir (n = 11) groups and were analyzed for virus levels in nasal secretions measured by TCID50 assay from the first day of treatment to the first day of negative result. The impact of age and treatment on the duration until defervescence and until loss of virus after initiation of treatment, and the duration from defervescence to loss of virus, was assessed. Results: The mean family infection rate was 8.9%. In cases of the index patients aged 0-6 and 7-12 years, the transmission rate to the family members of all ages was 12.5% and 7.7%, respectively, and the transmission rate to the same generation was 21.1% and 12.8%. The preschool investigation showed that a) 21.0% of the patients had no influence on transmission, b) 15.9% of the patients introduced the virus into a classroom, c) 61.6% of the patients were infected from earlier-infected patients at early phase of infection, and d) 1.4% of the patients were infected from earlier-infected patients after their recovery. At elementary school, the rates for a-d were 30.4%, 16.0%, 48.4%, and 5.2%, respectively. A statistically significant difference in the duration until loss of virus and until defervescence after initiation of treatment was detected between patients aged 3-6 years (n = 27) and those aged 7-12 years (n = 28). However, no difference in the duration from defervescence to loss of virus was observed between the groups. Also, we found a different treatment effect of the anti-influenza agents, but a significant difference was not observed. Conclusions: The highest transmission rate was observed in schoolchildren aged 0-6 years, suggesting that the virus spread among the children once it was introduced into a classroom. We also verified that a 2-day isolation period after defervescence was effective because fewer children were infected from recovered patients. Furthermore, we examined the virus titer over time and statisticall ysignificant differences were found in the time from the start of treatment to loss of virus (median) and the duration until defervescence between patients aged 3-6 years and those aged 7-12 years (3-6 years: 4.13 and 1.69 days, 7-12 years: 2.90 and 0.75 days, respectively). However, no difference was observed in the duration from defervescence to loss of virus between the groups (2.50 and 1.71 days). Thus, the requirement for being absent for 2 days after fever disappeared was validated.

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Haemagglutination inhibiting antibodies and protection against seasonal and pandemic influenza infection

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Background: Haemagglutination inhibiting (HI) antibodies are considered to be the main mediator of immunity against influenza infection but do not fully explain observed patterns of infection. We examined the role of HI antibodies in protection from natural infection in 940 unvaccinated individuals prospectively studied over three influenza seasons. Methods: Influenza illness was confirmed by RT-PCR, and HI assays were performed on at least 494 serum pairs spanning each season. Results: Increasing preseason homologous HI titres was significantly associated with a reduced risk of infection for H3N2 (OR 0.61, 95% CI 0.44-0.84) and B (0.65, 95% CI 0.54-0.80) strains. Preseason homologous H1N1 HI titres were not significantly associated with the risk of infection with H1N1 strains, whether re-circulated (OR 0.90, 95% CI 0.71-1.15), new seasonal (OR 0.86, 95% CI 0.54-1.36), or pandemic 2009 (OR 0.77, 95% CI 0.40-1.49). Increasing age was however associated with reduced risk of seasonal and pandemic H1N1 infection (both $p < 0.0001$), and prior seasonal H1N1 infection reduced the risk of pandemic H1N1 infection (OR 0.23, 95% CI 0.08-0.62), even though prepandemic HI titres against A/California/04/2009-like virus were below 20 in 98% of participants. All participants with RT-PCR-confirmed H3N2 and B infection had a four-fold or greater rise in homologous HI titre, compared with only 66% of those with RT-PCR-confirmed H1N1 infection. Conclusions: We found that the risk of H1N1 infection decreased with increasing age and prior H1N1 infection, but the effect of preseason HI titre was at best small and weak compared with that of H3N2

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and B. This suggests that antibodies targeting non-HI epitopes may be more important mediators of infection-neutralizing immunity in H1N1 compared with other influenza subtypes. This phenomenon could in part explain the slower rate of antigenic evolution of the HA1 domain of the haemagglutinin protein of H1N1 compared with H3N2.

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Characterization of respiratory droplets from community-acquired and experimental laboratory influenza infection in humans

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Background: We characterized virus shedding into respiratory droplets by both experimental donor volunteers and persons with community-acquired influenza infection as part of the EMIT (Evaluating Modes of Influenza Transmission) project. The objective of this characterization was to validate the experimental human infection model by comparison of respiratory droplet shedding by the two groups. Materials and Methods: Patients with community-acquired influenza were recruited from University of Maryland students and staff and residents of the community surrounding the College Park, Maryland, campus. We screened 355 people from December 4, 2012 through March 15, 2013 who volunteered with ILI (self-reported fever and either cough or sore throat). We enrolled 178 patients (50%) in respiratory droplet collection who, at the time of screening, were within 3 days after onset of symptoms and met one of the following conditions: 1) temperature > 37.8°C when measured in the research clinic or by a referring health care provider or 3) a positive influenza rapid test performed in the research clinic or by the referring provider. NP swabs were collected from each participant. The experimentally infected donors were studied at the quarantine facility operated by Retroscreen Virology Ltd. and the University of Nottingham. Healthy seronegative adults (donors) were infected with GMP A/Wisconsin/67/2005 by nasal instillation. Droplet samples were collected for donors on days 1-4 following inoculation. The first two quarantines included 32 donors. We collected exhaled breath by seating the patient in front of the intake for a human-source bioaerosol sampler (G-II), which allows natural breathing, coughing, speaking, and singing during collection. Each sampling session lasted 30 minutes. Two particle size fractions of exhaled influenza viral particles were collected by the G-II, ≥ 5 µm (coarse) and < 5 µm (fine). The number of coughs was also recorded. Coarse samples were extracted and frozen. Fine samples were collected in a buffered liquid, concentrated, cultured (community-acquired cases only), and frozen. Samples will be subjected to RT-qPCR for influenza viruses. Results: Of the 178 enrolled patients with community-acquired influenza infection, 120 had a positive influenza rapid test (66 for influenza A, 50 for influenza B, 3 for A and B, and 1 for a test that was not type specific). Of the 58 enrolled based on fever alone, preliminary tests of NP swabs from 22 by real-time RT-PCR showed that 18 (82%) were positive for influenza A. The 178 enrolled participants include 87 females and 91 males ages 15 to 86 years, with a mean age of 22.9 years and median age of 20 years. The mean body temperature at time of screening and droplet collection for those recruited was 37.4°C, median 37.2°C, and range 36.3°C to 39.6°C. The number of spontaneous coughs during the 30-minute collection ranged from 0 to 265, with mean of 27 and median of 17 coughs. Sneezes ranged from 0 to 4, with mean of 0.08 and a median of 0 sneezes per 30-minute collection. The experimental donors studied as of the submission deadline (the first two of three quarantine events) included 10 women and 22 men, with mean age of 30.3 years, median of 28 years, and range of 20 to 45 years. The number of spontaneous coughs during the 30-minute collections ranged from 0 to 35, with a mean of 1.3 and median of zero. No sneezes were recorded from the experimental donors. Statistical analysis and results of PCR and viral culture of respiratory droplets will be presented. Conclusion: Patients with community-acquired influenza virus infection showed a trend toward more frequent cough compared with the experimentally infected participants. This project was funded by CDC Cooperative Agreement 1U01IP000497. The findings and conclusions in this report are those of the authors and do not necessarily represent the official position of the funding agency.

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Repeat isolations of swine H1N1 pdm09 viruses with undetectable neuraminidase activity

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Background: The pandemic influenza A 2009 virus (pH1N1) was soon detected in pigs following its emergence in humans as a result of human to pig transmission. Here we describe two viruses ($pH1N1_{low}$ 1 and 2) isolated from nasal swabs obtained from two different pig farms. These pigs showed clinical symptoms of influenza (coughing, thumping, nasal discharge, and fever). These viruses were of interest because we were unable to detect any viral neuraminidase (NA) activity despite evidence for their spread within the infected herds. Materials and Methods: Viruses were propagated in swine testes epithelial (STE) cells and quantified by plaque assay using Madin-Darby canine kidney (MDCK) cells. NA activity was measured by serially diluting virus and detecting enzymatic activity via two methods, a fluorometric assay using MU-NANA as a substrate, and a colorimetric assay using fetuin as a substrate. Amounts of NA were determined using SDS-PAGE with clarified cell culture supernatants from infected STE cells. Binding of viruses to sialic acids was measured by solid-phase direct virus-binding assay by adding virus to fetuin immobilized on a solid phase followed by biotinylated glycans. Binding was measured colorimetrically using horseradish peroxidase-conjugated streptavidin and TMB substrate. Mammalian transmission experiments using ferrets were conducted by lightly anesthetizing 2 donor ferrets with isoflurane and inoculating them with 10^6 plaque forming units of virus in 0.5 mL of PBS. The next day donor ferrets were moved into separate cages with one direct contact and one aerosol contact ferret. The ferrets remained housed together to day 20 postinfection. Nasal washes were collected on days 3, 5, 7, 9, and 11 by flushing nostrils with a total of 1.0 mL of PBS, and viral titers were determined in MDCK cells. Results: The $pH1N1_{low}$ viruses showed almost undetectable NA activity, using both MU-NANA and fetuin as substrates. Compared with a control swine virus, a pH1N1 virus with 98% homology and significantly greater NA activity (pH1N1), there were no significant differences in growth kinetics in MDCK cells or in amounts of NA protein incorporated into virions as measured by SDS-PAGE. Somewhat unexpectedly, both $pH1N1_{low}$ viruses showed increased binding affinity to both α2,3- and α2,6-sialic acids compared with the control pH1N1 virus despite their low NA activity. In ferrets, the $pH1N1_{low}$ viruses were shed from donor ferrets up to 5 days postinfection but were not detected in direct contact or aerosol contact animals. The control pH1N1 virus was shed from donor ferrets up to 7 days postinfection and was also shed from direct contact and aerosol contact animals. Conclusions: The replication of $pH1N1_{low}$ viruses in vitro was similar to that of pH1N1, despite almost undetectable NA activity. $pH1N1_{low}$ was also shed from ferrets but transmission was abrogated. This study suggested that NA activity is important in supporting transmissibility of pH1N1 viruses between ferrets but may be less important for transmission in swine.

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Ancient investigation of yet unresolved questions on influenza-like illness: the experiments on prisoners during the Ming dynasty in China

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Background: The earliest descriptions of influenza-like illness (ILI) were written by Hippocrates, the first author to advocate that disease was not the result of supernatural forces. With few exceptions, ancient texts were the predominant source of knowledge in the Western world for more than 1300 years. Manipulative experimentation on respiratory diseases geared up only in the 19th century. Here we present a much earlier description of an experimental investigation that took place in the 15th century during the Ming Dynasty in China. This is the first known experimental investigation of respiratory infections. Despite its antiquity, it examined environmental drivers of the seasonality of ILI and other respiratory infections, an issue that remains unresolved nowadays. To our knowledge, this is also the first account of a manipulative experiment in the history of science. Materials and Methods: The bibliographical reference of this experiment is the book by Hong Zhang Liu and Bo Liu, 2006, Talk on Keeping Healthy by Imperial Doctor Liu, available at http://www.360doc.com/content/11/0319/23/6085925_102738542.shtml. There is no English version. Here we detail the ancient study as compared with current experimental methods. Results: To investigate the effects of thermal and nutritional regimens on respiratory infection incidence, official doctors in Nanjing designed a three-step procedure to induce prisoners to be sick with ILI during winter. Prisoners were randomly assigned to four test groups (A, B, C, and D), each including 50 individuals. Prisoners in group A were first administered oral limewater to cause lesions on their intestines and stomach, lowering digestive function. Next they were given only rice and vegetables to maintain a low nutritive status. Days afterwards, they were urged to step in and out of their warm prison rooms repeatedly to experience sharp changes in air temperature and humidity. Prisoners in group B were provided soup to increase digestive function followed by soup simmered with carp (vitamins and protein) to improve nutritional status. They were then asked to step in and out of their prison rooms repeatedly. Group C went through the same first two steps as group A but was urged to remain in a cell with sick prisoners for potentially infectious contacts. Group D went through the same first two steps as group B. They were next exposed to sick prisoners and stepped in and out of their warm prison rooms repeatedly. The authors reported that all prisoners in groups A and C developed ILI symptoms (fever, runny nose, and cough) within 3 days, whereas only a few prisoners in groups B and D showed ILI signs within the same period. Discussion: The experiments were motivated by the observation of seasonal respiratory disease and the potential of disease prevention and included four treatment groups. The experiments were conducted by imperial doctors in 1415-1416, four centuries ago. To our knowledge, this is the first manipulative experiment in the medical literature. In imperial China, the pressure for understanding disease mechanisms for therapeutic purposes was enormous because severe punishments could fall upon doctors who failed to please the emperor. Despite ethical issues, the experiments had an advanced design for the time, even providing valid information for questions that are not fully resolved now—such as the observation that nutrition has a noticeable effect on disease incidence. There are however limitations in the design (which, in fairness, might be due to the way information was recorded). First, we are reluctant to accept that outcomes were as sharply distinct as reported (“all” prisoners in groups A and C getting sick). Second, pseudoreplication cannot be ruled out because individuals from control and treatment groups may have been in more than one experimental batch. Still, this discovery constitutes an important mark in the history of scientific enquiry. It is also interesting to note that six centuries ago researchers were already investigating questions that remain open today. This description highlights the relatively slow progress in the understanding of the drivers of seasonal disease spread.

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Role of a hemagglutinin residue in the emergence of the 2009 H1N1 pandemic influenza virus

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Background: The 2009 H1N1 pandemic virus (H1N1pdm09) was a reassortant between a triple-reassortant swine (TRsw) H1N1/2-like virus and a Eurasian avian (EA)-like swine virus. Although the EA NA and M gene segments were required for airborne transmission of the H1N1pdm09 virus in ferrets, we noted that they alone were not sufficient to confer transmissibility to a North American TRsw H1N1 virus. We therefore hypothesized that additional HA changes were critical for the transmission phenotype of H1N1pdm09 virus and determined the impact of previously described HA mutations that differentially segregate in pandemic and swine H1 viruses. Materials and Methods: We created a panel of recombinant TRsw viruses with specific HA mutations and determined their phenotypes in vitro and in vivo. For determining transmissibility of the recombinant TRsw viruses, ferrets were inoculated intranasally with 10^6 EID_{50}/mL of each recombinant influenza virus and placed 24 hours later with naive direct and airborne contact animals. Transmission was determined by virus detection in nasal washes of contact animals. Virus receptor binding properties were measured by glycan array and a dose-dependent glycan binding assay. Results: Here we demonstrated that a single HA mutation distal to the receptor binding site of a TRsw H1N1 virus enhanced binding avidity to α2,6-sialic acid receptors, resulting in enhanced virus replication in vitro and increased contact transmission. Addition of the H1N1pdm09 NA and M gene segments to this virus resulted in airborne transmissible virus with properties similar to H1N1pdm09 viruses themselves. Conclusions: These results demonstrated that specific changes present in the HA of the H1N1pdm09 virus were important factors in its emergence.

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Investigation of a nosocomial influenza A(H3N2) outbreak among geriatric patients and health care workers

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Background: Hospital outbreaks are usually reported during the annual peak of community influenza activity. Infected patients, health care workers (HCW), or visitors can be sources of infection, transmission being facilitated by low vaccination coverage amongst HCW. Previous outbreak reports have shown that a precise epidemiological description of contacts combined with genotyping and

molecular phylogenetic analysis provide insights into routes of transmission. We conducted a prospective study during the 2011/2012 influenza season in the geriatric department of the Hôpital Edouard Herriot (Lyon, France). The aim was to analyze routes of transmission during the influenza outbreak among geriatric patients and HCW. Methods: From the 19th of February to the 15th of March, each patient included in the prospective study had a nasopharyngeal swab collected at the day of hospital admission. A second nasopharyngeal swab was collected in the case of development of signs of respiratory illness during hospitalization. Similarly, if HCW showed signs of respiratory illness, a specimen was collected. Samples were screened for influenza A/B, RSV, and human metapneumovirus and rhinovirus by real-time PCR. An infection was classified as nosocomial when symptoms were reported at least 72 hours after admission to the hospital. Results: Informed consent was obtained from all patients (n = 66) and HCW (n = 57). A total of 22 cases of influenza A(H3N2) were detected in 16 patients and 6 HCW, representing an attack rate of 24% for patients and 11% for HCW. Among the patients, 6 had acquired the infection nosocomially (attack rate of 9%). One patient died (CFR 6.3%). Eight patients (67%), but only 2 HCW (33%), were vaccinated with the 2011/2012 influenza vaccine. Based on epidemiological data, a synoptic chart could be drawn indicating 3 infection clusters: the first cluster involved one HCW and two patients, the second cluster involved one HCW and one patient, and the third 3 HCW and 3 patients. For each cluster, an HCW was the index case. One of the 3 HCW was vaccinated. We attempted to culture all PCR-confirmed samples, and NA (NA) and HA (HA) genes were subsequently sequenced. The phylogenetic analysis of concatenated HA and NA sequences confirmed the three separate outbreak clusters. All epidemiological linked cases from each cluster had either identical or very close HA and NA sequences, as opposed to patients with community-acquired influenza or between cases of the different clusters. Conclusion: The combined epidemiological and phylogenetic analysis revealed three independent nosocomial influenza outbreak clusters in a single geriatric department of the Hôpital Edouard Herriot in Lyon, France. NA and HA genotyping proved to be a useful tool for outbreak analysis in the hospital setting. The study showed that HCW were the index cases in all three clusters, emphasizing the need for increasing vaccination rates among the hospital staff to prevent influenza outbreaks.

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Animal-Human Interface

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Reduction in airborne virus using modifications of simulated home slaughter of asymptomatic H5N1 HPAI virus infected chickens

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Background: The majority of human infections with H5N1 high pathogenicity avian influenza (HPAI) virus have occurred in the village setting of developing countries with the primary exposure risk being direct contact with live or dead poultry in the household or neighborhood. In Egypt, the majority of the human H5N1 HPAI virus infections with clinical disease have been in women and children, and they are the high-risk group who tend, play with, slaughter and prepare the household poultry for consumption. Halal slaughter of poultry requires free movement of the bird during the death struggle, which tends to create a viral plume, thereby enhancing human exposure by inhalation. Experimentation was undertaken to develop a method for reduction of airborne virus by performing halal slaughter in a closed container with no further movement restriction. Critical elements are that the method be acceptable to Islamic authorities as proper halal slaughter, that it be affordable in terms of normal village income, and that it be simple and culturally acceptable. Previously, simulated home slaughter of asymptomatic H5N1 HPAI virus-infected chickens in a biosafety level 3 enhanced (BSL-3E) facility generated airborne virus, and chickens and ferrets housed in same airspace as the slaughter process became infected and died. Conducting the slaughter step of H5N1 HPAI virus-infected nonvaccinated chickens in a plastic bag greatly reduced the virus transmission through the air to ferrets. However, the availability and quality of plastic bags that can be access in Egypt are limited. A study was conducted to determine if a common household item, the hala pot with sliding lid (hala-slide-lid), hala pot slaughter with lifting lid (hala-lift-lid), and a modified plastic bucket slaughter with lid that allows scalding water to be poured through a hole in the lid (modified-bucket) could be used in place of a plastic bag to reduce airborne H5N1 HPAI virus following slaughter of H5N1 HPAI virus-infected chickens. Materials and Methods: Adult white Leghorn chickens were inoculated intranasally (IN) with 6 log10 mean embryo infectious doses (EID50) of HPAI A/Vietnam/1203/2004 (H5N1) and at 24 hours post inoculation (PI), six groups of chickens were slaughtered and air samples taken at 1½ feet from slaughter area. The standard slaughter protocol used: 1) kill step (1 min), 2) scald step (60-62°C) (1 min), 3) defeather step (2 min), 4) eviscerate step (2 min), and 5) cleanup step (1 min). The three experiments were conducted: 1) 5 hens slaughtered by hala-slide-lid compared with 5 hens slaughtered by standard method, 2) 5 hens slaughtered by hala-slide-lid compared with 5 hens slaughtered by hala-lift-lid, and 3) 5 hens slaughtered by modified-bucket compared with 5 hens slaughtered by hala-slide-lid. Results: The oral swabs for all 30 inoculated birds were positive for virus indicating infection in the chickens. The air sample taken during the slaughter of 5 chickens by standard method had high virus titers (2.9 log10 EID50's/1.0 mL large particles, 1.23 log10 EID50's/1.0 mL for small particles, no virus in fine particles) while hens slaughtered by hala-slide-lid had low virus titer (1.5 log10 EID50's/1.0 mL for large-sized particles; no virus in small or fine particles). The air sample taken during the slaughter of 5 chickens by hala-slide-lid had low virus titer (0.97 log10 EID50's/1.0 mL large particles, no virus in small or fine particles) while hens slaughtered by hala-lift-lid had slightly higher virus titer (1.5 log10/1.0 mL for large-sized particles). The air sample taken during the slaughter of 5 chickens by hala-slide-lid had low virus titer (1.7 log10 EID50's/1.0 mL large particles, no virus in small or fine particles), while hens slaughtered by modified-bucket had no virus recovery from air sample. Conclusions: Usage of hala pot or modified-bucket methods to contain birds during the kill step of halal slaughter reduced airborne H5N1 HPAI virus. Such modifications should reduce exposure and infections in women and children by H5N1 HPAI virus in Egypt and other enzootic countries.

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Surveillance for human infections with avian influenza A viruses among live bird market workers and their household members in Dhaka city area, Bangladesh

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Background: Live bird market (LBM) workers in Bangladesh are at risk of avian influenza A virus (AIV) infection due to ongoing circulation of these viruses among poultry in markets and their occupational exposure to poultry. LBM workers tend to handle, slaughter and process poultry and offal, many without using personal protective equipment. We initiated active influenza surveillance among LBM workers and their household members to identify human cases of AIV infection, to detect circulating AIVs and to assess serologic evidence of AIV infections. Materials and Methods: Surveillance was implemented in February 2012 in 16 LBMs in Dhaka with ongoing avian influenza surveillance of poultry since 2009. Among LBMs, four are wholesale (operating 24 hours/day) and 12 are retail. We enrolled 750 workers and 1830 household members proportionately from each market through random sampling, based on the number of LBM workers per market. Enrolled workers were visited twice a week at the market to determine if they or their household members experienced illness compatible with AIV infection since the last visit. A possible case of AIV infection was defined as: fever (subjective, measured or reported) and/or any respiratory signs or symptoms (cough, runny nose, sore throat, difficulty breathing, fast breathing, chest indrawing, stridor) or conjunctivitis. For all possible cases, nasopharyngeal and oropharyngeal and conjunctival (if conjunctivitis was present) swabs were collected in viral transport media either at the market or at the household. The samples were tested by real-time RT-PCR (rRT-PCR) for influenza viral RNA and further subtyped if positive for influenza A (H1N1pdm09, H3, H5, H9). Acute (< 7 days from illness onset) and convalescent serum (> 21 days after acute serum collection) were collected from laboratory-confirmed influenza A participants and tested for influenza H5N1 and H1N1pdm09 virus-specific antibodies by microneutralization and hemagglutination inhibition assays. An H5 seropositive was defined as a ≥ 4-fold rise in antibody titer in paired sera (seroconversion) or a microneutralization titer of ≥ 40 (equivalent to WHO criteria of ≥ 80) in both serum specimen and a positive result by other confirmatory tests (western blot and serum adsorptions). Results: In the first year of surveillance, 974 possible AIV cases were identified; including 817 (83%) workers and 157 (17%) household members. Of 974 swab samples tested, 74 (7.5%) tested positive for influenza A and 23 (2.5%) tested positive for influenza B by rRT-PCR. Of 74 samples positive for influenza A, 69 were from LBM workers and 5 from household members; all five household members had H1N1pdm09. Of 69 influenza A swab-positive LBM workers, 18 (27%) were subtyped as H1N1pdm09, 4 (6%) as H3, 13 (19 %) as H9N2, 10 (15%) as H5N1, 18 (27%) were unsubtypeable, and 4 (6%) were inconclusive. No worker with detectable H5 viral RNA developed severe illness. Detection of AIV RNA in symptomatic workers' swab samples coincided with circulation of AIVs among poultry in LBMs (October to April). Influenza A(H1N1pdm09) and A(H3) were also detected at that time, indicating co-circulation of human influenza viruses in workers and AIVs in poultry in LBMs. Among available paired serum from 15 participants, including two with H5 viral RNA detected in respiratory specimens, none demonstrated seroconversion to H5N1, four LBM workers had evidence of H5N1-specific antibodies suggesting previous infection, and three had evidence of seroconversion to H1N1pdm09 virus. Conclusions: Among symptomatic poultry workers in Bangladesh LBMs, we detected seasonal influenza A and B viruses, and viral RNA from AIVs, including H5 and H9. Use of serologic testing on paired serum can help to assess acute infection versus exposure to AIVs and inform the interpretation of detection of AIV RNA in LBM poultry workers exposed to AIVs. Surveillance in LBMs is useful for detecting and monitoring persons with occupational exposure to AIVs and increasing understanding of transmission of AIVs at the animal-human interface. To reduce the risk of coinfection with human and avian

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influenza A viruses and possible reassortment, practical interventions to minimize exposure in LBMs should be piloted and evaluated.

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TRPM8 and susceptibility to highly pathogenic avian influenza H5N1

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Background: The marked family clustering of human cases of highly pathogenic avian influenza H5N1 suggests that host factors may determine susceptibility to severe H5N1 disease. We report the results of work to test the hypothesis that host genetic factors are associated with susceptibility to H5N1 disease in humans. Methods: We conducted a genome-wide, case-control association study (GWAS) of 65 H5N1 cases and 2910 controls from Vietnam, China, and Thailand to identify single nucleotide polymorphisms (SNPs) associated with H5N1. The functional relevance of a putative susceptibility loci identified through the GWAS was validated in in vitro and in mouse models. Results: We found an association approaching genomewide significance between H5N1 and the SNP rs7560562 within the transient receptor potential melastin 8 (TRPM8) locus (minor allele odds ratio, 2.56; 95% confidence interval, 1.79–3.65; P = 7.37 x 10^{-8}; P after genomic control = 8.7 x 10^{-7}). No other locus within the human genome showed association P values of < 1 x 10^{-5}. Transfection of 239T cells with recombinant mouse-derived TRPM8 resulted in increased replication of highly pathogenic H5N1 influenza virus A/turkey/Egypt/7/2007 (peak viral titer 4.71 $\log_{10}$ TCID50/mL in transfected cells versus 3.25 in nontransfected cells). Mice lacking the TRPM8 gene were significantly more resistant than wild-type mice to mortality induced by H5N1 virus A/chicken/Egypt/1/2006 ($\log_{10}$ MLD_{50} 5.7 and 5.16, respectively; P = .03). Conclusions: We have identified an association between the sialated transmembrane ion channel TRPM8 and susceptibility to H5N1 disease. This association may be mediated at the level of viral replication.

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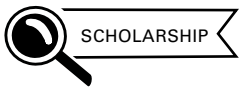

Promising Investigator Scholarship

Characterization of H3N2pM-like and novel reassortant H3N1 swine viruses with pandemic H1N1 2009-like segments isolated in Korea

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Background: Establishment of the pandemic H1N1 2009 [A(H1N1)pdm09] virus in pig populations worldwide resulted in reassortment events with currently endemic swine influenza viruses (SIVs) in North America, Asia, and Europe. In the United States, triple-reassortant H3N2 swine viruses containing the pandemic M segment (H3N2pM viruses) were predominantly found in North American pigs and were also found in humans (A[H3N2]v viruses), raising concerns for public health risks. Material and Methods: To examine whether the A(H1N1)pdm09 virus remains in circulation among

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Korean pigs and determine whether additional genetic reassortment with domestic SIV strains has occurred, lung tissue samples were collected from commercially slaughtered pigs from May 2011 through April 2012. Isolated SIVs were genetically analyzed and further characterized in vitro and in vivo. Results: A total of 19 SIVs were isolated that were subtyped as H1N1 (n = 5), H1N2 (n = 2), H3N1 (n = 4), or H3N2 (n = 8). The H1N1 and H1N2 SIVs are close descendants of A(H1N1)pdm09 and preexisting Korean H1N2 viruses, respectively, without any indication of genetic reassortment. In contrast to the intact H1 viruses, the H3 isolates had a more complex genetic phylogeny where the H3N2 viruses bear gene constellation identical to H3N2pM and A(H3N2)v strains from the United States, whereas the H3N1 SIV isolates reflect four distinct novel reassortant genotypes. The HAs of the four H3N1 isolates grouped together with contemporary cluster IV H3 viruses in North America and the NAs are A(H1N1)pdm09-like. The PB1/NP/NS of A/swine/Korea/CY02-07/2012(H3N1), PB1 of A/swine/Korea/CY02-08/2012(H3N1), and PB1/NS of A/swine/Korea/CY03-13/2012(H3N1) are also closely related to North American H3N2pM viruses while the other segments are A(H1N1)pdm09-like; the internal gene segments of A/swine/Korea/CY03-12/2012(H3N1) also clustered with A(H1N1)pdm09-like viruses. Selected H3 SIVs exhibited efficient replication in mice and ferrets, but the H3N1 reassortant strains indicated growth advantage over a representative H3N2pM-like virus (A/swine/Korea/CY02-10/2012) in human airway cells. Interestingly though, two H3N1 reassortants, A/swine/Korea/CY02-07/2012 and A/swine/Korea/CY03-13/2012, were more readily transmitted to respiratory droplet-contact ferrets compared with the H3N2pM-like isolate. Furthermore, ferret-raised H3 antisera showed poor cross-reactivities against contemporary reference human seasonal H3N2 vaccine strains in hemagglutination inhibition assays, indicating substantial antigenic divergence. Conclusions: We report the isolation of H3N2pM-like viruses outside North America and emergence of novel reassortant H3N1 SIVs possessing various combinations of segments derived from these H3N2pM-like strains and A(H1N1)pdm09-like viruses. Establishment of these viruses would further complicate the genetic diversity of influenza A viruses circulating in Korean swine herds. Indicating their potential to pose threat to public health, our data highlight the necessity of continuous virus monitoring in these ecologically important hosts.

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Human infections with avian influenza H7N1 and H5N2 strains during outbreaks in ostriches

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Human infections with avian influenza H7N1 and H5N2 strains during outbreaks in ostriches
Background: Several avian influenza outbreaks have been reported in ostriches over the last 10 years in South Africa, the most recent in the Western Cape being highly pathogenic influenza AH5N2 in 2004 and 2011, low pathogenic (LPAI) H7N1 in 2012 and H7N7 in 2013. In only one outbreak (HPAI 2004), an increase in ostrich deaths was reported. Large-scale culling followed the H5N2 outbreaks while only movement control was implemented for the H7N1 and H7N7 outbreaks in accordance with OIE guidelines for notifiable AI strains. These outbreaks resulted in a ban on export of ostrich meat to the European Union and have been devastating to the ostrich industry through economic and job losses. South Africa has the largest ostrich meat, feather and leather industry in the world, and vigorous surveillance exist for AI in birds due for slaughter and export. No evidence exists of H5N2 and H7N1 strains causing severe disease in humans, although a serosurvey in 2004 documented serologic evidence of H5N2 infection in 3 of 129 highly exposed persons. Recent emergence of LPAI H7N9 causing severe disease in humans in China and the continued threat of highly pathogenic (HPAI) H5N1 in Asia and other areas raise the question as to the risk of infection and possible symptoms due to other LPAIH7 or HPAI H5 strains in humans. To address this we conducted a serosurvey of at-risk persons during the 2011 and 2012 outbreaks, and tracked positive cases retrospectively to identify symptoms. Materials and Methods: In August 2011 and August 2012, sera

were collected from 207 and 66 people, respectively, with a history of direct contact with ostriches infected with AI H5N2 (2011) or H7N1 (2012) either through handling or slaughtering of infected birds. Sera were also collected from 38 state veterinarians from across the country at an annual congress in 2012, a proportion that had been involved in the AI outbreaks. All persons tested formally consented. Detailed questionnaires regarding demographic, occupational characteristics and clinical illness history were administered during the 2011 outbreak, while an abbreviated questionnaire and retrospective follow-up of seropositive people occurred in 2012. Hemagglutination inhibition assays (HAI) using turkey red blood cells and reference antigens from the World Health Organization Collaborating Centre, Centers for Disease Control and Prevention (CDC), Atlanta, USA, as well as outbreak-specific inactivated antigens obtained from the Department of Agriculture, Forestry and Fishery AI-approved laboratory (Deltamune)were run at the National Influenza Centre, NICD, against H5 and H7 AI and human influenza specific control antisera and serum samples from study participants. Results: Of 207 veterinarians, ostrich farmers, farm workers and abattoir workers exposed to avian influenza H5N2, and 66 exposed to H7N1 in the 2011 and 2012 outbreaks, 3 people with HAI antibody titers greater than 1:40 to influenza H5N2 and one person with H7N1 antibodies were identified. For H5N2 this included a veterinarian who was actively involved in postmortem investigations of ostriches, a farm worker and an abattoir worker. The H7N1-positive person was an abattoir worker who worked on the intestine and skin-sorting line. Reported symptoms included conjunctivitis and flu-like illness for two of the H5N2-seropositive cases, while the third H5N2 seropositive case and the single H7N1 seropositive case reported no symptoms. Conclusion: A low risk exists for human infection with influenza A H5N2 (1.4%) and H7N1 (1.6%) during outbreaks among ostriches, suggesting a need for increased biosecurity and surveillance of humans in contact with ostriches during avian influenza outbreaks, even if the strains are LPAI or the birds are asymptomatic.

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Risk analysis of H2N2 viruses from the avian reservoir

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Background: The H2N2 pandemic of 1957-58 caused significant morbidity, mortality and economic burden worldwide, but the H2 subtype disappeared from human circulation after only a decade. Historical evidence demonstrates the 1957 pandemic viruses arose from avian virus ancestors, and H2N2 infections persist in wild and domestic birds. The potential for reemergence in humans, particularly from the avian reservoir, must be addressed through surveillance, characterization, and antiviral testing. The goal of these studies was to understand the potential risk of avian H2 isolates to mammals by evaluating pathogenicity, virulence and transmission in multiple models, define antigenicity and receptor binding phenotypes, and test susceptibility to current antiviral drugs. Methods: We selected a panel of 22 avian H2N2 viruses isolated from wild and domestic birds over 6 decades. Antigenic characterization was carried out with a panel of ferret sera to avian, human and swine H2 viruses in the hemagglutination inhibition (HI) assay. Receptor specificity was determined by plate-based binding assays using biotinylated sialylglycopolymers. Viruses were tested for mammalian pathogenicity in a variety of ex vivo and in vivo models including differentiated normal human bronchial epithelial cells (NHBE), swine tracheal tissue explants, DBA2/J mice, and ferrets. An expanded panel of 59 viruses was tested for susceptibility to influenza antivirals. Adamantane susceptibility was assessed by sequence analysis of the M2 gene, and neuraminidase inhibitor (NAI) susceptibility (oseltamivir, zanamivir) was tested by fluorescence-based NA enzyme inhibition assay. Results: HI assays demonstrated that the avian H2N2 panel was closely related to all avian H2 isolates over the past 50 years and to human pandemic virus sera, but they rarely reacted with human sera to viruses toward the end of the H2N2 circulation period. All viruses showed a preference for the α2-3–linked sialylglycopolymers over those with α2-6–linkages. Nearly all (91%) of the isolates replicated in NHBE cells. All were pathogenic in mice, with varying degrees of virulence, and 5 isolates induced some degree of mortality. Replication in swine tracheal tissues was low or absent in the viruses tested. Of 9 avian viruses tested in ferrets, 5 replicated in the upper respiratory tract and 3 exhibited direct contact transmission. No aerosol transmission was observed. In all viruses tested, M2

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gene sequence analysis did not reveal amino acid residues known to confer resistance to adamantanes, and all were highly susceptible NAIs with IC50 values in the nanomolar range (0.1-7.0 nM). Conclusions: The data presented here represent a thorough risk analysis of H2N2 viruses from the avian reservoir. Despite antigenic and receptor binding patterns typical of avian viruses, most isolates were pathogenic in primary human cells and mice without prior adaptation. Though replication in swine tissues was nonproductive, several isolates were able to replicate in ferrets and transmit to cage mates. The pathogenicity of these avian H2 viruses in multiple mammalian models elevates their risk potential. However, all viruses remain highly susceptible to FDA-approved antiviral drugs, suggesting they may be used as a control strategy should H2 viruses emerge once again in humans.

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The avian-like PB1 gene of the 1968 pandemic influenza virus enhances viral replication and transmission

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Background: Pandemic influenza viruses originate from animal viruses by either adaptation of a whole animal virus to humans or reassortment between animal and contemporary human viruses. The 1957 and 1968 pandemic viruses emerged via reassortment, and both contained HA and PB1 gene segments of avian origin. It is generally believed that the acquisition of an antigenically novel avian HA allowed the pandemic viruses to overcome preexisting immunity in the human population. However, it is still not known whether the avian PB1 gene provided pandemic viruses with any replicative advantage. In this study, we wished to address this long-standing question using the 1968 pandemic virus as a model. Materials and methods: The H3N2/1968 pandemic virus contained HA and PB1 gene segments of an avian parent and remaining 6 gene segments of a contemporary human H2N2 parent. We decided to test whether substitution of the PB1 segment in the human parent by its avian counterpart will facilitate viral replication. Because the parent viruses and the original avian-human reassortant virus do not exist and their sequences are not known, we used the closest available relatives of these viruses. The avian-like gene segments (PB1 and HA) were taken from one of the earliest H3N2 pandemic virus strains A/Hong Kong/1/68 (HK68). To find an appropriate donor of human-like gene, we compared sequences of six human-like genes of HK68 with published sequences of human H2N2 viruses. Based on this analysis, the phylogenetically closest virus A/California/1/66 (H2N2) (Cal66) was used as a surrogate precursor of the 1968 virus. The genes of Cal66 and HK68 were cloned into reverse genetics vector pHW2000. The effects of PB1 of either virus on polymerase activity were determined by luciferase minigenome assay. Two pairs of recombinant reassortant viruses were generated, which differed by the origin of the PB1 gene (either from Cal66 or from HK68). The viruses of the first pair (rCal66 and rCal66-$PB1_{HK}$) shared 7 genes of Cal66. This pair was prepared to answer the question whether avian-like PB1 alone could enhance replication efficiency of a reassortant virus. The viruses of the second pair (rCal66-HA_{HK} and rCal66-HA_{HK}-$PB1_{HK}$) shared 6 genes of Cal66 and the HA gene of HK68. This pair was studied to account for potential interactions between avian-like HA and PB1 gene segments or/and their products. The viruses of each pair were compared for their replication kinetics in human epithelial cells (Calu-3). Viral competition in Calu-3 cells was studied by making consecutive passages starting from a 1:1 mixture of viruses and determining composition of the passage 3 material by sequencing. Competitive rescue experiments were performed by transfecting cells with a whole set of reverse genetics plasmids which included a 1:1 mixture of $PB1_{Cal}$ and $PB1_{HK}$ plasmids and determining composition of the rescued virus mixtures by sequencing. To study efficiency of contact transmission of the viruses, a group of four guinea pigs were inoculated with 10^4 PFU of an equal mixture of rCal66-HA_{HK} and rCal66-HA_{HK}-$PB1_{HK}$. Four naïve animals were co-housed with infected animals starting from day 1 post inoculation. At days 2, 4, 6 and 8 p.i. nasal washes were taken and characterized by sequencing. Results: A comparison of polymerase complexes of Cal66, HK68 and their reassortants in the luciferase minigenome assay demonstrated an enhanced polymerase activity of complexes with avian-like PB1 of HK68. The recombinant virus containing 7 genes of Cal66 and the PB1 gene of HK68 replicated more efficiently in Calu-3 cells than the parent rCal66. The difference in replication was even more pronounced if the viruses shared the HA gene of HK68. The virus with avian-like PB1 transmitted more efficiently in guinea pigs than its counterpart containing PB1 of Cal66. Conclusions: Our results show for the first time that the avian-like PB1 gene of the 1968 pandemic influenza virus served to enhance viral replication and transmission. An enhanced polymerase activity of avian-like PB1 and a potential cooperation between homologous HA and PB1 could be responsible for these effects.

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Avian influenza surveillance and public health education in Ghana

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Background: Influenza A viruses that cause highly pathogenic avian influenza (HPAI) also infect humans. In many developing countries such as Ghana, poultry and humans live in close proximity in both the general and military populations, increasing risk for the spread of HPAI from birds to humans. Respiratory infections such as influenza are especially prone to rapid spread among military populations living in close quarters such as barracks, making this a key population for targeted avian influenza (AI) surveillance and public health education. Methods: Twelve military barracks situated in the coastal, tropical rain forest and northern savannah belts of the country were visited, and the troops and their families educated on pandemic AI. Attendance at each site was obtained from the attendance sheet provided for registration. The seminars focused on zoonotic diseases, influenza surveillance, pathogenesis of AI, prevention of emerging infections and biosecurity. To help direct public health policies, a questionnaire was used to collect information on animal populations and handling practices from 102 households in the military barracks. Cloacal and tracheal samples were taken from 680 domestic and domesticated wild birds and analysed for influenza A using molecular methods for virus detection. Results: Of the 1028 participants that took part in the seminars, 668 (65%) showed good knowledge of pandemic AI and the risks associated with its infection. Even though no evidence of the presence of AI infection was found in the 680 domestic and wild birds sampled, biosecurity in the households surveyed was very poor. Conclusions: Active surveillance revealed that there was no AI circulation in the military barracks in the spring of 2011. Though participants demonstrated good knowledge of pandemic AI, biosecurity practices were minimal. Sustained educational programs are needed to further AI surveillance and prevention in military barracks.

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Avian influenza viruses acquire mutations to enhance pathogenesis during a single passage in ferrets

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Background: Highly pathogenic avian H5N1 and, more recently, H7N9 viruses have galvanised widespread attention due to their excessive mortality in humans. Sequence analysis of human isolates of these viruses often indicate the presence of PB2 627K, whereas avian isolates mainly possess PB2 627E. This raises the question of whether avian influenza viruses encoding PB2 627E are capable of initially establishing mild infections in humans before mutation to full virulence by acquisition of PB2 627K during viral replication in humans, or whether the input virus for severely infected humans already carries the PB2 627K mutation, prior to human infection. Given our lack of understanding regarding the adaptation of avian viruses to humans, we sought to examine this adaptation to mammals, using avian H5N1 virus and ferrets as a model of avian influenza infection of humans. Methods: Here we used ferrets to monitor the replication of an avian H5N1 virus encoding PB2 627E, A/Chicken/Laos/Xaythiani-26/2006 (A/Laos), following mammalian infection, and contrasted this with a human H5N1 virus encoding PB2 627K, A/Vietnam/1203/2004 (A/Viet). Results: On initial comparison, all ferrets inoculated with A/Viet experienced a highly pathogenic systemic infection accompanied by substantial weight loss. In contrast, while ferrets could be readily infected with A/Laos, the virus was confined to the respiratory tract and there were no signs of disease prior to day 5 post infection (pi). Thereafter, two of four ferrets cleared the virus and two developed substantial weight loss and a persisting infection that had spread beyond the respiratory tract. Virus

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from certain tissues of both systemically infected ferrets had acquired the PB2 E627K mutation. When a PB2 E627K-containing virus from the liver was used to infect additional ferrets, it produced a highly pathogenic infection, confirming that acquisition of PB2 E627K enhanced the pathogenicity of A/Laos in ferrets. Another isolate from the nasal wash of the systemically infected ferrets had retained PB2 627E but acquired two other novel mutations (PB2 S489P and NP V408I) and also produced a highly pathogenic infection upon infection of additional ferrets. In each case, enhanced pathogenicity was demonstrated by a more rapid onset and greater severity of disease symptoms associated with systemic virus spread. Finally, a reverse genetics system was used to generate isogenic H5N1 viruses encoding the PB2 E627K, PB2 S489P and NP V408I single amino acid changes. Inoculation of ferrets with each of these viruses confirmed that the PB2 E627K mutation dramatically enhanced the pathogenicity of H5N1 in ferrets. Whereas neither of the PB2 S489P or NP V408I mutations in isolation were sufficient to enhance the pathogenicity of H5N1 in ferrets, they likely only facilitate enhanced virus pathogenicity in a synergistic manner. Conclusions: Our findings highlight the remarkable ability of avian viruses to mutate toward increased pathogenicity during a single passage in mammalian hosts, through the acquisition of PB2 E627K in certain individuals. These results suggest that the PB2 E627K mutation likely similarly occurs regularly upon avian influenza virus infection in humans, increasing the chances of a more severe infection. Additionally, in the absence of a PB2 E627K mutation, alternative novel mechanisms (eg, combination of the PB2 S489P and NP V408I mutations) may similarly facilitate enhanced avian influenza virus pathogenicity in mammals. Recent serologic evidence of H5N1 infection in humans associated with mild or subclinical infection may reflect cases of infection where the infecting avian H5N1 virus fails to acquire PB2 E627K or a similar adaptive polymerase mutation. Regarding the recent H7N9 human infections in China, our findings suggest that the PB2 627K detected in human H7N9 isolates is likely acquired during viral replication in humans following infection, rather than in the avian source or an intermediate host. Investigating this rapid process of highly pathogenic avian virus evolution in mammals will significantly advance our understanding of the individual steps involved in the creation of pandemic viruses.

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P2-469 G FELLOWSHIP GATES Fellowship

Poultry-human interactions in a wholesale live bird market in Dhaka, Bangladesh: the potential for bird-to-human transmission of avian influenza viruses

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Background: In Bangladesh, H5N1 has been circulating since 2007, causing repeated outbreaks in poultry. Seven human cases have been reported, three of whom were live bird market (LBM) workers. In response to a suspected H5N1 outbreak among poultry workers in a large, wholesale and retail LBM in Dhaka in 2012, a team from icddr,b, in collaboration with the Institute of Epidemiology Disease Control and Research, conducted a qualitative study. This study describes the physical environment of the market to understand animal-human interactions with a focus on infection control practices among poultry workers and risk practices for avian influenza transmission from birds to humans. Methods: In May 2012, icddr,b anthropologists mapped the market and listed the total number of people working in this market. The market operated 24 hours a day and birds primarily arrived at night. We conducted 32 hours of observation in the LBM over the period of a week in 6 sessions: two were between 10 pm to 4 am and four occurred between 7 am to 2 pm. We counted the number of poultry workers, number of apparently sick and discarded dead birds, and recorded interactions between poultry and humans, including biosecurity practices. We interviewed transport drivers about the source and number of birds they carried each trip. We also conducted two group discussions with poultry workers to explore practices related to handling live, sick and dead birds. Results: The poultry market had an area of 4220 m^2 and housed 66 independent wholesale poultry shops and 37 retail poultry shops. The market operated 6 days a week and had approximately 1300 workers. The market

sold broilers, layers, backyard chickens, ducks, swans, and pigeons. The wholesale shops sold live poultry to 71 smaller retail LBMs and processed large amounts of chickens for catering companies and restaurants. The retail shops sold processed and live chickens to individual consumers on a smaller scale. Birds sold at the market were brought from 24 districts by buses, trucks and pick-up vans. Over two nights, a total of 70,559 birds entered the wholesale market; 79 were visually sick and 188 were dead on arrival and workers unloaded these birds from the vehicles and discarded dead birds either on the road or in a large open waste receptacle. Twelve chickens also died in the retail market and were discarded in small plastic bin. Poultry workers reported that birds arriving sick to the market were also processed and sold. The workers also reported that some people (desperate for ways to make money) collected, processed and sold dead birds to street food vendors and restaurants. To process the poultry, workers cut the chicken's throat with a small knife, then skinned the carcass, cleaned the gizzards and discarded the internals and offal in a small plastic bin. We observed 56 workers processing 3009 chickens without wearing gloves and each worker processed approximately two chickens per hour. They reported frequent finger cuts and skin injuries from the knives and chicken bones. There was no source of running water in the market. Water was stored in a plastic drum inside each retail poultry shop for rinsing hands, rinsing processed chickens and for washing the drum itself. We observed workers rinsing their hands directly in the drum 43 times and only 24 workers used cloth or surgical masks while handling poultry. At night, 35 workers slept either in a poultry shop or inside a large poultry cage. Conclusions: Influenza H5 viruses are frequently detected by poultry surveillance in this wholesale market. Poultry workers' intense daily interaction with birds and the high volume of different species of birds are favorable conditions for putative reassortment among influenza strains. Additionally, slaughtering and consuming sick and dead birds, limited use of personal protective equipment and lack of handwashing creates a high-risk setting for human infections with avian influenza viruses. To reduce the pandemic threat posed by H5N1, the market's physical environment and poultry workers biosafety practices should be improved.

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Influenza A virus surveillance in live bird markets in Bangladesh

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Background: Highly pathogenic avian influenza A (H5N1) virus poses a threat to the health of poultry and humans. In Bangladesh, between 2007 and 2012, more than 540 HPAI outbreaks in poultry and seven HPAI human cases, including one death, have been reported. Since August 2007, icddr,b has been conducting surveillance in live bird markets (LBMs) to identify avian influenza virus circulation among poultry in Bangladesh. Poultry raisers and local vendors sell chickens, quail, and pigeons alongside waterfowl such as ducks and geese at LBMs. From August 2007 to December 2010, we identified avian influenza virus A (H5) in 0.6% of waterfowl and 37% of pooled environmental specimens collected at LBMs. This report summarizes LBM-based surveillance conducted in Bangladesh from January 2010 to December 2012 to identify avian influenza virus A (H5) circulation in waterfowl and LBM environmental contamination. Materials and Methods: We conducted surveillance in one peri-urban LBM in each of four districts in Bangladesh (Chittagong, Rajshahi, Netrokona and Dinajpur) and 16 urban LBMs in Dhaka city. Surveillance teams visited each selected LBM on a monthly basis to collect samples from waterfowl and the environment. Each month veterinarians selected 20 ducks and/or geese by convenience from each of the four peri-urban LBMs to collect either cloacal or tracheal swabs, or freshly laid feces. We also collected swab specimens from seven environmental sources including poultry droppings, cages, feed, water, slaughtering sites, market floors and drains to prepare one pooled environmental sample monthly from each of the 20 LBMs. The icddr,b virology laboratory tested these samples using real-time reverse transcription polymerase chain reaction (rRT-PCR) assay to detect RNA for influenza A virus and subtype for H5. We interviewed poultry raisers of sampled waterfowl about the demographics, husbandry practices,

flock sizes and health status of all poultry. We used chi-square test and bivariate and multivariate logistic regression to identify demographics and exposure variables associated with rRT-PCR results. Results: We collected swab samples from 2347 ducks and 293 geese. Of the sampled waterfowl, 2459 (93%) were aged more than six months and 2627 (99%) appeared healthy during sample collection. Most of the sampled waterfowl (94%) were raised in backyard farms. The mean size of backyard poultry flocks was 18 (range: 1-99). Of 2640 waterfowl samples, 173 (7%) had detectable RNA for influenza A virus and 80 (3%) had H5 subtype-specific RNA. The prevalence of laboratory-confirmed influenza A /H5 was higher in ducks than geese (3% vs 1%, *P* = 0.01). All influenza A/H5 confirmed waterfowl were reared in a backyard facility. Of the 580 pooled environmental samples, 130 (22%) demonstrated detectable H5 subtype-specific RNA and specimens from urban LBMs were five times more likely to have evidence of avian influenza A/H5 contamination than peri-urban LBM specimens (95% CI: 2.5-11.6). Avian influenza virus A/H5 has been detected in both individual waterfowl and the LBM environment almost every month, but the highest proportion (13%) of samples with influenza A/H5 has been detected in March. Conclusions: Surveillance findings from 2010-2012 suggest that avian influenza A viruses, including H5, circulate year-round in domestic ducks and geese in Bangladesh. LBM environments contaminated with H5N1 may act as a potential source of infection in poultry that may increase the risk of avian-to-human transmission. LBMs should be targeted to provide intervention through improved biosecurity and disinfection for reducing avian influenza virus transmission. LBM-based surveillance should be continued for better understanding of influenza virus circulation in domestic poultry and may provide sentinel detection of novel avian influenza viruses of public health importance.

P2-472 G FELLOWSHIP GATES Fellowship

Sequence and phylogenetic comparison of human and avian influenza H3 strains reveals introduction of American and Eurasian lineages in Pakistan

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Background: Pakistan has been experiencing outbreaks of avian influenza viruses (AIVs) since 1995, and various subtypes reported so far include H7N3, H9N2 and H5N1. Both H7 and H5 subtypes have not been isolated from the country since July 2008; however, subtype H9N2 has become endemic in poultry across Pakistan. In March 2010, a low pathogenic AIV of H3N1 subtype was isolated for the first time in domestic chicken. On the other hand, a number of human H3N2 viruses are routinely reported. To understand the relationship between these two subtypes and the probability of subsequent transmission of avian viruses to humans, the sequences of HA and matrix genes of H3N1 were compared with human H3N2 strains isolated from Pakistan during 2008-2012. Furthermore, sequence and phylogenetic analyses were also carried out for the neuraminidase gene of the human H3N2 and H9N2 viruses already circulating in the country. Materials and Methods: Avian influenza virus was isolated from cloacal swabs collected from backyard poultry. The samples were inoculated in 9-day-old embryonated SPF chicken eggs for 2-3 days. Subtype identification of the isolated virus was carried out by hemagglutination (HA), hemagglutination inhibition and neuraminidase inhibition assays. Standard OIE procedures of real-time PCR and RT-PCR were applied for influenza typing and amplification. The human influenza viruses were detected from nasopharyngeal and throat swabs collected at sentinel surveillance sites across Pakistan. The samples were grown on Madin Darby Canine kidney (MDCK) cell lines, and subtyping for influenza A and B was carried out using a WHO influenza reagent kit supplied by the CDC Atlanta. For detection and characterization of influenza A or B, the same samples were also tested using the CDC real-time RT-PCR (RRT-PCR) protocol. The identified H3N2 viruses were then amplified by RT-PCR using the CDC protocols. For all viruses, the purified PCR products were sequenced using the chain termination protocol on an ABI-3130xl genetic analyzer. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 5.0. Results: Phylogenetic analysis of avian H3N1 revealed a new introduction of reassortant Eurasian avian strain that was clearly distinguishable from corresponding human H3 isolates. Both HA and matrix genes of Pakistani avian H3 clustered mostly with European isolates and specifically the HA gene was Nordic (Scandinavian) in origin, while the NA gene was Asian by showing maximum

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similarity (98%) with Indian H11N1. On the other hand, the HA, NA and matrix genes of Pakistani human H3N2 isolates showed 98%-99% sequence homology with American and Eurasian viruses. The HA and matrix genes of avian H3 showed nucleotide sequence identity at 79% and 87% with corresponding human H3s, while the NA gene of human H3 was distinct from avian H9N2 strains with sequence identity of only 79%- 81%. Sequence analysis of HA revealed that both avian and human viruses had an amino acid motif (PEKQTRG) at the cleavage site, which is indicative of low pathogenecity. Both avian and human H3 viruses retained conserved residues at positions 226 and 228 of the receptor binding site. The avian H3 possessed 6 N-linked glycosylation sites at amino acid positions 8, 22, 38, 165, 285 and 483, whereas human H3 had 11 potential glycosylation motifs with additional sites at positions 122, 126, 133, 144, and 246 in the HA gene. Furthermore, sequence analysis of NA and M genes of avian H3 viruses showed conservation of amino acids associated with sensitivity to amantadine and oseltamivir. On the other hand, Pakistani human H3N2 viruses possessed mutation S31N associated with resistance to amantadine, but remained sensitive to oseltamivir. Conclusions: Our study found no significant epidemiologic linkage between avian H3N1 and human H3N2 viruses from Pakistan. From sequence and phylogenetic analyses, it is obvious that the Pakistani avian and human H3 strains are similar to Eurasian and American H3 viruses, respectively, and were introduced into the country separately. The avian H3 reassortant Eurasian strain might have been introduced through migratory movements of wild birds, while the mechanisms for transmission of human H3 viruses require further studies.

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Seroprevalence survey of avian influenza viruses among poultry workers in Taiwan

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Background: Avian influenza viruses (AIVs) are zoonotic agents recognized as a continuing pandemic threat to human society based on their easily changing genetic nature. Because most confirmed cases in humans have involved exposure history to ill or dead poultry or a contaminated environment, poultry workers with intense occupational exposure are thought to be a high-risk group and serve as a bridge population between animals and human population. Six outbreaks of infection by highly pathogenic avian influenza (HPAI) H5N2 viruses occurred in 2012 in the central and southern regions of Taiwan. This study was carried out to estimate the infection rate for AIVs among Taiwanese poultry workers through a serological survey. Materials and Methods: We carried out a cross-sectional case control study in which the case group consisted of live poultry vendors (LPVs) and poultry farmers (PFs) who worked at 335 randomly selected live poultry stalls and 400 poultry farms in 22 different cities and counties as sources of members of the study population, choosing 2 workers from each stall or farm, and the control group consisted of non-poultry workers (NPWs), mostly government employees, who matched the poultry workers by sex, age, and administrative district of employment. The study was carried out from May 2012 to July 2012. After giving their consent, all participants completed a standardized questionnaire. We then collected a 7- to 10-mL sample of whole blood from each study participant for a hemagglutinin inhibition assay to titrate the serological titers against H5N2 and H7N3. The statistical analysis was carried out using SPSS software version 14. Results: *Demographics.* A total of 335 LPVs, 335 PFs, and 577 NPWs were enrolled for analysis. The majority of case subjects had worked more than 10 years (LPVs: 86.57%; PFs: 79.4%) and been in contact with poultry every day (LPVs: 90.45%; PFs: 94.03%). A review of their vaccination history revealed that most LPVs and PFs (73.13%-80.9%) had never received an H5N1 vaccine. The rate of vaccination with seasonal influenza vaccine was also low: only 19.1% to 38.21% among LPVs and PFs during the past 2 years. By contrast, the seasonal influenza vaccination rate exceeded 50% among NPWs during 2010 and 2011. *Seroprevalence.* The titers against H5N2 among LPVs and PFs were significantly higher than among NPWs (P = .000 and P = .001, respectively). When the titers against H7N3 among LPVs and PFs were compared with the titers in NPWs, however, the difference did not reach statistical significance. A total of 18 subjects had HI titers against H5N2 exceeding 1:80. If we use this value as the cut-off point, the seropositive rate was 2.99% among the LPVs and 1.79% among the PFs, both of which are significantly higher than among NPWs, with odds ratios of 8.85 and

5.24, respectively. A total of 7 subjects had HI titers against H7N3 of 1:40. If we use this value as the cut-off point, the seropositive rate was 0.6% among LPVs and 1.19% among PFs; these values are not statistically higher than among NPWs. We did not find a correlation between influenza vaccination history (seasonal or H5N1) and HI titers against H5N2 and H7N3. Furthermore, PWs who worked in administrative districts in which HPAI H5N2 poultry outbreaks occurred in 2012 had significantly higher antibody titers (P = .028, odds ratio = 5.574). Conclusion: We found that frequent AIV exposure correlated with seropositivity in PWs, especially LPVs. The number of PWs who were seropositive against H5N2 was higher than the number who were seropositive against H7N3; this is consistent with virus levels during the epidemic in Taiwan, when H5N2 infections were seen much more frequently than H7N3 infections. To reduce the incidence of human exposure to AIVs, we should continue to reinforce our surveillance systems for both animal outbreaks and human cases, increase influenza vaccination rates in high-risk groups, and strengthen the education of PWs regarding their risk of infection and how to reduce that risk.

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Species specific host factors of influenza A virus replication

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Background: Influenza A viruses (IAVs) are capable of infecting a wide range of animal hosts including humans, pigs, birds and dogs. However, some strains can only be maintained in the natural reservoir, aquatic birds, and transmission of these strains to another host could result in pandemics. Importantly, the strong host cell specificity of IAVs limits the possibility of interspecies transmission. Nevertheless, reassortant influenza viruses containing avian, human and/or swine genomic segments are frequently detected in pigs. Recently, it has been hypothesized that the differential host tropism observed between viruses could be partially due to differences in the requirements for host cell factors. In recent years, five RNA interference (RNAi)-based screens have identified several host factors essential for efficient viral replication in human cells. However, it still remains unclear if the identified host cell factors are required in other species as well. In the present study, targeted small interference RNA (siRNA) screens were performed in avian and porcine cells. Materials and Methods: DF-1 (chicken embryo fibroblasts) and PK-15 (porcine kidney) cells were reversely transfected in 384-well plates with custom-made siRNA (Microsynth) by lipofection using HiperFect (Qiagen). Fourty-eight hours post transfection, cells were subjected to either A) virus replication assay, B) cell viability assay, or C) interferon (IFN)-induction assay. For the replication assay (A), cells were infected with virus strains A/WSN/1933 or A/FPV/Bratislava/79. Then supernatants were transferred onto MDCK cells, incubated for 7 h, fixed, stained for viral protein and DNA, and analysed using Olympus Soft Imaging Solutions. For cell viability assay (B), cells were treated with WST-1 reagent (Roche) and the signal measured by Envision spectrophotometer (PerkinElmer). In case of the IFN induction (C), DF-1 or PK-15 cells stably expressing firefly luciferase under control of the IFN-stimulated response element (ISRE) were transfected and the luciferase signal measured. All multiwell pipetting steps were performed using a Biomek FXP Laboratory Automation Workstation and the assays were screened three times independently. Results: By using bioinformatic analyses of the consensus host-dependency factor hit list from the five previously published screens employing human cells, we found 112 genes which have orthologs in chicken and swine species. Using mRNA microarray analysis, we confirmed the expression of 94 of these in DF-1 and PK-15 cells. After excluding toxic and immunostimulatory siRNAs, Ingenuity pathway analysis revealed enrichment in gene categories associated with mRNA splicing, nuclear transport and cellular trafficking. To mimic in vivo conditions more closely, hit validation will be performed in primary chicken and pig epithelial lung cells. So far, chicken lung primary epithelial cells were successfully isolated and cultivated. Further characterization is currently ongoing. Conclusion: It is anticipated that this cross-species comparison of IAV host factor dependency will lead to a better understanding of viral infection mechanisms and will provide novel target candidates to support a host-directed approach to antiviral drug development.

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Biosecurity conditions and biosafety practices in the live bird markets of Dhaka city, Bangladesh, 2012

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Background: Highly pathogenic avian influenza A (H5N1) virus has been circulating since 2007 among poultry in Bangladesh. Poultry surveillance in live bird markets (LBMs) of Dhaka city has detected circulation of H5N1 almost year-round, with peak circulation during October to March. Three of the seven human H5N1 cases reported in Bangladesh have been poultry workers. Biosecurity and biosafety measures may help control the spread of avian influenza viruses in LBMs and prevent transmission to poultry workers. The government of Bangladesh, in partnership with UNICEF, FAO and WHO, is piloting a behavior change communication intervention in the LBMs. As part of this initiative, we performed a baseline assessment, exploring the biosecurity conditions of LBMs within Dhaka city and biosafety practices of the poultry workers. Methods: In August and October 2012, a qualitative research team conducted spot observations in 10 LBMs within Dhaka to record the location and condition of poultry keeping, selling and processing places. They conducted 8-11 hours of observations in each of four markets to explore personal hygiene practices during bird handling and waste disposal. They conducted 16 in-depth interviews with poultry workers to explore their practices of bird keeping, selling, slaughtering and handling, cleaning, waste disposal and personal hygiene while working with live birds. Results: The LBMs commonly sold chickens, including indigenous, broiler and layer chickens and/or cocks. Some (15% [23/194]) shops also sold ducks, geese and pigeons. Bamboo baskets were used for keeping backyard poultry and metal cages were used for broiler or layer chickens. Birds of different breeds and species were usually housed separately in stacked or adjacent cages or baskets without any divider. Metal cage and bamboo baskets were observed only to be dusted off four times. Live birds were slaughtered, defeathered or skinned, and eviscerated in places adjacent to the poultry housings. Processing birds on the floor and dry sweeping of the processing floor were commonly observed practices. Floors of shops and slaughtering places were mainly cemented and many shops had damaged flooring. Informants reported washing processing places and equipment with water at least once daily before closing at night. Use of detergent was never observed during 49 observed occasions of cleaning housing, floor and equipment. Processing places did not usually have running water or proper waste water drainage systems; workers used stored water for processing and cleaning activities. They stored offal in a container and skin and wings on the floor to sell later. Other waste, including beaks, feathers and feces, was observed to be disposed of directly on the ground and picked up by cleaners 1-2 times daily. Informants reported attempting to sell sick poultry quickly, even at a discounted price, or consuming the affected birds to recover their investment. Dead birds were discarded. Workers were observed to quickly slaughter and process birds with bare hands one after another and to smoke, eat or drink, or touch their face and body without first washing their hands. Rinsing hands with water was common during or after processing. Use of soap was observed in one of 48 occasions of hand washing. Workers wore open sandals or were barefoot. Use of personal protective equipment, such as gloves, masks, aprons or gumboots, was rarely observed or reported for any activity with healthy, sick or dead birds. Many child workers (5-15 years old) were observed participating in slaughtering and processing activities. Unavailability of water and soap or detergent was a major barrier for cleaning and hand washing. The most frequently reported constraint of using gloves, masks, or gumboots was inconvenience while performing their tasks, including carcasses slipping from hands and gloves tearing. Conclusions: Overall biosecurity conditions and biosafety practices among LBM poultry workers can facilitate transmission of avian influenza viruses to humans. Future interventions to improve biosecurity conditions and biosafety practices in these markets should consider improving the resource-poor infrastructures and taking into account the poultry workers' financial constraints and the nature and volume of work when designing intervention.

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Influenza A(H1N1)pdm09 225D/G variant virus replication and transmission in pigs

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Background: The human pandemic virus, "swine flu" A(H1N1)pdm09 with its putative origin in pigs, is now fully (re-)established as an endemic influenza virus infection of pigs worldwide. A range of variants and reassortants has evolved, including a mutation known to cause severe disease in humans (HA D225G) as a result of its increased α-2,3 sialic acid binding in the lower respiratory tract (LRT). These 225G variants have also been isolated from European pigs. We have assessed the impact of these mutations in the swine host reservoir in relation to the disease pathogenesis and as a potential source of severe influenza infection in humans. Materials and Methods: Plaque purified D225 (MDCK) and G225 (MDCK/Egg) variants of A/Hamburg/05/09 were used as either single isolates or as a 50:50 mixture to study their infectivity in ex vivo respiratory organ cultures, and in vivo for pathogenesis and transmission. Following intranasal aerosol inoculation ($10^{5.6}$ PFU) of 12 animals, they were monitored for clinical signs, nasal shedding of virus and two were removed at 0/1/2/3/5/8 dpi for post mortem. The transmission element included 4 infected pigs + 4Contacts-R1 + 4C-R2 + 4C-R3 model, with contact at dpi 2-4 for each of the virus groups. Two animals from each contact round underwent post mortem at dpc 5. Virus (4 animals) from each of the scenarios was sequenced to detect the presence and/or change in the occurrence at the HA-225 site. Results: D225, G225 and mixed population viruses replicate at similar rates in ex vivo trachea, bronchi and lung cultures. However, in vivo the mixed infection was similar to that of the 225D but both were more productive than the 225G in relation to virus shedding. In the transmission rounds, this trend across the variants continued and varied little within the three transmission cycles. Shedding of the 225G mutant was delayed by 1-3 days in relation to the mixed population, but was not observed for the 225D variant. Lung gross pathology scores were highest in the 225G and lowest in the mixed infection animals. Gross lesions were minimal in contact animals irrespective of the variant. Virus immunohistochemistry (IHC) scores for the URT were highest in the mixed infection and lower in the two single variants; for the LRT mixed infection had the highest score followed by the 225G and 225D variants. The dpi/dpc 5 IHC comparisons indicated that both single variants transmitted less well to the lungs during the contact rounds than did the mixed virus. Viral RNA in the tissues at dpi/dpc 5 indicates that there was little difference between the three virus inocula (U&LRT); however, differences became enhanced as the viruses moved through the transmission rounds. This was particularly evident for the 225G variant from R2 onward in the LRT. Output virus sequence from the 225D or 222G variant virus infected and contact pigs was found to be unchanged. Only the 225D variant was shed into the environment from 225D/G mixture (dpi 2-7) infected pigs as well as their contact animals. Whereas, in the turbinate tissues, a double peak 225D>222G (75-90%) was observed in the infected pigs but not the contact animals, which showed 100% selection of the 225D genotype. Mid-lung tissue from infected animals (dpi1&3) showed a mixed population, predominantly 225D>225G (50-95%). Mid-lung tissue from 1/5 contact pigs showed selection of the 225G>225D (10%). Conclusions: Experimentally infected pigs are capable of being infected and transmitting A(H1N1)pdm09 225D/G variants and mixtures thereof; the 225G variant does not appear to cause more substantial disease in pigs as has been observed in human cases. The single and mixed population studies have shown that the disease is relatively mild and the rapid selection of the 225D variant following mixed population infection and transmission. It seems unlikely that pigs will represent a reservoir of the 225G more virulent strain of influenza in relation to public health issues. Funding - EU FP7 projects: FLUPIG #258084, ESNIP3 #259949 649.

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Investigating mechanisms of influenza polymerase host adaptation

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Background: Typical avian influenza A viruses do not replicate efficiently in mammals. Many host adaptive mutations map to the polymerase complex (PB1, PB2 and PA); however, the mechanism/s of host adaptation are unknown. Polymerase activity is often measured using a cell-based assay in which the active enzyme is reconstituted from its constituent parts expressed from plasmids. Using this approach we suggest that different PB2 humanising mutations do not enhance polymerase activity by a universal mechanism and furthermore are not all host specific, as some enhance activity in avian as well as mammalian cells. Methods: We wished to address why certain mutations that enhanced activity in the reconstituted polymerase assay are not selected for in nature and to explore whether the polymerase assay truly reflects viral fitness. To investigate this we used reverse genetics to create a series of viral variants carrying mutations in the PB2 gene that enhanced polymerase activity in the in vitro assay. We carried out virologic assays as well as measures of transcription and replication in the context of replicating virus and also analysed the effects of the mutations in vivo. Result: Some mutations that increased in vitro polymerase activity lead to attenuated virus replication that resulted from an increase in interferon activation. Conclusion: These data help risk assess the likelihood of different avian influenza viruses crossing the host range barrier and increase our understanding of the relative balance between virus replication and the host innate response.

P2-478

The NS1 protein of avian influenza viruses inhibits the major cellular signaling integrator c-Abl

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Background: The nonstructural protein 1 (NS1) of influenza A viruses (IAV) modulates immune signaling by direct binding to cellular factors. The protein encodes several src homology (SH) binding motifs (bm) (one SH2bm and two SH3bm), mediating interactions with host cell proteins. In contrast to NS1 of human IAV, NS1 of avian strains possess the second SH3bm (SH3(II)bm) consensus sequence with the special position of H5N1 isolates not carrying this motif. Our former studies showed that NS1 binding to CRK proteins is mediated via this motif. In the current study we addressed the regulatory properties of this SH3bm for cellular signaling and asked for the functional consequences for the IAV life cycle. Materials and Methods: Activity of the c-Abl tyrosine kinase was analyzed in an in vitro kinase assay upon c-Abl immunoprecipitation using MBP as substrat. Samples were subjected to SDS-PAGE and Western blot, and MBP phosphorylation was detected using an antiphosphotyrosine antibody. Additionally, analysis of the phosphorylation status of the c-Abl substrates CRKL and CRKII by phosphospecific antibodies was conducted. In addition to influenza A virus isolates, specific mutant strains carrying alterations in the SH3(II)bm consensus sequence of the NS1 protein were created using standard reverse genetic approaches. NS1-c-Abl interaction was investigated in a c-Abl pull-down assay upon GST-c-Abl and NS1 overexpression or influenza A virus infection. Coprecipitation of NS1 was analyzed using an anti-NS1 antibody in SDS-Page and Western blot. The impact of altered c-Abl signaling on influenza A virus replication was conducted by growth kinetics in human lung epithelial cells. Therefore, either growth abilities of virus mutants carrying different c-Abl inhibitory capacity were compared or chemical inhibition of c-Abl using imatinib was

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conducted. Viral titers were determined by standard plaque assay. For gross determination of cell morphology, infected or uninfected cells were analysed by light microscopy. For indirect immunofluorescence analysis, the influenza nucleoprotein was stained in addition to nuclear and actin staining. Stained cells were either analysed using epifluorescence or confocal microscopy. Confocal microscopy was used for z-stack analyses and 3D reconstructions as well. Results: In NS1-CRK interaction studies, we observed that the basal phosphorylation level of CRK was clearly reduced upon infection with avian IAV harboring an NS1 with an SH3(II)bm. This contrasts with results using human IAV. Reduced activity of the tyrosine kinase c-Abl was identified to be the cause for reduced CRK phosphorylation. Direct binding of NS1 to c-Abl was determined, and mutational manipulation of the SH3(II)bm clearly illustrated the necessity of this motif for Abl kinase inhibition. Avian NS1-induced kinase inhibition resulted in impaired avian IAV propagation, which was also detectible after chemical inhibition of c-Abl. Mutational analysis correlated diminished inhibition of c-Abl with strongly reduced cytopathic effects upon avian IAV infection, linking the pronounced inhibition of c-Abl to cytopathic host cell alterations during avian IAV infections. Finally, confocal microscopic analyses revealed gross alterations of the actin cytoskeletal network accompanied by strongly diminished detection of polymerized actin filaments and massive occurrence of filopodial structures in cells infected with IAV carrying c-Abl inhibitory capacity. Taken together, NS1 proteins of avian IAV interfere with the kinase activity of c-Abl leading to a massive reorganization of the actin cytoskeleton, resulting in strong cytopathologic alterations of the infected cell. Conclusions: In consideration of the still-existing threat of pandemics occurring from avian reservoir, we identified c-Abl, a major cellular signaling integrator that controls multiple signaling processes and cell fate regulations, as a new protein differentially regulated by avian and human IAV. These c-Abl regulations are potentially being involved in mediation of host range restrictions and influenza A virus pathogenicity, even in the context of highly pathogenic H5N1 infections, by controlling replication properties in epithelial cells.

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Pathogenicity and fitness of recent reassortant avian H5N1 viruses

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Background: Highly pathogenic avian influenza (HPAI) viruses of subtype H5N1 were identified in healthy domestic ducks during systematic surveillance in Lao People's Democratic Republic during 2010. Among the identified HPAI H5N1 viruses were natural reassortants (clade 2.3.2.1 x clade 2.3.4 NP; clade 2.3.4.2 x clade 2.3.2.1 PB2; clade 2.3.2.1 x H9N2 PB1). The genotypes of these viruses did not contain the major known mammalian pathogenicity/adaptation markers such as PB2627E or 701N, or HA222L. The phenotype of the reassortant HPAI H5N1 viruses and of a clade 2.3.4.1 virus isolated from an asymptomatic domestic duck was assessed in animal and cell culture models. Materials and Methods: The reassortant H5N1 viruses were inoculated into adult mallards (ocular, nasal, oral with 4 log EID_{50}). One reassortant was also assessed for the ability to transmit to direct-contact mallards and chickens. Differentiated normal human bronchial epithelial (NHBE) cells were inoculated apically with a multiplicity of infection of 0.001. The inoculum was removed with PBS washes after 1 hour incubation at 37°C. Three-month-old outbred male ferrets were inoculated intranasally with 6 log$TCID_{50}$. Naïve ferrets were brought into direct contact with donor ferrets one day post inoculation. Results: Adult mallards inoculated with the three reassortants showed symptoms ranging from none to transient conjunctivitis. Virus shedding was observed with differing preference for tracheal and cloacal shedding among the reassortants. One reassortant was assessed for transmission and found to transmit to 2/3 direct-contact mallards. The same reassortant was also assessed in adult chickens and induced mortality (100%) on day 3 post inoculation without prior symptoms. Chickens transmitted the virus to 3/3 direct-contact animals with 2/3 direct-contact animals surviving.In NHBE cells inoculated with a multiplicity of infection of 0.001, the three reassortants and a clade 2.3.4.1 duck isolate(A/duck/Lao/1023/2010) grew to low titers (3-4 log $TCID_{50}$/mL) compared with control A/Brisbane/59/07(seasonal H1N1; 9 log $TCID_{50}$/mL). The three reassortants and the

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clade 2.3.4.1 duck isolateA/duck/Lao/1023/2010 were inoculated into ferrets (n = 2). Different levels of pathogenicity (nonpathogenic to lethal) and virus shedding (none to 6 log TCID50/mL) were observed. The clade 2.3.4.1(A/duck/Lao/1023/2010) virus showed some ability to transmit to direct-contact animals. Most surviving inoculated ferrets seroconverted to low titers (5/6; detectable with horse red blood cells only). Conclusions: Ongoing assessments of novel genotypes of H5N1 indicate a relatively low ability to replicate in NHBE cells and intermediate to nonpathogenic phenotype of the reassortants in ferrets. One virus, A/duck/Lao/1023/2010 (clade 2.3.4.1), was highly pathogenic in ferrets and showed some ability to transmit via direct contact. All of the tested viruses were able to replicate in mallards with none to mild symptoms. Despite the absence of known pathogenicity markers, two of the viruses examined showed potential to cause disease in humans as assessed in the ferret model, and one virus showed potential for mammalian transmission. These results reiterate the call for systematic pathogenicity assessment of novel H5N1 viruses even in the absence of known pathogenicity markers.

P2-480

Maintenance of influenza A viruses by domestic ducks in West Java, Indonesia

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Background: As the reservoir for influenza A viruses, waterfowl typically are asymptomatic or show only mild clinical disease when infected, even when infected with viruses that are highly pathogenic in poultry. This may allow influenza virus outbreaks in waterfowl populations, including domestic duck populations, to go undetected by flock owners and government livestock officials. The role that domestic ducks play in the maintenance and spread of influenza viruses, particularly highly pathogenic H5N1 avian influenza virus (HPAIV H5N1), is poorly understood. Indonesia has a large domestic duck population, estimated at 34.3 million ducks. The vast majority of domestic duck flocks are raised on smallholder farms for meat and egg production. Materials and Methods: Domestic duck flocks were categorized as fully free-grazing, partially free-grazing or confined, based on production practices. Flocks in three districts of West Java were selected for cross-sectional and longitudinal studies. Flock owners were surveyed to assess flock husbandry, production practices, bird movement, flock housing and environment, biosecurity practices and flock health. For the cross-sectional study, oropharyngeal and cloacal swab samples as well as blood samples were collected from 30 flocks in each district (90 total flocks). For the longitudinal study, 6 flocks in each district (18 total flocks) were selected and 30 study ducks were placed in each flock for 7 months. Oropharyngeal and cloacal swab samples as well as blood samples were collected from the study ducks monthly. Swab samples were tested for influenza A virus and H5 subtype virus by real-time reverse transcription polymerase chain reaction (rRT-PCR). Serum samples were tested for anti-influenza virus antibodies using a blocking ELISA. A subset of 150 rRT-PCR positive samples was inoculated into 9- to 11-day-old specific pathogen-free embryonated chicken eggs and 38 influenza viruses were isolated. The hemagglutinin gene was sequenced for 33 of the isolated viruses. Results: Results from both studies show a high prevalence of influenza virus infection in domestic duck flocks in Indonesia. In the cross-sectional study, 39% of the flocks were positive for influenza virus and just over 10% were positive for H5 virus. The flock-level seroprevalence was very high, with 97% of flocks positive for influenza virus antibodies. During the seven-month longitudinal study, 17/18 (94.4%) flocks were positive for influenza virus at least one month during the study. Overall, 9/18 (50%) flocks were positive during the study for H5 subtype virus. The prevalence and incidence of influenza virus were significantly higher in the fully free-grazing flocks than the partially free-grazing or confined flocks. HPAIV H5N1 was isolated from three clinically normal partially free-grazing flocks. H2, H3 and H7 viruses were isolated from five different flocks, including one flock that was coinfected with HPAIV H5N1, H3 and H7 viruses. Conclusions: These results provide evidence that domestic ducks may play an important role in the maintenance of influenza viruses, including HPAIV H5N1, and could potentially be a source of infection to animal and human populations in Indonesia. Fully free-grazing flocks may have the highest prevalence and incidence of influenza virus due to a number of factors, including longer

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production cycles of up to three years, extensive movement of the flock throughout the production cycle, frequent commingling with other free-grazing flocks and high levels of contact with wild birds. Domestic duck production and product distribution systems in Indonesia are very complex and involve an intricate network of producers, middlemen and purchasers who all contribute to moving the ducks and duck products over a widespread geographical area. This movement, combined with the difficulties in detecting influenza virus outbreaks in domestic duck flocks and poor biosecurity practices of the flock owners, provides opportunities for dissemination of influenza viruses to animal and human populations.

P2-481

Development of a methodological risk assessment framework to assess the pandemic potential of animal influenza strains

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Background: The fact that an influenza virus of swine origin unexpectedly became the most recent human pandemic virus and that a low pathogenicity avian influenza virus H7N9 is currently causing human health concerns in Asia highlights deficiencies in current influenza pandemic preparedness. With the aim to systematically assess the potential public health threat posed by animal influenza viruses, the European Food Safety Authority (EFSA) has commissioned the FLURISK project. The project's main objective is to develop an influenza risk assessment framework (IRAF) for the ranking of animal influenza A strains in their potential to cross the species barrier and cause human infection. Materials and Methods: The project developed a prototype spatial epidemiologic model to answer the risk question: "What is the relative likelihood of a specific animal influenza A virus (compared with other influenza A viruses) currently present in animal populations to infect humans?" The model framework includes both virologic and epidemiologic components and data input is either generated through different specific activities of the project (global surveys, literature reviews, expert elicitation, etc) or derived from existing databases (eg, FAO's EMPRES-i database; animal species population density by production system from the FAO's Gridded Livestock of the World). Through expert opinion elicitation of international experts using an online questionnaire, weights of specific virus characteristics (eg, receptor preference, reassortment) were derived for calculating a virologic score, a component of the IRAF. The output of the overall IRAF model will be a list of ranked animal viruses according to their potential to infect humans. Suitability maps are generated to highlight high-risk regions. The ongoing validation of the IRAF makes use of genetic and epidemiologic data on historic animal influenza isolates selected among strains that have already caused infections in humans (eg, HPAI H5N1) and strains that are regarded as very unlikely to cause public health concern (eg, H13N8). The project has identified a number of scientific gaps concerning both epidemiologic and virologic factors potentially influencing the jump between animal species and from animals to humans. As a result of the gap analysis, priority research areas have been identified and will be put forward to the scientific community. Results: The prototype epidemiologic binomial model includes elements correlated with the properties or attributes of the virus, estimates of animal and human species populations and the transmission components β and V. Here, β is the "probability of an effective contact" between animals and humans and is derived from integrating data on human and animal density (stratified by production system, ie, intensive versus extensive). V is the "virus suitability score" for infecting humans and is based on specific virus characteristics identified through an expert elicitation process, such as preference toward α 2-6 receptors or presence of human adaptive markers in the replication proteins. In addition, results from a global survey on surveillance strategies for detection of animal influenza viruses contributed to estimate the uncertainty of the model

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estimates. Gap analysis indicates that there is clearly more knowledge on risk factors for spread of influenza viruses within animal populations rather than the jump from animal to humans, but it is important to note that much of this knowledge is restricted to H5N1 HPAI in poultry populations and not necessarily transferable to other viruses or hosts. Very little information is available regarding swine-to-human transmission or the risk factors involved. Considering the role of swine as a hypothetical bridge species and/or mixing vessel, the importance of this limitation is evident and further research strongly encouraged. Conclusions: The preliminary results from the model and the virus score validation are plausible. Nevertheless, areas for further development and validation of the IRAF tool are being identified and will be addressed before the model can be released for regular use. Findings from the FLURISK project will also help prioritize research areas and encourage animal influenza surveillance at high-risk areas of the animal-human interface globally.

P2-482

Playing hide and seek—understanding the role of collectins and pentraxinsin limiting novel and seasonal influenza viruses

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Background: Influenza A virus (IAV) pandemics are derived from avian or swine viruses that acquire relevant gene segments and/or mutations to infect and transmit efficiently in humans. Over time, seasonal IAV strains acquire additional mutations in the HA (and other genes) associated with further adaptation to the human host and/or escape from immune pressure. Common changes include substitutions that modify HA receptor specificity for sialic acid (SA), as well as the addition of glycans to the head of the viral HA. Soluble extracellular proteins of the innate immune system, including members of the collectin and pentraxin superfamilies, are present in airway fluids and can limit the ability of particular IAV to infect host cells. In general terms, these molecules act as (i) lectins, that bind to glycans expressed on the viral HA, or (ii) “receptor decoys” that express SA and compete with sialylated host cell receptors. Our recent studies have defined mechanisms by which collectins and pentraxins act against IAV and investigated their roles in (i) innate immune defense against IAV infection, (ii) limiting interspecies transmission of IAV, and (iii) driving the evolution of HA receptor specificity. Materials and Methods: Purified human collectins and pentraxins, as well as whole airway fluids from humans, mice and ferrets, were used. The viral determinants recognized by collectins and pentraxins were identified using mutant viruses generated by reverse genetics. Finally, mouse and ferret models of IAV infection were used to investigate pathogenesis and disease. Results: Animal IAV and novel IAV entering the human population carry little glycosylation on the viral HA but acquire glycans as they circulate in humans. Consistent with this, avian IAV and IAV strains that had recently emerged in humans were resistant to the antiviral activities of collectins, whereas seasonal IAV strains were sensitive. We identified the pentraxins PTX3 and serum amyloid P (SAP) as “receptor decoys,” expressing α (2,3)- and α (2,6)-linked SA, respectively, and demonstrated that the activities of both the viral HA and the NA were critical in determining anti-IAV activity. PTX3-resistant mutants (H3N2) selected under experimental conditions were characterized by a single amino acid change in the receptor-pocket of HA and similar substitutions were associated with the natural evolution of H3N2 IAV from PTX3-sensitive to PTX3-resistant soon after their emergence in humans in 1968. Virus mutants selected in the presence of purified SAP were characterized by distinct substitutions in the receptor pocket of HA and these were not common in circulating IAV strains. Conclusions: Given the limited glycosylation expressed by pandemic and animal IAV, sialylated “receptor decoys” in airway fluids may provide initial protection following exposure to novel IAVs. As PTX3 expresses α (2,3)-linked SA, it may limit interspecies transmission and/or moderate disease when a novel IAV enters the human population, however IAV can evolve resistance to PTX3 while retaining specificity for human cellular receptors. In contrast, SAP (and other α (2,6)-rich soluble glycoproteins) may represent “true” neutralizing inhibitors of IAV and resistant mutants are unlikely to emerge, as they would exhibit poor recognition for human receptors and likely reduced fitness.

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The first reported fatal pediatric case of H5N1 with atypical symptoms, Bangladesh

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Background: Although highly pathogenic avian influenza (HPAI) A(H5N1) virus has been circulating widely among poultry in Bangladesh since 2007, only six human cases of HPAI A(H5N1) virus infection have been detected and all experienced mild illness. On February 12 2013, a previously healthy boy aged 23 months was admitted for febrile seizures at a tertiary care hospital in Comilla. He was enrolled as a control subject in a icddr,b-led study on vitamin D and respiratory virus infection as he did not present with respiratory symptoms; throat and nasopharyngeal swabs and blood were collected during enrollment. Later on, the icddr,b's virology laboratory detected A(H5) and A(H1N1)pdm09 viral RNA in the swabs using real-time reverse transcription polymerase chain reaction (rRT-PCR) assay. We conducted investigations to explore the clinical presentation, outcome and possible exposures of this case. Methods: A multidisciplinary team investigated the event. Epidemiologists interviewed family members and hospital physicians, reviewed hospital records to collect detailed clinical history, and assessed whether any secondary cases had occurred among the child's family members and health care providers. Anthropologists used qualitative interviews to investigate possible routes of exposure and obtained detailed illness and care-seeking history from family members. Veterinarians collected specimens from a frozen processed chicken obtained from the case's household. Both human and poultry samples were tested at icddr,b and Institute of Epidemiology, Disease Control and Research (IEDCR) virology laboratories using rRT-PCR for influenza A virus and subtyped for A(H5). Finally, Centers for Disease Control and Prevention (CDC), Atlanta, Georgia, performed confirmation, virus isolation and molecular characterization. Results: The child developed fever on February 11 and was taken to the Comilla hospital on February 12 when he developed convulsions. When seizures subsided after treatment with rectal diazepam, the parents decided to discontinue hospital treatment and returned home with the boy. On February 13, he presented to another hospital with respiratory distress. Laboratory tests demonstrated microcytic anemia and lymphocytosis (total white blood count = 9000/mm^3, lymphocytes = 60%). A chest X-ray on February 15 revealed opacities in the upper lung fields suggestive of pneumonia. He was transferred to a Dhaka hospital on February 16 for intensive care support. Cerebrospinal fluid examination performed at the hospital suggested pyogenic meningitis but bacterial culture was negative. On February 17, the patient developed disseminated intravascular coagulation and died on February 18. Respiratory swab samples were tested a month later—on March 12 and 16 in icddr,b and IEDCR respectively—as hospital-based influenza surveillance samples are shipped fortnightly to icddr,b and testing of case samples is prioritized over controls. On April 6, CDC confirmed HPAI A(H5N)1 virus, clade 2.3.2.1. The parents reported that several days prior to their child's illness onset, 35 chickens from their backyard poultry flock got sick and a few died. Household members slaughtered, cooked and consumed the remaining sick chickens. While his mother processed the carcasses, the child played with the uncooked chicken organs. The family froze processed chickens for future consumption. Tissue samples obtained from one such processed chicken had detectable HPAI A(H5N1) virus. Sequence analysis identified the isolate as clade 2.3.2.1 and the haemaglutinins, neuraminidase and matrix genes showed 99.9% homology with those genes identified in the patient's sample. No close contacts of the slaughtered chickens or the child developed influenza-like illness in the two months following the child's death. Conclusions: This is the first known fatal HPAI A(H5N1) human case in Bangladesh and evidence suggests he was infected through exposure to HPAI A(H5N1)-infected poultry. None of the treating hospitals had viral pathogen detection capabilities. This case did not present with typical respiratory symptoms and was detected by chance through a research study. Without routine etiologic investigations, similar cases will remain undetected in Bangladesh. Alerting local physicians about atypical presentations and screening

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patients with severe illness for exposure to sick poultry may help identify further cases. Precautions during slaughtering or processing sick chickens may prevent future HPAI A(H5N1) cases in the community.

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Comparison of virulence and transmissibility of two canine H3N2 influenza viruses isolated in South Korea

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Background: Canine influenza outbreaks have raised concerns over the potential of dogs to mediate adaptation of influenza A viruses, which may become a threat to public health. Interspecies transmission of a wholly avian-origin H3N2 was first documented in South Korea in 2007, causing clinical disease ranging from mild respiratory disease to fatality in dogs. Genetically similar viruses were also reported in some regions of China. Since then, canine influenza viruses (CIVs) have been sporadically detected in different provinces of South Korea. We now report the results of the comparison of virulence and transmissibility of two CI H3N2 viruses isolated in South Korea in 2009 and 2012, respectively. Materials and Methods: Phylogenetic and genetic analyses of individual gene segments were performed to determine the genetic origin of A/canine/Korea/DJ-cd1/2009 [DJ-cd1/09] and A/canine/Korea/NS-S4/2012 [NS-S4/12] or presence of any virulence markers. To investigate pathogenesis, beagles, White Leghorn chickens, ducks and ferrets were inoculated with the two H3N2 CIVs and further compared with a closely related reference avian H3N2 virus (A/duck/Korea/LPM91/2006 [LPM91/06]). Serologically naive counterparts were placed in each of the inoculated group of test animals as contacts after one day to examine transmission. Viral titers were measured in both infection and contact groups for 14 dpi. Results: Phylogenies of viral segments revealed that DJ-cd1/09 and NS-S4/12 are closely related to previous H3N2 CIVs in Korea since 2007 that originated from avian species. Molecular characterization revealed nonsynonymous mutational differences between the two H3N2 CIVs, particularly in the putative H3 antigenic sites of the 2012 isolate and the acquisition of an N-linked glycosylation site at the globular head of the H3 HA molecule. In domestic poultry, none of the inoculated chickens could shed the DJ-cd1/09, NS-S4/12 nor LPM91/06 virus, whereas only LPM91/06 was able to successfully replicate in ducks and transmit to co-housed contact birds despite similar avian virus ancestry. The two H3N2 CIVs, but not LPM91/06, were transmissible between dogs, but NS-S4/12 caused more severe disease with mortality. Similarly, NS-S4/12 induced higher nasal wash titers than the other viruses in ferrets that persisted up to 5 days post infection. Moreover, the 2012 H3N2 CIV established transmission between ferrets via aerosol droplets as early as 3 days post exposure. Conclusions: We provide evidence that H3N2 CIVs are constantly evolving in canine species and slowly gaining foothold of mammalian hosts. Since dogs are common companion animals of humans, they represent an alternative hosts for influenza virus zoonosis. Thus, our results add weight to the importance of continuous surveillance of CIVs in the country.

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Evolutionary properties of HA and NA genes of H7 and N9 subtype avian influenza viruses isolated from wild and domestic waterfowl in Taiwan

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Background: A novel influenza subtype H7N9 virus coursed caused human fatal cases in Feb of 2013 in China. The hemagglutinin (HA) and neuraminidase (NA) genes of the H7N9 virus maybe origin formoriginate from the viruses of wild birds. Taiwan is located about 400 miles away from the H7N9 outbreak regions in Shanghai City and Hangzhow City of Zhejiang Province. Thousands of wild ducks and shorebirds, which carry influenza A viruses, fly to Taiwan through the East Asian–Australasian flyway. The study aimed to realize determine the evolutionary relationship of these Taiwanese H7 and N9 subtype AIVs with the novel avian-origin H7N9 in China, to assess the potential risk of wild birds in the transmission of avian influenza. Materials and methods: Forty-five of avian influenza viruses isolated in Taiwan in 1998 to 2013 were selected for this study. All the viruses were proliferation proliferated in specific- pathogen-free chicken embryonated eggs. The viruses were subtyped using the hemagglutination inhibition test. The NA subtypes of the AIV isolates were subtyped using a neuraminidase inhibition test. The HA and NA subtypes of the isolates were also confirmed using RT-PCR, with H- subtype-specific primers and with N subtype-specific primers, respectively. Twenty-seven of them were the H7 subtype of wild waterfowl viruses, six 6 of them were the H7 subtype of domestic duck viruses, and 12 of them were the N9 subtype of wild waterfowl viruses. All of HA genes of H7 viruses and NA genes of N9 viruses were sequenced and analysis. The viral genomes were amplified by RT-PCR. RT-PCR products were purified and cloned into the pCR2.1-TOPO vector. Recombinant plasmids were purified and sequenced. Sequence alignment and phylogenetic analysis were performed. The neighbor-joining method was used in PHYLIP to build the tree. Results: The phylogenetic tree of the HA gene of H7 viruses showed that a correlation existed between the isolation years and the evolutional distances. The HA gene of HA of H7 and NA gene of N9 of waterfowl in Taiwan were clustered together with the viruses of waterfowl in Japan, Korea, Siberia, and China. The blast result of all gene segments of two 2 Taiwanese H7N9 strains viruses were high similarity to the viruses of other aquatic birds, indicated indicating that no reassorttment reassortment event occur. Conclusions: The HA gene of H7 viruses and NA gene of N9 viruses in waterfowl in Taiwan are highly relationship correlated with the novel avian-origin H7N9 in China. There is still have the potential risk of wild birds in the transmission of H7N9 avian- influenza virus transmissiones from wild birds.

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Consequences of improper control of avian influenza in Egypt: Evidence from systematic surveillance

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Background: Seven years after the onset of avian H5N1 influenza in Egypt, no signs of its eradication are visible. The virus has become endemic and Egypt has reported more human cases than any other country. Materials and Methods: Since the reservoirs for the continuing H5N1 outbreaks in Egypt are ill-defined, we conducted active surveillance in various geographic regions and poultry production sectors to detect influenza viruses. The survey included Cairo and 6 governorates that had previously reported H5N1 activity. qRT-PCR was used to type and subtype influenza A viruses. Sequencing of the HA gene and hemagglutination inhibition assay were used to characterize the viruses genetically and antigenically. Western blotting, immunofluorescence, and virus culture were used to study

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coinfections with different virus subtypes. Results: Between August 2010 and January 2013, 11,452 swabs were collected from domestic poultry; 10% tested positive for influenza A virus. Between August 2010 and November 2011, all detected influenza A samples were of the H5N1 subtype. In December 2011, we detected the first H9N2 virus. In March 2012, we detected the first samples with H5N1 and H9N2 coinfections. Between September and November 2012, we detected a sudden increase in incidence of avian influenza viruses. H5N1 and coinfections constituted the majority of subtypes detected in this outbreak. By December 2012, H5N1 detection decreased and the majority of samples were H9N2. Avian influenza viruses appeared to be circulating in various poultry production sectors and did not adhere to a seasonal or geographic pattern. All species of domestic poultry were prone to infection, but the incidence was highest in turkeys. We detected influenza, including H5N1, in apparently healthy birds. The phylogenetic analysis of H5 genes revealed that in the early months of our surveillance, clade 2.2.1 and 2.2.1.1 viruses co-circulated. However, as of 2011, clade 2.2.1 viruses dominated. This was also shown to be true when we characterized the H5N1 viruses antigenically. H9N2 viruses belonged to the G1 group and showed little variation over time. We then focused on H5N1 and H9N2 coinfections. The presence of the two viruses in three samples collected in 2012 was verified by RT-PCR, qRT-PCR, HA sequence analysis, immunofluorescence, and Western blotting. Next, the samples were propagated in embryonated chicken eggs. H9 grew at a higher rate than H5 after 1 passage even though cycle thresholds for both viruses were the same for the original swab. We then conducted a plaque purification assay in an attempt to separate the viruses. All selected and propagated plaques were found to be H9N2. To understand our inability to isolate H5N1 by plaque purification, we conducted an experimental coinfection time course analysis of both viruses at an equal dose in eggs and MDCK cells. Our results show that both viruses grow to similar titers in cells and the presence of the other virus in the culture did not affect propagation. In eggs, H9N2 grew more efficiently but the presence of H5N1 was not affected. Conclusions: Through our systematic surveillance program of avian influenza in Egyptian poultry, we were able to detect 3 major events: the emergence of H9N2, coinfection of singles hosts with H9N2 and H5N1 viruses, and an increase in influenza virus infection incidence over baseline incidence as of September 2012. We determined that the reservoir for influenza viruses in Egypt is not localized in a specific poultry production sector, not specific to a single species, and is geographically widespread. Co-circulation and coinfection of avian influenza viruses is a major concern due to the high potential of reassortment that can lead to virus progeny with novel characteristics that can threaten not only avian but human health as well. Indeed, Egypt remains a hot spot for avian influenza circulation, and vigilant surveillance remains the single most important tool to keep track of these viruses.

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Triple reassortant internal gene cassette from 2009 H1N1 influenza A virus could increase public health risks of canine influenza viruses

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Background: First identified in 2003-2004, H3N8 canine influenza A viruses (CIVs) of equine origin have been isolated from dogs in the UK and US; whereas avian origin CIV H3N2 strains, identified in 2006-2007, have circulated in both pet and farmed dog populations in China and Korea. The 2009 novel H1N1 triple reassortant between avian, swine, and human influenza caused the first pandemic since 1968. It has been speculated by many researchers that the triple reassortant internal gene (TRIG) cassette is highly stable and conducive to easy surface gene reassortment. We hypothesize that the TRIG cassette from 2009 H1N1 influenza A virus could reassort with H3 CIVs to generate strains that pose a risk to public health. Materials and Methods: Serological assays using ferret generated antisera demonstrated that both H3N8 and H3N2 CIVs are antigenically distinct from contemporary human H3N2 viruses, suggesting currently there would be no human herd immunity to the CIV H3 subtypes. The following reassortant viruses were generated using reverse genetics

techniques: A/canine/Guangdong/1/ 2006(HA,NA)xA/California/4/2009(H3N2) and A/canine/Iowa/13628/2005(HA,NA)xA/California/4/2009 (H3N8). Results: The replication kinetics of these 2 reassortant viruses were compared with their wild-type CIVs, respectively, in both MDCK cells and in human alveolar epithelial (A549) cells. The reassortant CIVs replicated to significantly higher titers in vitro than the wild-type CIVs. Conclusions: Humans are suspected to be a naive population to wild-type CIVs and our results suggest the pH1N1/CIV reassortant viruses may increase the risk of CIVs to public health. The pathogenicity and transmissibility of the reassortant viruses will be evaluated in vivo in the ferret animal model.

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International collaboration on the characterization of H5N1 highly pathogenic avian influenza viruses isolated from humans in Indonesia during 2011-2012 for developing the candidate vaccine viruses

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Background: Highly pathogenic avian influenza (HPAI) H5N1 viruses currently circulating in birds have caused hundreds of human infections and pose a significant pandemic threat. In Indonesia, after the first human case of H5N1 reported in 2005, HPAI H5N1 viruses have spread among poultry, with sporadic cases of avian-to-human transmission. The rapid evolution of H5N1 viruses has resulted in genetic diversity with distinct antigenic characteristics. In this study, to understand the current situation of H5N1 influenza viruses in Indonesia, we analyzed H5N1 viruses isolated recently from humans in Indonesia. Also, we report here the evaluation of the immunogenicity of a new candidate vaccine virus, NIIDRG-9, developed by reverse genetics with the hemagglutinin and neuraminidase genes of an H5N1 virus isolate and six internal genes from A/Puerto Rico/8/34 (PR8). Materials and Methods: Five H5N1 human isolates, A/Indonesia/NIHRD11767/2011, A/Indonesia/NIHRD11771/2011, A/Indonesia/NIHRD11931/2011, A/Indonesia/NIHRD11949/2012 and A/Indonesia/NIHRD12379/2012 were used in this study. These isolates were propagated in the allantoic cavities of 10-day-old embryonated chicken eggs to produce viral stocks. Full genome sequencing and phylogenetic analysis of the viruses were performed. Antigenic characterization of the viruses was done by hemagglutination inhibition (HI) assay with a panel of postinfection ferret sera. A reassortant virus, termed NIIDRG-9, was generated by using the 12-plasmid reverse genetics (RG) system (kindly provided from Dr. Kawaoka). NIIDRG-9 contained six internal genes from A/Puerto Rico/8/34 as well as the hemagglutinin (HA) and neuraminidase (NA) gene segments from the A/Indonesia/NIHRD11771/2011 virus. The multibasic amino acid residues at the cleavage site of HA were removed to reduce virulence. To rescue and propagate the reassortant virus, after 48 hours post transfection of 12 plasmids into LLCMK2-D cells, the supernatant was harvested and inoculated into 10-day-old specific pathogen-free embryonated chicken eggs. Results: Phylogenetic analysis of the HA genes showed that the five human isolates were closely related to the viruses of clade 2.1.3.2 isolated in Indonesia previously. The amino acid sequence at the cleavage site of HA was PQRESRRKKR/GLF, which is characteristic of HPAI viruses. The receptor-binding pocket of HA1 of all isolates retained residues Q222, and G224, which are known to be related to the preferential binding to avian α2,3-NeuAcGal receptors. The mutations at antigenic sites were found at positions 183N, 184G, E or D as compared to that of A/Indonesia/5/2005. Sequence analysis of these viruses revealed no mutation that is associated with a known reduced sensitivity to NA inhibitors. Notably, one of these isolates, A/Indonesia/NIHRD12379/2012, had a 701N mutation in PB2, suggesting that this isolate had adapted to an extent to mammalians. Antigenic analysis showed the postinfection ferret antiserum raised against the current candidate vaccine virus, A/Indonesia/5/2005 (CDC-RG2) poorly reacted with these isolated viruses, while the postinfection ferret serum against A/Indonesia/NIHRD11771/2011 and NIIDRG-9 reacted well with these isolates. Conclusions: These results indicate that the recent H5N1 viruses isolated from humans in Indonesia had distinct antigenic properties, compared with that of A/Indonesia/5/2005. Our finding suggests that NIIDRG-9 is suitable for use in vaccine manufacturing and as a reference vaccine virus against the H5N1 clade 2.1.3.2

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viruses in Indonesia. Continuous monitoring of the prevalence of H5N1 viruses is needed for pandemic preparedness and risk assessment of a pandemic.

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Poultry H5N1 outbreaks on the countryside, human deaths in Jakarta: are mega-cities sinks for avian influenza viruses?

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Background: Within a two-week period in January 2007, five people in the greater Jakarta area became infected with H5N1 virus, despite the absence of concurrent outbreaks in neighboring poultry. This spatiotemporal phenomenon was observed again in January-February 2008. More recently in 2013, within a few weeks, more than 130 people in the greater Shanghai and other mega-urban areas in China were infected with H7N9 virus. In an exhaustive search for the virus, it was detected in only a few samples, mainly from live bird markets, while no virus was found on poultry farms. A source-sink accumulation mechanism is proposed as a possible model to explain the phenomena observed. Poultry outbreak data from the Participatory Disease Surveillance and Response (PDSR) surveillance system, initiated in Indonesia in 2006, have shown a high incidence of HPAI H5N1 in poultry in rural areas, including areas producing commercial poultry. Human H5N1 incidence in these areas has been low. The highest human incidence has been reported from Jakarta Province, which has the highest human population density and the lowest ratio of chicken population to human population in Indonesia. Materials and Methods: An assessment of commercial poultry movements into the greater Jakarta metropolitan area indicated that live birds were sourced from many areas across Java Island, as well as southern Sumatra Island, for consumption in Jakarta. The majority of this poultry supply was marketed through live bird markets (LBMs) in which birds are slaughtered at or near the point of sale. A LBM environmental surveillance system was established in 2009 covering all known LBMs in the greater Jakarta area. Each month five environmental swabs from each market were pooled and tested by PCR for influenza A and H5 viral antigen. Results: Over the first year of sampling, a monthly average of 80% of LBMs tested positive for influenza A and 50% for H5. The monthly prevalence in LBMs showed a seasonal pattern, similar to the seasonal pattern observed in the PDSR data and in human cases. Observation of sick or dead poultry in LBMs remained low relative to the H5 prevalence observed. Additional environmental surveillance at LBMs in less densely populated areas found lower influenza A and H5 prevalence than in Jakarta. H5 prevalence in LBMs showed a significant downward trend between 2009 and 2012. A similar downward trend was also observed in poultry outbreak incidence measured through PDSR surveillance, and in human cases, although the spatial discordance between poultry cases and human cases continued. Conclusions: A modified source-sink accumulation model may explain the H5N1 viral and disease patterns observed in Indonesia. Within this model, avian influenza virus replication occurs in poultry production areas (source) with subsequent accumulation along the market chain, leading to increased human exposure in the mega-urban areas (sink). Areas with the highest demand for poultry from the most sources would be expected to show the highest LBM virus contamination and the highest human disease incidence. In avian influenza virus endemic areas, the establishment of a standardized LBM environmental surveillance system in major sinks is important to assess human risk levels in sink location and monitor overall impact of poultry disease control at source. The system could also provide a more cost-efficient and more sensitive surveillance method for detecting avian influenza viruses with a low prevalence in the field, such as the recently emerged H7N9 virus.

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Human, environmental and animal surveillance of influenza A(H5N1) virus in Cambodian live poultry markets

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Background: Since 2004, A(H5N1) virus has been detected in Cambodia in 39 poultry outbreaks and in 32 patients (27 deaths) to May 1st, 2013. Outbreaks in Cambodia are associated with main festivals (Chinese/Lunar New Year, Khmer New Year, etc) due to increased demand for poultry. In most countries, animal A(H5N1) virus surveillance often only relies on virus detection in randomly selected live poultry populations. In Cambodia, as in other countries, relying on identifying A(H5N1) virus in poultry has many limitations resulting in the virus rarely being detected in poultry populations and often detected in humans followed by poultry flocks in surrounding areas. Previous environmental studies in live poultry markets (LPMs) and human seroprevalence studies in Cambodia have shown that H5N1 virus is circulating in markets, but is rare among close contacts of human H5N1 cases. Here we report the preliminary results of a second study that combines human, animal and environmental surveillance of A(H5N1) virus circulation in LPMs. Materials and Methods: Since January 2013, weekly collection of environmental specimens has been ongoing in 4 LPMs. In each market, environmental specimens (eg, drinking water used by poultry, water on the floor/sewage, soil/mud collected on the floor, poultry feathers, poultry faeces) as well as tracheal and cloacal swabs from live poultry are collected. After sample processing and virus concentration, samples are tested by real-time RT-PCR for influenza A, A(H5N1) and A(H7N9) virus detection. Specimens tested positive are inoculated into embryonated eggs for virus isolation. A subset of the strains isolated is partially or fully sequenced including unsubtypable influenza A strains. To monitor the exposure of poultry workers (poultry sellers, butchers, middlemen) to the virus, human serum samples are collected every 3 months throughout 2013 (starting in January 2013). Sera are tested by hemagglutination-inhibition assay (HIA) and microneutralization test (MN) to detect anti-H5 and anti-H7 virus neutralizing antibodies. Results: Preliminary results indicate that from January to May 2013, of the 528 LPM environmental samples tested, 151 (29%) tested positive for A(H5N1) virus by qRT-PCR and an additional 114 samples (21%) tested positive for Influenza A virus. The detection rate of A(H5N1) virus by RT-PCR was higher in environmental specimens (43% in water, 40% in soil/mud, 34% in feathers) than in poultry swabs (16%). The positivity rate in environmental specimens was highest (81%) during the Chinese/Lunar New Year festival (Feb 2013). Serology screening of the two first series of sera obtained from 125 poultry workers found no recent H5N1 infections. Conclusions: Past and current avian influenza virus surveillances at LPMs in Cambodia demonstrate that the LPM environment is highly contaminated with A(H5N1) virus, particularly before and during the main national festivals. In contrast with traditional surveillance, virus circulation can be very efficiently monitored by environmental samples analysis. The risk for poultry workers to develop infection is currently being evaluated.

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Analysis of the swine movement network in a province of Northern Vietnam: implications for swine influenza surveillance

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Background: South-East Asia, and especially Vietnam, is known for its high human, poultry and pig population density. The Red River Delta region alone includes about a quarter of the human, pig and poultry population of Vietnam in just 6.4% of the area of the country. This high density along with familial production practices could put the area at a higher risk for interspecies influenza virus transmission and emergence of reassortant viruses. Live animal movements are one of the most important factors for disease transmission between holdings and geographic areas, and its description is required to study disease spread and design surveillance. The objective of the present study was to describe the swine movement network in the Red River Delta, and to draw implications for local surveillance of swine influenza viruses. Materials and Methods: The study was carried out in Vietnam, in two communes of Hung Yen province. Structured interviews of farmers were performed to collect data on animal production and live pig movements from January 2011 to June 2012. All the large familial farmers (100-1000 fattening pigs per cycle and/or 10-250 sows) were interviewed in the two selected communes, as well as all the small familial farmers (< 100 fattening pigs per cycle and/or < 10 sows) in one selected village in each of the communes. Based on the farmer questionnaires, a sample of the mentioned middlemen, slaughterhouses and also industrial farms were chosen for interview. Data were entered into a computerized database and analyzed using R 2.15.3. Results: A total of 88 small farmers, and 50 large farmers were interviewed, ie, over 95% of the farms fitting the selection criteria. Thirty middlemen and slaughterhouses and two industrial farmers were interviewed. Trades of live pigs from interviewed farmers represented 49,000 pigs and 1600 trades, and the involvement of a high number of traders and different farmers. However, about 85% of the actors were mentioned only 1 or 2 times. Also, important differences were observed for pig trading between the two communes. Farmers in Me So bought preferentially from large and industrial farms while Dinh Du farmers bought from middlemen and small farmers. Overall, large farmers in Me So commune traded 78% of the pigs. The pigs originated from height provinces in Northern Vietnam and three in Center and South Vietnam, but were sold only in the Red River Delta. The traders and other industrial farmers who were subsequently interviewed mentioned the trade of 550,000 pigs over 22,000 trades. Weaners and breeders were purchased mostly from familial farms in 11 provinces in Northern Vietnam and one in the South. They were then sold to other farms over 16 provinces in Northern Vietnam. Fattening pigs sold to slaughterhouses were mostly purchased in Hanoi and Hung Yen, most of the pigs coming from large and industrial farms. Conclusions: The study shows the complexity of the pig movement network in Vietnam composed by many small private businesses and involving many provinces. This pattern in trading is a challenge for influenza surveillance design and also food safety control. In particular, influenza spread in this area among swine and other species could differ substantially from the dynamics in other countries with generally larger farms and tighter biosecurity. In further work, we hope to explore the implications on influenza transmission dynamics of the different network structures in the Dinh Du versus Me So communes. In particular, the feasibility and added value of surveillance at different points in the swine supply chain, including slaughterhouses, live pig markets, farms, and the approach to selection of animals for sampling. Strategies adapted to the local context must be developed to increase the chance of influenza virus detection while keeping surveillance at a low cost.

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Haemagglutinin activation by human transmembrane protease serine 2 or by human airway trypsin-like protease is necessary for the production of high titre influenza A virus pseudotypes that can be employed for the evaluation of pandemic potential

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Background: The monomer of influenza haemagglutinin (HA) is synthesized as a single polypeptide precursor that during maturation is cleaved by proteases into two active subunits. Recent studies have demonstrated that the human transmembrane protease serine 2 (TMPRSS2) and the human airway trypsin-like protease (HAT) can activate, by cleavage, the HA of human seasonal influenza viruses. As a model of activation of all influenza subtypes (human H1, H2, H3 viruses and other representative viruses from group 1 and group 2 avian subtypes), we have investigated the use of human TMPRSS2 and HAT to produce high titre influenza HA lentiviral pseudotypes. Such pseudotypes represent powerful and safe tools to study viral entry mechanisms and zoonotic potential. Materials and Methods: Influenza pseudotype particles are obtained by cotransfecting human embryonic kidney HEK293T/17 cells using plasmids coding for the influenza HA, HIV gag-pol and a retroviral vector incorporating firefly luciferase. To investigate the role of these proteases in the HA activation necessary for infective particle generation, a plasmid expressing TMPRSS2 or HAT was cotransfected during pseudotype production. The HA lentiviral pseudotypes produced were used to investigate cell entry potential via transduction of target cell lines and as surrogate antigens in neutralization assays. Results: Influenza pseudotype particles produced by cotransfection of these proteases can transduce HEK293T/17 cells with high efficiency compared with the pseudotypes produced in the absence of proteases. The high titre of these influenza pseudotypes permits their use as surrogate antigens in neutralization assays and subtype-specific sera can readily neutralize them. Conclusions: TMPRSS2 and/or HAT can activate, in vitro, both the HA of human seasonal influenza and also other avian HA influenza strains in a pseudotype particle production system. Furthermore, this panel of influenza pseudotypes can be used in neutralization assays to study heterosubtypic antibody responses and pandemic potential.

P2-494

Avian influenza at the animal-human interface: investigations among poultry workers in live bird markets in Dhaka city, Bangladesh, 2012-13

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Background: Since 2007, highly pathogenic avian influenza (HPAI) H5N1 virus has circulated among Bangladesh poultry. Workers in live bird markets (LBMs) have intense occupational exposure to poultry with minimal use of personal protective measures. In February 2012, we started surveillance to identify human cases of infection with avian influenza A viruses (AIVs) among poultry workers in 16 LBMs of Dhaka. Whenever poultry workers with detectable AIV RNA by rRT-PCR were identified, we

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initiated an outbreak investigation in the affected market to enhance case detection, ascertain clinical history and health status of suspected cases, and explore possible sources of infection. Details regarding index cases that triggered investigations are reported separately; here we report on investigations at markets with suspected cases from February 2012 to February 2013. Methods: We defined suspected AIV cases as influenza-like illness (fever with cough or sore throat) for the first LBM investigation and any respiratory symptoms subsequently. In response to any AIV RNA-positive case, we screened all workers in that market to identify further suspected AIV cases for up to three weeks after detection of the index case. Nasopharyngeal (NP) and oropharyngeal (OP) swabs were collected from suspected cases and tested by rRT-PCR for influenza viral RNA and further subtyped (H1N1pdm09, H3, H5, H7 and H9) if positive for influenza A virus. Acute (< 7 days post illness onset) and convalescent serum (> 21 days after acute serum collection) were collected from workers with detectable AIV RNA and tested for H5-specific antibodies by microneutralization assay. An H5 seropositive was defined as a ≥ 4-fold rise in antibody titer in paired sera (seroconversion) or a neutralizing antibody titer of ≥ 40 in any serum specimen with confirmation by Western blot assay and serum adsorptions. Starting with the second investigation, NP and arm swab specimens were collected from asymptomatic workers matched 1:1 to suspected cases by timing and market location. Results: Nine investigations were launched within eight markets, all during months of peak AIV circulation among poultry (November-March). We screened 3111 workers, of whom 836 (27%) had respiratory symptoms. Respiratory swabs were collected from 778 (93%) symptomatic workers. Of 778, 69(8.9%) had detectable AIV viral RNA but frequently with high Ct values (> 37): 25 (3.2%) for H5, 35 (4.5%) for H9, 9 (1.2%) for H5 and H9. A further 59 (7.6%) had detectable influenza ARNA but unsubtypeable. Attempts to culture H5N1 virus from 12 positive swabs were unsuccessful. Of 25 paired sera tested to date, no seroconversions to H5N1 virus were identified, but sera from five workers were H5 seropositive in both specimens. Four suspected AIV cases missed work due to illness, but none were hospitalized and all recovered. Among 371 asymptomatic workers, 17 (4.5%) had detectable AIV RNA in NP swabs and 67/361 (18.5%) had detectable AIV RNA in arm swabs; H5 viral RNA was detected in 7 NP and 37 arm swabs, and H9 viral RNA was detected in 8 NP and 11 arm swabs. The proportion of H5 viral RNA-positive NP swabs was similar among symptomatic (23/778; 3.0%) and asymptomatic (7/371; 1.9%) workers. Conclusions: In Dhaka LBMs with suspected AIV cases, we identified 145 poultry workers with detectable AIV or influenza A/unsubtypeable viral RNA by rRT-PCR in respiratory specimens, often with high Ct values, during months of peak AIV circulation among poultry. Five workers were seropositive for HPAI H5N1 virus antibodies, suggesting prior infection. Among symptomatic workers, no severe illness was reported and no H5-specific seroconversion was identified. Frequent detection of AIV RNA among poultry workers and in NP swabs and on arms of asymptomatic workers suggests that detection of AIVs in symptomatic workers without serologic confirmation could represent viral contamination rather than active infection. LBM markets are an ideal setting to perform surveillance for zoonotic influenza A virus transmission, but interpretation of rRT-PCR findings is challenging and highlights the need to discriminate between viral contamination of the human respiratory tract and infections with AIVs. Further research should investigate why severe illness is not detected among highly exposed poultry workers.

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P2-495 SCHOLARSHIP International Research Scholarship

Did intensified surveillance lead to increased detection of human infections with highly pathogenic avian influenza A(H5N1) virus in East Jakarta, Indonesia?

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Background: Indonesia has reported the most human cases of highly pathogenic avian influenza (HPAI) A(H5N1) virus infection worldwide, with 192 cases and 83% mortality since 2005. Cases are detected through health care facility notifications, in which patients meeting the World Health Organization suspected case definition for A(H5N1) virus infection are tested. To assess whether

there is underdetection of human cases, enhanced influenza surveillance was implemented in East Jakarta District. The district was chosen because 12 confirmed human HPAI A(H5N1) cases have occurred there since 2005, and periodic outbreaks of HPAI A(H5N1) virus have been identified in backyard poultry and through ongoing sentinel live bird market (LBM) surveillance. The district also has an extensive poultry trade, with 63% (n = 114) of poultry collector yards, 79% (n = 926) of slaughterhouses and 37% (n = 314) of LBMs in Jakarta province located in this district. This study reports the findings of enhanced influenza surveillance conducted during August 2011 through March 2013. Materials and Methods: Enhanced surveillance was conducted by designating teams at four government health centers and six of the largest hospitals in East Jakarta District to actively screen and enroll patients. At the health care centers, all outpatients with influenza-like illness (ILI), defined as fever ≥ 38°C and either cough or sore throat, seeking care from Monday through Friday, were enrolled. At the hospitals, all inpatients with severe acute respiratory infection (SARI), defined as sudden onset of fever in the last five days or history of feverishness and either cough or sore throat or shortness of breath, were enrolled 7 days a week. For all ILI and SARI patients, demographic and poultry exposure data were collected through a questionnaire. Nasal and throat swabs were collected at enrollment and tested by real-time reverse transcription polymerase chain reaction for influenza viruses, including A(H5). Data were analyzed to determine the proportion testing positive for influenza viruses, types of poultry exposure and number of HPAI A(H5N1) cases detected. Results: Of the 269,188 outpatient visits and 105,905 hospitalizations during August 2011 through March 2013, 4572 (2%) and 2692 (3%) met the definition for ILI and SARI, respectively. All of these were tested, of which 33% ILI cases and 15% SARI cases tested positive for influenza viruses. For ILI cases, the proportion of specimens testing positive for influenza viruses was highest in children aged 5 to 17 years (50%), whereas for SARI cases, the highest percentage of influenza positive specimens was identified in children aged < 5 years (33%). Of the 1882 influenza positive cases, 57% were positive for influenza A and 43% positive for influenza B. Among patients positive for inflIuenza A, 56% were positive for A(H3), 44% were positive for A(H1N1pdm09), and none were positive for A(H5). No HPAI A(H5N1) infections were detected (prevalence 0%, upper limit 95% confidence interval = 0.05%) despite 30% of ILI and SARI cases reporting poultry exposures such as visiting LBMs (12%), owning poultry (13%) and handling poultry products (7%). Conclusions: Despite enhanced influenza surveillance conducted over 19 months in which thousands of ILI and SARI case specimens were tested, including patients with poultry exposures known to be risk factors for HPAI A(H5N1) virus infection, no human HPAI A(H5N1) virus infections were identified from the enhanced system or from the routine national surveillance system. This suggests that clinical HPAI A(H5N1) virus infections among the East Jakarta District population under surveillance are rare. In contrast, a substantial number of seasonal influenza A and B virus infections were documented, suggesting that the public health impact of seasonal influenza may be underrecognized among this population. For HPAI A(H5N1), further research, including assessing the suitability of the surveillance sites for detection of HPAI A(H5N1) virus infections and the health care utilization practices of HPAI A(H5N1) cases, and determining the impact of preventive measures such as poultry vaccination and poultry movement control, is needed.

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P2-496 SCHOLARSHIP Promising Investigator Scholarship

Risk analysis of a novel avian H3N8 virus isolated from harbor seals

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Background: The recent isolation of avian H3N8 influenza viruses from New England harbor seals that naturally acquired several of the changes associated with increased transmissibility of H5N1 viruses generated a great deal of scientific and public interest. To elucidate the potential human health threat from these viruses, we evaluated the virulence and transmissibility of a panel of avian and mammalian H3N8 viruses in vitro and in vivo, including a panel of avian H3N8 viruses genetically related to the seal virus. Materials and Methods: Viruses were subjected to a full risk analysis panel including replication assays in MDCK, A549 and NHBE cells, pathogenicity in mice and

transmissibility in ferrets. In addition, viruses were analyzed by ELISA against different sialic acids to determine receptor specificity. Finally, to determine the baseline immunity to the seal H3N8 virus, HI assays were performed with a panel of sera from vaccinated adults. Results: Our studies demonstrate that although the seal and genetically similar wild-bird viruses replicate faster in human lung cells compared with other mammalian (canine and equine) and avian H3N8 viruses, only the harbor seal virus caused severe disease in mice. More importantly, the seal and genetically similar wild-bird viruses readily transmit via direct contact in ferrets and the harbor seal virus transmitted by aerosol droplet to 2 of 3 contacts. Sequence analysis of the seal viruses from aerosol contacts revealed genetic changes that may increase transmissibility. Receptor binding specificity revealed that the seal virus had increased specificity for a2,6 sialic acids. Importantly, none of the human sera used in our studies had neutralizing antibodies against the seal H3N8 virus, suggesting that there is little population-wide immunity to this virus. Therefore, it is imperative to understand how this particular clade of viruses may have evolved over time to become readily transmissible in ferrets. Conclusions: Our results suggest that the avian H3N8 viruses warrant closer examination and enhanced surveillance to monitor potential risks to human health, as they exhibit a number of prepandemic characteristics. Further study will attempt to define why this particular clade of H3N8 viruses seems to be able to readily adapt to mammalian hosts.

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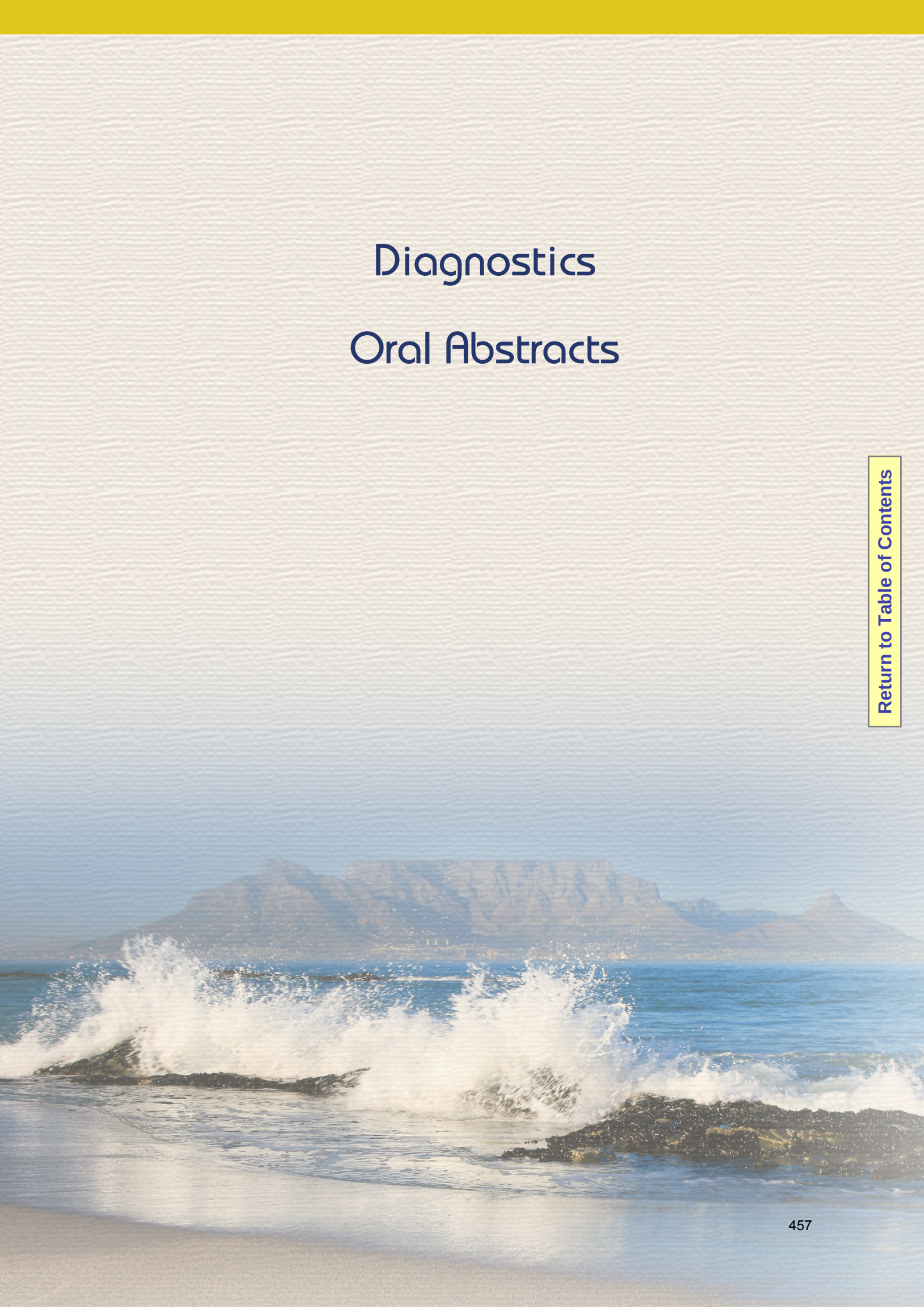
Diagnostics

Oral Abstracts



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H5N1-SeroDetect EIA and rapid test: a novel differential diagnostic assay for serodiagnosis of H5N1 infections and surveillance

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Background: Pandemic influenza is a persistent threat to global health. The continuing evolution of highly pathogenic (HP) H5N1 influenza viruses in wild birds with transmissions to domestic poultry and humans poses a pandemic threat. There is an urgent need for a simple and rapid serologic diagnostic assay which can differentiate between antibodies to seasonal and H5N1 strains that could provide surveillance tools not dependent on virus isolation and nucleic acid technologies. Methods: We constructed whole-genome-fragment phage display libraries (GFPDLs) expressing fragments of 15-350 amino acids covering all the open reading frames of the H5N1-A/Vietnam/1203/2004 strain. These GFPDLs were used to elucidate the epitope repertoire recognized by the antibodies in the sera of five individuals who had recovered from H5N1 infection. Three of these immunodominant epitopes, mapping to conserved regions in H5N1 HA2, M2e, and PB1-F2, were further evaluated as the basis for development of a serodiagnostic assay in ELISA as well as a rapid test platform. Results: Following affinity selection using the H5N1-GFPDL, we identified a diverse array of epitopes recognized by the H5N1 convalescent sera, including several potentially protective targets in HA1, NA catalytic site, and M2 ectodomain. In addition, for the first time in humans, we identified strong reactivity against PB1-F2, a putative virulence factor, following H5N1 infection. Importantly, few novel epitopes were identified, which were recognized by H5N1-convalescent sera but did not react with sera from control individuals (H1N1 or H3N2 seropositive but H5N1 negative). Here we describe the establishment of H5N1 SeroDetect ELISA and rapid test assays based on three peptides in HA2 (488-516), PB1-F2 (2-75), and M2e (2-24) that are highly conserved within H5N1 strains. To date, both platforms demonstrated high levels of sensitivity and specificity in detecting H5N1 infections (clade 1 and clade 2.3.4) in Vietnamese patients as early as 7 days and up to several years post infection. H5N1- uninfected individuals in Vietnam and the US, including subjects vaccinated with seasonal influenza vaccines or confirmed seasonal infections, did not react in the H5N1-SeroDetect assays. Moreover, sera from individuals vaccinated with H5N1 subunit vaccine with moderate anti-H5N1 neutralizing antibody titers did not react positive in the H5N1-SeroDetect ELISA or rapid test assays. Conclusions: Our data support the use of peptide-based H5N1-SeroDetect ELISA and rapid tests for serodiagnosis of H5N1 exposure in humans that can be easily adapted for surveillance of H5N1 infection in birds as a POC test for detection of H5N1 infection. The simple H5N1-SeroDetect ELISA and rapid tests could provide an important tool for large-scale surveillance for potential exposure to HP H5N1 strains in both humans and birds.

O-883

Poor correlation of influenza viral load between swabs of clinical samples from the 2011-2012 influenza season in Belgium highlights the importance of standardized sample collection

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Background: Respiratory tract infections (RTIs) have an enormous social economic impact, with high incidence of hospitalization and high direct and indirect costs. Because of similar clinical symptoms and simultaneous circulation of several different viruses, the etiology is often unknown. Timely detection and discrimination of the infecting pathogens are crucial to optimize treatment and care, to prevent unnecessary antibiotic use, and to prevent secondary spread of infection. Adequate specimen

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collection has a high impact on the correct diagnosis of influenza and other respiratory infections. In this study, the influenza A viral loads of two midturbinate swabs (one for each nostril) per patient were compared. Material and Methods: Belgian patients suffering from RTI presented themselves at a primary care physicians' office during the 2011-2012 influenza season. The patients were enrolled regardless of age and were symptomatic for less than 3 days at presentation. Sampling on patients with acute RTI was done using midturbinate flocked swabs (Copan Flock Technologies) and the collected pathogens were stored in universal transport medium (UTM). Two swabs per patient were collected (one swab per nostril). One swab was resuspended in 1 mL of UTM and the other swab in 3 mL of UTM. The influenza A viral load of all samples was determined with qRT-PCR assay using universal influenza A primers/probe (CDC). Results: A total of 175 patients were enrolled in this study during the period of Influenza epidemic peak. One hundred and one samples (57.7%) of the 1-mL aliquots were identified as influenza A positive (viral load range 3.64-9.34 log copies/mL with an average of 6.63 log copies/mL) and 74 samples (42.3%) as influenza A negative. Only 94 samples (53.7%) of the 3-mL aliquots were recognized as influenza A positive (viral load range 3.24-9.00 log copies/mL with an average of 6.12 log copies/mL) and 81 samples (46.3%) as influenza A negative. Of them, 86 were concordantly positive, and 66 concordantly negative for influenza A. The remaining 23 samples (13.1%) gave discordant results (either positive in 1 mL and negative in 3 mL, or the inverse). The 1-mL aliquots have a statistically higher viral load than the 3-mL aliquots (mean of differences = .5377; paired *t* test, *P* = .0003), which could imply that using 1-ml collection volume could also increase the probability of a correct influenza diagnosis. However, there was a poor correlation between the concordant positive aliquots per patient (R^2 = .3142). On an individual basis, even a difference in viral load up to 5 log copies/mL was observed. In this study, it was not known in which order the swabs were taken (first the 3-mL or the 1-mL aliquot). Therefore, this study was expanded to the 2012-2013 influenza season whereby two groups of clinical samples were made. In the first group, the 3-mL aliquot will be first collected and in the second group the 1-mL aliquot. Conclusions: Collection of swabs in a lower collection volume resulted in a higher viral load of these samples, and this could have implications for influenza diagnosis. However, a poor correlation between two swabs per patient was observed. Discordant results were observed for 13.1% of the samples. This study highlights the importance of proper specimen collection.

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Quantitative influenza diagnostic testing (QIDT) as a novel biomarker for the monitoring of disease activity at the point-of-care

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Background: Influenza infections create considerable disease burden in infants and children, who also tend to exhibit higher viral loads than adults. WHO and CDC guidelines state that antiviral treatment may need to be extended beyond 5 days, in case of ongoing viral replication. RT-PCR cycle thresholds (CT) and viral load (VL) assessments have been used to direct duration of treatment in immunosuppressed patients, but quantitative tests that can be performed at the point-of-care (POC) are currently lacking. Rapid Influenza Diagnostic Tests (RIDT) are widely used for the initial diagnosis of influenza infections. Second-generation fluorescence-based RIDT such as the SOFIA™ Influenza A+B FIA, show increased sensitivity while providing a user-independent qualitative read-out within 15 minutes. SOFIA™ quantitative immunofluorescence signals may be used to establish a novel biomarker for the longitudinal monitoring of influenza infections at the POC. Materials and Methods: The study was performed in the context of an IRB-approved quality management (QM) program at the Charité Department of Paediatrics (ChILD = Charité Influenza Like Disease Cohort). Patients with influenza-like illness were screened prospectively using nasopharyngeal swabs. Fluorescence-based RIDT and QIDT (SOFIA™) were performed at the POC, in addition to (blinded) RT-PCR at the National Reference Centre for Influenza at the Robert Koch Institute. Patients with laboratory-confirmed influenza were followed prospectively until a first negative RIDT or RT-PCR was obtained.

Follow-up parameters included RIDT, QIDT, and RT-PCR. QIDT-values were calculated, based on the relationship between the influenza A and B test lines' fluorescence intensities and the cutoffs for each analyte with the SOFIA™ test. CT values were obtained from qualitative RT-PCR. In RT-PCR-positive samples, VL was measured using genome equivalents. Samples with quantifiable VL (ie, above 103 cp/ml) were included for the calculation of viral clearance rates over time. Influenza subtyping (HAI or sequencing) was performed with all PCR-positive samples. Results: During the 2 consecutive influenza seasons, 2011/2012 and 2012/2013, a total number of 1821 infants and children participated in the QM program. Overall, 277 (15.2%) patients showed laboratory-confirmed influenza infection. Of these, 36 patients were treated with oseltamivir. Subtyping of PCR-positive samples revealed 26% influenza A(H1N1)pdm09, 40.4% influenza A(H3N2), and 33.6% influenza B. During longitudinal follow-up visits, the first negative RIDT result was consistently observed either prior to the first negative RT-PCR result, or simultaneously. The "switch" from the last positive to the first negative RIDT occurred around day 8 (range = 2-19) after symptom onset, with a median CT of 36 and a median VL of 5.6*103 cp/ml. During follow-up, no cases of viral rebound with newly positive RIDT were observed. QIDT values showed a significant correlation with CT values of 0.67 (Spearman, $P < .001$). Median VL clearance rates (CLVL) were 1.36/day for treated patients and 0.63/day for untreated patients ($P = .035$). QIDT-based clearance rates (CLQIDT) revealed similar ranges in both groups, with median rates of 1.41/day and 0.75/day respectively, thus discriminating between treated and untreated patients ($P = .06$). Conclusions: Regular use of second-generation rapid influenza diagnostic testing (RIDT) may provide a first, immediate assessment of influenza disease activity. A "switch" from positive to negative RIDT results during follow-up indicates that viral loads may have dropped below a critical threshold, when viral rebound is no longer expected. Second-generation rapid testing may also be used to obtain quantitative results. QIDT provides excellent tools to estimate virus burden at the time of disease onset, as well as during follow-up, with the advantage of obtaining results immediately at the POC. QIDT may also be able to discriminate between treated and untreated patients, ie, to estimate viral clearance as an effect of antiviral therapy. Minor significance levels with CLQIDT compared with CLVL suggest that further analyses in larger groups of treated patients may be required. Large-scale studies investigating influenza disease severity in relation to CT, VL, RIDT, and QIDT will reveal the full impact of this innovative and personalized approach to the clinical and therapeutic management of influenza infections in infants and children.

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O-885 SCHOLARSHIP International Research Scholarship

Do case definitions affect the prediction of influenza virus in different seasons in north India?

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Background: A variety of case definitions have been used to screen for influenza disease, but the sensitivity and specificity of these vary. Influenza has also been shown to have different seasonality in different parts of India. While influenza infection reaches a peak in July-August in and around Delhi, the peak of respiratory illnesses usually occurs in December-February. This can result in change in validity parameters of a testing strategy based on symptoms and signs. We evaluated the performance of five screening definitions for detection of influenza among hospitalized patients in India during different seasons of the year. Materials and Methods: From January 2009 through December 2012, patients of any age with any acute medical illness (resident of Ballabgarh Health and Demographic Surveillance System, rural community near Delhi) were enrolled at 33 primary- and secondary-level medical facilities from January 2010-December 2012. Patients hospitalized for trauma, accident, poisonings, obstetric causes, or elective surgeries were excluded. Data were collected on patients' medical histories and illness signs and symptoms, and respiratory specimens (nasopharyngeal swab) were collected for influenza virus testing by real-time reverse transcription polymerase chain reaction. The clinical details of the patients were assessed by a doctor who visited

these facilities, and the condition on admission was accessed through hospital records. Clinical histories and examination were used to classify the patient in one of the following categories: acute respiratory infections (ARI), febrile ARI (FARI), severe ARI (SARI), influenza-like illnesses (ILI) and presence of any respiratory symptoms. Diagnostic odds ratio (DOR), sensitivity and specificity were calculated for screening definitions during winters (December to March), summers (April to June), and monsoon-post monsoon (July to November) over a period of three years from January 2010 to December 2012. DOR is the ratio of the odds of positivity in disease relative to the odds of positivity in the nondiseased; it rises steeply when sensitivity or specificity becomes nearly perfect. Results: During 2009-2012, 1852 patients hospitalized with acute medical illness were enrolled and tested for influenza, of which 110 (5.9%) tested positive. The median age of enrolled patients was 26 years (IQR: 10-45). Of 110 patients with laboratory-confirmed influenza, the most common symptoms were fever (79.5%) followed by cough (51%). Overall, FARI had the highest DOR of 7.0 (4.1-12.2) with a sensitivity and specificity of 85% and 54%, respectively, compared with DORs of 6.9 (sensitivity = 89%; specificity = 45%) for ARI, 6.4 (sensitivity = 83%; specificity = 57%) for ILI, 3.6 (sensitivity = 95%; specificity = 16%) for any respiratory symptoms and 2.1 (sensitivity = 40%; specificity = 76%) for SARI. However, when stratified by season, this order changed; in the summer, ARI definition had the highest DOR (14.7, 95% CI 1.9-113.0), whereas in the monsoon and postmonsoon period, both ARI and ILI definitions had maximum DOR of 9.3 (95% CI 4.4–19.5), and in winter, any respiratory symptom had the highest DOR (4.9). Presence of any respiratory symptom had the highest sensitivity (94%) in the monsoon period, while SARI (33%) had the least. In winters the DOR for different case definitions ranged from 1.8-4.8, while in summers and monsoon, it ranged from 4.7–14.7 and 1.7-7.4. SARI had a lower DOR in all seasons. Conclusions: With the changing season the accuracy (DOR, sensitivity and specificity) of various definition changes. This needs to be kept in mind by researchers while planning studies or comparing studies from different settings or seasons. Another implication of changing accuracy of different definitions in various seasons would be assigning correction factors to various definitions (depending on the seasons) while estimating the burden of influenza. This study was supported in part by cooperative agreement U01 IP000206 from the Centers for Disease Control and Prevention, Atlanta, Georgia, United States.

O-886

Diagnosis of influenza A/H1N1pdm virus genome by hairpin-type peptide nucleic acid chromatography

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Background: The diagnosis of infectious diseases is increasingly important for preventing pandemics and prescribing the correct treatment to patients. Although DNA microarrays and PCR analyses are well established, they require genome amplification and fluorescence detection. The development of a rapid and accurate diagnostic kit requires a methodology that can directly capture the viral genome and visualize it without requiring advanced technical skills or expensive equipment. Peptide nucleic acid (PNA) is an RNA/DNA analogue in which the sugar-phosphate backbone is replaced by a neutral amide backbone. PNA is advantageous for the diagnosis of diseases from clinical samples because it is nuclease and protease resistant and recognizes RNA/DNA without electrostatic repulsion. Hairpin-type PNA comprising two complementary strands against the target sequence forms a stable triplex with the target oligonucleotide. Here, we synthesized hairpin-type PNAs that have different types of spacer molecules linking the two PNA strands and studied their binding properties against a conserved sequence of the NS genome of influenza A/Osaka/180/2009/H1N1pdm. We also immobilized hairpin-type PNA in a well or on a chromatography strip to visualize the target gene by the naked eye. Materials and Methods: Single-stranded PNA (ssPNA) and hairpin-type PNA tethered with either an ethylene glycol linker (hairpin-type PNA) or a thioazobenzene linker (hairpin-type PNA-AZO) were synthesized to be complementary against a conserved sequence of the NS genome of influenza A/Osaka/180/2009/H1N1pdm virus. The ssPNA, hairpin-type PNA, and hairpin-type PNA-AZO sequences were K_3-$(AEEA)_3$-CCTTCTCTTCCAGGA-K-$(AEEA)_2$-K, K_3-TCTCTTCC-$(AEEA)_3$-

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CCTTCTCTTCCAGGA-K-$(AEEA)_2$-K, and K_3-TCTCTTCC-(AZO)-CCTTCTCTTCCAGGA-K-$(AEEA)_2$-K, respectively, where AEEA is 2-(2-aminoethoxyethoxy) acetic acid, AZO is thioazobenzene, and K is lysine. In each well of the ELISA plate, 0.5 µg of PNA was immobilized via primary amine cross-linking. The well was prewarmed with 0.05% Triton-X100-PBS solution at 50°C for 15 min to prevent inter- and intramolecular aggregation of PNA. At the same time,90 µL of influenza virus containing specimens were mixed with 10 µL of 10% lysis buffer and incubated on ice for 30 min. After removing the Triton-PBS solution from the wells, 100 µL of the lysis buffer-treated virus were added to each well and incubated for 1 h at r.t. Then, the wells were washed with 0.05% Triton-X100-PBS and were loaded with 20 µL of the manufacturer's recommended dilution of the anti-influenza A virus nucleoprotein and 20 µL of goat antimouse IgG secondary antibody conjugated with alkaline phosphatase in 2% BSA-PBS at a final dilution of 1/20. The wells were placed on an orbital shaker at r.t. for 2 h. After washing the wells with 0.05% Triton-X100-PBS (300 µL × 3), the wells were incubated with a BICP/NBT (5-bromo-4-chloro-3'-indolyphosphate/nitro-blue tetrazolium) assay solution for 1 h. Alkaline phosphatase conjugated with the antimouse IgG secondary antibody produces a stable purple dye. Images of the plates were taken using a digital camera. For each strip of the PNA chromatography, 0.5 µg of PNA was immobilized and the virus gene detection was performed in a similar manner, except that the visualization step was accomplished by gold-conjugated anti-NS antibody. Results: Hairpin-type PNA-AZO selectively recognized the NS gene of influenza A/H1N1pdm among other seasonal strains such as influenza A/H3N2 and B virus. The NS gene of A/H1N1pdm and nucleoprotein complex in PNA-immobilized wells was detected by an antibody-enzyme linked system for virus concentrations between 10^4 and 10^6 pfu/mL. Further, hairpin-type PNA-AZO on a chromatography strip also captured the A/H1N1 virus gene and allowed it to be visualized using gold-nanoparticle conjugated anti-NP antibody for virus titers of 10^{4-5} pfu/mL with the naked eye. Conclusions: Introduction of AZO linker into hairpin-type PNA enhanced its affinity to the virus gene and enabled us to develop a diagnostic for influenza virus A/H1N1pdm that can be read by the naked eye. This technology can be applied to the diagnosis of emergent viruses such as highly pathogenic avian influenza viruses and drug-resistant influenza viruses at any place without requiring the use of expensive equipment or electricity.

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Better tests, better guidance, better practice: outcomes of a strategy for improving rapid influenza diagnostic tests

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Background: Rapid influenza diagnostic tests (RIDTs) using immunoassays to detect influenza A and B antigens are widely used; however, a rising number of reports indicate that RIDTs have low sensitivity (≈ 60%), leading to misinformed clinical management. Suboptimal RIDT performance may be due to several factors beyond the performance of the test device itself, including specimen collection, timing of testing during the season, patient demographics, and clinician knowledge of test performance and interpretation. Materials and Methods: Beginning in 2010, the Centers for Disease Control and Prevention (CDC), along with private and government partners in clinical medicine, device manufacturing, and public health, developed and implemented a strategy for improving clinician and laboratory use of RIDTs with the goals of 1) better guidance on RIDT use, 2) better practices for optimal use of RIDTs, and 3) better tests available for clinicians and laboratories. Evaluation of current RIDT use practices was done using nationally representative medical care claims data, surveys and online voting of representatives from clinician and laboratory organizations, and through a representative sample of approximately 5000 outpatient facility providers (OFPs) comprised of physician offices, community health centers, and emergency departments. Finally, a comparison of 11 commercially available RIDTs was performed using 23 representative influenza viruses to determine analytic sensitivity. Results: Medical care claims data revealed a 17-fold increase

in RIDT use between 2000 and 2009; by 2011, use persisted at around 12-fold higher. Stakeholder engagement revealed need for improved, detailed RIDT use algorithms, improved communication of influenza surveillance data to effect clinical management, and strong support for regulatory agencies to participate in guiding test manufacturers toward improved test performance. A representative survey of clinical laboratories showed 88% of 566 respondents used RIDTs; 49% used RIDTs as the only influenza diagnostic, and most (60%) did not include information on test performance/sensitivity to clinicians in the reported results. The OFP survey revealed that RIDTs are commonly used, but 55%-75% of OFPs do not use any supplemental testing (culture, PCR, DFA) to confirm RIDT results, and OFPs relied on positive RIDT results to determine whether antivirals were prescribed. Based on stakeholder and study findings, an online clinician continuing education Web site was established. An evaluation of 330 clinicians completing the course showed 99% had an improved understanding of RIDT use, and 47% planned to change their practice. Instructional YouTube videos demonstrating appropriate specimen collection were posted in October 2012, and by June 2013, 11,606 viewings of the 1-2 minute videos had occurred. Algorithms for optimum RIDT use and influenza clinical management were developed in collaboration with clinical professional societies and were posted on the CDC Web site. Results comparing the analytic performance of 11 commercially available RIDTs were posted to the CDC Web site and shared through multiple communication paths to clinicians and laboratorians. The study showed that positivity varied considerably among RIDTs by virus concentration and by strain of influenza; only one RIDT detected at least 50% of all influenza A viruses at lower virus dilutions. Conclusions: Efforts to improve RIDT use require addressing not only the test devices themselves, but also providing better guidance and tools to improve clinician and laboratory practices. The combined findings of the strategy described here were provided to US regulators overseeing clearance of RIDTs and are informing ongoing efforts for regulatory enhancements and incentives for improved RIDT development.

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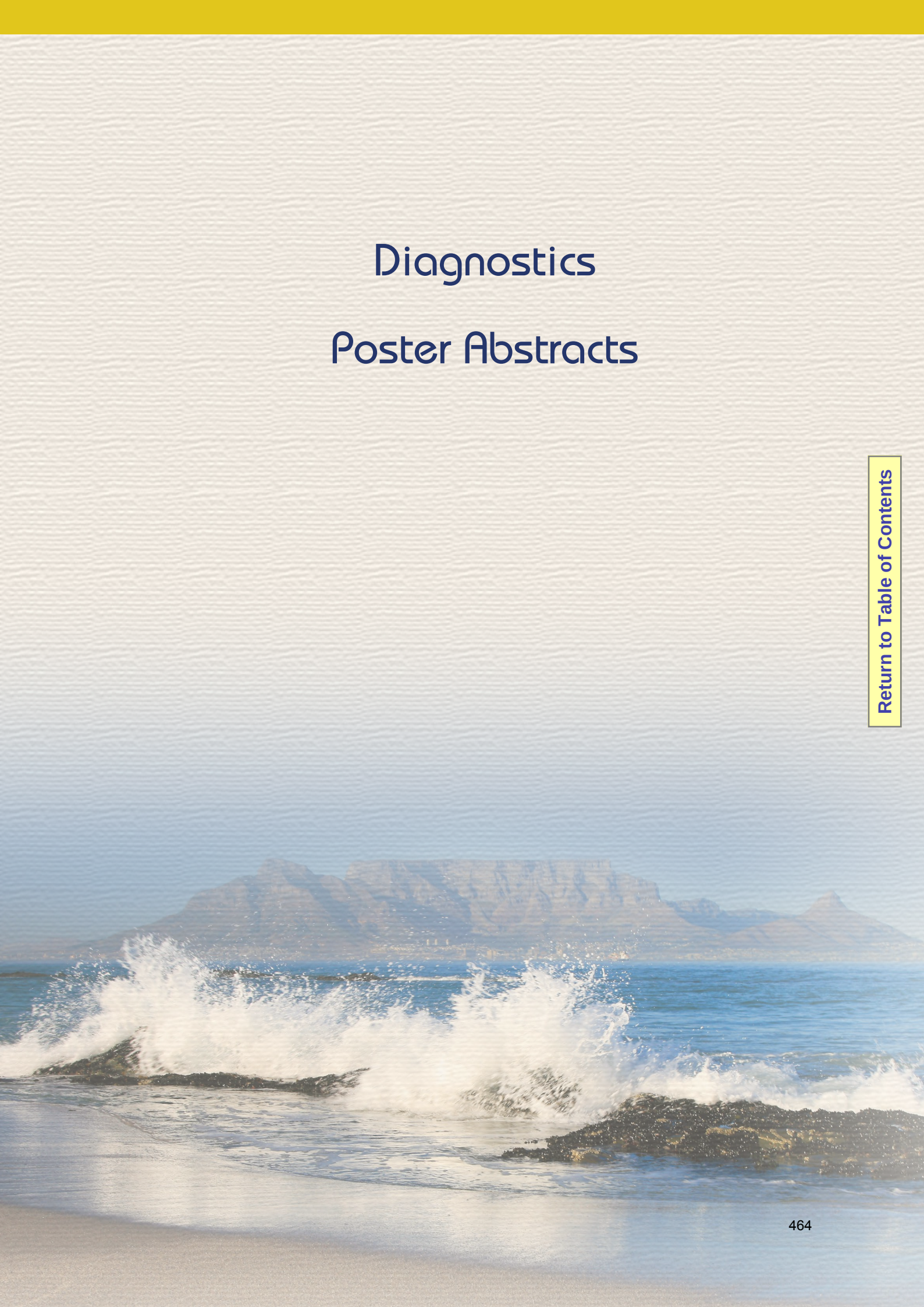
Diagnostics

Poster Abstracts



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Application of single-strand conformational polymorphism for differentiation of influenza A strains

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Background: Mixed infections of a single host with different genetic variants of influenza A virus are the main source of reassortants, which may have unpredictable properties when they establish themselves in a human population. We describe here a method for detection of minor genetic influenza variants in a host with sensitivity down to 0.1% of total genomic material. Different A/H5N1 strains as well as the seasonal A/H1N1/ human strain and the swine-origin pandemic A/H1N1/v strain were used to validate the method. Materials and Methods: The influenza A strains were characterized by a multitemperature single-stranded conformational polymorphism (MSSCP) method. Single-stranded DNA fragments attain a number of conformations that may be separated by electrophoresis, giving a characteristic pattern of bands. Minute sequence changes (eg, point mutations) and physicochemical conditions (eg, temperature) may have a significant effect on electrophoretic mobility of single-stranded DNA. A series of primers were synthesized after the comparison of the hemagglutinin gene sequences of different A/H1N1/ and A/H5N1 strains. RT-PCR reactions were run using these primers and, after denaturation, single-stranded DNA fragments were subjected to MSSCP electrophoresis at strictly controlled temperatures. Results: The MSSCP gel patterns of hemagglutinin gene fragments originating from different H5N1 and H1N1 strains were easily distinguishable two hours after completion of PCR. Coinfections with different variants of the A/H1N1/ subtype were identified in swabs diagnosed by real-time RT-PCR assay as a single strain infection. The sensitivity of the MSSCP-based minor genetic variant detection was 0.1% as determined by a mixture of DNA fragments obtained from amplification of two different hemagglutinin genes. Conclusions: This technique makes possible fast characterization of new viral variants, circulating, for example, in birds or pigs, long before they become dominant in the mixture of strains. We anticipate its possible use in the detection of early stages of a minor variant in mixed infections in swine reactors, where the human-to-swine transmission of the pandemic A/H1N1/v is gaining importance and may give rise to future reassortments with unpredictable outcome.

P2-551

Influenza A(H1N1)pdm09 virus as the cause of lethal pneumonia in Russia, 2009-2013: epidemiological, biological, and genetic properties

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Background: The autopsy materials (bronchi, trachea, and lungs) from 127 patients who succumbed to lethal pneumonia caused by influenza A(H1N1)pdm09 virus during 2009-2013 were examined in the DI Ivanovsky Institute of Virology, Moscow. Most of the cases were registrated in the European part of Russia (87%): Moscow, Lipetsk, Yaroslavl, Vladimir, Novgorod Velikiy, Nizhniy Novgorod, Cheboksary, Orenburg, Penza, etc. The mean patient age was 42.7 years (from 4 months to 79 years); 47% of the patients were women; 10% were pregnant. Many patients (34.4%) had chronic diseases, mainly metabolic (11.5%), cardiac (8.2%), and renal (9.4%). The mean duration of the illness was 10.2 days (from 2 to 26 days). No one among these patients was vaccinated. Materials and Methods: We used RT-PCR, virus isolation on MDCK and embryonated eggs (EE), hemagglutination inhibition, sequencing, and in-cell ELISA to study the properties of A(H1N1)pdm09 viruses. Results: Thirty-seven strains of A(H1N1)pdm09 were isolated: 25 on EE (68%), and 12 on

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MDCK (32%). Most isolates were characterized as A/California/7/2009-like, the virus used as the influenza A(H1N1)pdm09 component of the 2009-13 influenza vaccines for the Northern Hemisphere. One virus (2.7%) showed reduced titers with antiserum produced against the reference virus. All viruses tested were susceptible to the arbidol and neuraminidase inhibitors, oseltamivir and zanamivir, respectively, and were rimantadine resistant. We detected mutations in the HA1 receptor-binding site in 103 samples (37% of total; 29/42 [69%] samples collected in 2009-2010, 5/45 [11%] samples collected in 2010-2011, 4/16 [25%] samples collected in 2012-2013) and in 71% of 34 strains isolated from these materials. D222G and Q223R were the most common mutations found in 69% and 30.8% of the strains accordingly. Only in 11 of the 61 samples (18%) revealed coinfection of influenza A(H1N1)pdm09 virus and other pathogens, including BoV (1 case), adenovirus (2 cases), parainfluenza (1 case), rhinoviruses (6 cases) and Streptococcus (1 case). Conclusion: Our findings demonstrate that future monitoring of influenza viruses from patients with lethal pneumonia will provide the most important trends in A(H1N1)pdm09 virus evolution and the effectiveness of control/treatment /prevention measures. Funding: Our investigations were supported by the Centers for Disease Control and Prevention, Atlanta, Georgia, United States. CoAg: U51IPOOO527-02.

P2-552

Molecular transport media for inactivation of influenza virus and other respiratory viruses allows safe transport and molecular analysis for global surveillance

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Background: Emergence of novel and highly pathogenic respiratory viruses requires safe and efficient collection and transport for effective global surveillance. Specimen collection is the first and most critical step in robust and sensitive detection using downstream nucleic acid testing (NAT). Sensitive real-time RT-PCR detection assays hinge on the quality and preservation of nucleic acids from collected specimens. PrimeStore MTM is a collection and storage solution that safely inactivates bacteria and viruses, including influenza virus, and preserves and stabilizes released RNA/DNA at ambient temperature or higher for prolonged periods. These studies analyzed the inactivation and killing of influenza A H1N1-2009 pandemic virus and the newly emerging H7N9 strain, and SARS coronavirus. Materials and Methods: Swabs were loaded with 0.1 mL of virus grown to 10^6 $TCID_{50}$/mL or viral storage buffer (negative control), placed into PrimeStore MTM and held for 10, 30, or 60 minutes at 25°C (ambient temperature). Samples were cultured for 96 hours and visually examined for CPE with total $TCID_{50}$/mL determined. *Results*: At the earliest sampling time (10 minutes), no viable influenza viruses were detected. Because MTM is designed to destroy lipid membranes of cells and microbes, MDCK cells were destroyed until the test samples were diluted 1:1000, LOD 10^{-3}. Greater than 4-log reduction, > 99.99% viral killing was demonstrated in 10 minutes at ambient temperature. Conclusions: Previous studies have shown that MTM rapidly kills a wide variety of microbes, including many respiratory pathogens such as *Mycobacterium tuberculosis* and MRSA. This report extends the respiratory virus data to include additional emerging and pandemic influenza A strains that continue to circulate around the world. New viral pathogens often emerge in areas endemic for other highly infectious agents, such as *M tuberculosis*. In addition, safe specimen transport and analysis may be a challenge. Global surveillance of emerging respiratory pathogens increasingly utilizes molecular diagnostics and sequencing. Safe, efficient, and cost-effective collection and transport of specimens for molecular analysis are important to improve identification and tracking of these new pathogens. PrimeStore MTM provides a collection and transport solution that kills pathogenic microbes while preserving high-quality RNA/DNA for downstream molecular testing.

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Comparison of influenza diagnostic results among seven United States Department of Defense laboratories

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Background: Naval Health Research Center (NHRC) provided annual quality assurance (QA) panels for influenza testing to 6 other Department of Defense laboratories. All laboratories used the Applied Biosystems 7500 Fast real-time PCR platform to test for influenza using reagents provided by the CDC Influenza Reagent Resource (IRR). Materials and Methods: Blinded QA panels containing 8 samples each were prepared by NHRC in 2011 and 2012. Panels were identical within each year and consisted of influenza A/H3, A/H1N1(09), B and negative samples. Influenza positive samples were a 1:100 dilution of infected cell tissue culture isolates. Panels were shipped frozen on dry ice and tested by all laboratories in both 2011 and 2012. Results: All laboratories correctly identified 100% of the samples in both years. The mean and maximum crossing thresholds (CTs) for influenza positive samples were 24.8 and 32.2, respectively, in 2011 and 23.9 and 30.5 in 2012, well under the positive threshold value of 38. There was strong correlation among laboratory CT values, with all 7 having significant ($P < .05$) Pearson correlation coefficients of 0.85 or more in 2011 and 6 of 7 in 2012. Conclusions: The Department of Defense laboratories in this program successfully identified 100% of blinded samples, providing confidence in their influenza diagnostic capabilities. The reproducibility and high correlation of CT values among different laboratories suggest that the Applied Biosystems 7500 Fast real-time instrument and CDC IRR primers comprise a robust and reliable diagnostic platform for detection of influenza.

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Comparison of Quidel rapid diagnostic influenza tests, 2012-13

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Background: National borders do not prevent the transmission of pathogens and associated vectors among border populations. The Naval Health Research Center (NHRC) has collaborated with the Mexican Secretariat of Health, the US Department of State's Biosecurity Engagement Program and the US Centers for Disease Control and Prevention in concert with local health officials to conduct influenza-like illness (ILI) surveillance since 2004 in the border region. Participating clinics have tested each ILI patient with a commercial (Quidel QuickVue Influenza A + B) rapid influenza diagnostic test (RIDT). This past influenza season NHRC provided three of four clinics with the Quidel Sofia Fluorescent Immunoassay Analyzer to replace the RIDT used in the past (clinics A, B and C). One clinic continued to use the Quidel QuickVue Influenza A + B test (clinic D). Materials and Methods: Demographics were determined for all clinics. Respiratory swabs were collected from patients with ILI (fever ≥ 100˚F, and sore throat or cough). A nasal swab was used to run the RIDT. Specimens were tested with molecular techniques, viral and bacterial culture. RIDT results were compared with PCR results to determine sensitivity and specificity for each RIDT. Results: At clinic D, 102 ILI patients were tested. Clinics A, B and C tested 372 ILI patients. Clinic D tested a somewhat older ILI population: 30% of their patients were aged 25 years or older, while clinics A, B and C had 8% of their patients in this age group ($P < .0001$). Overall sensitivity/specificity of the Quidel Sofia was 83% (95% CI: 77-88)/81% (95% CI: 75-86) and the Quidel QuickVue was 59.0% (95% CI: 46-72)/100% (95% CI: 91-100). Influenza A sensitivity/specificity: Quidel Sofia—82% (95% CI: 73-90)/98% (95% CI: 94-99); Quidel QuickVue—57% (95% CI: 39-74)/100% (95% CI: 91-100). Influenza B sensitivity/specificity: Quidel Sofia—84% (95% CI: 75-91)/83% (95% CI: 77-88); Quidel QuickVue—62% (95% CI: 41-80)/100% (95% CI: 91-100). Conclusions: The Quidel Sofia Fluorescent Immunoassay Analyzer had higher sensitivity than the Quidel QuickVue Influenza A + B test for influenza A + B, influenza A and

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influenza B in this population. It had a decreased specificity for influenza A + B and influenza B. The results for the QuickVue test are very similar to what we have observed for this test over the past 9 influenza seasons in the same population, that is, poor sensitivity and very good specificity. The large amount influenza B false-positive results seen with the Quidel Sofia (compared with the amount of influenza A false-positives on this instrument) resulted in a decreased specificity. Therefore, an influenza B–positive Quidel Sofia result should be evaluated with this in mind.

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Enhanced RNA preservation may be critical for molecular detection, tracking and surveillance of influenza cases

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Background: The threat of new and highly pathogenic respiratory pathogens emerging around the world requires continuous global surveillance. In the past decade there has been the rapid spread of SARS, pandemic H1N1-09, the continued threat of H5N1, emergence of a novel SARS-like-coronavirus, and a new H7N9 influenza strain. Specimen collection is the first and most critical step in downstream nucleic acid testing (NAT). PrimeStore Molecular Transport Medium (MTM) is a collection and storage solution that safely inactivates bacteria and viruses, and preserves and stabilizes released RNA/DNA at ambient or higher temperatures for prolonged periods. Clinical studies were combined with in vitro laboratory studies to analyze the relationship between influenza virus titers in collected specimens, RNA preservation, and testing the capability to accurately detect influenza. Materials and Methods: A prospective family influenza transmission study was conducted at Wilford Hall Medical Center (WHMC) in San Antonio, Texas, during the 2007-08 influenza season. Nasal wash specimens (N = 222) were obtained from pediatric patients with influenza-like-illness (ILI), and symptomatic and asymptomatic accompanying family members. Nasal wash aliquots were placed into commercial universal transport medium (UTM) and PrimeStore MTM. Specimens in UTM were analyzed for influenza virus using PrimeMix RT-PCR, rapid antigen test (Remel), and culture. The specimens in PrimeStore MTM were analyzed using PrimeMix RT-PCR. Additional laboratory studies evaluated influenza virus detection from various collection media and the level of RNA degradation over time. Influenza A virus was inoculated into PrimeStore MTM, commercial UTM, RNALater, or water control. Media were incubated at various temperatures and times, and analyzed by real-time RT-PCR. Results: In the family study, influenza was detected in 66/222 (31%) enrolled subjects. Of these, 11 were PCR positive from PrimeStore MTM collected specimens, but culture negative and PCR negative from UTM. Additionally, PrimeStore MTM specimens also detected 3 asymptomatic family members not detected from UTM specimens. Controlled laboratory studies demonstrated that influenza virus may be destroyed and RNA degraded during transport or storage and that PrimeStore MTM, in contrast to other transport media, enhances RNA preservation at temperatures up to 40°C. Conclusions: For a variety of reasons, collected specimens containing influenza virus often have low viral loads. To optimize detection and surveillance, preservation of specimen RNA may be essential to identify asymptomatic cases and individuals with low levels of influenza virus.

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Assessment of needs and opportunities across the landscape of influenza testing

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Background: Timely and accurate laboratory diagnosis of influenza infection is important in both patient care and surveillance. The results of influenza testing inform clinical management decisions and infection control efforts. In addition, data produced from influenza testing performed for both clinical and public health purposes are integral to influenza surveillance and pandemic response activates. Despite their suboptimal performance compared with reference standard methodologies, antigen-based rapid influenza diagnostic tests (RIDTs) remain in common use in many clinical settings and also as a component in some surveillance programs. Current reference testing, particularly reverse transcriptase (RT-PCR) or eukaryotic cell culture, offers great advantages over RIDTs in terms of test performance and their capacity for allowing further characterization such as subtyping. However, reference testing is more costly, requires a longer time to result following specimen collection, and must typically be performed in specialized laboratory settings by trained laboratorians. Finally, process and system requirements, such as specimen collection and handling, are considerations that directly impact performance of influenza tests regardless of the diagnostic device used. In the present study, we sought to evaluate the current landscape of influenza testing, technical requirements for influenza diagnostic tests used in different settings, and opportunities for improvement in testing. Material and Methods: A comprehensive landscape analysis of published data was conducted in conjunction with qualitative research activities. This included interviews with US and international subject-matter experts and test users as well as process mapping at multiple sites in Washington State (United States) that perform influenza testing for either clinical or surveillance purposes. A map of where influenza tests are used across both clinical and surveillance domains was developed and used in conjunction with collected data to inform inputs of target product profiles for influenza tests used for patient management or surveillance purposes and in clinical or laboratory settings. Finally, the general influenza testing process was plotted and used in an assessment of gaps and opportunities. Results: Test performance was considered a key characteristic for test users across all domains. However, in point-of-care clinical settings, additional requirements for test complexity, time-to-result, and cost were also critical considerations. The ability to batch and detect additional analytes was desirable for referral laboratory settings in both the clinical and surveillance domains. Low cost and minimal complexity of the tests were also important considerations for commercial tests used as part of surveillance programs in low-resource, international settings. Analysis of requirements at different stages of the testing process indicated system improvements including efforts to better stabilize testing components and inputs during transport as opportunities that could be emphasized to further improve influenza testing. particularly in remote, resource-poor locations. Furthermore, connectivity for data transfer following performance of the test was another area of improvement identified. Conclusions: A new influenza test that offers performance similar to RT-PCR but like RIDTs is low cost and offers convenience in terms of both speed and complexity would improve both clinical and public health decision-making and be attractive for uptake and adoption by users across multiple domains of influenza testing. However, advancements that improve the stability of test reagents and sample inputs are important considerations that still must be addressed to improve the quality and robustness of influenza testing, particularly in low-resource settings where infrastructure challenges exist. Finally, a growing area of emphasis for improvement in new devices is connectivity. Real-time data access to influenza results is already a desirable attribute for global surveillance applications, due to its ability to reduce response times. In clinical domains within the US health care system, connectivity is not currently a key consideration, but it could become an area of future importance as test devices become more integrated into information management systems to improve efficiency.

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Influenza viral shedding in HIV-infected and HIV-uninfected individuals, South Africa, 2012

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Background: Influenza virus is often shed in respiratory secretions for 5-7 days after symptom onset in otherwise healthy individuals. HIV-infected individuals infected with influenza may be more likely to transmit the virus due to shedding of a higher load or for a longer time. In South Africa > 10% of the adult population is infected with HIV. To assess the potential for influenza viruses to be shed for a prolonged period in HIV-positive patients in South Africa, we conducted a pilot study in HIV-infected and -uninfected individuals with influenza, comparing the viral load and duration of influenza viral shedding. Materials and Methods: Children and adults of all ages with severe acute respiratory illness (SARI) or influenza-like illness (ILI) who presented with symptoms for ≤ 3 days at two surveillance clinics in Klerksdorp, Northwest Province, and Pietermaritzburg, KwaZulu-Natal Province, South Africa, were enrolled. Individuals ≥ 5 years of age had a nasopharyngeal swab and throat swab taken for influenza diagnosis. Paediatric patients < 5 years of age had a nasopharyngeal aspirate performed. Patients were screened for influenza using a rapid Directigen EZ Flu A+B (Becton-Dickinson) test, and patients testing influenza positive were enrolled in the study. Patients were asked to return on days 2, 4, 6, 8, 10, 12, 14, 21, and 28 for repeat specimen collection until two consecutive negative influenza tests results were obtained. All samples were confirmed and typed at the National Institute for Communicable Diseases (NICD). Influenza viral load was measured using real-time reverse transcription polymerase chain reaction (rRT-PCR) assays. Viral load was estimated by Ct values relative to a standard curve. Patients enrolled in the study were offered a rapid HIV test if HIV status was unknown. Date of onset of influenza symptoms was determined on history. Results: Eleven persons met the enrollment criteria and tested positive for influenza on the rapid influenza test between May and October 2012. The median age was 13 years (range 3-38 years). Eight (73%) were male. All patients had ILI, and one was later hospitalized with SARI. HIV infection status was known for all patients; two (18%) of the 11 individuals were HIV infected. No other underlying medical conditions were reported. Three (27%) of the 11 individuals were infected with influenza A(H3N2) and the remaining eight were infected with influenza B/Victoria-lineage. Influenza was detected for a median of 9 days (range 2-14 days) from onset of symptoms for HIV-infected and -uninfected patients. As the number of days of influenza viral shedding increased, the influenza viral load decreased. The mean influenza Ct value and viral load on day 0 were 25.5 and 85,375,705 copies/mL, respectively; day 2, 28.3 and 6,583,750; day 4, 31.0 and 3,306,881; day 6, 35.1 and 127,895; day 8, 35.0 and 38,833; day 10, 34.7 and 42,850; day 12, 36.4 and 27,060; and day 14, 38.5 and 1811. No significant differences in terms of duration or intensity of influenza shedding were observed between HIV-infected and -uninfected individuals. Conclusions: Influenza virus is shed in highest quantities in the first two days of illness, declines gradually over the first week, and can be found for up to 14 days after symptom onset. The lack of association with HIV infection could be attributed to the small number of individuals enrolled in the study. This study will be continued in the current influenza season and more individuals enrolled in both groups.

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Understanding the process of influenza testing in clinical management of influenza patients in urgent and outpatient care clinics

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Background: The development of diagnostic tests often occurs in a silo, separate from the end-use scenario for which the tests themselves are intended. Although this can lead to appropriate and successful tests, many tests fail to meet specific attributes required for uptake by their targeted end-user groups. It is important to understand the environment the tests will eventually be used in to ensure that products are viable in those settings. In the United States, specific recommendations for the use of influenza tests are provided by the manufacturer, through health care organizations, and through agencies including the CDC and the FDA. However, it is through the firsthand experiences of end-users that an accurate picture can be developed of how recommendations are translated into real-world practices and policies of test use in clinical management. In this study, we sought to explore how influenza testing is used in different clinical settings and what factors influence end-users' choice of which test to use, when, and by whom. This information can be used to aid in the development of future tests that better encompass features that will allow for successful adoption and sustained use. Materials and Methods: We performed a series of site visits and interviews with providers, lab managers, and test users at primary and urgent care centers in western Washington. Data from these visits and interviews were used to develop process maps to visualize the processes, subprocesses, and decision-making points. We classified common clinic flows in primary care and urgent outpatient settings to identify the most important decisions that clinicians make when diagnosing and treating influenza patients. We then incorporated this information with feedback from the interviews to understand the most important trade-offs in product requirements for an influenza diagnostic test. Results: Although the overall activities and flow of urgent and primary care clinics are analogous, the realities of their patient population and the intended use of the clinic create differences in diagnostic needs. Urgent care settings are characterized by a high throughput of patients particularly during the influenza season, which requires an efficient clinic flow with minimal waste. Similarly, primary care settings have high numbers of patients during the influenza season; however, providers have an existing relationship with these patients, and this changes the dynamics considerably. Familiarity with patients and their families also has implications on care decisions including the use of diagnostic testing. Participants were asked to rank the most important attributes of a future influenza test. Accuracy was highly ranked although not the most important feature. A CLIA waiver was deemed critical to perform influenza testing on site. Insurance reimbursement rates for influenza tests were a major factor that influenced the selection of a particular test as costs must be kept low, particularly in clinics where patients are more likely to be uninsured. Finally, the algorithms that providers use when making decisions on testing and treating patients with influenza-like illness were explored. The decision to test is primarily influenced by the social setting in which the patient lives. Treatment for influenza was discussed during all interviews as one of the bottlenecks in care. In this context, the lack of treatment options became a major contributor in the decision to test. Conclusions: By identifying and qualifying the current realities of primary and urgent clinic flow during clinical management of influenza, we were able to better understand what tests fit best into different settings. While accuracy remains an important requirement for an influenza test, cost is an equal, if not more important, determinant of uptake and widespread use. As discussed, the most important decisions when a patient presents with influenza-like illness are the decision to test and decision to treat. These two decisions are not mutually exclusive and often occur simultaneously during patient management. By identifying the factors influencing the use of influenza testing in clinical care, the needs of the end-user will be better understood and can be incorporated into product development.

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Preliminary assessment of the performance of the Sofia Analyzer in an outpatient clinic

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Background: Rapid diagnostic tests are frequently used to allow a quick laboratory diagnosis of influenza virus infections, informing clinical management and infection control measures. The objective of our study was to make a preliminary assessment of the diagnostic accuracy of the Sofia Influenza A+B Fluorescent Immunoassay Analyzer (Quidel, San Diego, CA) with RT-PCR in an outpatient clinic. Materials and Methods: We recruited patients between January and April 2013 in a large outpatient clinic in Hong Kong, as part of a larger study investigating influenza virus concentrations in exhaled breath. Subjects were eligible to participate if they were aged 11 y or older with acute respiratory illness onset within 72 hours. Acute respiratory illness was defined as having at least two of the following signs or symptoms: fever ≥ 37.8°C, cough, sore throat, runny nose, headache, myalgia and phlegm. A nose swab was collected from each participant for testing by the Sofia Analyzer according to the manufacturer's instructions, and a separate nose swab was collected, transported at 4°C and stored at -70°C for subsequent testing by quantitative RT-PCR for influenza A and B (matrix gene). We assessed the sensitivity and specificity of the Sofia Analyzer compared with RT-PCR as the gold standard, and used bootstrapping with 1000 resamples to estimate 95% confidence intervals. Results: We recruited 78 subjects with median age 35 y (range 12-84) and 29% were male. Thirty-four (44%) had illness onset within 36 h. A total of 12 subjects were positive for influenza A and 3 for influenza B by the rapid test. For influenza A, 8/12 rapid test positives were confirmed by RT-PCR, while 0/3 rapid test positives were confirmed by RT-PCR for influenza B. We estimated the sensitivity of the Sofia Analyzer for influenza A to be 80% (95% CI: 50%, 100%). We could not estimate sensitivity for influenza B. We estimated the specificity of the Sofia Analyzer to be 90% (95% CI: 82%, 96%). Among 10 subjects positive for influenza A by RT-PCR, the geometric mean viral load was 5.5 $\log_{10}$ copies/mL for the subjects negative by rapid test compared with 7.0 $\log_{10}$ copies/mL for the subjects positive by rapid test (P = .46). Conclusions: The diagnostic accuracy of the Sofia Analyzer measured in our study was within the manufacturer's product insert (sensitivity 89%-90; specificity: 95%-96%). Larger studies are needed to confirm whether the Sofia Analyzer has improved diagnostic accuracy compared with other point-of-care rapid tests in community settings.

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Clinical performance of the Sofia Influenza A+B FIA to detect influenza A virus in adults

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Background: The rapid influenza diagnostic test (RIDT) has been used widely for early diagnosis of influenza. However, unsatisfactory sensitivity of conventional RIDT is one of the clinical issues. The objective of this study was to evaluate the performance of Sofia Influenza A+B FIA in adult patients with influenza-like illness (ILI). Materials and Methods: From 23 Dec 2012 to 11 Apr 2013, nasopharyngeal swab specimens were obtained from 394 adult patients with ILI in three teaching hospitals in South Korea. The performance of the Sofia Influenza A+B FIA was compared with that of the SD Bioline Influenza Ag A/B/A(H1N1/2009). A specimen was regarded as true positive when the real-time RT-PCR test was positive. Results: Median age of the patients was 40 years (IQR 30-59) and median time to sample collection from symptom onset was 1 day (IQR 0-2). Among 394 specimens, 196 (49.7%) samples were influenza A virus and 149 (37.8%) were H3N2 influenza virus. The Sofia Influenza A+B FIA showed 74.0% sensitivity, 95.4% specificity, 94.2% positive predictive value, and 78.5% negative predictive value for influenza A virus. The Sofia Influenza A+B FIA yielded higher sensitivity than the SD Bioline Influenza Ag A/B/A(H1N1/2009) for both influenza A virus (74.0% vs 54.1%, $P < .01$) and H3N2 influenza virus (73.8% vs 57.1%, $P < .01$). The Sofia Influenza A+B FIA showed higher sensitivity in patients who visited the hospital within two days from symptom onset than in patients who visited the hospital after two days from symptom onset (for influenza A, 77.4% vs 56.3%, $P = .01$). Test sensitivity was decreased in patients over age 50 years than in younger patients; however, this was not significant. Conclusions: The Sofia Influenza A+B FIA showed a relatively high sensitivity to detect influenza A virus. It would be useful for rapid diagnosis of influenza, especially in patients who visit the hospital within two days from symptom onset.

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Association of cycle threshold values with disease severity in HIV-infected and HIV-uninfected patients with influenza-associated respiratory illness, South Africa

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Background: It is possible to obtain semiquantitative data on the amount of nucleic acid present in a clinical sample based on the cycle threshold (Ct) value as measured by real-time polymerase chain reaction (RT-PCR) amplification curves. If the Ct value is associated with disease severity, this would be valuable in a diagnostic setting because the Ct value is the output of all diagnostic RT-PCR assays, regardless of whether a standard curve was used. Our aim was to determine if there is an association of Ct values with disease severity of influenza viruses in different age groups and in specimens from HIV-infected and HIV-uninfected patients collected as part of a severe acute respiratory infection (SARI) surveillance programme in South Africa. Materials and Methods: Surveillance for patients hospitalized with SARI is conducted at 6 hospitals in 4 provinces in South Africa. At 3 hospitals in 2 of those provinces, surveillance of patients with outpatient influenza-like illness (ILI) and enrollment of patients without fever or respiratory symptoms (controls) are conducted. Patients meeting the case definition for SRI, ILI and controls were systematically enrolled.. Nasopharyngeal aspirates were collected from children < 5 years of age, and nasopharyngeal and oropharyngeal swabs were collected from individuals ≥ 5 years of age. All respiratory samples were tested by multiplex RT-PCR for 10 respiratory viruses: influenza A and B, respiratory syncytial virus, adenovirus, enterovirus, human metapneumovirus, rhinovirus and parainfluenza virus 1-3. Children and adults of all ages with laboratory-confirmed influenza (Ct value ≤ 40) and a valid Rnase P (human housekeeping gene) amplification were included in the analysis to look at pathogen-specific Ct values. A logistic regression model was used to model the association between influenza Ct values and disease severity. Because age is the most important potential confounder, separate analyses were done for children < 5 years of age and older children and adults ≥ 5 years of age. HIV status, age, symptom duration, and presence of underlying medical condition were explored as potential factors associated with Ct values. Results: From 2009 to 2013, 21,745 patients were enrolled, of which 1379/21,745 (6%) had ILI, 18,621/21,745 (86%) had SARI, and 1073/21,745 (5%) died. Of these patients, 1599/21,745 (7%) had influenza Ct values available for analysis. Of those, 292/1599 (18%) had influenza Ct values < 25. For ILI cases, the median influenza Ct value was 30.5 (range 20.2-39.3), for SARI cases 31.0 (range 17.7-41.0), and for deaths 35.7 (20.0-41.4). On multivariable analysis controlling for ILI and SARI case status, influenza Ct values ≥ 25 were associated with older age individuals (odds ratio [OR] 0.3, 95% confidence interval [CI] 0.19-0.43 for 5-64 year olds and OR 0.4, 95% CI 0.20-0.83, for those 65 years old and older, compared with children < 5 years of age]and HIV infection (OR 0.5, 95% CI 0.31-0.76) for HIV-infected individuals compared with HIV-uninfected individuals). Conversely, influenza Ct values < 25 were associated with death compared with ILI cases (OR 3.0, 95% CI 1.02-8.79). Conclusions: Higher influenza Ct values were found in patients who died and in younger children. HIV-infected patients had lower Ct values, compared with HIV-uninfected patients, which may suggest lower shedding or that HIV patients may present later.

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Evaluation of a new lateral flow immunoassay for rapid and differential detection of RSV, influenza type A and type B viruses

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Background: Respiratory syncytial virus(RSV) is the one of the most important respiratory viruses such as influenza viruses, especially for children and for high-risk patients. Early diagnosis of influenza and RSV infections may help to select appropriate treatment for patients with respiratory tract infection and may also be helpful for infection control. In this study, we have evaluated QuickNavi™ FLU+RSV (DENKA-SEIKEN Co., Ltd. Tokyo, Japan), a new multiline lateral flow immunochromatographic assay for rapid and differential detection of antigens of influenza A(FluA), B(FluB), and RSV. Materials and Methods: Vaccine strains and clinical isolates were used for evaluation of detection limits of QuickNavi™ FLU+RSV. Nasopharyngeal aspirate specimens were collected from children and adults with respiratory tract infection for clinical evaluation. The performances of QuickNavi™ FLU+RSV were compared with viral isolation in cell culture for influenza and RT-PCR for RSV and also compared with other rapid antigen tests (QuickNavi™ FLU and CHECK RSV). The sensitivity and specificity of the test were determined by using viral isolations and RT-PCR as a reference standard. Results: Detection limits of QuickNavi™ FLU+RSV were 2.5×10^2 to 5.0×10^4 pfu/mL for Flu A and B viruses, and 2.5×10^3 to 2.0×10^5 $TCID_{50}$/mL for RSV. The sensitivity and specificity of QuickNavi™ FLU+RSV using nasopharyngeal aspirate specimens were 95.4% and 99.4% for flu A, 89.4% and 100% for flu B, and 79.8% and 98.3% for RSV respectively. Conclusions: Our findings suggest that QuickNavi™ FLU+RSV have high sensitivity and specificity, and may be useful for early diagnosis of influenza and RSV infections in the clinical setting throughout the year.

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Use of influenza duplex PCR to improve response to influenza pandemic and epidemic surge in a hospital virology department

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Background: During the 2012-2013 influenza season, the Lyon hospital virology department received an average of 6000 respiratory samples a year, ie, as many as during the 2009 pandemic. Since 2009, we moved from in-house real-time PCR assays (1 for FluA, 1 for FluB, 1 for RSV, 1 for rhinovirus [Rh]) to the Argene-bioMérieux Respiratory MWS r-gene system duplex real-time PCR (FluA/FluB; RSV/metapneumovirus; adenovirus/bocavirus; parainfluenza/coronavirus, Rh/cell control performed in 96 well plates). This study aimed to demonstrate the improvement in our lab workflow after introduction of this system by comparing two representative weeks of 2009 and 2013. Methods: We compared two sets of data collected during two representative weeks of 2009 and 2013 seasons, which represented a similar number of respiratory samples. For these two periods, we retrieved the number of PCR and batches done, and calculated the percentage of empty wells. The turnaround time (TAT) for influenza and the others respiratory viruses was defined as the time spent between registration and result transfer to electronic medical record. Results: From the 16th of November to

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the 22nd of November 2009 (week 47), we received 414 respiratory samples, compared with 429 samples from the 18th of February to the 24th of February 2013 (week 8). In week 47 of 2009, we performed 717 PCR tests on 10 plates (38.8% of empty wells per plate), and searched for 717 respiratory viruses (2.8 viruses sought per sample). In comparison during week 8 of 2013, we performed 814 PCR tests (12.3% more than week 8/2009) on 10 plates (16.0% of empty wells per plate) and searched for 1570 viruses (45.7% more than week 8/2009). The TAT (time between arrival of the sample and release of the results) for influenza was consistent with the lab objectives (an average of 11 hours in 2009 and 17 hours in 2013) and was shortened for RSV (an average of 37 hours in 2009 versus 17 hours in 2013). Conclusions: Implementation of the respiratory multi-well system r-gene Argene-bioMérieux in our laboratory gave us the opportunity to (i) provide a more complete panel of respiratory viruses, while reducing the number of PCR runs, and (ii) reduce the TAT for others respiratory viruses. Quick expansion of the surge capacity is an important issue for laboratories involved in influenza diagnosis, to be able to cope with increased demand and early response for disease management.

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Implementation of an external quality assessment for the evaluation of the real time rt-pcr influenza diagnosis performance in the Argentinean surveillance network

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Background: Argentinean influenza (FLU) surveillance pursues the reduction of the disease burden associated with FLU by collecting and exchanging timely information on FLU activity. It provides relevant information about FLU to health professionals and the general public, and contributes to the revision of the FLU vaccine formulation. FLU surveillance in Argentina is based on a laboratory network, a sentinel units system and clinical surveillance. The success of the laboratory networks can be measured through the implementation of standardized procedures and assays, and specific training programs as well as a reliable technical proficiency test. A surveillance network with strong detection capability is required for the early detection of new viral variants that could emerge as the next epidemic and/or pandemic strain. PCR, as a diagnostic tool, requires a high degree of technological expertise including operational skills and procedures, and appropriate facilities in addition to good quality reagents. Therefore, quality assessment is required to evaluate network performance. In 2010 the National Influenza Centre (NIC) organized the first external quality assessment (EQA) to evaluate the performance of the real-time RT-PCR (rtRT-PCR) technique implemented. In the National Influenza Laboratories Network during the 2009 pandemic (9 labs) and during 2010 and 2011, a total of 35 labs were incorporated into the new platform. We designed and distributed proficiency panels containing influenza A virus subtypes H1N1pmd09 and H3N2 and influenza B virus to evaluate their performance to carry out the detection of influenza A and B viruses by rtRT-PCR. Materials and Methods: The designed panel included the first isolate of the influenza A(H1N1)pdm09 in Argentina, (A/Argentina/17/2009) full genome sequenced, and two reference viruses, A/NewYork/55/2004 (H3N2) and B/Brisbane/60/08 (Victoria lineage). The viruses were propagated in MDCK cells. Virus titers were tested using hemagglutinin assay with guinea pig red blood cells. The RNA copy number was measured by rtRT-PCR using a quantified commercial positive control. The 2012 panel consisted of 6 samples (S) containing 560 µL of AVL buffer (QIAamp Viral RNA Mini kit, QIAGEN) and 140 µL of the following viruses: S1: A/Argentina/17/2009 (H1N1)pdm09 1.7 x 10^4 copies/µL (cp/µL); S2: A/NewYork/55/2004(H3N2) 2.3 x 10^3 cp/µL; S3: B/Brisbane/60/08; S4: negative; S5: A/Argentina/17/2009 (H1N1)pdm09 256 cp/µL. S6: A/NewYork/55/2004(H3N2) 2.2 x 10^4 cp/µL. The panel was distributed to 35 lab members of the National Laboratory Network. Results: 24 of 35 labs that received the panel performed the EQA and returned the results to the NIC. For S1, 100% of the labs identified this sample correctly; for S2, 95.8% (23 labs); S3, 94.7% (18 labs); S4, 100%; S5, 83.3% (20 labs); and S6, 95.8% (23 labs). All the participants were able to correctly detect the M gene of the influenza A viruses, 100% in medium

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viral loads to H1H1pdm and 95.8% for influenza A(H3N2). The negative sample was correctly identified by 100% of the labs. Conclusions: In 2010, after the 2009 pandemic, as a result of three training courses given by the NIC with the aim to transfer the rtRT-PCR methodology for influenza detection, 9 lab members of the National Laboratory Network had implemented this technique and participated in the first EQA. Two years later, 24 of 35 participated in this proficiency test and almost all of them were able to correctly detect the positive samples A(H3N2) and A(H1N1)pdm09 with medium viral load and the negative sample; 20 of 24 correctly detected the A(H1N1)pdm09 with low viral load. The effort to expand the molecular diagnosis of influenza during and post pandemic helped to incorporate molecular platforms for influenza detection in provincial labs and provided the foundation for further expansion of this new technology into their diagnostic repertoire. These benefits clearly extend beyond influenza testing and have strengthened laboratory networks in Argentina. One of the missions of our NIC is transferring new technologies to the national network to improve diagnostic capability and to evaluate their performance in order to be prepared for an effective response to public health emergencies.

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Towards the development of alternative methods for determining antibody to influenza

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Background: Diagnosis of influenza infection is usually based on detection of virus in respiratory secretions during the acute phase (days 1-7 post onset) of illness using molecular, biochemical or culture methods. Acute viral infection is followed by the development of strain-specific antibodies from about 10 to 14 days post illness onset, and detection of such (mainly anti-HA) antibody in convalescent serum has been the basis of traditional serologic methods for identifying influenza infections. Serology detects symptomatic and asymptomatic infections and can thus aid estimates of disease burden and severity in epidemics and pandemics. However, in the 2009 pandemic, very few serologic studies were undertaken globally. One of the main obstacles for serologic investigations is the requirement for venous blood; collection of this specimen type requires a network of trained staff while members of the public are typically reluctant to give blood samples. Therefore, the majority of samples used for influenza seroepidemiology have been opportune sera collected for other investigations. Samples collected through less invasive methods (oral fluids or dried blood spots) for use in antibody detection have enormous value in public health programmes for infectious disease control, as they are inexpensive, user friendly, and allow self-sampling in population studies. They could also be useful for influenza serology. Here we report the use of an ELISA format to detect anti A(H1N1)pdm09 antibodies in serum, plasma and oral fluids. Materials and Methods: We designed IgG antibody capture ELISAs utilising recombinant haemagglutinin proteins derived from influenza A(H1N1)pdm09 (HA1 and HA0 GACELISAs). The test format was validated using sera from individuals after laboratory-confirmed natural infection (collected 1-13 months post diagnosis) and pre-/postimmunisation sera by comparison with traditional neutralisation (MN) and haemagglutination (HAI) tests. Furthermore, the ELISAs were used to analyse sera and plasmas collected in October and December 2009 from blood donors (age 17-71 years) and oral fluids collected using Oracol collection kits and extracted as described in Brown et al (1994). Two hundred and seventeen anonymous residual oral fluids (collected January-December 2007 from three age groups: < 5 years, 10-19 years and ≥ 50 years) and 152 oral fluids from 125 individuals receiving monovalent A(H1N1)pdm09 vaccine (GlaxoSmithKline, Pandemrix) November 2009-February 2010 in the UK were analysed. The later panel included paired pre- and postimmunisation samples from 25 individuals for whom we had received both oral fluids and sera. Results: The analysis of 54 sera from individuals with laboratory-confirmed A(H1N1)pdm09 infection shows good agreement of T:N ratios

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09HA1 ELISA and 09HA0 ELISA and results by HAI, MN. The relationship between time since diagnosis and antibody titres or T:N ratios indicates that antibody detectable by the 09HA1 GACELISA is short-lived, whereas that detected by the other three assays persists beyond 12 months after infection. Comparing HAI titres and T:N ratios for 389 samples indicated 55% vs 77% sensitivity as well as 97% vs 77% specificity for the 09HA1 ELISA and 09HA0 ELISA, respectively. Comparing correlation of HAI result for serum with ELISA result for the paired oral fluids from the same individual, we found correlation between the two sample types with an R^2 of 0.6537 and 0.6236 for the 09HA1 and 09HA0 assays, respectively. Conclusions: Use of the assay on sera and plasma indicated that there was potential for using the GACELISA to carry out seroepidemiologic studies. Although the ELISA formats described here would benefit from improvements in sensitivity and specificity through further development, we also demonstrated that if the GACELISAs were employed, very similar results would be obtained using oral fluid or serum/plasma. This is particularly useful in children and in settings where blood collections by venipuncture are impractical or difficult.

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Subtyping and H275Y oseltamivir-resistant H1N1pdm09 influenza virus detection by xTAG fluorescent beads technology

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Background: The spread of oseltamivir-resistant H1N1pdm2009 strains is still low, but their appearance requires monitoring. Multiplex testing for subtyping of human influenza virus A and simultaneous detection of a genetic marker of oseltamivir-resistant virus were developed using xTAG fluorescent beads technology. Materials and Methods: Six PCR primer pairs for detection of H1, H1pdm09, H3, N1, N1pdm09, N2 genes, one universal primer pair for detection of the M gene and two allele-specific primer pairs for detection of the H275Y (H1N1pdm09) mutation were used in a one-tube PCR reaction. PCR products were hybridized to appropriate xTAG beads and analyzed on a Luminex200 instrument. The test developed was used to subtype 20 influenza virus strains isolated in MDCK cell lines in 2012-2013 and to test autopsy lung specimens taken from 24 patients who died of viral pneumonia caused by H1N1pdm09 virus. Twelve lung samples were sequenced by next-generation sequencing technology (Illumina platform). Results: Eighteen H1N1pdm09 and two H3N2 isolates were detected. One H1N1pdm09 isolate with an H275Y mutation, conferring oseltamivir resistance, was found. It was confirmed by sequencing of the NA gene and neuraminidase inhibition (NI) assay. Only 16 lung specimens were successfully subtyped by the test developed despite the fact that all samples were positive for H1N1pdm09 in a real-time RT-PCR (rtRT-PCR) test. Only high-titer (Ct < 30 in rtRT-PCR) samples were subtyped. Therefore sensitivity the test developed is lower than rtRT-PCR. No cases of H275Y mutation were found among 16 positive specimens, which was confirmed by sequencing. Mutations D222G, D222E or D222G/N in the HA gene of the H1N1pdm09 genome were found in four samples. Thus, the H1N1pdm09 influenza mortality was not related to the oseltamivir-resistant strains. Conclusion: The test developed using xTAG fluorescent beads technology can be used for rapid influenza virus subtyping and H1N1pdm09 oseltamivir-resistant mutation detection in clinical samples including autopsy specimens.The work was supported by the Centers for Disease Control and Prevention, Atlanta, Georgia, United States. CoAg: U51IPOOO527-02.

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Development of monoclonal antibodies specific for H5N1 virus HA and their application to rapid detection of influenza A/H5N1 virus diagnostics

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Background: The highly pathogenic avian influenza A/H5N1 (HPAI H5N1) virus infection is sporadic persistent in humans and has the potential to cause a serious future pandemic. There are a number of commercially available rapid influenza diagnostic tests for influenza A and B virus infections. However, these are indistinguishable from seasonal influenza and HPAI H5N1 influenza. To develop a simple and rapid diagnostic system with high sensitivity and specificity for HPAI H5N1 virus detection by chemiluminescent immunoassay, we attempted to produce monoclonal antibodies (mAbs) recognizing linear epitopes specific for HPAI H5 hemagglutinin (HA). Materials and Methods: To obtain mAbs for specific HPAI H5 HA, mice were immunized with five synthesized peptides conjugated to keyhole limpet hemocyanin (KLH). These amino acid sequences of five peptides were highly conserved among HPAI H5HAs.The screening of mAb was conducted by ELISA using denatured HA antigens of A/Vietnam/1194/2004 (NIBRG-14) virus, which was lysed with 1% Triton X-100 treatment. The lysates were diluted with ELISA-coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6) and coated ELISA plates. The ELISA method was followed according to the conventional protocol. To assess the mAb for specificity for H5HA antigen, these mAbs were tested by ELISA using H1 and H3 virus antigens. Because 1% Triton X-100 was insufficient to lyse H5HA antigens in the ELISA system, we determined the most suitable concentration of detergent to detect lysed NIBRG-14 virus. Results: When five peptides conserving to linear epitopes of H5HA immunized to mice, nine mAb clones were obtained by the screening ELISA that reacted to one of the peptides. These clones reacted specifically with H5 virus antigen, and not with H1 and H3 virus antigens. The most appropriate composition of detergent with maximum denaturing of HA molecules and conserving the mAb binding capacity was 0.05% SDS and 0.01% Empigen. Conclusions: Nine mAb clones were obtained from mice immunized with peptides corresponding to linear epitopes conserved among H5HA molecules. These clones were specific for H5N1 virus, and the highest reactivity in ELISA was shown in a buffer with 0.05% SDS or 0.01% Empigen. It was presumed that in these lysis buffer conditions, virus antigens were lysed sufficiently without affecting the binding capacity of mAb. Development of a highly sensitive HPAI H5N1 virus detection system is in progress using these mAbs and chemiluminescent immunoassay.

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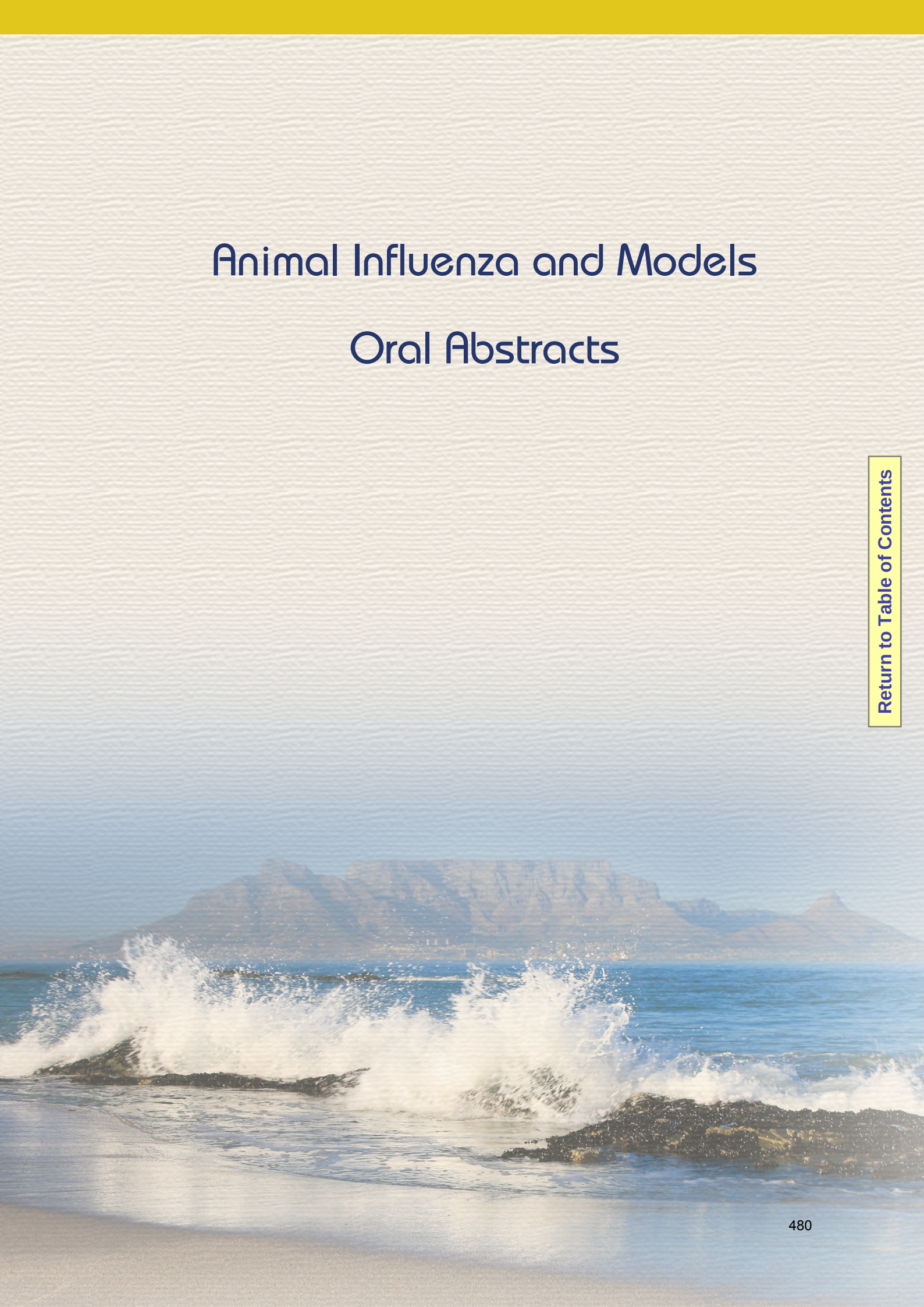

O-888

Quantifying the global antigenic and genetic evolution in swine influenza A viruses and evaluating the relative zoonotic potential to humans

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Background: Swine are important hosts for influenza A viruses where outbreaks of disease have a significant economic impact. Variants arising in pigs can be zoonotic and may cause epidemics and pandemics. The 2009 H1N1 pandemic in humans was caused by a virus that might have originated in swine. That outbreak highlighted our lack of knowledge about the circulation of influenza A viruses among pigs worldwide, the relative threat of these viruses to human health, and the limitation of vaccination as a method of disease control in pigs. The zoonotic potential of H1 and H3 viruses in pigs is difficult to assess, being complicated by marked heterogeneity among some of the H1 and H3 strains currently in circulation globally. Virus control is hampered further by the lack of a formalized method of monitoring and selecting specific vaccine strains. Pigs are routinely vaccinated against influenza A virus in the US swine industry, where fully licensed commercial vaccines or autogenous vaccines are available. The use of autogenous vaccines continues to rise because of the diversity of viruses co-circulating among North American pigs and the inability of the animal biologics industry to change vaccines as rapidly as the viruses change. The more limited use of swine influenza vaccine in the European Union and elsewhere increases the possibility of substantial differences in immune selection pressure in an already diverse antigenic landscape. The key to assessing the zoonotic potential of swine influenza A viruses, which may improve our ability to develop more effective vaccines, is to improve our understanding of both the phenotypic and molecular characteristics of the virus throughout its evolution across the globe. Materials and Methods*:* Antigenic drift is usually assessed using the hemagglutination inhibition (HI) assay, but the semi-quantitative data it provides can be difficult to interpret. We used antigenic cartography to quantify and map antigenic differences among viruses in different parts of the world. We integrated differences in antigenic measurements with genetic sequence data to investigate the molecular basis for the evolution of antigens into endemic influenza A viruses in swine. We also followed amino acid (AA) substitutions that occurred in swine viruses to make them antigenically similar to the human influenza A viruses that were circulating during the previous season. Using experimental cross-protection data in swine, we identified antigenic distances associated with a reduction in vaccine efficacy. Results: The global evolution of antigens to swine influenza A viruses clustered around both H1 and H3 subtypes within subtype clades. We found substantial antigenic and genetic heterogeneity among viruses that are endemic in different geographic regions, with substantial differences between the H1 viruses endemic to swine in North America versus Europe. We found that a single AA substitution can alter the antigenicity of swine influenza A H3 viruses significantly and that 6 AA substitutions in HA1 may be responsible for antigenic differences among swine influenza A H3 viruses. We also evaluated the relative cross-reactivity between endemic swine influenza A viruses and selected seasonal human influenza A viruses to determine whether it has any implications for our understanding of the genetics

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and antigenic characteristics of these viruses and for disease control. Conclusions: We have provided a basis for the quantitation and monitoring of antigenic variability among endemic swine influenza A viruses. Our findings could be used to determine the relative risk of swine infection to humans. Given that no single vaccine is sufficient to protect against all circulating swine influenza A viruses in a specific region, and given the increasingly global nature of livestock movement that allows for continued variations in antigens and the risk of re-introducing seasonal influenza A viruses from humans to pigs, there is still a risk for large-scale dissemination of the virus. With further study and refinement of the methods described in our study, we may eventually be able to follow the evolution of this virus globally and develop effective vaccines while minimizing the extent and severity of infection.

O-889

Ocular-only aerosol inoculation of ferrets with H7 and H5 influenza viruses

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Background: The study of influenza A viruses has traditionally been performed by studying virus exposure to and infection of respiratory tract tissues. However, these studies typically overlook the role of ocular surfaces, which represent both a potential site of influenza virus replication and a portal of entry for establishment of a respiratory infection. The ability of influenza viruses to engage both tissue systems is best exemplified by H7 influenza viruses, which frequently cause conjunctivitis in infected individuals but also can cause severe and fatal respiratory disease, as evidenced in the recent emergence of H7N9 viruses in humans. While ocular disease has not been reported among H7N9 virus–infected individuals, there is a need to understand the capacity of this virus to employ transocular entry to cause respiratory disease in mammals as a potential route of human exposure. Here, we established a ferret model to study the ability of human and avian influenza viruses to cause disease and be transmitted to naive contact animals following ocular-only exposure to aerosolized virus compared with ferrets inoculated intranasally or by aerosol inhalation. This allowed us to assess differences in virus infectivity, disease progression, and transmissibility between respiratory and ocular routes of virus exposure. Materials and Methods: Influenza A viruses of the H7N3, H7N7, H7N9, H5N1, H1N1, and H3N2v subtypes were used in this study. To inoculate ferrets by the ocular aerosol route, sedated ferrets were fitted with aerosol-delivery goggles that formed a close seal around each eye to exclude respiratory exposure to virus. Aerosolized influenza virus was passed through the goggles for varying times and concentrations. Ferrets were observed for clinical signs and symptoms, and nasal washes were collected postinoculation to measure viral replication. Transmission was assessed by placing naive ferrets in direct contact with inoculated animals or in adjacent cages with perforated side walls to evaluate transmission by respiratory droplets. Parallel studies were conducted in ferrets inoculated intranasally or by aerosol inhalation. Results: All human and avian influenza viruses tested were capable of mounting a productive respiratory infection following ocular aerosol exposure. Strikingly, ferrets succumbed to fatal H5N1 infection following ocular-only aerosol exposure of < 10 PFU of virus or for as little as 2 minutes of virus exposure time, demonstrating comparable infectivity and lethality of this virus subtype to ferrets inoculated by the respiratory route. The pathogenesis of H7N9 viruses was assessed in the ferret following traditional intranasal aerosol inhalation or ocular-only aerosol exposure. H7 influenza viruses associated with human conjunctivitis (H7N7, H7N3) and respiratory disease caused by the novel H7N9 virus replicated to a high titer in the upper respiratory tract of ferrets following ocular-only aerosol exposure, albeit with slightly delayed kinetics compared with intranasal or aerosol inhalation inoculation. Ferrets inoculated by the ocular aerosol route with H7 viruses associated with human infection were capable of transmitting virus to naive animals in a direct contact model, but transmission efficacy was reduced compared with traditional intranasal inoculation. Similarly, transmission of seasonal H1N1 and H3N2v viruses by respiratory droplets by ferrets inoculated via the ocular route was detected, but with reduced frequency compared with intranasal inoculation. Conclusions: We established a novel inoculation method that delivers an influenza virus aerosol inoculum exclusively to the ocular surface of ferrets. Our results reveal that the ocular surface represents a potential entry point for human and avian influenza viruses to mount a productive and potentially fatal respiratory infection in ferrets, even

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among viruses that do not typically demonstrate an ocular tropism. Divergent patterns in respiratory disease and virus transmissibility following ocular as compared with respiratory routes of inoculation highlight the modulation of virus infectivity and disease progression, depending on the route of virus exposure. These findings allow for a greater understanding and risk assessment of emerging influenza viruses, such as H7N9, that pose a threat to human health and underscore the importance of eye protection during occupational exposure to aerosols containing influenza viruses of all subtypes.

O-890

Characterization of the 2012 highly pathogenic avian influenza H7N3 virus isolated from poultry in Mexico: pathobiology and vaccine protection

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Background: In June 2012, a highly pathogenic H7N3 avian influenza (HPAI) virus was identified as the cause of a severe disease outbreak among commercial laying chicken farms in Mexico. Vaccination was rapidly implemented as a control measure, along with increased biosecurity and surveillance. However, the virus was not contained and has spread to other areas of Mexico. At the time of the initial outbreaks, the H7 AIV vaccine was not commercially available in North America. A recent H7N3 wild bird isolate from Mexico (A/cinnamon teal/Mexico/2817/2006 H7N3) was selected for use as the vaccine seed strain. Material and Methods: We characterized the Mexican 2012 H7N3 HPAI virus (A/chicken/Jalisco/CPA1/2012) genetically and biologically and determined the degree of protection conferred against the virus by different H7 vaccines in chickens. Results: Chickens inoculated intranasally with the virus became infected and died 2 to 4 days after inoculation; this was expected with an HPAI virus infection. High virus titers and viral replication in many tissues was demonstrated in infected birds. The Jalisco virus had high sequence similarity (> 97%) with viruses in wild birds from North America in all 8 gene segments. The hemagglutinin gene of the virus contained a 24-nucleotide insert at the hemagglutinin cleavage site that had a 100% sequence identity with chicken 28s ribosomal RNA; this suggests that the insert was a result of nonhomologous recombination with the host genome. In protection studies using experimental oil emulsion vaccines, low pathogenic (LP) H7 AI viruses from the United States and a 2006 Mexican H7 LPAI virus antigen were injected as a challenge into chickens 3 weeks prior to testing. All H7 vaccines tested provided >90% protection against clinical disease after the Mexican H7N3 virus challenge and decreased the number of infected birds and the amount of virus shedding. In another study, laying chickens were vaccinated with a recombinant fowlpox virus vaccine containing a low pathogenic LPAI H7 gene insert (rFPV-H7-AIV), and an inactivated oil-emulsified H7N3 AIV vaccine and were subsequently challenged with the Jalisco H7N3 HPAIV. All vaccine combinations provided similar and significant protection against mortality, morbidity, and shedding of the challenge virus from the respiratory and gastrointestinal tracts. In a third study, the potency and efficacy of a vaccine seed (A/cinnamon teal/Mexico/2817/2006 H7N3) was evaluated in chickens against a challenge with Jalisco H7N3 HPAIV. A vaccine dose of 256 and 102 hemagglutinating units (HAUs) reduced mortality and morbidity significantly compared with sham vaccinates, but a dose of 512 HAUs was needed to prevent mortality and morbidity completely. Conclusions: The Mexican H7N3 virus represents a new HPAI virus that probably mutated from an LPAI virus. The exact timing of movement of the LPAIV from wild birds to poultry cannot be determined, because a closely related LPAI virus was never identified. The virus was associated with rapid and severe disease in naïve chickens. Several vaccines were identified, including the killed oil adjuvant vaccine using the Mexican 2006 virus, which was used as a seed in a widespread vaccination effort in the affected Mexican states. Initially, the vaccine program appeared to control the outbreak. Unfortunately, the virus re-emerged in March 2013 and continues to cause outbreaks in Mexico.

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O-891

The first detection and isolation of avian influenza viruses in Antarctica

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Background: Avian influenza viruses (AIVs) typically cause asymptomatic infections in wild aquatic birds; occasionally, highly pathogenic AIVs, such as H5N1, cause large-scale bird mortality. The movement of AIVs around the world occurs predominantly through migratory dabbling ducks, gulls, and shorebirds. As a result, distinctly different AIVs exist in birds along major migratory bird flyways, eg, North American and Eurasian lineage strains. Antarctica is the most southern and isolated continent on earth and is home to at least 45 species of birds. Some of these birds are migratory and, thus, may facilitate the movement of avian influenza viruses to and from the Antarctic continent. Previous studies of birds and penguins in Antarctica have detected antibodies against influenza, but none has detected any AIVs. Given the importance of understanding the ecology of avian influenza viruses in Antarctica, particularly with respect to the potential movement of highly pathogenic AIVs into this fragile environment, we collected swabs and blood from nearly 400 penguins in Antarctica to test for the presence of AIV or antibodies to AIV. Materials and Methods: In January and February 2013, we sampled penguins from 2 locations in Antarctica: Admiralty Bay on King George Island (62°9'35"S; 58°28'17"W) and Kopaitik Island on the Antarctic Peninsula (63°19′15″S; 57°53′55″W). A total of 150 Adelie penguins (*Pygoscelis adeliae*) from King George Island (110 adults and 40 chicks) and 151 Adelie penguins (110 adults and 40 chicks), 74 Gentoo penguins (*P papua;* 10 adults and 64 chicks) and 18 Chinstrap penguins (*P antarcticus;* all adults) from the Antarctic Peninsula were sampled. Blood was collected from each bird for serological analysis and was tested using a universal influenza A ELISA test and hemagglutination-inhibition assay. Combined cloacal and tracheal swabs were taken for virological analysis using various RT-PCR assays and Sanger sequence analyses. Samples with a matrix gene real-time RT-PCR assay cycle threshold (Ct) of <38 were considered presumptive positives. They were tested further using gene sequencing analysis and by inoculating embryonated hens' eggs to confirm the presence of live/infectious influenza virus. Results: Of the 393 combined cloacal/tracheal swabs from penguins, 14 had a matrix gene real-time RT-PCR (rRT-PCR) Ct value of <38. Six of these samples were subsequently confirmed as being influenza-positive following successful RT-PCR amplification and sequencing of the HA2 region of the hemagglutinin gene. BLAST analysis of these sequences revealed they were all the influenza A subtype H11. All 6 viruses were identified in samples obtained from Adelie penguins on Kopaitik Island. Four of the 6 samples were successfully isolated in embryonated hens eggs, with HA titers ranging from 8 to 64. Through subsequent full HA and neuraminidase (NA) sequence analysis of the isolates, we determined that the full subtype of the virus was H11N2 and that the HA and NA sequences of the 4 viruses were virtually identical, differing by only 1 or 2 nucleotides. Phylogenetic analysis of the H11 HA gene with sequences on public databases revealed that these viruses were distinct from other H11 AIVs currently circulating in the southern or north hemispheres and, instead, were most closely related to strains circulating in Eurasia during the 1960 and 1970s. The N2 gene was distantly related to all other N2 sequences on public databases, including Eurasian and North American AIV sequences and human H2N2 and H3N2 NA sequences. The lack of close similarity with either early or contemporary N2 sequences suggests that an evolutionary divergence took place prior to the 1960s. Internal gene analysis and serological analyses will be completed shortly. Conclusions: We present the first description of AIVs in penguins and the first detection of AIVs in any bird in Antarctica. Phylogenetic analysis of the H11N2 viruses revealed that the genetic sequences were highly divergent from those recently isolated on any other continent, which suggests that these viruses may have been evolving independently in Antarctica for at least half a century.

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O-892

Detection of multiple genotypes of swine influenza viruses in southern China, 2009-2012

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Background: Pigs may act as “mixing vessels” to facilitate the genesis of novel variants of influenza viruses and as intermediate hosts for zoonotic transmission to humans. All major existing swine influenza virus lineages--classical swine (CS), Eurasian avian-like swine (EA), and triple-reassortant swine (TR)--have co-circulated in southern China during the last decade. With the introduction of the pandemic H1N1 2009 (pdm09) virus into pigs, frequent reassortment with enzootic swine influenza viruses have occurred. To investigate the impact of the pdm09 virus on swine influenza, an active influenza surveillance program has been conducted on slaughtered pigs at abattoirs in Hong Kong, Guangdong, and Guangxi provinces since 2009. Materials and Methods: Between August 2009 and September 2012, 387 swine influenza viruses were isolated from nasal and tracheal samples taken from slaughtered pigs, and the full genomes of these viruses were sequenced. Complete genome sequences of all swine influenza viruses available in GenBank and GISAID EpiFlu databases were combined with our swine influenza virus sequences and analyzed with reference avian, human, and equine influenza viruses. Sequence alignments were carried out using MUSCLE. Maximum likelihood phylogenies of each viral gene segment were inferred using RAxML with the GTR+I+Γ4 substitution model and Shimodaira-Hasegawa-like branch tests. Swine influenza genotypes were characterized by lineage of origin of each of their gene segments, based on the phylogenetic analyses. Results: Most of the contemporary swine influenza isolates were reassortant variants. Approximately 60% contained at least one gene segment from the human pdm09 lineage. The proportion of pdm09 reassortants in our surveillanced regions increased from 16% in 2010 to 68% in 2011 and decreased slightly to 55% in 2012. A total of 29 distinct “genotypes” were identified throughout our surveillance period. Of these, 23 were novel genotypes, with 17 containing at least one segment of pdm09 origin. Nine novel genotypes were isolated on different sampling occasions over an extended period, which suggests sustained onward transmission of these variants in the swine population. Conclusion: Repeated introduction of human pdm09 viruses to the swine population has led to an expansion of the genotypes of swine influenza viruses in southern China. The high prevalence of pdm09 reassortant viruses suggests that pdm09 genes are highly compatible with current swine influenza viruses and may confer a fitness advantage. Some of the newly generated reassortant variants may be establishing themselves in pigs and might lead to a threat to the human population.

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Coordinated surveillance of influenza viruses in European pigs: enhanced virological and epidemiological analysis from the European Surveillance Network for Influenza in Pigs (ESNIP3)

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Background: Since its detection, the human influenza pandemic H1N1 2009 virus has spread around the world infecting pigs. Although the virus did not emerge in pigs in Europe, its appearance highlighted the fact that there were gaps in surveillance for influenza in pigs that caused concern for global veterinary and public health officials. As a result, the European Union (EU) funded a network to address the epidemiology, identification, and detailed characterization of influenza viruses circulating in European pig populations. The primary aims of the network were to develop programmes of harmonized surveillance across Europe and to coordinate the appropriate identification and characterization of viruses. Materials and Methods: The methods of surveillance include the collection of epidemiological meta-data to understand the distribution and spread of the viruses, the basic virological characterization of virus subtypes, antigenic cartography, and the full genome genetic characterization of selected strains. Results: An extensive virological surveillance system has been harmonized and established in 14 countries. Influenza virus has been detected in approximately 30% of the herds examined, in which approximately 750 viruses have been identified and subtyped and approximately 180 fully sequenced. Influenza A (H1N1)pdm09 has been detected in pigs in at least 7 EU member states. The epidemiology of swine influenza across Europe continues to vary among regions. Detected viruses include the endemic strains: avian-like swine H1N1, swine H1N2 and human-like swine H3N2, and H1N1pdm09, which now holds endemic status in many countries. These viruses continue to co-circulate but with a different incidence in each country. Enhanced analysis of these viruses has revealed low levels of first-generation genetic reassortment involving H1N1pdm09. Preliminary antigenic cartography maps reveal substantial geographical and temporal differences. Conclusions: The epidemiology of influenza in pigs in Europe is complex and involves several co-circulating strains. The unique emergence of several first-generation reassortments between endemic swine strains and H1N1pdm09 strains will require continued surveillance to determine if any of these become established in pig herds with implications for veterinary health. Preliminary antigenic maps will be informative for vaccine strain selection in the European context and can be cross-referenced for worldwide scenarios. The potential emergence of some of these novel strains may have implications for zoonotic infection and public health in the future.

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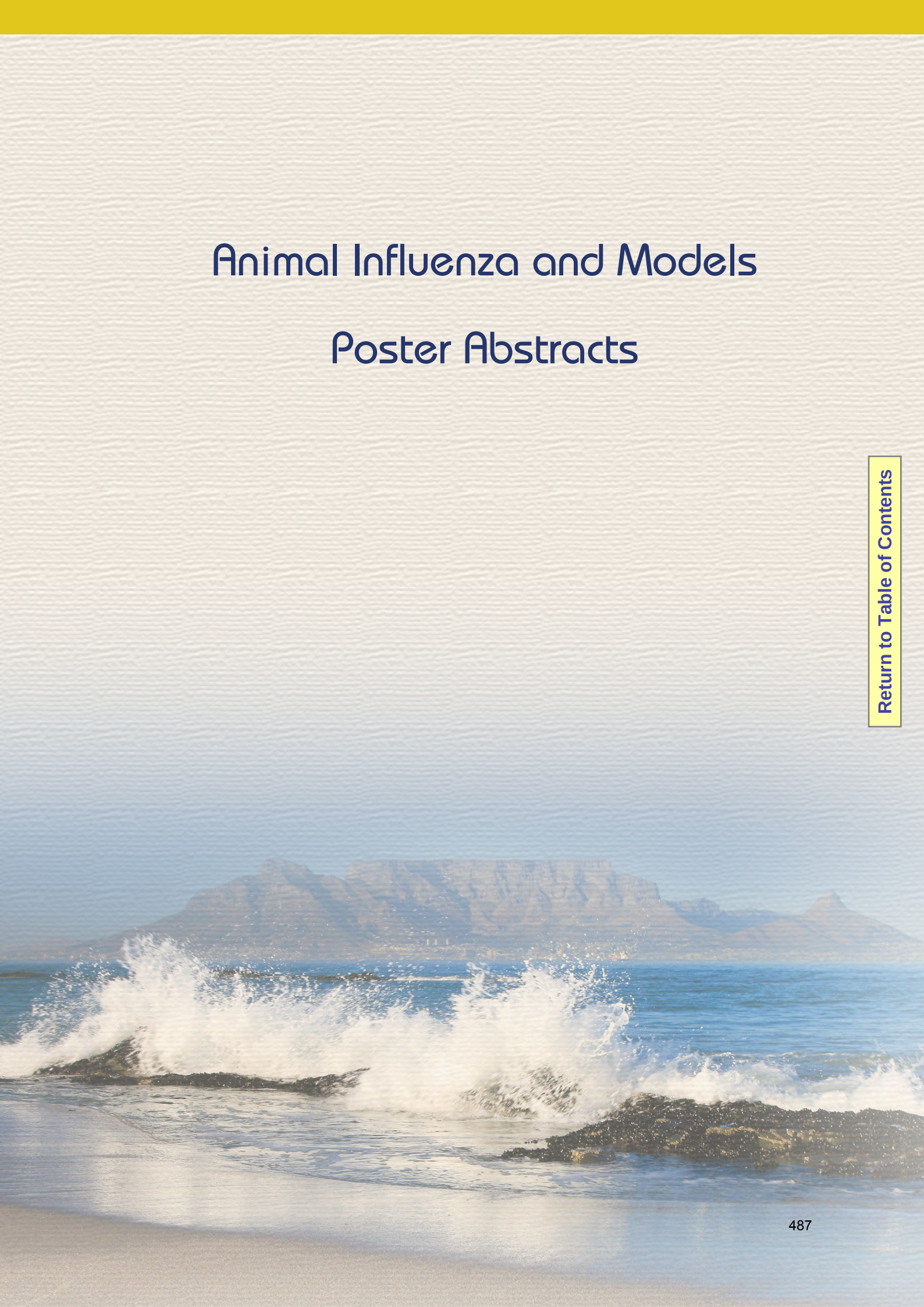

P2-571

Swine and human influenza viruses

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Background: An H1N1 subtype of swine origin caused the first influenza pandemic in Accra, Ghana. This pandemic strain was a reassortant of avian, swine, and human influenza viruses. Many diagnostic laboratories were overwhelmed by the testing demands related to this pandemic. Nevertheless, there remains the threat of other animal influenza viruses, such as the highly pathogenic H5N1 virus. An essential part of pandemic preparedness is the ability to identify the diagnostic challenges that will accompany that pandemic. Content: We discuss the natural reservoir of influenza viruses and the possible role of livestock in the emergence of pandemic strains. Current molecular tests commonly used for influenza diagnosis or surveillance are also briefly reviewed. Some of these approaches are also used to detect animal viruses. Unfortunately, owing to the lack of a systematic method of surveillance of animal influenza viruses, established tests may not be able to detect pandemic strains that have yet to emerge from the animal reservoir. Thus, multiple strategies need to be developed for better identification of influenza viruses. Molecular assays for mutations associated with antiviral resistance and for the reassortment of viral segments should also be encouraged. **Conclusion:** Influenza viruses are highly dynamic. Regular and systematic surveillance in both humans and animals is essential to provide a more comprehensive picture of prevalent influenza viruses. To be better prepared for the next pandemic, we should develop simple and easy-to-use tests for characterizing newly emerging influenza viruses.

P2-572

Phylogenetic analysis of surface and internal protein genes of influenza A H3N6 and H3N8 virus subtypes isolated from wild birds in Kazakhstan

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Background: The natural reservoir of influenza A is wild avifauna, mainly migratory birds occupying water and wetland complexes. Influenza A virus overcomes the species barrier between birds and mammals through long-term adaptation, after which it circulates through the ecological niche of the “new” species for decades as an endemic pathogen. In swine, horses, and dogs and in most cases of human infection, influenza can be attributed to such endemic infections. The H3 subtype of the influenza virus belongs to one of 3 variants that caused a pandemic during the 20th century. There is a considerable amount of interest in carrying out a comparative phylogenetic study of A/H3 influenza viruses isolated from wild birds in different regions of Kazakhstan with other viruses of this subtype. Materials and methods: Four isolates of influenza A/H3 virus—A/garganey/Korgalzhyn/865/04 (H3N6), A/teal/Korgalzhyn/1797/06 (H3N8), A/graylag goose/Korgalzhyn/1867/06 (H3N6), and A/teal/South Kazakhstan/8048/08 (H3N8)—isolated from cloacal swabs of waterfowl in the Central and Southern Kazakhstan were cloned by limited dilution in 10- to 11-day-old chicken embryos using a standard technique. We then isolated their RNA using a QIAamp Viral RNA Minikit (Qiagen GmbH, Hilden) following the manufacturer’s instructions. Complementary DNA was derived from the RNA using reverse transcription with help from a uni-12 universal primer for influenza A viruses (First Strand cDNA Synthesis kit; Fermentas) following the manufacturer’s instructions. DNA sequencing was carried out by identifying terminal dideoxynucleotides on an automatic 96-capillary ABI 3730xl DNA sequence analyzer (Applied Biosystems). Alignment of influenza A virus gene sequences with full nucleotide sequences of analogous viral genes obtained from an international data base GenBank was carried out using BioEdit computer software. The sequences were compared by creating phylogenetic trees containing an "accession of neighbors" with 1000 bootstrap replications using

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MEGA software. Result: Phylogenetic analysis of HA, NP, M, and NS genes of Kazakhstan isolates of influenza A virus containing subtype H3 hemagglutinin revealed the heterogeneity of these populations. The HA gene of the A/garganey/Korgalzhyn/865/04 (H3N6) isolate is similar to that of the A/mallard/Netherlands/05/2001 (H3N6) virus and is included in the cluster of European strains. By contrast, the A/teal/Korgalzhyn/1797/06 (H3N8) isolate demonstrated a high-degree relationship with A/aquatic bird/Korea/CN-1/2004 (H3N6) and is included in a cluster of Asian viruses in the Euroasian line. The A/teal/Korgalzhyn/1797/06 (H3N8) virus, having an NP gene, is considered a member of a separate cluster, unlike the A/garganey/Korgalzhyn/865/04 (H3N6) and A/graylag goose/Korgalzhyn/1867/06 (H3N6) strains, which have similar isolation periods and regions of occupancy. A close phylogenetic relationship was identified between isolates from Central Kazakhstan on the M gene; differences between this strain and the A/teal/South Kazakhstan/8048/08 virus were revealed. Nucleotide sequencing of NS genes in isolates from the A/teal/Korgalzhyn/1797/06 and A/teal/South Kazakhstan/8048/08 viruses revealed that they are considerably different from the A/common tern/Korgalzhyn/847/04 and A/graylag goose/Korgalzhyn/1867/06 viruses, and they were assigned to A and B genetic alleles, respectively. Conclusion: The H3 subtype influenza A virus remains one of the most widespread and quickly changing infection agents. The data from our study confirm the important role of Kazakhstan migratory birds in the evolution of influenza A virus.

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P2-574

Five known molecular determinants of virulence correlate with the virulence of strain A/swan/Mangistau/3/06 (H5N1) in birds and mammals

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Background: A number of determinants of genetic virulence are associated with the virulence of the avian influenza virus and its ability to replicate in the cells of mammals and birds. Our study was carried out to determine the correlation between known molecular determinants of virulence in different avian influenza virus (AIV) genes and the results of experimental infection of birds (chickens, ducks, geese, turkeys) and mammals (mice, guinea pigs, rabbits, cats, dogs, pigs) with AIV strain A/swan/Mangistau/3/06 (H5N1), which was isolated in March 2006 from a dead swan in the Mangistau region of the Republic of Kazakhstan. Materials and Methods: The nucleotide sequences of the HA (Genbank Accession No FJ436942), NA (FJ436943) and NS (JF262041) genes and the PA, PB1, PB2, NP and MP genes (not yet published in Genbank) were determined with dideoxy sequencing techniques using the ABI Genetic Analyzer 3130 xl. In 29 previous studies of the pathogenicity of avian influenza virus A/H5N1 strains, investigators found that strains containing amino acid (AA) substitutions in 11 genes were associated with virulence in mammals and birds. The intravenous pathogenicity index (IVPI) was determined according to WHO protocol. The pathogenicity of A/swan/Mangistau/3/06 strain (H5N1) in ducks and geese (age: 6 weeks), turkeys (age: 4 weeks), mice (age: 5-6 weeks), guinea pigs (age: 8 weeks), cats and dogs (age: 8-12 weeks), rabbits (weight: 2.5-3.0 kg), and pigs (age: 12-20 weeks) was assessed by determining the duration, severity, and outcome of infection; the severity of pathological changes; and accumulation of the virus in animals that died or were euthanized in an agonal state. Animals were infected intranasally, intramuscularly, or subcutaneously with doses of $10^{4.00}$ EID_{50}/50 µL (mice), $10^{4.29}$ EID_{50}/0.5 mL (geese, ducks, turkeys, and guinea pigs), $10^{4.59}$ EID_{50}/0.5 mL (cats and dogs), $10^{5.29}$ EID_{50}/mL (pigs), or $10^{5.95}$ EID_{50}/0.5 mL (rabbits). The specificity of disease was confirmed by the presence of the hemagglutinating agent using the hemagglutination reaction (HAI), by identification of the virus in biomaterials from euthanized animals using ELISA, or by detecting antibodies in the recovered animals using the hemagglutination inhibition (HI) reaction. Results: The avian A/swan/Mangistau/3/06 (H5N1) strain was highly pathogenic (IVPI = 2.34), being fatal in turkeys, ducks, mice, and dogs, which died within 48 to 72, 120 to 144, 48 to 72, and 72 to 120 hours post infection, respectively. Hemagglutinating agents (titer: 2.67 ± 0.33 – 6.67 ± 0.33 $\log_2$) and H5 subtype-specific antigens (titer: 4.93 ± 0.33 – 8.65 ± 0.33 $\log_2$) were detected in suspensions of lung tissue taken from euthanized mammals and birds using HI and ELISA analyses, respectively. The

virus could be released into the environment by infected turkeys, ducks, mice and dogs, given that it was isolated from tracheal and oropharyngeal swabs (titer: 4.65 ± 0.00 to 7.32 ± 0.33 $\log_2$ per ELISA). Anti-hemagglutination antibodies were detected by seral HI in geese, guinea pigs, rabbits, cats, and pigs, which did not get sick after infection. Titers ranged from 3.32 ± 0.00 to 9.32 ± 0.00 $\log_2$ when measured 14, 21, and 28 days after infection. Molecular determinants of the virulence of the strain A/swan/Mangistau/3/06 (H5N1) were identified in *HA* (HA-140), *NS1* (V149A, ESEV motif), *NP* (M105V, A184K), *M1* (15T, 30D, 215A) and *M2* (31S). Other known molecular determinants were not identified, including those in *HA* (multiple basic AAs or insertion of AAs at the HA cleavage site, HA-102, HA-154 , HA-172, HA-228, and HA-279), *NA* (deletion of AAs 49-68), *PA* (T515A, V127A, and L336M), *PB1* (M317I), *PB1-F2* (N66S), *PB2* (E627K, D701N , Q591K, R318K, R355K), *NS1* (A42S, D92E, D97E [with a deletion], T127N, D189N, S195T, and S228P), and *NS2* (M31I and H56Y). Conclusions: AA substitutions in *HA, NS1, NP, M1* and *M2* in A/swan/Mangistau/3/06 (H5N1) are responsible for the virulence of this strain in chickens, turkeys, ducks, dogs, and mice. This study provides new information about the influence of molecular virulence determinants on the pathogenesis of A/H5N1 infections in birds and mammals.

P2-575

Nomadic duck flock management practices and their potential relevance to the spread of avian influenza A viruses in northeastern Bangladesh

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Background: Avian influenza A (H5N1) virus has been known to be circulating among domestic poultry in Bangladesh since 2007. Nomadic duck flocks may be important carriers of this virus, facilitating its persistence in this country by shedding the virus into the environment. We surveyed nomadic duck flocks to characterize their demographic characteristics and management practices and to determine the proportion with avian influenza type A infection and antibody positivity in northeastern Bangladesh. Materials and Methods: We defined nomadic duck flocks as groups of domestic ducks moving intentionally to access feeding sites beyond village boundaries, not returning home for extended periods of time, and housed overnight in temporary enclosures in the scavenging areas. From December 2011 to March 2012, trained field workers interviewed 62 nomadic duck flock owners using structured questionnaires. They also collected eggs and fecal samples early in the morning from the night shelters of randomly selected nomadic duck flocks in northeastern Bangladesh. Egg yolk specimens were tested for the H5 antibody using antibody-capture ELISA, and fecal samples were analyzed for influenza A virus RNA and subtype for H5 using real-time one-step reverse transcriptase polymerase chain reaction (rRT-PCR). Results: The median age of the ducks in the nomadic duck flocks was 24 months (range: 8-36 months) and the median flock size was 300 (range: 105-1100 ducks).The majority of the duck flocks (63%, n = 39) consisted of multiple breeds of ducks (Khaki Campbell and local breeds). All of the ducks were used for commercial egg production and were housed overnight in temporary enclosures in scavenging areas. Most of the duck flock owners (74%, n = 46) did not provide litter as bedding material in the duck night shelter. The median length of time the duck flocks stayed in scavenging areas was 30 days (range: 15-99 days). The main reason for moving the duck flocks to a new scavenging area was a scarcity of feed in the current scavenging area (100%, n = 62). The median distance duck flocks moved in a year was 7 km (range: 1 - 150 km). Most duck owners moved their duck flocks outside their village (58%, n = 36); 16% and 28% of owners moved their ducks outside their subdistrict and district, respectively. All of the duck eggs were sold in markets. The majority (92%, n = 57) were sold to a vendor; the remainder (8%, n = 5) were sold directly to retail customers. Most of the flock owners (94%, n = 58) reported that their duck flocks shared their feeding ground with wild waterfowl. The majority of the duck flocks (58%, n = 36) were vaccinated against either duck plague or duck cholera. We collected 1860 egg yolks and

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496 pooled fecal samples, of which 886 (48%, 95% CI: 42%-53%) were positive for antibodies to avian influenza (H5). Sixty flocks (97%) produced at least one egg with antibodies against avian influenza type A (H5). Of the 496 pooled fecal samples, 131 (26%) had detectable RNA for avian influenza type A virus, but none had detectable RNA for A/H5. Thirty-three flocks (54.8%, 95% CI: 42%-68%) had at least one pooled fecal sample that was positive for avian influenza type A virus. Conclusions: Nomadic duck flocks in northeastern Bangladesh frequently shed avian influenza type A virus and had evidence of exposure to avian influenza type A (H5) virus. Developing and evaluating interventions to reduce influenza A virus infection and shedding in these birds should be a priority for research. This process should include an investigation of their interactions with wild birds and other domestic birds to determine the potential for influenza transmission.

P2-576

Complete genome sequence analysis of avian-origin H3N2 influenza viruses isolated from dogs and cats in South Korea

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Background: In South Korea, an avian-origin H3N2 influenza virus strain designated A/canine/Korea/01/2007 (H3N2) was isolated in 2007 from nasal swabs of pet dogs exhibiting severe respiratory syndrome. At the beginning of 2010, another avian-origin Korean H3N2 influenza virus strain (A/feline/Korea/01/2010 [H3N2]) was isolated from a lung specimen taken from a dead cat that had had a severe respiratory disease. To date, complete genome sequences containing 3′ and 5′ noncoding regions (NCRs) of influenza viruses have rarely been reported, despite multiple known functions of NCRs in the replication of influenza viruses. Therefore, it is necessary to analyze the complete genome sequence—including both 3′ and 5′ NCRs—of the A/canine/Korea/01/2007 (H3N2) and A/feline/Korea/01/2010 (H3N2) strains and elucidate their molecular characteristics. Materials and Methods: Viral RNA was extracted from the allantoic fluid of embryonated eggs infected with the A/canine/Korea/01/2007 (H3N2) or A/feline/Korea/01/2010 (H3N2) strains and circularized using T4 RNA ligase. To determine the complete genome sequence containing 3′ and 5′ NCRs, the PCR products of RNA ligation-mediated reverse transcriptase PCR (RT-PCR) were purified, cloned, and sequenced using an automated DNA sequencer with universal primer sets containing slight modifications and newly designed segment-specific primers. Results: The complete genome of the A/feline/Korea/01/2010 (H3N2) is 13,629 nucleotides (nt) long, making it identical in length to A/canine/Korea/01/2007 (H3N2). Segments 1 through 8 (Seg-1 – Seg-8) contain 2341, 2341, 2233, 1765, 1565, 1467, 1027, and 890 nts, respectively, and encode 12 viral proteins containing the following number of amino acids: PB2, 759; PB1, 757; N40, 718; PB1-F2, 90; PA, 716; HA, 566; NP, 498; NA, 469; M1, 252; M2, 97; NS1, 230; and NS2, 121. The lengths of the 3′ and 5′ NCRs in the viral RNA of the A/canine/Korea/01/2007 (H3N2) and A/feline/Korea/01/2010 (H3N2) strains varied (19-45 and 20-58 nt at the 3′ and 5′ NCRs, respectively) in different genome segments, and the terminal 12 and 13 nt of the 3′ and 5′ ends, respectively, were highly conserved (3′-UCG(C/U)UUUCGUCC- and -GGAACAAAG AUGA-5′) compared with all 8 genome segments; this is consistent with the findings of previous studies. Surprisingly, 1 nt changed between the start codon (UAC) and the conserved region [UCG(C/U)UUUCGUCC] in the 3′ NCR of Seg-1 and in Seg-6 of A/feline/Korea/01/2010 (H3N2) compared with A/canine/Korea/01/2007 (H3N2). Conclusion: This is a report of the complete genome sequence containing the 3′ and 5′ NCRs of avian-origin H3N2 influenza viruses isolated from dogs and cats in South Korea. We hope these data will provide important insight into the molecular basis of the pathogenesis, transmission, and evolution of influenza viruses.

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Antigenic drift of the pandemic 2009 A(H1N1) influenza virus in a ferret model

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Background: Surveillance data indicate that most circulating A(H1N1)pdm09 influenza viruses have remained antigenically similar since they emerged in humans in 2009. However, antigenic drift is likely to occur in the future in response to increasing population immunity induced by infection or vaccination. The potential for antigenic drift of A(H1N1)pdm09 viruses was assessed in the ferret model of influenza virus infection and immunization. Materials and Methods: Ferrets were suboptimally vaccinated with (i) monovalent pandemic vaccine (MIV) alone, (ii) adjuvanted MIV (MIV+IFA) or (iii) adjuvant (IFA) alone or were not vaccinated (naive). A(H1N1)pdm09 virus was sequentially passaged by contact transmission seven times through two independent series of ferrets of each group. Daily samples were collected from the upper respiratory tract of all ferrets. Kinetic parameters of growth and transmission were analyzed. Viruses that were transmitted were subject to detailed genetic, antigenic and receptor binding analyses. Results: A variant virus, with an amino acid substitution (N156K, H1 numbering without signal peptide; N159K, H3 numbering without signal peptide; N173K, H1 numbering from first methionine) in a known antigenic site of the A(H1N1)pdm09 viral HA emerged only in the MIV+IFA passage line. The N156K HA variant replicated and transmitted efficiently between naive ferrets and outgrew wild-type virus *in vivo* in ferrets in the presence and absence of immune pressure. *In vitro*, in a range of cell culture systems, the N156K variant rapidly adapted, acquiring additional mutations in the viral HA that also potentially affected antigenic properties. The N156K escape mutant was antigenically distinct from wild-type virus and had altered receptor binding preferences based on HA-specific antibodies and glycans, and these findings were also supported by computational modeling predictions. The N156K substitution, and culture adaptations, have been detected in human A(H1N1)pdm09 viruses with N156K preferentially reported in sequences from original clinical samples rather than cultured isolates. Conclusions: This study demonstrates the ability of the A(H1N1)pdm09 virus to undergo rapid antigenic change to evade a low-level vaccine response while remaining fit in a ferret transmission model of immunization and infection. Furthermore, the potential changes in receptor-binding properties that accompany antigenic changes highlight the importance of routine characterization of clinical samples in human A(H1N1)pdm09 influenza surveillance.

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Proteomics analysis of mouse lung tissue proteins caused by H9N2 avian influenza virus PB2 K627E

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Background: Although the influenza virus was one of the first human viruses isolated, it continues to pose a challenge each year with newly acquired genetic variation. H9N2 subtype avian influenza viruses (AIVs) have shown expanded host range and can infect mammals, such as humans and swine. Our previous studies had demonstrated that PB2 amino acid 627 is critical for pathogenicity and viral replication of H9N2 viruses in mice lungs and brains. Furthermore, the increased virulence induced by PB2 E627K of H9N2 AIV was associated with the activated hypothalamus-pituitary-adrenal (HPA) axis and sympathetic nervous system (SNS) response, which contribute to the immunosuppression. Materials and Methods: To gain more information about the increased pathogenicity for H9N2 AIV PB2 E627K, we compared the difference in mouse lung proteins expression caused by a single amino acid in PB2 627. Four-week-old Balb/C mice were infected with wild H9N2 AIV PB2 K627 and recombinant H9N2 AIV PB2 K627E. The lungs were harvested, respectively, at 12 h and 72 h post infection. Results: The results revealed that the mutation K627E contributed to a set of differentially expressed lung proteins, including 6 up-regulated proteins and 10 down-regulated proteins in 12 h post infection; 12 up-regulated proteins and 29 down-regulated proteins in 72 h post infection. These changed proteins were mainly invoved in the formation of cytoskeleton and the regulation of cell cycle, mRNA transcription, and protein translation, as well as biochemistry metablism and transport of cellular protein. Some changed proteins caused by PB2 K627E were also proved in A549 cells. The mutation K627E recovered the distribution of Moesin and Ezrin in A549 cells. Moreover, the over-expression of surfactant proteins, SP-A in A549 cells could decrease the efficiency of virus (PB2 K627) replication. The knockdown of SP-A in A549 cells could increase the virus titer. Conclusions: The changed pathogenicity caused by PB2 K627E may be associated with the increased level of sp-A, Moesin and Ezrin in the lungs. These data can provide insight into the potential mechanism of increased pathogenicity for H9N2 AIV PB2 E627K.

P2-579 GATES Fellowship

Isolation and sequence analysis of reassortant low pathogenic avian influenza virus H4N6 from ducks and chickens in the live bird markets of Pakistan

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Background: Live birds markets (LBMs) are considered major centers of avian influenza A virus dissemination and, possibly, reassortment. In Pakistan, bird markets are abundantly filled with captured birds during the winter. Pakistan is home for a wide variety of migratory birds that travel from Siberia and Russia during that time of year. The largest group of visitor-birds is the waterfowl group, most commonly ducks. During routine avian influenza (AI) surveillance in Pakistan, the avian influenza virus subtype H4N6, which is not very pathogenic, was isolated for the first time in Khaki Campbell ducks (*Anas platyrhynchos*) during 2010 and in broiler chickens during 2011 in the LBM in Karachi. To determine the evolutionary status and genetic makeup of this virus, we subjected both isolates to whole-genome sequencing, phylogenetic analysis, and serological evaluation. Materials and Methods: Avian influenza surveillance was carried out by analyzing and monitoring specimens obtained by cloacal swabbing of ducks and chickens with mild respiratory symptoms. These samples

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were processed and subjected to virological evaluation by inoculating embryonated SPF chicken eggs. Subtype identification of the isolated virus was determined by standard hemagglutination assessment (HA), hemagglutination inhibition, and neuraminidase inhibition tests. Reverse transcription polymerase chain reaction (RT-PCR) and real time PCR analyses were carried out using sequence-specific primers and probes. The purified PCR products were subjected to cycle-sequencing reactions then sequenced in a genetic analyzer. Phylogenetic and molecular evolutionary analyses were conducted using *MEGA* version-4. Results: Whole-genome sequencing of these viruses revealed that a new reassortant of a Eurasian avian strain had been introduced. Phylogenetically, the HA, neuraminidase (NA), matrix (M), nucleoprotein (NP), and polymerase basic 2 (PB2) genes were Asian in origin and clustered mostly with Russian strains of influenza viruses. By contrast, nonstructural (NS) and PB1 genes showed maximum homogeneous sequence identity with the Pakistani H3N1 virus, which was isolated from backyard poultry during 2010. The polymerase acidic (PA) gene was European in origin and clustered with AI isolates from The Netherlands. No significant relationship was observed between these and the previously reported AIV subtypes (H9N2, H7N3, and H5N1). Sequence analysis revealed a low pathogenic amino acid (AA) motif (PEKASR) at the HA cleavage site; avian-like receptor specificity achieved by retaining the AAs Q239 and G241; conservation of AAs at the receptor binding sites; and 5 N-linked glycosylation sites at AAs 18, 34, 178, 310, and 497. The NA gene possessed 9 N-linked glycosylation sites at AAs 28, 32, 51, 54, 67, 70, 86, 146, and 201; one glycosylation site was lost due to a mutation in N62S. The AAs known to be associated with sensitivity to antiviral drugs (oseltamivir, zanamivir, and amantadine) were conserved. The C-terminal of the NS gene was unique, containing an ESEI PDZ domain motif in duck viruses due to mutation. Seroprevalence of H4N6 was recorded in both commercial and domestic poultry. Domestic poultry showed more seroconversion against H4N6 than other birds, especially in the bird populations of the provinces of Sindh and Khyber Pakhtoon Khawa (KPK), where bodies of water serve as attractive birding sites, with better opportunities for the wild birds and migratory water fowls to move frequently and intermingle with local domestic and commercial poultry. Conclusions: Our observations suggest that these LP H4N6 strains continue to circulate among wild and domestic bird populations in Pakistan. Isolation of the H4 subtype, first from ducks and subsequently from chickens, indicates that the mingling of bird species in LBMs provides an excellent opportunity for the transmission of avian influenza viruses between bird species. The seroprevalence of the H4N6 virus among domestic birds is noteworthy, in that it represents a risk for inoculation with reassortant AIVs in local bird species. The point mutations in these H4N6 isolates and the close relationship of Pakistani H3N1 and other Eurasian strains from Russia and Europe also reflect the prevailing diversity among AI viruses circulating in local communities.

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Transcriptome analysis of influenza-infected mouse lungs reveals the kinetics of the host response

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Background: To capture the kinetics of the host response after influenza infection, we studied the transcriptome in mouse lungs after infection with a nonlethal H1N1 influenza A virus (PR8) dose using Agilent´s microarrays. Materials and Methods: Global analyses as well as cell specific gene profiles were applied followed by a network analysis. Flow cytometry and immunohistology were performed to correlate gene expression to the cellular immune response. Results: Distinct phases of the host response were observed and specific pathways could be identified in the transcriptome of the whole lung (eg, the increase in interferon genes and upregulation of a defined NK-specific gene set revealed the initiation of the innate immune response phase). Infiltration and activation of T and B cells could be seen by an augmentation of T- and B-cell specific expression signatures. Our results demonstrate that the permanent changes in expression profiles were still evident at 60 days after infection.

Furthermore, the network analysis based on the kinetics of gene expression changes revealed a model which also includes genes that are not well studied or have not yet been annotated. We also compared the expression profiles from wild type with Rag2$^{-/-}$ mice, demonstrating that the deficiency of T and B cells in Rag2$^{-/-}$ mice is well reflected within the transcriptome. Finally, we compared the gene expression from resistant C57BL/6J mice with the susceptible DBA/2J mouse strain. Conclusions: In conclusion, our comprehensive gene expression study describes the entire host response to an acute influenza A infection at the transcriptome level and is a suitable method to unravel the biological mechanisms for phenotypic alterations in genetically different mice.

P2-581

Unusual mortality of crows and ducks associated with an outbreak of H5N1 clade 2.3.2.1 in Bangladesh: a study in comparative pathology

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Background: Two large bird die-offs were investigated in disparate parts of Bangladesh. The first occurred from January to March 2011 and involved sick crows in Patuakhali and Dhaka city in the southern and central parts of the country; and the second occurred in June and July 2011 and involved ducks in the northern city of Netrokona. In each case, a significant percentage of backyard-raised poultry was found to be sick or dying 1 to 3 weeks prior and within a 3- to 5-kilometer radius of these events. In both die-offs, sick crows and ducks displayed unusual neurological signs, and test results indicated a pronounced concentration of viral antigen and extensive pathology within the central nervous system (CNS). Materials and Methods: In both events, die-offs were reported and investigated by field teams consisting of veterinarians and veterinary technicians from the Department of Livestock Services and the International Centre for Diarrheal Diseases Research in Bangladesh. Oropharyngeal and cloacal swabs were obtained from sick and dying birds, and postmortem examinations were performed. Tissue samples were obtained from the eye, lung, trachea, pharynx, gizzard, skeletal muscle, peripheral nerve, gall bladder, brain, liver, pancreas, intestine, kidney, bursa of Fabricius, and spleen of each bird and placed in 10% buffered formalin in preparation for histopathology and immunohistochemistry testing. The swabs were placed in viral transport media. Virus isolation and real-time RT-PCR analysis allowed investigators to identify influenza A and determine the specific subtype of this virus in each specimen. Results: In crows and ducks (*Anas platyrhynchos domesticus*), common clinical signs consisted of pronounced opisthotonus and torticollis, incoordination, and sudden death. The virus was successfully amplified in oropharyngeal/cloacal swabs from 100% of the crows—both *Corvus splendens* and *C macrorhynchos*—from Dhaka city and Patuakhali and 89% of the ducks from Netrakona. Histopathology tests revealed significant CNS lesions confined to the cerebellum, brain stem, and medulla oblongata and characterized by severe enlargement, degeneration, and necrosis of neurons, including Purkinje cells, as well as diffuse gliosis. Occasional perivascular cuffing was also noted, with mild hemorrhaging seen within the neuropil. The meninges were thickened due to infiltration by lymphoid cells. Necrotizing pancreatitis (acinar cells) was evident in both crows and ducks, as was diffuse splenitis (plasmacytosis) with hemosiderosis and multifocal necrosis and degeneration of hepatocytes associated with lymphocyte and macrophage infiltration. Kidney and heart tissue specimens both lacked evidence of inflammation, but multifocal acute necrosis was seen in the tubular epithelium of the kidney and multifocal degeneration was seen in the myofibers of the heart. Necrotizing enteritis was noted only in ducks. Immunohistochemistry studies demonstrated pronounced levels of viral antigen in the brain, acinar cells of the pancreas, hepatocytes, lamina propria of the intestine, myocytes of the heart, and epithelial cells of the bursa of Fabricius. Antigen was also seen in the trachea and in the tubular epithelium of the kidneys, although with less intense staining. No lesions were seen in specimens from the eyes, lungs, trachea, pharynx, gizzard, skeletal

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muscle, peripheral nerves or gall bladder. Sequence analysis indicated that the viral isolate was influenza A/H5N1; phylogenic analysis of HA gene sequences indicated the isolates were specifically from clade 2.3.2.1. Conclusions: These observations indicate the introduction of a new clade of H5N1 virus (subtype 2.3.2.1) into Bangladesh in 2011. This is of considerable concern, given the unusual neurological signs observed in these birds, which signal the introduction of a virulent neurotropic variant of influenza virus into Bangladesh. Continued vigilance in surveillance platforms is warranted, given that Bangladesh continues to be at risk for the introduction or evolution of new influenza viruses in both animals and humans.

P2-582

Influenza A H3N2 viruses from duck farms near Poyang Lake could infect mice without adaption

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Background: Poyang Lake is an important stopover and winter residence for migratory birds in China. The exchange of influenza viruses between wild birds and domestic poultry is monitored in that area. In March 2011, we conducted an active avian influenza virus survey near Poyang Lake in Jiangxi, China. In this study, we obtained 2 isolates of the H3N2 subtype and one isolate of the H3N8 subtype of the avian influenza virus (AIV) from a duck farm. A sequence analysis of the 3 viruses showed that they were all new genotypes from different origins. A duck-origin virus was supposedly the donor of the 1968 H3N2 pandemic strain. Therefore, a study of the biological characteristics of these H3 viruses would be meaningful. Materials and Methods: We infected mice with the 3 viruses to assess their pathogenesis and infectivity in this mammalian model. The mice were inoculated intranasally with a 10^6 50% egg infectious dose (EID_{50}) of the H3 virus. Clinical signs and weight change were recorded daily. Mouse tissues were collected on day 3 after the infection, and virus replication in the tissue was titrated in the eggs. Blood samples were collected 14 days after infection. Results: None of the mice demonstrated significant clinical symptoms. Both H3N2 isolates were replicated efficiently in the mouse lung tissue without adaption, and pathological changes were exhibited in the lungs. No viruses were detected in the spleen and brain of these animals. By comparison, the H3N8 virus could not be detected the lungs or any other organs of these animals after a high-dose infection. Conclusions: The 3 new genotypes of influenza H3 subtype viruses isolated from ducks varied in their ability to induce an infection in mice. The H3N2 duck-derived viruses were able to infect respiratory tissue in mice with adaptation. AIVs with different genotypes should be investigated systematically for their biological characteristics to determine their mammalian infection potential.

P2-583

Phenotyping of influenza A virus–infected mice with respect to different genetics of virus and host using hematologic and immunologic parameters

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Background: The outcome of an influenza A virus (IAV) infection depends on the viral subtype and the genetics of the host—here inbred mouse strains. We compared C57BL/6 (B6) mice infected with two different variants of PR8 and a relative-resistant B6 mouse with susceptible DBA/2 (D2) mice. Materials and Methods: By analyzing body weight loss, virus titers in the lung and oxygen saturation in the blood, we could identify a clear correlation between these parameters and severity of the

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disease. By using flow cytometry, we could measure the kinetics of single-cell populations in IAV-infected lungs. Results: While the amount of phagocytes increased strongly in the case of severe infections until the animals succumbed, an increase in T and B cells could be measured starting on day three postinfection in the lungs of surviving mice. To verify how the infection process in the lung is reflected in peripheral blood, hematologic parameters were analyzed and flow cytometric analyses of the blood were conducted. We found significant differences in the relative distribution of the main immune cell populations in peripheral blood as early as day two postinfection, revealing this as an early and sensitive marker. The susceptible mouse strain and the lethally infected B6 mouse exhibited strong decreases in lymphocyte and very intense augmentation of granulocytes after infection. In comparison, the hemograms of surviving mice changed to a much lesser extent. We demonstrated that the ratio of lymphocytes to granulocytes is a strong indicator of infection outcome. Conclusions: In summary, peripheral blood parameters correlate well with the clinical course and outcome of IAV infection in mice. Therefore, these parameters (eg, the lymphocyte/granulocyte ratio) can indicate an IAV infection in the lung very early and indicate that the severity of the infection, independent of these parameters, is due to the pathogenicity of the virus or to the susceptibility of the host.

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Avian influenza A viruses in wild birds and domestic ducks in Bangladesh

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Background: Wild waterfowl are the ancestral reservoirs for avian influenza A viruses and support the persistence of these viruses in nature. Domestic ducks, as well as resident and migratory wild birds, inhabit the freshwater wetlands in northern Bangladesh during the winter months (November to March). The coastal areas of the Bay of Bengal are also wintering grounds for a number of migratory waders and shorebirds. We conducted a study of wild birds and domestic ducks in these areas to assess the prevalence of avian influenza A viruses using satellite telemetry to quantify flight distances and trace migratory routes of influenza-virus–infected waterfowl in these areas. Materials and Methods: We sampled wild birds from the Hakaluki and Tanguar *haors* of northern Bangladesh and 2 coastal sandbars in southeastern Bangladesh. Mist nets, noose traps, and dazzling techniques were used to capture wild birds. Oropharyngeal and cloacal swabs from each bird were placed in viral transport media before being stored in liquid nitrogen. Cloacal swabs were also obtained from domestic ducks (*Anas platyrhynchos*) from Hakaluki *haor*. All swab samples were tested using real-time reverse transcription-polymerase chain analysis with influenza A specific primers and probes at the icddr,b laboratory prior to confirmation at the CDC. Argos (9-12 g) and GPS (22 g) satellite transmitters were used to provide flight data from March 2010 through April 2012. Results: A total of 1172 wild birds representing 95 species and 23 families were sampled from March 2010 to April 2012; of these, 285 were resident birds and 887 were migratory birds. Among them, 564 were from Hakaluki *haor*, 290 from Tanguar *haor,* and 318 from coastal sandbars. Nine birds (0.76%, CI 0.35%-1.45%) tested positive for avian influenza A virus, including 8 migratory waterfowl from Hakaluki *haor:* 4 northern pintails (*A acuta*), 1 ferruginous pochard (*Aythya nyroca*), and 3 garganey (*A querquedula*) ducks. Of the 4 influenza-A–positive northern pintails, one was positive for both H5 and H9 subtypes and another for H5 only. Among birds from the coastal area, one lesser sand plover (*Charadrius mongolus*), a migratory wader, also tested positive for influenza A virus. Among the 800 domestic ducks at the Hakaluki *haor* from which samples were obtained, 16 tested positive for influenza A (1.86%, CI 1.05%-3.07%). We affixed satellite transmitters on 43 migratory waterfowl (16 during 2010 and 27 during 2011). The marked birds consisted of 17 ruddy shelduck (*Tadorna ferruginea*), 11 northern pintail, 8 garganey, 6 northern shoveler (*A clypeata*), and one gadwall (*A strepera*) animals.

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The median distance covered by these birds was 2390 km (IQR 554-4370). The median days of signal transmission after satellite deployment was 110 days (IQR 26-235). Among the marked birds, 38 (88.37%) were negative and 5 (11.63%) were positive for influenza A virus; these included 1 northern shoveler, 2 northern pintails, and 2 garganey birds. Of the 2 marked northern pintails, 1 (distance covered: 7188 km) tested positive for H5 and another (distance covered: 2805 km) tested positive for both H5 and H9. Transmission signals were maintained in 8 waterfowl as far as Assam (India), 6 as far as Lhasa (China), 2 as far as south China, 7 as far as Qinghai Plateau (China), 1 as far as Myanmar, and 1 as far as Siberia. The median distance covered by influenza A–positive birds did not differ significantly with that covered by influenza A–negative birds (2738 km vs 2387 km, P = .95). Conclusion: Both migratory wild birds and domestic ducks in Bangladesh can harbor and shed influenza A viruses, including the highly pathogenic H5N1 subtype. The migratory waterfowl routes connect Bangladesh with other regions in south and central Asia. Any attempt to model the geographic distribution of avian influenza A viruses should consider the fact that infection with these viruses does not hamper long distance migration of wild waterfowl. Continuous surveillance of migratory wild birds to identify and characterize avian influenza viruses would allow us to determine whether they play any role in the dissemination of influenza A viruses among domestic ducks of Bangladesh and throughout their migratory flight ranges.

P2-586

The survey of avian influenza viruses in wild birds in eastern China, 2013

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Background: H7N9 is a serotype of the Influenza A virus (avian influenza virus, or bird flu virus). Avian influenza A H7 viruses normally circulate among avian populations with some variants that are known to occasionally infect humans. An H7N9 virus was first reported to have infected humans in 2013 in China. From March to May 2013, 4 Chinese provinces (Shanghai, Zhejiang, Jiangsu, and Shandong) ended their emergency response to H7N9 bird flu. Chongming Island, which is located in the Yangtze River estuary in Shanghai, is the third largest island in China. It has a north subtropical humid temperate climate, with 4 distinct seasons and many species of wild birds and waterfowls. Materials and Methods: We conducted a survey of avian influenza viruses in Chongming Island and Zhejiang in April 2013 by collecting 1555 feces samples from 3 species of wild birds (Calidris alpine, Charadrius alexandrines, and Anas platyrhynchos [mallard]) and using AIV-M PCR testing to identify the virus. The test was positive for 15 birds (1.8%). We inoculated 9-day-old SPF chicken embryos with the positive samples to culture and isolate the virus. Results: We identified the genome for 3 avian influenza viruses: one for the H9N2 virus, isolated from samples taken from A/Mallard/Zhejiang/136/2013, and 2 for H5N2, isolated from samples taken from A/Mallard/Zhejiang/9/2013 and A/Mallard/Zhejiang/16/2013. All of the isolates were genetically and phylogenetically characterized. Phylogenetic analysis of the H9N2 viral genomes showed 8 gene segments with a recent H9N2 relationship very close to those in the Yangtze River delta region. We also identified 6 internal genetic evolutionary trees within a group and 2013 H7N9 coexistence. Conclusions: These findings highlight the importance of surveillance in this region for potential recombinant avian influenza viruses.

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Viral kinetics and pathogenesis of intranasal and intratracheal infection with influenza A (H1N1)pdm09 virus in isogeneic guinea pigs (*Cavia porcellus*)

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Background: Animal models for influenza virus infections have their respective advantages and limitations. The most appropriate animal model for any given study should therefore be selected based on the research question. In studies of transmission, outbred ferrets and guinea pigs are the preferred species. Mice do not transmit influenza, but the availability of inbred syngeneic mice permits investigation of the role of different cellular subsets in immunity through, for example, adoptive transfer of lymphocytes. In studies of both cellular immunity and viral transmission, inbred isogeneic guinea pigs uniquely combine the virtues of the ferret and the mouse model. In order to pave the way for new lines of research into viral transmission and cellular immunity, a baseline study was performed to assess replication kinetics and pathogenesis of influenza A(H1N1)pdm09 virus infection in strain 2 isogeneic guinea pigs. Materials and Methods: Twenty-two female 12-16-week-old strain 2 guinea pigs were inoculated with 300 µl of 2 x 10^6 $TCID_{50}$ pandemic H1N1 (A/Netherlands/602/09) on day 0. Twelve animals were inoculated intranasally. Nasal, pharyngeal and rectal swabs were taken on days 1, 2, 3, 4 and 7. On days 4 and 7, six animals were euthanized. Ten animals were inoculated intratracheally; all were swabbed daily as described, and 2 animals were killed on days 1, 2, 3, 4, and 7. Full necropsy was performed, and the entire respiratory tract and other parenchymatous tissues were collected and either frozen at -80ºC or fixed in 4% buffered formalin. Formalin-fixed tissues were embedded in paraffin, cut to 4-µm sections, and stained with haematoxylin and eosin for histological evaluation or stained immunohistochemically (IHC) with primary antibody directed against influenza A nucleoprotein. Infectious virus titres in tissue samples and swabs were determined on Madin-Darby Canine Kidney (MDCK) cells according to standard protocols. Results: Intranasally inoculated animals shed virus from days 1 to 4, with a peak in viral titres in nasal secretions on day 2 postinoculation (pi). By day 7 pi, infectious virus was no longer detectable (< 0.75 $TCID_{50}$ per ml) in nasal secretions. Pharyngeal swabs were variably virus positive until day 4, and rectal swabs were uniformly negative. Nasal epithelium collected on day 4 pi showed viral titres ranging from 2.7 to 5.6 log $TCID_{50}$ per gram tissue. Virus could not be isolated from the remaining tissues, with the exception of one animal that had low titres in the cerebrum on day 4. Histological evaluation revealed mild to moderate multifocal acute to subacute purulent rhinitis, tracheitis, bronchitis and bronchiolitis as well as multifocal mild broncho-interstitial pneumonia with type II pneumocyte hyperplasia on day 7. IHC demonstrated moderate numbers of viral antigen–positive cells in the nasal epithelium on day 4 and fewer on day 7. Intratracheal infection resulted in more diffuse pneumonia and higher numbers of IHC-positive cells. Conclusions: Isogeneic guinea pigs show great promise as a model for cellular immunity and transmission of influenza viruses. However, further studies, including studies of adoptive transfer of lymphocytic subsets, is required to fully assess the suitability of this model.

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Pathogenicity of an H5N1 highly pathogenic avian influenza virus isolated in the 2010-2011 winter in Japan to wild ducks and herons

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Background: Many highly pathogenic avian influenza (HPAI) outbreaks occurred in Japan during the 2010-2011 winter. H5N1 HPAI viruses were isolated from 63 wild birds, including migrating and resident birds, and caused HPAI outbreaks in 24 chicken farms by the end of March. It has been suggested that migratory waterfowl flying from their nesting lakes in Siberia play a role in dissemination of H5N1 HPAI viruses to domestic poultry and resident birds, and that dead wild birds act as a source of infection to birds of prey such as Falconiformes. Elucidating the susceptibility of each wild bird to HPAI viruses should be needed to prevent future HPAI outbreaks. In the present study, we examined the pathogenicity of causal H5N1 HPAI virus to several wild duck and heron species, which are commonly observed in Japan. Materials and Methods: Anatidae (mallards, wigeons, pintails, tufted ducks, and mandarin ducks) and Ardeidae (grey herons, intermediate egrets, little egrets, and black-crowned night herons) were intranasally inoculated with 200 µl of allantoic fluid containing $10^{6.0}$ 50% egg infectious dose (EID50) of A/mandarin duck/Miyazaki/22M-807/2011 (H5N1), which belonged to clade 2.3.2.1 and are mainly circulating in East Asia. The birds were observed for clinical signs for 10 days, and checked for viral shedding in laryngopharyngeal and cloacal swabs at 0, 1, 2, 3, 5, 7, and 10 days post inoculation. Results: A tufted duck (1/2: indicates 1 of 2 birds), a mandarin duck (1/3), grey herons (2/4), little egrets (2/2), and a black-crowned night heron (1/3) showed clinical signs such as depression and neurologic symptoms. All the birds presenting disease signs terminated in death except 1 tufted duck and 1 grey heron. Viruses were mainly recovered from the laryngopharyngeal swabs of mallards, tufted ducks, mandarin ducks, and Ardeidae until 5-7 days post inoculation. On the other hand, viruses were rarely recovered from the swabs of wigeons and pintails. Further study revealed that the H5N1 HPAI viruses were transmitted between Anatidae or grey heron populations. Conclusions: The results showed that pathogenicity of the H5N1 HPAI viruses differed by wild bird species while all the experimentally-inoculated ducks and herons were susceptible to the recent H5N1 HPAI virus. The viruses were mainly recovered from laryngopharyngeal swabs of some birds with subclinical infection, indicating that they have the potential to disseminate the virus to other birds. Therefore, attempts should be made up to keep wild birds out of poultry farms. Furthermore, the number and species of wild birds around farms need to be continuously monitored to reduce the risk of viral transmission to poultry populations.

P2-589 GATES Fellowship

Characterization and pathogenicity of highly pathogenic H5N1 avian influenza virus strains isolated from migratory wild birds in Mongolia (2005-2010)

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Background: Since its emergence in 1997 and re-emergence between 2002 and 2003, highly pathogenic avian influenza (HPAI) infections caused by the H5N1 virus have spread to most of the countries in east and south Asia. A new outbreak of morbidity and infection due to AI in wild birds, domestic birds, and humans has been observed worldwide. Mongolia is the second country to report wild birds infected with the H5N1 virus after the Qhinghai Lake outbreak in China in 2005. H5N1 HPAI viruses were isolated from whooper swans and other migratory wild birds during 8 reported outbreaks

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in Mongolia since that time. A combination of geographical features makes Mongolia an ideal location for studying the epidemiology of avian influenza viruses in wild birds. For example, 4 main migration routes pass through Mongolia (East-Asia/Australia, Central-Asia/India, West-Asia/Africa and Mediterranean/Black-Sea), and approximately 391 species of migratory birds arrive in Mongolia. Mongolia also represents an important molting site for the *Anseriformes* species. Materials and Methods: We isolated the virus from a homogenate of tissue from the brain, lungs, and spleen of 20 carcasses of bar-headed goose (*Anser indicus*), whooper swan (*Cygnus cygnus*), common goldeneye (*Bucephala clangula*), and ruddy shelduck (*Tadorna ferruginea*) birds, which were found in the Khunt (Saikhan soum, Bulgan Province), Erkhel (Alag-Erdene soum, Khuvsgul Province), Doityn Tsagaan (Ugiinuur soum, Arkhangai Province), Doroo (located at the boundary of Rashaant soum in Khuvsgul Province and Tsetserleg soum in Arkhangai), and Ganga Lakes (Dariganga soum, Sukhbaatar province) in Mongolia in July 2005, May 2006, May 2009, July 2009, and May 2010. The virus was isolated using the egg inoculation method, and the RT-PCR, qRT-PCR, and neuraminidase inhibition tests were used to identify subtypes. Confirmation of the isolated virus as the highly pathogenic avian influenza virus (HPAIV), based on OIC criteria, as well as determination of the intravenous pathogenicity index (IVPI) and preparation of the phylogenetic analysis, were carried out in the OIE Reference Laboratory for HPAI at Hokkaido University, Japan. Results: In July 2005, H5N1 viruses were isolated from tissue homogenates and swab samples taken from a bar-headed goose and a whooper swan in the Khunt and Erkhel Lakes. Similarly, H5N1 viruses were isolated from a whooper swan and a common goldeneye in May 2006 in the Khunt and Erkhel Lakes. These viruses were classified as clade 2.2 Qinghai Lake-type. In May 2009, H5N1 viruses were isolated from 3 whooper swans in Doityn Tsagaan Lake. In late July 2009, H5N1 viruses were isolated from dead wild birds, 3 bar-headed geese, 2 ruddy shel ducks, and 2 common goldeneyes in Doroo Lake. In May 2010, H5N1 viruses were isolated from 4 whooper swans in Ganga Lake. These viruses were classified as being part of the clade 2.3.2. The IVPI was high /2.97-3.00/. Conclusions: All H5N virus infections identified in 2005, 2006, 2009, and 2010 occurred in May and July, when wild waterfowl migrate from southern Asia to their nesting lakes in Siberia, and all HPAI outbreaks were restricted to wild birds in north central and eastern Mongolia. Additionally, H5N1 viruses that are genetically related to isolates found in Mongolia were prevalent among domestic poultry in southern Asia, although no HPAI outbreak has been reported in poultry in Mongolia so far. The H5N1 HPAI viruses were isolated only from migratory waterfowl returning to their northern territory and not from those flying south from Siberia in autumn. This suggests that H5N1 HPAI viruses had not perpetrated their nesting lakes in Siberia until then. Finally, phylogenetic differences among H5N1 isolates identified in 2005, 2006, and 2009, 2010 indicate that the role of the migratory birds in Mongolia in the AIV mutation should be clarified. Therefore, studies of avian influenza in wild birds should be continued.

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Poultry vaccination and H5N1 occurrence in Indonesia

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Background: HPAI due to H5N1 virus was first recognized in Indonesia in 2003 and soon became enzootic in poultry throughout much of the country. Vaccination was introduced as one measure of controlling HPAI. Homologous and heterologous vaccines from both local and international production laboratories were used. Vaccine was provided free of charge by the government to sector 4 poultry keepers. Commercial companies were permitted to use the registered vaccine of their choice, but overall coverage was low. Structured HPAI surveillance began in 2006 with the introduction of the Participatory Disease Surveillance and Response (PDSR) system, which mainly detected outbreaks in poultry sectors 3 and 4. Beginning in 2009, HPAI environmental surveillance was introduced in live bird markets. Materials and Methods: Since 2007, H5N1 field strains have been monitored under an OIE/FAO network of experts on animal influenza (an OFFLU programme) to screen for antigenic

variants, to obtain a representative sample of circulating viruses, and to identify potential strains for use in vaccines or challenge testing. Two challenge virus strains and 4 candidate vaccine strains, all in sub-clade 2.1.3, were identified and provided to vaccine manufacturers to develop and register vaccines against H5N1 in Indonesia. Results: As a result of these collaborative efforts, efficacious vaccines with antigenically relevant strains were produced locally, with sales initiated in 2009. Simultaneously, vaccine policy and advocacy was refocused on commercial production, with sector 4 vaccination de-emphasized. From 2009 to 2012, sales of poultry vaccine based on antigenically similar viral strains derived from local field isolates increased steadily in Indonesia, progressively replacing vaccines based on poorly matched foreign virus strains as the most common vaccine sold in Indonesia. The vaccination rate using local vaccines in the commercial sector increased, but there was no evidence of significant improvement in biosecurity practices in the commercial sector. Over this same time period, the number of outbreaks of HPAI detected by the PDSR system decreased. A significantly negative correlation was also observed between the prevalence of H5-subtype positive animals in live bird markets and the dose of locally produced antigenically relevant vaccine sold. The number of cases of H5N1 infection in humans also decreased over this time period, indicating a negative correlation between human infection and the proportion of locally produced antigenically relevant vaccine sales. Conclusion: Use of locally produced, antigenically relevant vaccines significantly improved control of H5N1 HPAI in poultry in Indonesia, thereby reducing urban environmental contamination and the number of cases of infection in humans. To reduce the risk to both the public and animal health, vaccination of commercial poultry using antigenically relevant virus strains is strongly recommended in areas where H5N1 HPAI is endemic. Adoption of this practice by farmers may be boosted further if antigenically relevant vaccines can be produced locally by existing poultry vaccine manufacturers, rather than being imported. Surveillance systems designed to monitor strain antigenic characteristics in the field and monitor poultry outbreaks and environmental contamination in live bird markets are strongly recommended in endemic areas. The efficacy of candidate vaccine master seeds should be verified through challenges by antigenically representative field strains.

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H3N2 canine influenza virus with the matrix gene of the pandemic A/H1N1 virus: infection dynamics in dogs and ferrets

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Background: Canine influenza virus (CIV) is a newly emerging agent that causes acute respiratory diseases in dogs. Interspecies transmission of H3N2 CIV from dogs to domestic cats with high mortality also occurred at the beginning of 2010. After a recent event caused by the pandemic influenza A/H1N1 (pH1N1) virus, the emergence of a recombinant H3N2 canine influenza virus (CIV) representing the pH1N1 virus and the classical H3N2 CIV was reported. Our continuing surveillance resulted in the isolation of another reassortant H3N2 CIV containing the matrix gene of the pH1N1 virus in 2012. Materials and Methods: As part of the canine influenza surveillance program of the Korean National Veterinary Research and Quarantine Service (NVRQS), 8 nasal swab samples were collected from sick dogs showing influenza-like symptoms in animal hospitals. Three of these samples were determined to be positive for the influenza A virus based on the findings of a commercial rapid detection influenza virus A antigen kit, and only 1 of the 3 was found to be positive based on the findings of virus isolation (A/Canine/Korea/MV1/2012 [CE/Kor/MV1/12; H3N2]). All 8 segments of the virus were identified using reverse transcriptase polymerase chain reaction (RT-PCR) assays followed by sequence analysis. The dynamics of infection by the CE/Kor/MV1/12 virus were investigated in dogs and ferrets through experimental infection and transmission. Results: Dogs and ferrets infected by the CE/Kor/MV1/12 virus demonstrated symptoms typical of respiratory disease, efficient virus shedding, and seroconversion, as previously shown in classical H3N2 CIV infections. Direct contact transmission of the CE/Kor/MV1/12 virus was detected in both animal models, although this type of transmission occurred more efficiently in ferrets. Interestingly, transmission by the

CE/Kor/MV1/12 virus appeared to be revitalized compared with transmission by the classical H3N2 CIV. Transmission via respiratory droplets, which was performed only in the ferret model, failed. Conclusion: We isolated and characterized a strain of the H3N2 CIV containing the M gene segment of the pH1N1 virus. Our CE/Kor/MV1/12 virus demonstrated efficient replication and triggered contact transmission in dogs and ferrets. Most importantly, it showed augmented transmissibility in the ferret model when compared with the classical H3N2 CIV, which was hypothesized to be conferred through the presence of the M gene segment of the pH1N1 virus. To prove this hypothesis, additional studies using reverse genetics will be needed.

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The mechanisms of route dependent immunodominance to influenza A virus: a strategy for optimizing CD8+ T cell vaccine efficacy

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Background: Creating a successful vaccine requires understanding the optimal conditions for generating the best immune response for a particular physiological site. The immune response to influenza A virus (IAV) involves multiple arms of the immune system, including responses to numerous CD8+ T cell epitopes. CD8+ T-cell responses offer cross-reactive protection against multiple strains of IAV due to conserved epitopes. Because of this, CD8+ T cells are excellent vaccine candidates against IAV. CD8+ T-cell responses can be combined collectively into an immunodominance hierarchy based on magnitude. Interestingly, the immunodominance hierarchy to IAV differs depending on the administration site of infection. Materials, Methods, and Results: Using a murine IAV model, we show that intranasal (IN) infection results in enhanced responses in the lung to several subdominant CD8+ T-cell epitopes, especially PB1/703, when compared with intraperitoneal (IP) immunization. Alveolar macrophages have been proposed to be a depot of antigen in the lung following local infection. Through ex vivo antigen presentation studies, we have shown that alveolar macrophages have the ability to present multiple CD8+ T-cell epitopes to monospecificity CD8+ T-cell cultures within hours of infection. Using BFA antigen presentation kinetics assays of in vitro cultured macrophages from various anatomical sites, we have also shown that alveolar macrophages have enhanced antigen presentation kinetics of subdominant epitopes when compared with bone marrow derived dendritic cells and macrophages from alternate sites. Conclusions: To that end, we propose a role for macrophages in antigen presentation leading to enhancement of immune responses observed during IN infections. Targeting antigen not only to DCs but also towards macrophages may provide an excellent vaccine strategy to direct a more balanced T-cell response to IAV.

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Infectivity and transmissibility of avian H9 influenza A virus in pigs

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Background: Interspecies transmission of avian H9N2 influenza viruses to mammalian hosts has been reported sporadically over the last 2 decades. These viruses are widely prevalent in land-based poultry in Asia; therefore, understanding the infectivity and transmissibility of the virus in pigs, a potential "intermediate host" for transmission of influenza viruses to humans, is of importance to evaluate the pandemic potential of this virus. Materials and Methods: We collected sera from 4500 pigs from 2002 to 2012 in 3 cities in southern China and tested them against a panel of antisera from antigenically different H9 viruses from the major H9 lineages. Six H9 strains from 3 major lineages

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(G1, Y280, and Y439) that are established in domestic poultry in China were selected to study the infectivity of the virus in pigs. Two strains that infected pigs were used for a transmission study in pigs. Results: We found that 10.42% of the pig sera we collected was seropositive for H9 viruses. These samples were tested against viruses of the H9N2 Y280 or G1 lineage that have been established in land-based poultry for more than a decade. No sera were positive for viruses of aquatic bird Y439 lineage. A similar pattern was observed for infectivity in pigs. Viruses from the G1 and Y280 lineage were able to infect pigs, in which nasal shedding of the virus and seroconversion were detected. Neither aquatic bird H9 virus from the Y439 lineage was able to induce a productive infection in pigs. No transmission of the H9N2 viruses to physical or airborne contact animals was detected. Conclusion: This retrospective serologic study revealed that interspecies transmission of avian H9 viruses to pigs has occurred frequently in the field. The H9N2 G1 and Y280 lineage viruses established in land-based birds were able to infect pigs, whereas the viruses from aquatic birds could not. This suggests that adaptation in land-based poultry led to the ability of the viruses to infect pigs. Although H9N2 viruses do not appear to be able to be transmitted to pigs, repeated introduction of H9 viruses to pigs will increase the risk that adapted or reassorted variants will be generated and, thus, pose a threat to humans. In view of the wide prevalence of H9 viruses in poultry, surveillance of H9 viruses in pigs should be strengthened.

P2-594 G FELLOWSHIP GATES Fellowship

Evolution and diversity of HPAI H5N1 clade 2.3.4 in Vietnam from 2005 to 2010

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Background: The highly pathogenic avian influenza A H5N1 virus has caused poultry outbreaks in Vietnam since 2003. Although the number of reported outbreaks has decreased substantially, H5N1 continues to circulate despite national control and prevention efforts, such as poultry vaccination campaigns. A total of 123 laboratory-confirmed cases of H5N1 infection in humans, including 61 deaths (case fatality rate: 50%), have been reported in Vietnam. From 2003 to 2010, clades 1 and 2.3.4 were predominant, although their distribution has changed over time. Clade 2.3.4 was first identified in poultry in early 2005, and the first human case was reported in August 2005. Clade 2.3.4 expanded across northern Vietnam and replaced clade 1 in 2006, but essentially disappeared in 2011 following the introduction of a variant clade 2.3.2.1. We conducted molecular characterization and whole genome sequencing studies of human and poultry clade 2.3.4 isolates to better understand the evolution and diversity of this clade in northern Vietnam. Materials and Methods: All poultry and human H5N1 isolates of clade 2.3.4 obtained from animal and human virological surveillance programs in Vietnam from 2005 to 2010 were classified according to time (year) and place (province). Isolates (18 human and 30 poultry) were amplified in eggs for sequencing. All isolates underwent full genome sequencing using the Sanger method and antigenic analysis by using the hemagglutination-inhibition assay based on a panel of 3 reference ferret antisera provided by the US Centers for Disease Control and Prevention and chicken antisera provided by the Vietnam National Center for Veterinary Diagnosis. For all data sets, phylogenetic alignments were determined using Geneious 6.0.6, and neighbor-joining trees were inferred using a general time reversible model. Reassortment analysis was carried out for concatenated whole-genome isolates. Results: H5N1 viruses in Vietnam have diverged genetically and antigenically since 2003. Viruses derived from a clade 2.3.4 ancestor circulated in northern Vietnam from 2005 to 2010 and belonged to 3 sub-clades--2.3.4.1, 2.4.3.2, and 2.4.3.3--as determined by genetic analysis of the hemagglutinin gene. Sub-clades were identified as having incongruent tree topologies in 8 different segments in some poultry and human isolates, suggesting reassortment. Reassortment of clade 2.3.4 was determined in sub-clades 2.3.4.2 and 2.3.4.3 only, occurring more frequently in the NA and MP genes (7/28 viruses in each gene) and less frequently than in NP genes (4/28 viruses) and the PB2 gene (1/28 viruses). Reassortments between clades 2.3.4 and a newly identified variant clade 2.3.2.1 were observed in the NA and MP genes of 2

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isolates belonging to sub-clades 2.3.4.2 and 2.3.4.3. Reassortment of the NS, PB1, and PA genes was not identified in this study. Antigenic cartography of HI data for clade 2.3.4 viruses and its sub-clades indicated antigenic similarity among the 3 sub-clades. An adequate reaction between the H5N1clade 2.3.4 isolates and the corresponding H5N1 poultry vaccination antisera was shown with the hHI titer ranging from 40 to 160. Conclusions: Over a 5-year period, H5N1 clade 2.3.4 viruses exhibited marked genetic diversity in northern Vietnam, with 3 sub-clades in both human and animal isolates. Reassortments in the NA, MP, NP, and PB2 genes were identified and related to viruses clustering in sub-clades 2.3.4.2 and 2.3.4.3. Although there is no evidence of antigenic changes following genetic diversity, reassortment between clade 2.3.4 and clade 2.3.2.1 was identified in this study. These findings suggest continued diversification and evolution of HPAI H5N1viruses in Vietnam and indicate that continued surveillance for pandemic preparedness is warranted.

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Neuraminidase-dependent multi-organ replication of H7 influenza viruses

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Background: Influenza virus replication is initiated by the cleavage of the major surface glycoprotein hemagglutinin (HA). Tissue-specific trypsin-like proteases are essential for the replication of low pathogenicity. By contrast, the HA of high-pathogenicity viruses harbors a multibasic motif that allows proteolytic cleavage by various proteases that facilitate systemic spread. Alternative mechanisms of HA protein cleavage and activation have been demonstrated by different viruses. One classic example is the facilitation of efficient viral replication of the A/WSN/33 (H1N1) virus through activation of plasminogen in a neuraminidase (NA)-dependent manner. We report a novel NA-dependent mechanism for replicating an avian H7N6 influenza virus (A/Mallard duck/Korea/6L/07 [A/Md/6L/07]) in vitro and in vivo. Materials and Methods: During routine avian influenza activity surveillance in wild migratory birds, we isolated A/Md/6L/07 from dead mallard ducks in December 2007. This virus can grow without trypsin in culture media. Viral replication kinetics and pathogenesis were measured in vitro and in vivo in mice, chicken, ducks, and ferrets. To determine the details of the trypsin-independent growth mechanism for A/Md/6L/07, we generated various combinations of reassortant HA (H1, H3, H5, H7, H9, H10) and NA (N1-N9) viruses in the backbone of A/Md/6L/07 using reverse genetics. Rescued viruses were evaluated for their growth kinetics with and without trypsin or various protease inhibitors. Results: Genetic characterization revealed that A/Md/6L/07 is a low-pathogenicity virus possessing a monobasic cleavage motif in its HA gene. Despite its low pathogenicity, A/Md/6L/07 grew efficiently in vitro in the absence of exogenous trypsin, thereby producing an influenza virus-specific cytopathic effect (CPE). Animal studies demonstrated that the A/Md/6L/07 virus can replicate in multiple organs (lung, spleen, kidney, and brains) apart from the respiratory tract. Among the recombinant viruses, only the N6-NA was able to cleave glycine (G) specifically from the P2 position of the H7 HA0 glycoprotein. The results of the molecular biological study revealed that this novel mechanism is partially associated with thrombin, a serine protease, with A/Md/6L/07 replication significantly attenuated in media containing the direct thrombin inhibitor agatroban. Conclusion: We report for the first time a means by which influenza H7 viruses promote viral replication in a thrombin-dependent manner. This mechanism appears to be associated with a specific N6 NA gene constellation. We propose that thrombin-dependent cleavage of the HA protein is an emerging novel mechanism by which H7 virus replication is facilitated in various hosts.

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Infection and transmission of emerging swine H3N2 reassortant influenza viruses in ferrets and pigs

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Background: Following the initial outbreak in humans, the pandemic 2009 H1N1 influenza virus (pdm/09) has been repeatedly introduced back to pigs and reassorted with the enzootic swine influenza viruses to generate an array of novel variants. In North America, a swine-origin H3N2 virus containing the pandemic matrix gene has caused more than 300 human infection cases since August 2011. In southern China, H3N2 reassortants with pdm/09-like internal genes (rH3N2pIG) were first detected in 2010 and were subsequently found to be established in the swine populations across several provinces. This raised concerns that the prevailing rH3N2pIG viruses might also infect humans. Here we used ferret and pig models to investigate the infectivity and transmissibility of this reassortant virus. Materials and Methods: Influenza-free pigs (4-6 weeks old, n = 6) were intranasally inoculated with 10^6 times the median tissue culture infectious dose (TCID50) of the rH3N2pIG virus A/Swine/Hong Kong/NS2811/2011 (Sw-NS2811) and co-housed with four physical contact– and three airborne-exposed pigs and three airborne-exposed ferrets at 1 day postinoculation (dpi). Ferrets (6-8 months old, n = 2) intranasally inoculated with 10^6 TCID50 of Sw-NS2811 were co-housed with two physical contact– and two airborne-exposed ferrets at 1 dpi. Another two naive ferrets were introduced as secondary airborne exposed ferrets at 4 dpi. Clinical signs, body weights and body temperatures were recorded daily, and nasal swabs (for pigs) or nasal washes (for ferrets) were collected for virus titration. Seroconversion was determined with a hemagglutination inhibition (HI) test. The prototype pdm/09 virus A/California/07/2009 (CA07) and a contemporary human seasonal H3N2 virus A/Shantou/546/2010 (ST546) were used as control viruses. Results: The novel swine H3N2 reassortant Sw-NS2811 could replicate and transmit efficiently via both direct contact and airborne exposure in swine, with a disease profile and virus shedding kinetics similar to those of the CA07 virus. Sw-NS2811 could also be efficiently transmitted from pigs to airborne-exposed ferrets. All tested animals developed mild pneumonia and seroconverted. Ferret-to-ferret transmission of the Sw-NS2811 virus was efficient via direct contact but was limited via airborne exposure. Only one of the four ferrets airborne-exposed to Sw-NS2811 seroconverted, but without detectable virus being shed from the nasal route. All ferrets airborne-exposed to CA07 shed virus and seroconverted by 35 dpi, while those airborne-exposed to ST546 shed virus at marginal levels and seroconverted by 14 dpi. Conclusions: The novel swine H3N2 reassortant virus established recently in southern China could transmit and cause mild disease within experimentally exposed pigs. It could also cross the species barrier to infect ferrets via airborne exposure. Though efficient by direct contact transmission in ferrets, it only has limited airborne transmissibility from ferret to ferret. The potential for this virus to infect humans cannot be excluded, but its airborne transmissibility is expected to be lower than that of the contemporary human seasonal H3N2 and pdm/09 viruses.

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Changes in the prevalence of H5N1 influenza viruses in Hong Kong

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Background: The highly pathogenic Asian H5N1 avian influenza virus first emerged in 1997 and by 2006 had spread in 3 major transmission waves to more than 60 countries across Eurasia and Africa. During each wave transmission, a different H5N1 clade or genotype was disseminated, which widened the geographic range affected by the viruses and led to some viruses becoming enzootic in affected regions. More recently, clade 2.3.2.1 H5N1 viruses have been transmitted to many Eurasian countries and are forming different subgroups. Introduction of live poultry market management measures in Hong Kong has meant that H5N1 viruses are mainly isolated from migratory wild birds. The presence of clade 2.3.2.1 viruses in wild birds in Hong Kong could be part of the widespread dissemination of these viruses. Materials and Methods: Avian influenza H5N1 viruses were isolated from dead wild birds or poultry carcasses found in Hong Kong and identified using real-time PCR and hemagglutinin inhibition tests at the laboratory of the Agriculture, Fisheries and Conservation Department of the Hong Kong SAR Government. Three human viruses were isolated from specimens taken from RT-PCR–confirmed H5N1 human infections in Hong Kong and Shenzhen, Guangdong, China. Genetic and antigenic analyses of these viruses were performed as described previously. A phylogenetic tree of each gene segment was generated using a maximum likelihood model, and the robustness of the tree topologies was evaluated with the approximate likelihood log ratio test, implemented in PhyML (version 3.0). Results: We characterized 71 H5N1 viruses isolated from poultry, wild birds, and humans in Hong Kong and Shenzhen from June 2008 to June 2013. One third of the viruses belonged to clade 2.3.4; the others belonged to clade 2.3.2.1 viruses that had diverged into 2 phylogenetic subgroups: Barn-swallow-like and 6841-like. The last virus belonging to clade 2.3.4 was isolated in January 2011; clade 2.3.2.1 viruses were last isolated in January 2013. H5N1 viruses showed a distinct preference for the winter season in Hong Kong. Viruses from clades 2.3.4 and 2.3.2.1 were antigenically distinct. However, the internal genes of the clade 2.3.2.1 viruses were mainly derived from clade 2.3.4 H5N1 viruses that were present in southern China from 2006 to 2008. All clade 2.3.2.1 viruses had a T156A substitution (most also had an N154D substitution) in the HA, which removed the glycosylation site at position 154. Loss of this site was implicated in the recent attainment of airborne transmission by H5N1 viruses in ferrets. S223R in HA, which may confer an increasing binding affinity for α-2, 6-NeuAcGal receptors, was found in 13 of the 2.3.2.1 viruses. Conclusions: Clade 2.3.2.1 viruses appear to have replaced clade 2.3.4 viruses in Hong Kong as part of the apparently new transmission wave of clade 2.3.2.1 viruses throughout Eurasia. Wild bird 2.3.2.1 viruses from Hong Kong belonged to 2 of the 4 subgroups in which this clade appears to be evolving. Possible antigenic and dissemination differences among these subgroups need to be evaluated.

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Immune responses against H5N1 highly pathogenic avian influenza virus induced by intradermal immunization of inactivated viral antigen with CpG-ODN adjuvant in ducks

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Background: Because waterfowls are considered a natural reservoir for avian influenza viruses (AIV), AIV could circulate silently among *Anseriformes* birds, such as ducks, geese, and swans. Raising ducks in Asia has been found to play an important epidemiological role in the transmission of HPAI H5N1 to domestic poultry. Therefore, the effective control of H5N1 virus in ducks has important implications for the eradication of H5N1 influenza virus infection in poultry. To prevent AIV infection in ducks, various types of AIV vaccines are currently under development. The only vaccines currently available to control AIV in ducks are inactivated vaccines that demonstrate variable efficacy. It has been widely proposed that delivery of the vaccine antigen to the dermis and epidermis rather than to muscle or subcutaneous tissue could induce superior protective immune responses and have an antigen dose-sparing effect. We compared hemagglutination inhibition (HI) titers induced by vaccination through the intradermal (ID) web skin route and the intramuscular (IM) route using various dose of vaccine concentrations in commercial ducks. We also used a well-established toll-like receptor agonist, CpG-motif oligodeoxynucleotides (CpG-ODN), as an ID vaccine adjuvant to evaluate the adjuvant effect in ducks. Materials and Methods: A reverse genetics-derived H5N1 virus containing the modified H5 and N1 of A/chicken/Korea/ES/03 and the internal genes of influenza A/PR/8/34 was kindly provided by Dr BL Seong (Yonsei University, Korea). Virus was propagated in embryonating chicken eggs and formalin-inactivated for vaccine antigen preparation. A total of 56 one-week-old influenza virus seronegative domestic ducks were divided into 10 groups. To measure antibody responses induced by specific doses of the vaccine, with or without CpG-ODN, we vaccinated 6 groups of ducks with escalating doses of antigen ($10^{4.3}$, $10^{5.3}$, and $10^{6.3}$ EID_{50}/bird) through the web skin route with or without CpG-ODN. To compare the effectiveness of the ID route with the IM route, we vaccinated another group of ducks with oil-emulsified vaccine through the IM route. After 4 weeks, boost immunization was done using same vaccine preparations. To determine the immunogenicity of vaccine, we collected serum samples 1, 2, 3, 5, 6, and 7 weeks after prime immunization for HI test. Results: After prime immunization, ducks receiving the highest antigen dose ($10^{6.3}$ EID_{50}/bird) did not exhibit an immune response, whether they were immunized through the ID route or IM route. After boost vaccination, ducks immunized through the ID web skin route showed higher HI titers than those immunized through the IM route. Ducks who also received CpG-ODN exhibited its antigen dose-sparing effect compared with non–CpG-ODN vaccinated groups. Conclusion: ID immunization is an emerging technique in vaccine delivery. In previous studies, researchers investigated ID vaccine delivery, driven by the fact that the dermis and epidermis are rich in antigen-presenting cells, which, in turn, suggests that the delivery of vaccines to these tissue layers, could induce an immune response with smaller amounts of vaccine antigen compared with the amount required when the vaccine is delivered through muscle or subcutaneous tissue. In our investigation of ID delivery of vaccine to commercial ducks, we found that prime-boost immunization by ID route resulted in a superior immune response compared with immunization through the IM route. We also found that a significantly higher mean antibody titer could be achieved using a reduced dose of antigen when CpG-ODN as administered than a high dose of vaccine without CpG-ODN. Thus, ID vaccine delivery and use of CpG-ODN adjuvant may be an attractive vaccine strategy to better control AIVs in commercial ducks.

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The use of equine influenza pseudotyped lentiviruses for serological screening

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Background: The H3N8 subtype of equine influenza virus continues to produce outbreaks worldwide, with resultant economic and agricultural impact. The surface hemagglutinin (HA) glycoprotein elicits neutralizing antibody responses following a natural infection or vaccination. Unfortunately, the standard assays used for influenza serology present certain practical issues, such as interlaboratory variability, complex protocols, and the necessity to handle certain virus strains in high biological containment facilities. To address this, avian and human influenza HA pseudotyped retroviruses have been used successfully in antibody neutralization assays. In this study, we have, for the first time, generated equine influenza pseudotyped lentiviruses to detect HA-directed neutralizing antibodies in equine sample sera. Materials and Methods: Stocks of H3N8 A/equine/Sussex/89 were grown in 10-day-old embryonated eggs, with viral RNA extracted and HA amplified using RT-PCR and custom primers. The product was cloned into the pI.18 expression vector. Pseudotyped viruses were then produced through co-transfection of HEK293T cells with plasmids expressing the HA, HIV gag-pol, and firefly luciferase reporter genes and harvesting of virus from the supernatant. To produce infective pseudotype particles, it was necessary to also cotransfect a plasmid encoding the TMPRSS2 endoprotease to cleave the HA. High-titer pseudotype virus (PV) was then used in PV antibody neutralization assays (PVNAs); a luminometer was used to quantify PV infectivity (luciferase expression). Sera from vaccinated (n = 21) and influenza-naïve (n = 2) equines were tested. These samples were also screened using the single radial hemolysis (SRH) assay. Correlation and significance were determined by Pearson analysis using GraphPad Prism software. Results: Pseudotype viruses were generated successfully, with a titer of 1 x 10^9 relative luminescence units per mL. The TMPRSS2 protease was essential for this process, with no significant PV generation in the absence of the protease expression plasmid. The PVs thus produced were used in PVNAs to distinguish all test sera from vaccinated versus nonvaccinated equines. All vaccinated equines exhibited PVNA antibody titers of IC_{50} >1100 (positive control IC_{50}: >40,000; negative sera: <80). Antibody was detected by SRH in 18/20 of the vaccinated equines (range: 61-207 mm^2). There was a 65% correlation between results from the 2 assays (r = 0.65, *P* = .002). Conclusion: H3N8 equine influenza pseudotyped lentiviruses have been generated for the first time. These were successfully used to detect anti-HA neutralizing antibodies in serum samples. We were also able to use the pseudotype virus neutralization assay to distinguish sera from vaccinated versus influenza-naïve (by SRH) equines. The correlation between PVNA and SRH assays was good (65%), with the pseudotype assay exhibiting slightly more sensitivity. Future work will require more extensive testing of the PVNA using a larger number of serum samples to assess sensitivity/specificity and inter/intralaboratory variability and to define a protective antibody titer.

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Mutation from arginine to lysine at the position 189 of hemagglutinin contributes to the antigenic drift in H3N2 swine influenza viruses

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Background: In the North American swine population, the current predominant H3N2 influenza A virus (IAV) was associated with a “spillover” of human seasonal H3N2 IAVs to pigs in 1990s. Two distinct antigenic clusters were previously identified among the H3N2 swine IAVs and were designated H3N2SIV-alpha and H3N2SIV-beta (Feng, et al. *J Virol.* 2013;87(13):7655-7667). A consistent mutation was observed at the position 189 of hemagglutinin (R189K) between H3N2SIV-alpha and H3N2SIV-beta fair isolates. Our previous study also showed that the viruses in the antigenic cluster H3N2SIV-beta cross-reacted with ferret antisera produced against several seasonal human influenza viruses with HA carrying R189K mutation. However, little is known about the contribution of R189K mutation to the antigenic drift from H3N2SIV-alpha to H3N2SIV-beta, and also to the cross-reaction. Materials and Methods: Four reassortant viruses with HA carrying189R or 189K were generated by reverse genetics and designated as 09SW64_189R (wt), 09SW64_189K(mt), 11SW347_189K(wt), and anti11SW347_189R(mt). The antigenicity of these viruses was characterized by HI test and antigenic cartography. Results: The HI and antigenic cartography demonstrated that the R189K mutation in the hemagglutinin of H3N2 SIV contributed to the antigenic drift, separating these viruses into H3N2SIV-alpha to H3N2SIV-beta. This R189K mutation was also found to contribute to the cross-reaction with several ferret sera raised against historical human IAVs with hemagglutinin carrying 189K. Conclusions: This study suggests that the R189K mutation plays a vital role in the antigenicity of swine and human H3N2 IAVs and identification of this antigenic determinant will help us rapidly identify antigenic variants in influenza surveillance.

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A mechanism of antigenic escape: generation of a neuraminidase-negative H5(N1) HPAIV (and its characterization)

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Background: To model an escape from immunogenic pressure, the H5N1 HPAIV strain A/*Cygnus cygnus*/Germany/R65/06 (H5N1) (R65) was passed through embryonated chicken eggs in the presence of chicken serum. The escape variant (EscEgg50A), which was obtained after 50 passages, acquired extensive alterations in its NA-encoding segment, which resulted in the loss of sialidase activity. Material and Methods: We used a neutralizing polyclonal serum originating from chicken after vaccination with an inactivated H5N2 vaccine followed by a challenge with HPAIV H5N1 to provide antigenic pressure. Reverse genetics was used to generate a recombinant virus (EscEgg50Arec) consisting of a wild-type R65 backbone and the shortened NA-segment. Results: In addition to 12 amino acid substitutions scattered across its genome, whole-genome sequencing of EscEgg50A revealed deletions and rearrangements within the NA-encoding segment. Loss of the NA protein of EscEgg50A and EscEgg50Arec was confirmed using indirect immunofluorescence and Western blotting; a neuraminidase activity assay verified the complete loss of enzymatic activity. After

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inoculation of MDCK cells at MOI 1, EscEgg50A and EscEgg50Arec revealed a delay until 48 h pi but replicated to similar titers as R65 at 72 h pi. Replication after the application at MOI 0.01 led to a more pronounced delay, which, together with a severely reduced plaque size, indicated an impaired cell-to-cell-spread of NA-deleted variants. Two additional escape variants (EscEgg50B, EscEgg50C) were generated from 2 independent repetitions starting from the 30th passage in the presence of different polyclonal chicken sera. These variants also demonstrated similar partial deletions and shuffling events within the NA segment that resulted in the loss of NA activity. In contrast to R65, which has an IVPI of 2.97, the NA-negative variants EscEgg50A, EscEgg50B, and EggEsc50C were attenuated in chickens and did not cause any clinical signs, resulting in an IVPI of 0. Almost all of the chickens (27 out of 30) developed antibodies against NP and H5, indicating viral replication. After the oronasal administration of NA-deleted mutants, the chickens remained healthy and were challenged with a lethal dose of R65. All of the chickens that tested positive for H5 antibodies (3, 4, and 1 out of 10 for EscEgg50A, EscEgg50B, and EscEgg50C, respectively) survived, whereas the remaining animals succumbed to the disease. Conclusions: We present a replication-competent virus without NA activity and propose that the NA of HPAIV H5N1 may not be essential for in vitro replication and assembly, but is necessary for efficient cell-to-cell-spread and host entry. Because independent truncation and segment shuffling events in segment 6 occurred repeatedly during parallel passages under multiple immunogenic pressures, we claim this to be a general mechanism of antigenic escape. Our work provides a unique tool for studying the role of NA in the replication cycle and pathogenesis of this virus. It also serves as a new approach for developing attenuated live vaccines.

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Shedding duration: the key risk factor for the spread of highly pathogenic H5N1 avian influenza viruses over long distances by ducks?

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Background: Since their reemergence in humans in 2003, highly pathogenic avian influenza (HPAI) H5N1 viruses have been detected in avian species in Asia, Europe, and Africa. Only specific H5N1 clades have spread outside Asia: clade 2.2 viruses, which first reached Europe and Africa in late 2005 to early 2006; and clade 2.3.2 viruses, which reached Europe in early 2010. Their route of transmission across continents is still a subject of controversy, although wild birds have been implicated. Clade 2.3.2 viruses have been repeatedly isolated from wild birds, whereas clade 2.3.4 viruses, which circulated contemporaneously, have not, which suggests that a virologic property may influence their ability to establish an infection and spread in this host population. Our aim in this study was to clarify the putative (selective) role of wild birds in virus spread using representative viruses from clades 2.3.2 and 2.3.4. Materials and Methods: We compared the pathogenesis and transmission patterns of 4 HPAI H5N1 viruses in the mallard duck. Two of the viruses were from clade 2.3.2—A/barn swallow/Hong Kong/1161/2010 (HK1161) and A/buzzard/Bulgaria/38WB/2010 (Bulg38WB)—and 2 were from clade 2.3.4—A/duck/Lao/LH2/2010 (LaoLH2) and A/chicken/Hong Kong/AP156/2008 (HKAP156). Adult (3-month-old) mallards were selected as age-relevant for migrating birds. To better assess any differences in pathogenicity among these viruses and to compare their transmission patterns, we also inoculated 4-week-old mallards. Donor birds were inoculated with 10^4 EID_{50} of one of the viruses through the nasal, ocular, and oral route One day after the infection was established, each donor bird was housed with a naïve bird that had been infected by direct contact and a naïve bird that had been infected through airborne contact Thus, a total of 12 young mallards were used to test each virus: 4 donor (D) birds, 4 direct contact (DC) birds, and 4 aerosol contact (AC) birds. Results: In adult mallards, clinical signs of infection were only observed in birds that had been inoculated with clade 2.3.2 viruses. All 4 ducks inoculated with HK1161 had cloudy eyes, as did half of the ducks infected with Bulg38WB by day 5 post infection. In all, 75% of

the mallards inoculated with HK1161 succumbed to the infection, whereas all of the birds inoculated with Bulg38WB survived. No morbidity or mortality was observed in adult ducks infected with either 2.3.4 virus. Virus shedding was the highest in birds inoculated with HK1161 (2.3.2), followed by Bulg38WB (2.3.2), LaoLH2 (2.3.4), and HKAP156 (2.3.4). In the young mallards, clinical symptoms were more severe. Cloudy eyes and neurological signs were observed in some inoculated birds in all virus groups. Every donor bird inoculated with the Bulg38WB (2.3.2) or HKAP156 (2.3.4) virus survived the infection, whereas 25% and 0% of donors inoculated with LaoLH2 (2.3.4) and HK1161 (2.3.2), respectively, survived. All 4 viruses were transmitted efficiently to each direct contact and aerosol contact, with the highest mortality, morbidity, and virus shedding titers seen in birds inoculated with HK1161 (2.3.2), followed by LaoLH2 (2.3.4), Bulg38WB (2.3.2), and HKAP156 (2.3.4). Interestingly, Bulg38WB (the virus that reached Europe) donor and contact birds shed the virus for longer periods of time than birds infected with the remaining 3 H5N1 strains (mean shedding duration by Bulg38WB-infected ducks: 7.5 ± 1.6, 10.4 ± 1.3, and 5.0 ± 2.4 days ± SEM for D, DC, and AC birds, respectively). Conclusion: The pathogenicity and shedding of HPAI H5N1 viruses are highly variable both between and within each virus clade. It has been proposed that an intricate interplay of virus shedding and a low level of pathogenicity is required for each virus spread via migratory birds. We found a relatively low degree of pathogenicity in mallards accompanied by robust virus shedding in a virus that can be transmitted across continents. Further assessment of viral phenotype, particularly for HPAI H5N1 viruses seen more frequently isolated from wild birds, is warranted to identify fitness markers for these viruses in mallards.

P2-603

Susceptibility of bank voles (*Myodes glareolus*) to infection with H5N1 and H7N1 highly pathogenic avian influenza viruses

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Background: Direct transmission of avian influenza (AI) viruses to humans and other mammals that are not traditionally considered susceptible to AI through natural exposure has been reported, particularly for highly pathogenic AI (HPAI) viruses. This finding has led to an increase in research efforts to better understand the mechanisms of pathogenicity and transmission for the HPAI virus in mammals and the role of non-avian species in AI epidemiology. The laboratory mouse has been widely used to investigate AI virulence and pathogenesis, but only a few studies have explored the potential role of wild rodents in the AI ecology. This study was designed to evaluate the infectivity and transmissibility of H7N1 HPAI A/ostrich/Italy/2332/2000 (Os/2332) and H5N1 HPAI A/turkey/Turkey/1/2005 (Tk/05) viruses in the bank vole (*Myodes glareolus*), a wild rodent common throughout Europe and Asia. Material and Methods: To assess nasal shedding, we infected 2 groups with 12 animals each intranasally with $10^{4.4}$ and $10^{3.75}$ EID_{50} of Tk/05 and Os/2332 strains, respectively, corresponding to 10 x MLD_{50} in BALB/c mice. On days 3, 5, 7 and 9 post infection (pi), nasal washes (NW) and brains from 3 subjects in each group were tested using real-time RT-PCR (RRT-PCR) and virus isolation (VI). Lungs were processed for immunohistochemical (IHC) examination. We then assessed virus pathogenicity and transmissibility to contact sentinels (CS) by infecting 12 voles of each strain and moving them (24 h pi) into a clean cage hosting 12 AI-seronegative CS. Lungs, NW, brain, spleen, kidney, and intestinal tissue samples from 2 infected voles and 2 CS were tested by RRT-PCR and VI on days 3 and 4 pi and post contact (pc), respectively. Seroconversion of the remaining animals was assessed using ELISA and hemagglutination inhibition (HI) tests on day 30 pi/pc. Results: Nasal shedding was detected by RRT-PCR in 1/3 asymptomatic Os/2332-infected voles on days 3, 5 and 7 pi, and viable virus was isolated from one NW on day 5 pi. All brain samples tested negative by RRT-PCR and VI. Positive staining was revealed in the trachea and lungs of 2 voles on day 3 pi. In Tk/05-infected voles, nasal shedding was detected only by RRT-PCR in 3/3, 1/3, and 2/3 voles on days 3, 5 and 9 pi, respectively. Only one vole showed mild respiratory signs and tested positive by RRT-PCR in brain tissue on day 7 pi. No virus was detected in the remaining brains and lungs by RRT-PCR, VI and IHC. The results

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obtained in the transmission experiment demonstrated that all lungs collected from asymptomatic Os/2332-infected voles were positive by RRT-PCR and VI on days 3 and 4 pi. Viral RNA was also detected in the NW and extra-respiratory organs of infected voles on day 3 pi. Six out of 8 infected voles seroconverted. CS were asymptomatic; however, the lungs of 1/2 sentinels tested positive by RRT-PCR and VI on day 4 pc and 4/8 CS seroconverted. In Tk/05-infected voles, all lungs and NW tested positive by RRT-PCR on days 3 and 4 pi, and 4/4 lungs and 2/4 NW were positive by VI. Moreover, one brain tested positive by RRT-PCR on day 4 pi. Only one infected vole showed severe respiratory signs; it died on day 2 pi, testing positive by RRT-PCR in the lungs, NW, and spleen. All surviving infected voles seroconverted. CS animals were asymptomatic; however, the lungs of 1/2 sentinels tested positive by RRT-PCR and VI on day 4 pc and 1/8 CS seroconverted. Conclusions: Two non-rodent–adapted HPAI viruses caused asymptomatic infections in bank voles, which shed viable virus capable of infecting in-contact sentinels. Infection of BALB/c mice with the same strains in previous experiments caused 100% mortality and severe clinical signs. Thus, our findings suggest that voles are more resistant to HPAI viral infection but may still transmit the virus. Additional studies may elucidate the potential role of wild rodents in the epidemiology of HPAI infections.

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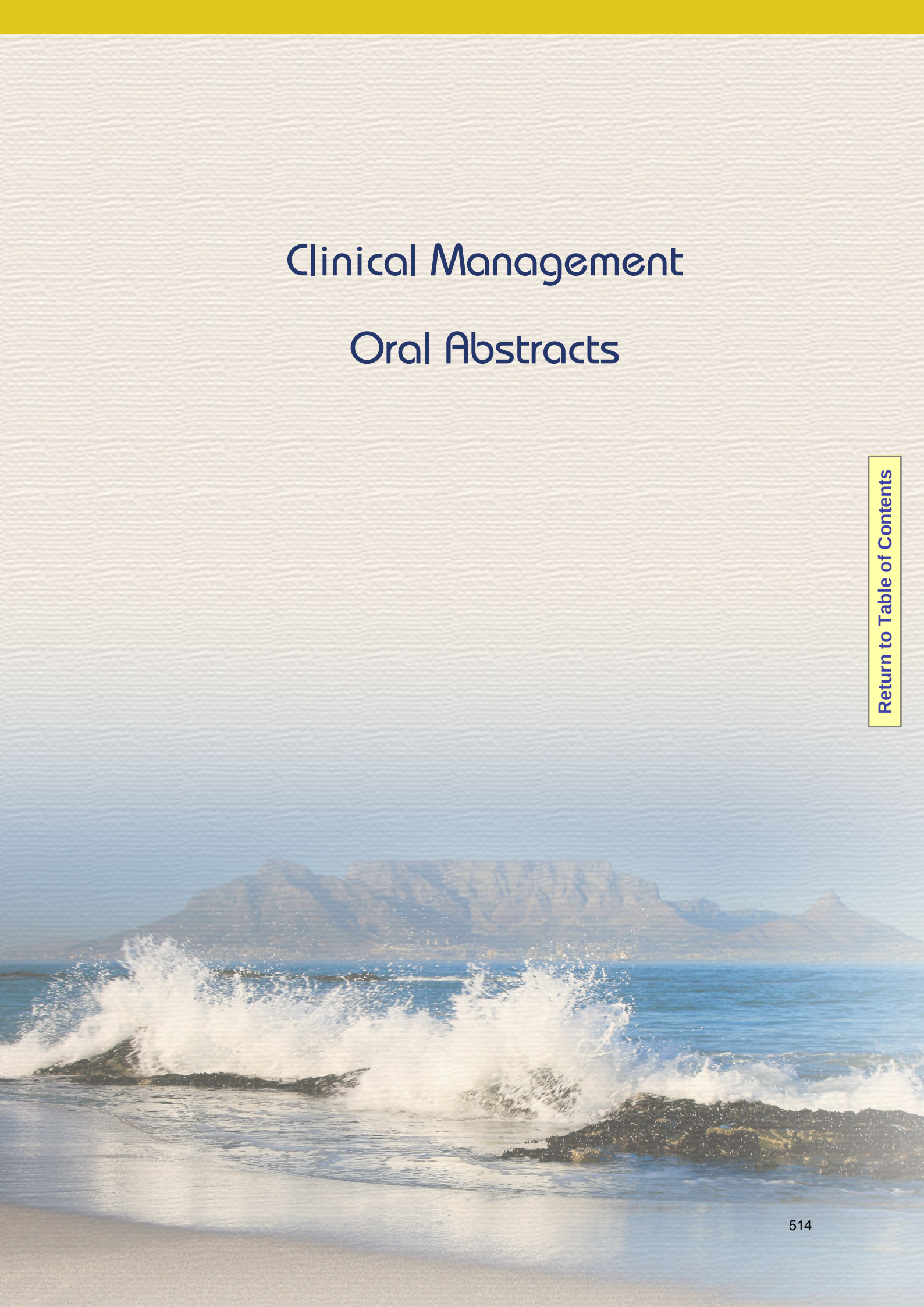

O-900

Post-pandemic review of anti-influenza drug effectiveness (PRIDE study): an individual patient data (IPD) meta-analysis investigating the impact of antiviral use on severe patient outcomes during the 2009-2010 influenza A(H1N1)pdm09 pandemic

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Background: The impact of neuraminidase inhibitor (NAI) treatment on clinical outcomes of public health importance during the 2009-2010 pandemic has not been firmly established. Materials and Methods: We conducted an individual patient data meta-analysis of data on hospitalised A(H1N1)pdm09 pandemic influenza cases (n = 29,235) contributed by 78 research groups from 38 countries and 6 WHO regions. We considered severe outcomes, which included need for critical care and/or mortality. We conducted multilevel logistic regression modelling, with each research site included as a random intercept. We adjusted for propensity scores (based on age, sex, comorbidity and disease severity at admission) and for treatment with antibiotics and steroids. We conducted further analyses by age and pregnancy status. Results are presented as adjusted odds ratios (ORs) and 95% confidence intervals (CIs) unless otherwise stated. Results: After adjustment, we observed a significant decrease in mortality associated with NAI treatment (at any time) versus no treatment (OR, 0.83 [95% CI, 0.72 - 0.97]) and early treatment (≤ 48h after symptom onset) versus late (OR, 0.47 [95% CI, 0.41 - 0.55]) or no (OR, 0.50 [95% CI, 0.37 - 0.67]) treatment. Preliminary unadjusted results for admission to critical care suggest a reduced likelihood of admission to critical care with early treatment compared with late treatment (OR, 0.59 [95% CI, 0.53 - 0.66]). Conclusions: During the 2009-2010 influenza A(H1N1) pandemic, early initiation of NAI treatment reduced the likelihood of the need for severe outcomes (critical care and/ or mortality) compared with late treatment. *Prospero Registration.* CRD42011001273.

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O-901

Indirect effects of oseltamivir treatment of an influenza-infected index case-patient on secondary household transmission: a randomized placebo-controlled clinical trial in a crowded urban area in Bangladesh

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Background: Prophylactic use of oseltamivir can prevent influenza infection and illness when given prior to exposure or immediately after exposure to an infectious person. However, during a pandemic, a large drug supply would be needed if prophylactic use was recommended. Oseltamivir treatment reduces virus shedding and may reduce infectiousness and subsequent virus transmission to susceptible individuals. However, studies supporting a reduction in transmission are limited. Prior to the 2009 pandemic, we initiated a double-blind, randomized, placebo-controlled trial in a crowded area in Dhaka, Bangladesh, to determine the indirect effects of treatment of an index case-patient on secondary household transmission.
Materials and Methods: From May 11, 2008 through December 31, 2010, we enrolled ill index patients with a positive rapid influenza test result and randomized them 1:1 to placebo to oseltamivir. The index patient and household members were monitored daily for illness. Household members with one major symptom (eg, fever, tachypnea, breathing difficulty) or two minor symptoms (eg, cough,

sore throat, chills, myalgia, rhinorrhea) were sent to the clinic for collection of a nasal wash specimen. All specimens were tested for influenza with reverse transcriptase–polymerase chain reaction (PCR). We compared the secondary attack rate (SAR) due to illness and PCR-confirmed influenza between households with an index patient randomized to oseltamivir treatment versus placebo. Effectiveness = 1-SARo/SARp estimated from a log binomial model adjusted for household clustering. Index patients and their households were pre-specified to be stratified by onset of the index case-patient's major symptoms relative to enrollment, < 48 hours or 48-120 hours ("≥ 48 hours"). All analyses were intent to treat, except where noted. Results: Among 6544 households under surveillance, 1190 index case-patients and their households were enrolled in the study; 794 (67%) index case-patients were enrolled < 48 hours and 396 (33%) were enrolled ≥ 48 hours after symptom onset. Overall, 2292 and 2402 household members were monitored from placebo- and oseltamivir-treated index case-patient households. The median age of index case-patients was 5 years (interquartile range [IQR] 2, 9), and infecting viruses included influenza A(H3N2) (35%), seasonal influenza A (H1N1) (11%), A(H1N1)pdm09 (18%), and influenza B virus (33%). The median household size, excluding index patients, was 4 (IQR 3, 5), and the median age of household members was 24 years (IQR 10, 34). The median interval from index enrollment to household illness was 1 day; 32% of household illnesses occurred before or on the same day as index enrollment, and 27% occurred 1 day after enrollment. There were no differences between groups. The SAR for illness was less in the oseltamivir group (196/2402 [8.1%]) compared with the placebo group (233/2292 [10%], *P* = .02; effectiveness = 0.20; 95% confidence interval [CI] = 0.03, 0.34). The SAR for PCR-confirmed illness was not statistically different between placebo (4.5%) and oseltamivir (3.8%) groups (*P* = .30); however, only 40% of ill household members had a respiratory specimen collected for PCR. Among the group with index case-patients enrolled < 48 hours after illness onset, SAR for household illness was less in the oseltamivir group (8.5%) compared with the placebo group (10.2%, *P* = .09), but not statistically significant. Among the households with index case-patients enrolled ≥ 48 hours from illness onset, SAR for illness was less in the oseltamivir group (7.4%) compared with the placebo group (10%, *P* = .07), but the difference was not statistically significant. In a post hoc analysis, households with an illness before or on the same day as the index case-patient enrollment, or with incomplete household illness onset dates, were excluded (n = 208 households). Among households with index case-patients enrolled ≥ 48 hours after illness onset, the SAR for illness was less in the oseltamivir (3.2%) compared with the placebo (5.7%) group (*P* = .04; effectiveness = 0.43; CI = 0.02, 0.67); the < 48 hour groups were not significantly different (oseltamivir 5.7%, placebo 6.8%; *P* = .21). Conclusions: In a crowded low-income setting, oseltamivir treatment of an index case-patient resulted in a 20% reduction in secondary illness among household members when treatment was started within 120 hours after illness onset. Future studies will explore these results and their relevance more completely.

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O-902

Impact of pneumococcal co-infection on disease severity and antibiotic prescriptions in infants and children with influenza: an inception cohort study

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Background: Respiratory tract infections are common diagnoses in the paediatric health care setting. Even though influenza-like illness (ILI) is predominantly viral in origin, antibiotics are used frequently. Antibiotic overuse contributes to the development of multidrug-resistant bacteria. Ever since the 1918 influenza pandemic, coinfections with *Streptococcus pneumoniae* among other bacterial pathogens are considered major contributors to influenza morbidity and mortality. Material and Methods: The study was performed in the context of the Charité Influenza-Like Disease (ChILD) cohort, a real-time surveillance program at the Charité Departments of Paediatrics and Microbiology in collaboration with

the National Reference Centre for Influenza at the Robert Koch Institute (RKI). This prospective in-/outpatient cohort encompasses the full spectrum of disease presentations, from mild forms of ILI to severe to fatal cases. In addition to routine medical care, a specifically trained quality management team performs daily screening exams and standardized disease severity assessments on all patients fulfilling pr-defined case criteria (ChILD score; based on 22 WHO case criteria). Nasopharyngeal swabs are sent to the RKI for independent real-time PCR, as well as to the Department of Microbiology for culture-based microbiologic analysis: Swabs were spread onto routine agar plates and read after first and second days of incubation at 37°C. Identification of pneumococci was using classical optochin test, biochemical analyses (StreptRapid or Vitek2, bioMérieux), or mass spectrometry (VitekMS, bioMérieux). Bacterial coinfection was defined as culture positivity for any bacterial pathogen in a patient with evidence of ILI or laboratory-confirmed influenza. Statistical analyses were performed using Kullback-Leiber (KL) divergence as a nonsymmetric measure probability distributions and the Fisher exact test for significance testing of categorical data. Results: From December 2011 through April 2013, a total of 2574 patients presenting with ILI were enrolled, with an average age of 39 months (median: 20, range: 0-224). Influenza infection was confirmed in 408 patients (16%), 238 of whom showed evidence of bacterial coinfection. In this inception cohort setting, pneumococcal coinfection was detected in only 20/408 patients with laboratory-confirmed influenza. This may be attributed to adequate immunization coverage with pneumococcal conjugate vaccines (87.1%) and comparatively low influenza vaccination rates (6.0%). Other types of bacterial coinfection were more common in influenza patients, namely *Staphylococcus aureus*, (21.3%) *Haemophilus influenzae* type non-B (15.7%) and *Enterobacteriaceae* (12.8%). Using standardized disease severity assessments (ChILD score), pneumococcal coinfection had no significant impact on disease severity in influenza patients. The same holds true for other forms of bacterial coinfection in the ChILD cohort, including staphylococcal and *Haemophilus* coinfections. During feature analysis, fever was shown to be the only clinical sign that significantly correlated with pneumococcal coinfection (P = .0162). Using fever (≥ 38°C) and hypoxia (O_2 saturation < 93%) as key criteria in decision tree analyses, the predictive value for pneumococcal coinfection can be increased to 66.4%. At the time of presentation to the hospital, 12% of children with influenza were already on antibiotics, most commonly (68%) 2nd-generation cephalosporins. In ≈ 20% of all patients with influenza and ILI, new antibiotic prescriptions were issued before bacterial culture results were available. If antibiotic prescriptions had been delayed until results were available, the physicians would have learned that (amino-) penicillins could have sufficed in the majority of cases (76%). KL-divergence statistics revealed that antibiotic prescriptions were associated with disease severity (= elevated ChILD scores; KL 6.41), followed by abnormal haematocrit and CRP values (KL 3.51 and 3.35, respectively), as well as need for hospitalization (KL 1.25). Conclusions: Antibiotic prescriptions in influenza and ILI patients should be restricted to patients with laboratory evidence of bacterial coinfection. Antibiotics should be withheld until bacterial culture results are available, along with standardized assessments of disease severity, such as the ChILD score. Patients with influenza-bacterial coinfection exceeding a critical level for disease severity should receive individualised antibiotic therapy based on culture and sensitivities, using the narrowest spectrum possible. In the tertiary care setting described above, the proposed standard operating procedure would reduce inappropriate use of antibiotics by more than two thirds.

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O-903 SCHOLARSHIP International Research Scholarship

Drug dispensing practices for acute respiratory infections and availability of oseltamivir through pharmacies in Dhaka, Bangladesh

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Background: Drug sellers comprise 16% of primary healthcare providers in Bangladesh and often serve as first point of care for the poor and less educated. During the 2009 influenza pandemic, a survey in Dhaka reported that 48% of respondents who had influenza-like illnesses (ILI) used

pharmacies as the first point of care. Therefore, drug sellers have an important influence on public health through their health advice and treatment recommendations. No published data are available in Bangladesh on drug dispensing practices for acute respiratory infection (ARI) or on the availability of oseltamivir in pharmacies, an antiviral drug used to treat and prevent influenza virus infection. We investigated drug sellers dispensing practices for ARI and the availability of oseltamivir in pharmacies throughout Dhaka city, Bangladesh. Methods: During May to October 2012, we conducted a cross-sectional study among 100 pharmacies: 10 pharmacies from each of the 10 zones of Dhaka city which were selected using random points on GPS and identifying the closest pharmacy to that point. Using a structured questionnaire researchers collected data on demographics, availability of oseltamivir and knowledge of oseltamivir from the drug seller who spent the most time in the pharmacy. Additionally, 6 field research assistants (FRAs) visited all the selected pharmacies and acted as relatives of patients with ARI. FRAs sought drug sellers' advice about clinical management and did not carry a prescription from a physician. FRAs described to drug sellers one of three clinical symptoms for either children or adults: acute onset of cough with runny nose, cough with fever, or respiratory distress. FRAs recorded health advice provided and drugs dispensed by the drug sellers for each visit. We compared dispensed drugs with ARI management guidelines (Government of Bangladesh, Unicef, and WHO) to assess the appropriateness of drug seller recommendations. According to the guidelines acetaminophen was considered the only appropriate choice of drug for patients with common cold and fever; antibiotics (erythromycin, azithromycin, amoxycillin, penicillin) were appropriate for pneumonia; whereas steroids, nonsteroidal anti-inflammatory drugs (NSAIDs) and pseudoephedrine were inappropriate and potentially harmful. Results: Among 100 selected pharmacies, none had oseltamivir available for sale during the survey. Ninety-nine (99%) drug sellers had no knowledge of oseltamivir and only 1 (1%) drug seller dispensed oseltamivir as recommended by the government of Bangladesh during the 2009 influenza pandemic. Drug sellers dispensed drugs for the patient in 383 (64%) of 600 FRA visits, asked FRAs to take the patient to a physician or hospital in 184 (31%) visits and refused to give drugs without the patient present in 33 (6%) visits. In 84 (14%) of the 600 visits, drug sellers dispensed drugs at the behest of FRAs despite referral or refusing to treat the patient. Among all the dispensed drugs, acetaminophen was dispensed in 238 (40%) visits, antibiotics in 224 (37%), antihistamines in 177 (30%), bronchodilators in 103 (17%), cough expectorants in 83 (14%), , herbal cough syrups in 52 (9%), gastric ulcer healing drugs in 30 (5%), prophylactics for bronchial asthma in 25 (4%), cough suppressants in 20 (3%), steroids in 12 (2%) and other drugs in 15 (3%) visits. An average of 1.6 drugs was dispensed per visit. According to the guidelines, 687 (70%) of the dispensed drugs were inappropriate but not harmful, 225 (23%) were appropriate, 52 (5%) (herbal medicines) could not be judged appropriate or inappropriate and 15 (2%) were inappropriate and potentially harmful. In 150 (25%) visits, advice was provided by drug sellers during consultation. The most common advice provided was to take antibiotics if the patient was not cured within 3-5 days in 69 (46%) visits, avoidance of cold drinks in 22 (15%) visits, and to take antibiotic according to physician if not cured in 19 (13%) visits. Conclusion: Drug sellers were unfamiliar with and did not stock oseltamivir. Their dispensing practices for ARI were often inappropriate. Formal training on the empirical management of acute respiratory illness may promote safer dispensing practices.

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O-904

INSIGHT FLU002 and FLU003, an international cohort study of influenza A(H1N1)pdm09 virus infection, 2009-2012

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Background: Influenza causes significant morbidity and mortality; however, prospective multicenter follow-up studies assessing rates and risks for disease progression are rare. Following the emergence

of influenza A(H1N1)pdm09, two international cohort studies were initiated to estimate morbidity and mortality due to A(H1N1)pdm09 and assess predictors of progression. Methods: From October 2009, patients > 17 years were enrolled in an outpatient (FLU002) or inpatient (FLU003) protocol, using a Northern and Southern Hemisphere network (INSIGHT) encompassing 95 sites in 18 countries, if there was clinical or laboratory evidence of influenza infection. Demographics, clinical data, and upper respiratory tract (URT) swabs were collected. Swabs were tested centrally for influenza detection and subtyping using RT-PCR. For outpatients, disease progression (hospitalization or death) through 14 days follow-up was determined; for hospitalized patients disease progression (hospitalization for ≥ 28 days, progressing to ICU or death) through 60 days follow-up was determined. Cross-sectional comparisons of baseline characteristics of patients in FLU003 (hospitalized) versus FLU002 (not hospitalized at enrollment) were conducted with logistic regression controlling for demographics, symptom duration and medical history. Similar multiple logistic models, pooled across both studies, examined baseline predictors of disease progression. Adjusted odds ratios are cited for cross-sectional (OR_{CS}) and cohort (OR_{CO}) analyses. In a substudy, a random sample of 333 participants (235 from 002, 98 from 003) with confirmed A(H1N1)09pdm were analyzed for viral and bacterial copathogens using RT-PCR on URT samples. Results: Through December 2012, 2602 patients were enrolled in FLU002 and 749 in FLU003. One thousand and sixty-two (40.8%) patients in FLU002 and 395 (52.7%) patients in FLU003 had confirmed influenza A infection: 589 (55.5%) in FLU002 and 294 (74.4%) in FLU003 had A(H1N1)pdm09. The percentage of patients with influenza A virus subtyped as A(H1N1)pdm09 was greatest during the first year of enrollment (92.8% for FLU002 and 94.8% for FLU003). The average age of patients with A(H1N1)pdm09 was 33.6 years in FLU002 and 47.1 years in FLU003 (OR_{CS} associated with 10 years older = 1.49 (1.30-1.72; $P < 0.001$); approximately 50% in both studies were female (adjusted OR_{CS} =1.37; $P = 0.11$); 6.8% of patients in FLU002 reported a history of asthma or COPD, 9.3% reported HIV or other immunosuppressive conditions, and 2.2% reported a history of other CVD or diabetes. These percents were higher for FLU003 patients (28.2%, 14.6% and 21.8%). OR_{CS} for COPD/asthma [OR 3.62 (2.17-6.03; $P < 0.001$)] and diabetes/cardiovascular/disease [OR 5.54 (2.63-11.69; $P < 0.001$)] were significant; the OR_{CS} for HIV/other immunosuppressive conditions was not (OR_{CS} = 0.88; 95% CI: 0.48-1.59; $P = 0.67$). Median (IQR) duration of influenza symptoms at enrollment was 2 (1-3) days in FLU002 and 5 (3-9) days in FLU003 (OR_{CS} per 1 day longer symptoms = 1.51; 95% CI: 1.39-1.63; $P < 0.001$). Twenty-nine patients (5.1%; 95% CI: 3.4%-7.2%) in FLU002 and 60 in FLU003 (21.9%; 95% CI: 17.1%-27.3%) had disease progression; 23 in FLU003 died (8.5%; 95% CI: 5.5%-12.5%). In the cohort analysis HIV/other immunosuppressive conditions was significantly associated with disease progression [OR_{CO}: 2.32 (1.22-4.42; $P = 0.01$)]. Associations with age (OR_{CO}=1.13; 95% CI: 0.94-1.35; $P = 0.19$), sex (OR_{CO} = 1.30; 95% CI: 0.79-2.15; p=0.31), symptom duration (OR_{CO}=1.05; 95% CI: 0.99-1.11; p=0.13) and other medical conditions (ORco=0.64; 95% CI: 0.32-1.31; $P = 0.22$ for COPD/asthma and OR_{CO} = 1.47; 95% CI: 0.73-2.98; $P = 0.28$ for diabetes/CVD) were not significantly associated with disease progression. Prevalence of other viruses and potentially pathogenic bacteria in a random sample was 15% and 60%, respectively. Conclusions: During this 3+ year enrollment period (where most influenza A(H1N1)pdm09 cases presented in the first year after this strain's emergence), hospitalized patients were older, had more chronic health conditions, and had longer symptom duration than outpatient enrollments. In the cohort analysis, risk factor associations with progression were weaker and only HIV infection or other immunosuppressive condition was significantly associated with an increased disease progression. Differences between the cross-sectional and cohort analyses demonstrate the difficulty of using cross-sectional comparisons to identify prognostic variables. The rates of disease progression observed in both studies over a relatively short follow-up period underscore the importance of effective control strategies.

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O-905

A phase 2, randomized, double-blind, placebo-controlled, multicenter study evaluating the safety and pharmacokinetics of different dosing regimens of favipiravir (T-705) in adult subjects with uncomplicated influenza

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Background: Favipiravir (T-705) is a novel antiviral agent discovered by Toyama Chemical Co., Ltd. Mechanistic studies have revealed that T-705 is converted to T-705 ribosyl triphosphate (T-705RTP) by host cellular enzymes and that T-705RTP selectively inhibits viral RNA polymerase. Preclinical studies have demonstrated its effectiveness against all tested influenza strains, including H5N1. Combination studies with various influenza strains have demonstrated synergy with oseltamivir. We present results from a double-blind, randomized, placebo-controlled study conducted during the 2012-2013 influenza season. Materials and Methods: This phase 2 study was designed to evaluate the safety and efficacy of a BID and TID regimen, including virologic response and clinical outcomes. Subjects were eligible for inclusion if they were between the ages of 18 and 80 years, had uncomplicated influenza with symptoms of less than 48 hours duration, and were otherwise healthy. Of six influenza symptoms (fatigue, cough, body aches and pains, nasal congestion, headache, sore throat), two or more had to be of at least moderate severity (disturbing symptoms, partially interfere with daily activities), and the subject had to have had a fever during the site visit or within the six previous hours if they had taken an antipyretic during that interval. A positive rapid antigen test (RAT) for influenza was required unless the subject had been in close contact with an influenza-infected person with a diagnosis made by RAT or real-time polymerase chain reaction (PCR) or there was a local influenza outbreak. After eligibility was confirmed, subjects were randomized to one of two active treatment arms (a loading T-705 dose of 2400 mg followed by 600 mg approximately every 8 hours for a total of 14 doses or 1800 mg approximately every 12 hours for two doses followed by 800 mg every 12 hours for a total of eight doses), or matching placebos, in a 2:2:1:1 ratio. The first dose of study drug was administered at the study site. Subjects returned to the study site on Days 2-5, 8, and 15. Tests included nasal swabs for virology testing (quantitative PCR, viral culture, viral typing, resistance studies) and laboratory safety studies. Subjects discussed their daily body temperature and influenza symptoms with the investigator, who reported them in the case report form. Adverse events and concomitant medication use were collected at each study visit. Results: Five hundred forty-eight eligible subjects were randomized and received study drug. The two placebo groups with a BID and TID regimen were prospectively pooled and treated as one single placebo arm in the final analyses: 183 subjects were randomized into the active BID regimen and 184 into the placebo regimen. The intent-to-treat infected (ITTI) population consisted of 101 BID subjects and 88 placebo patients—the primary population for efficacy analyses. Subjects were generally comparable across groups for most demographic criteria in both the safety and ITTI populations. In the ITTI population, statistically significant differences were seen in time to alleviation of the six clinical symptoms plus resolution of fever (P = .01), time to alleviation of each of the six individual symptoms (cough: P = .034; fatigue: P = .005; headache: P < .001; nasal congestion: P = .012; body aches and pain: P < .001; sore throat: P = .048), change from baseline in median tissue culture infective dose at Days 3 and 4 (P = .0005 and .0035, respectively), and change from baseline in quantitative PCR at Days 3, 4, and 5 (P = .016, .043, and .004, respectively) for the BID compared with placebo group. Significant differences were also observed in select intent-to-treat population analyses. Adverse events were mild and, except for transient asymptomatic elevations of uric acid in the favipiravir arms, comparable across all groups in the safety population. Conclusions: T-705 is a promising novel anti-influenza agent with both clinical and anti-viral activity, as demonstrated in the 2012-2013 influenza season in the United States. Phase 3 studies are planned for the 2013-2014 influenza season. This study was partially funded by the Joint Project Manager Medical Countermeasure Systems (JPM-MCS), Department of Defense, USA under HDTRA1-12-C-0031.

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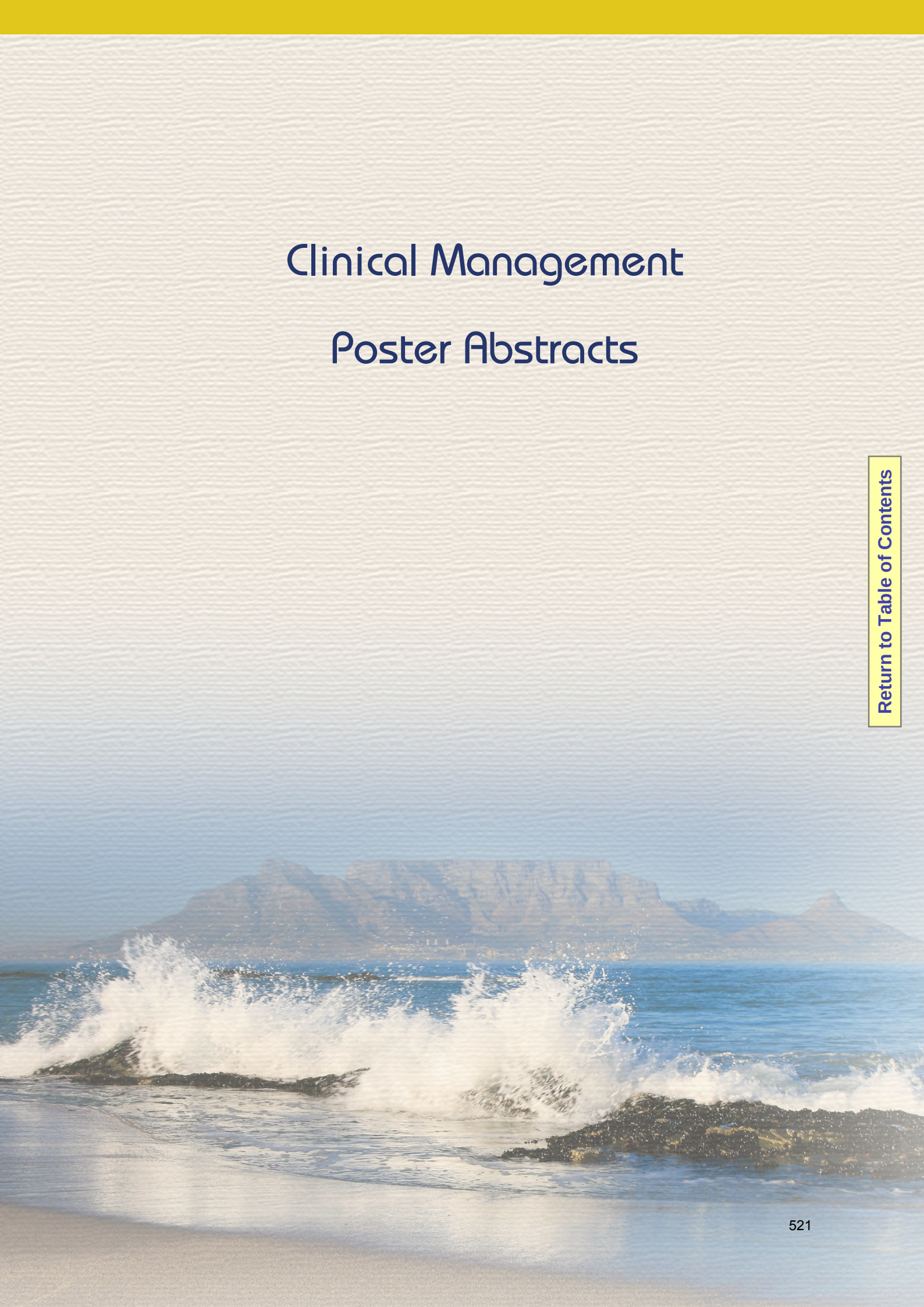
Clinical Management

Poster Abstracts



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Development of a decision making model on the management of influenza. A proposal of diagnostic policy based on phenomena and therapeutics

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Background: Management of influenza dramatically changed since the introduction of rapid influenza diagnostic test (RIDT), and neuraminidase inhibitors (NIs). However, it is still far from optimal due to low sensitivity of RIDT and problems involving NIs such as side effects and potential emergence of resistant virus. Therefore, we developed a decision-making model on the management of influenza, including Kampo medicines (Chinese herbal medicines) on the strategy. Methods: Related literatures were reviewed on the diagnostic values of RIDT, as well as the efficacy of NI and Kampo medicines. After the literature review, a decision making model on the diagnosis and treatment of influenza was developed using Delphi methods among authors. Results: In the model developed, severity of patients will be evaluated first. If a patient was judged as severe or having high-risk, intravenous NI, such as peramivir would be the main component of the treatment. If a patient was neither in severe condition nor having high-risk, the patient would be asked to choose either NI or a Kampo medicine. We advocate the use of oseltamivir or zanamivir, but not peramivir or laninamivir available in Japan, at outpatient settings. The formers were chosen based on the available evidence and safety data. For Kampo medicines, we chose either Maoto (Ma Huang Tang) or Makyokansekito (Ma Xing Shi Gan Tang) for the treatment of influenza, depending on the condition of the patient. If one chose to use NIs, RIDT would be used only when pretest probability was less than 50%, but it would not be used when the pretest probability was more than 50%, based on the lack of influence on the posttest probability. If one chose to use Kampo medicines, RIDT would be not used in general as Kampo targets “phenomena” only, and the result of RIDT would not change the management. Conclusions: This model enables us to optimize the use of RIDT by selecting out the appropriate patients based on the characteristics of RIDT, and to avoid unnecessary tests and its misinterpretation.

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Study of viremia in immunocompetent infants and young children with lower respiratory tract infection

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Background: Viral infections of the lower airways are a major cause of morbidity and even mortality in infants and young children. Viremia has been implicated in the spread of many viral infections from the upper to the lower respiratory tract. However, data regarding risk factors and clinical significance of viremia in immunocompetent patients are not available, and the role of viremia in the pathogenesis of many respiratory infections remains unknown. The aim of this work was to study the frequency of occurrence of viral dissemination (viremia) in immunocompetent infants and children with acute lower respiratory tract infections (ALRTIs), and to investigate whether viremia has an impact on the disease course and prognosis. Materials and Methods: The study population included infants and children

below the age of 5 years who were admitted to a tertiary hospital with ALRTIs from May 2010 through May 2011. Respiratory symptoms and signs were recorded on a standardized form during the emergency department visit. Clinical data were retrieved via a retrospective review of medical records. Blood samples were obtained immediately following hospital admission. Six different multiplex real time PCR reactions (Sacace Biotechnologies, Italy) were used to detect the following viruses: respiratory syncytial virus (RSV), parainfluenza viruses 1- 4, all 100 seroptypes of the rhinovirus, adenoviruses B,C, and E, and the new respiratory viruses: human metapneumovirus (hMPV), human bocavirus (hBoV), and human coronavirus (hCov) CoNA , OC43, E229, NL63, and HKUI. Results: Of the149 participants, 47 (31.5%) demonstrated viremia caused by the different studied viruses. Adenovirus was the most frequently detected in serum of children with viremia (19/47, 40%), followed by rhinovirus (11/47, 23 %). Viremia due to other studied viruses included: hMPV (n = 5), hBoV (n = 5), CoHKU (n = 4), CoNA (n = 2), parainfluenza (n = 3), and RSV (n = 2). Age (except with rhinoviremia), prematurity, presence of underlying illness, presence of fever, and important respiratory symptoms and signs were not significantly associated with viremia. Apart from the high frequency of rhinoviremia among patients with history of bronchial asthma, as well as with asthma exacerbation diagnosis, no correlation was found between viremia and the severity of illness, and the occurrence of blood stream dissemination did not always lead to a significantly worsened outcome. This could be related to individual variability in the control of viral replication, the later sampling in relation to symptom initiation, viral load below the detection limit. Conclusion: The data obtained suggest that viremia due to the studied respiratory viruses does occur during the course of infection in immunocompetent infants and young children. Viremia does not seem to be implicated in the severity of illness nor the untoward prognosis.

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Recognizing true H5N1 infections in humans during confirmed outbreaks

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Background: The highly pathogenic avian influenza virus of subtype H5N1 remains prevalent in many countries, causing human illness that is frequently fatal. The goal of this study was to compare the demographic and clinical characteristics of confirmed and possible cases of human H5N1 infection at initial presentation, and to compare antiviral treatment effectiveness in the 2 groups. Methods: For patients of any age with data recorded in the Avian Flu Registry (www.avianfluregistry.org) during H5N1 outbreaks between 2005 and 2011, information was extracted on clinical signs and symptoms when patients initially presented for medical attention, treatments received, and disease outcomes, using a standardized data collection form. Both laboratory-confirmed cases and “possible” cases (those who presented during a confirmed local outbreak through the same medical channels as confirmed cases, but who had either a negative or indeterminate H5N1 test result, were not tested, or died before testing) were analysed. The analysis was restricted to countries where confirmed and possible cases presented at similar times during known H5N1 outbreaks, ie, Azerbaijan, Indonesia, Pakistan, and Turkey. This produced a sample of 353 cases (total cases in registry = 647 in 12 countries as of 30 April 2012). Results: The sample comprised 153 confirmed cases (44.4% male; median age, 17.0 years [range, 1.5-67.0]) and 200 possible cases (57.5%; 13.0 years [0.4-80.0]). Possible cases were more likely to present at an emergency room than confirmed cases (45.0% vs 28.1%) and to present later (median of 2 vs 0 days after symptom onset). Confirmed cases had a substantially higher case fatality rate (79.1% vs 12.0%, P < .01). At presentation, 2 symptoms were significantly more frequent in confirmed cases than possible cases (P ≤ .05): fever (87.8% vs 76.8%) and headache (43.5% vs 29.5%). Four symptoms were more frequent in possible cases (P < 0.01):

unexplained respiratory illness (82.8% vs 65.7%), sore throat or pharyngitis (62.4% vs 44.1%), excessive sputum production (37.8% vs 4.4%), and rhinorrhea (29.1% vs 11.2%). The survival benefit in patients who received oseltamivir treatment was significantly higher in confirmed cases than in possible cases (10.4% and 3.2% reductions in case fatality, respectively; P < .01). The largest survival benefit was seen in confirmed cases who were treated with oseltamivir within 2 days of symptom onset; the relative risk of survival compared with no antiviral treatment was 5.51 (95% CI, 2.54-11.94; P = .002). Conclusion: Since prompt treatment with oseltamivir significantly prolonged survival in patients with confirmed H5N1 infection, presumptive antiviral treatment should be considered for all possible cases presenting during an outbreak of H5N1 as a potentially life-saving measure.

P2-608

Developing a practical and truly global response to the next pandemic

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Background: Current pandemic preparedness plans are focused on vaccines and antivirals, yet in 2009 more than 90% of the world's people had no access to timely and affordable supplies of these agents. For the foreseeable future, it is unlikely that the response to the next pandemic will much better. Thus, health officials need to ask if there is anything physicians could use to initiate treatment in patients who develop severe illness following infection with a new pandemic virus. Findings: Clinical investigators have developed a set of modern medicines that improve outcomes in patients with cardiovascular diseases and diabetes—statins, angiotensin converting enzyme inhibitors (ACEIs), angiotensin receptor blockers (ARBs), and peroxisome proliferator-activated receptor (PPAR) and adenosine monophosphate-activated protein kinase (AMPK) agonists (glitazones, fibrates and metformin, respectively). Each of these agents has anti-inflammatory and immunomodulatory (pleiotropic) activities, and although their mechanisms of action are incompletely understood, the phenotypic benefits of treating pneumonia patients with some of these agents have been unmistakable. Statins have received the most attention from clinicians and epidemiologists. Many observational studies have shown that outpatient statin treatment is associated with reductions in pneumonia hospitalizations and deaths. In these studies, statins have acted as prophylactic agents. For inpatient treatment of pneumonia patients, 3 observational studies have reported that statins reduced 30-day or hospital mortality by approximately 20%-30%. Another study showed similar mortality reductions in hospitalized pneumonia patients treated with ACEIs and ARBs. These studies provide indirect evidence that inpatient treatment with these agents is effective, although none of the studies showed that treatment was effective if it was initiated after ICU admission. There have been no reports of randomized controlled trials of the efficacy of immunomodulatory treatment of hospitalized pneumonia patients. However, a recent randomized controlled trial in 100 statin naive patients hospitalized with early sepsis showed that treatment with atorvastatin (40 mg/day) reduced the development of severe sepsis from 24% to 6% (P < .007) (Patel JM, et al. *Crit Care.* 2012;16:R231). This important study suggests that instead of limiting immunomodulatory treatment to patients requiring ICU admission, treatment should be administered to all sepsis (and pneumonia) patients admitted for hospital care. Only one study has reported the effectiveness of statin treatment in patients hospitalized with influenza (Vandermeer ML, et al. *J infect Dis.* 2012;205:13). This study included 3043 older adults who were hospitalized with laboratory-confirmed seasonal influenza in 2007-2008. In a multivariable logistic regression analysis that adjusted for demographic factors, high-risk conditions, previous influenza vaccination and antiviral treatment, 1013 (33.3%) statin-treated patients had a 41% reduction in mortality within 30 days of a positive influenza test (adjusted odds ratio [OR] 0.59; 95% CI 0.38-0.92). These results have been criticized because it is thought that patients who received statins were "healthy users." Yet, statin-treated patients had almost twice as many high-risk conditions as those who were not given statins. More important, if receipt of influenza vaccine defines a "healthy user," this potential bias was accounted for by the investigators in their adjusted analysis: the 41% reduction in mortality in statin-treated patients was in addition to any reduction that might have been attributable to previous influenza vaccination and antiviral treatment. Conclusion: For the next pandemic, vaccines and antiviral agents will be unable to meet the needs of

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most of the world's people. In contrast, statins and the other immunomodulatory agents mentioned above are currently produced as inexpensive generics in developing countries. Global supplies are huge, the cost of treating an individual patient would be approximately $1.00, and treatment would be available to anyone with access to basic health care on the first pandemic day. If any of these agents could be shown to be effective, ordinary physicians working in ordinary health care systems throughout the world would have the means to reduce pandemic mortality.

P2-609

The severe cases of influenza infection in Vietnamese children—from the Cases of PICU in NHP-Hanoi

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Background: Clinically, ARDS associated with H5N1 avian influenza virus (H5N1) infection is much more severe compared as general ARDS (Kawachi, et al. *JID.* 2009). However, not only in H5N1 but also in other types of influenza, ARDS sometimes develop in children and the ARDS with influenza were always sever. It is an urgent mission to elucidate the mechanism of influenza-ARDS and to develop a therapeutic strategy. Methods: We have been cooperating the study for ARDS with National Hospital of Pediatrics–Hanoi (NHP-Hanoi) from October 2007. The entry criteria are as follows; 1)The patients who admitted the PICU-NHP with ARDS under intra-pulmonary reason (pneumonia), and needed mechanical ventilation. 2) The patients aged over 1 month. 3) P/F ratio (PaO2/FIO2 ratio) ≤ 100 mmHg in arterial blood gas analysis during the stay in the PICU. Serum, tracheal lavage fluid and/or nasopharyngeal aspirate samples were collected on admission. Viral genomes were examined by PCR or RT-PCR method with TLF/NPA samples of each patient. In 55/84 patients we tried 2 gram /kg gamma globulin therapy after informed consent. All patients were tracheal intubated and mechanical respiratory support was performed basically according to the AECC strategy (1998). Results: Eighty-four patients were diagnosed as ARDS matched in the criteria of prospective study. In 8 patients among 84, influenza viruses were detected: H5N1 (3), A(H1N1)pdm09 (4), H3N2 (1). Also, before 2007, 10 H5N1-ARDS patients were admitted in PICU of NHP-Hanoi. We analyzed the clinical data in totally 18 cases of influenza associated ARDS (Influenza group(I group)). In 22 patients among 84, the following viruses were also detected; rhinovirus (10), adenovirus (5), CMV (4), RSV (4), measles (1) (non-I group). In the other 54 patients, viral genomes were under detection limit. When compared between these 2 groups, we observed significant differences in survival time (days) (I group: 13.85 vs non-I group: 31.14) and survival probability (P = .0016 by log-lank test, P = .0129 by Wilcoxon test), PH and PaCO2 in arterial blood gas analysis, body temperature, and white blood cell and platelet counts. Conclusions: In Vietnam, severe ARDS associated with influenza had higher mortality rate than the other viral infections (I group: 83%, non-I group: 64%). These results reinforce the importance of further research on the etiology of influenza virus infection induced ARDS. Our study will provide some clinical information for development of the future therapeutic options.

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Influenza baseline disease severity does not differ between virus types A and B in the first 4 years of the Influenza Resistance Information Study (IRIS)

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Background: Since 2009, two influenza A viruses (H1N1 and H3N2) and two influenza B viruses (Yamagata and Victoria lineages) have circulated among humans. The Influenza Resistance Information Study (IRIS; NCT00884117) was initiated in December 2008 to study global neuraminidase inhibitor (NAI) resistance and the clinical and virological course of influenza infection. Materials and Methods: Patients aged ≥ 1 year with influenza-like illness and/or a positive rapid test result for influenza were enrolled between 2009 and 2012. On patient agreement, baseline nasal and throat swabs were conducted for real-time RT-PCR analysis to determine influenza type, subtype, viral load, and NAI resistance. Baseline influenza disease severity scores were recorded on diary cards by the physician. Multivariate statistical analysis was performed to compare baseline virus characteristics and clinical parameters between influenza A and B virus infections. Results: Of 2262 RT-PCR influenza-positive patients, 1565 had an influenza A virus infection (581 H3N2, 937 H1N1pdm2009, 47 seasonal H1N1) and 697 had an influenza B virus infection (98 Yamagata, 564 Victoria, 35 undetermined). Patients with incomplete baseline data (n = 144) were excluded from the multivariate analysis. No NAI-resistant viruses were detected at baseline after 21 September 2009, when the last patient with seasonal A/H1N1 virus infection was enrolled. Viral loads were higher in patients infected with B/Yamagata viruses compared with B/Victoria viruses ($P < .001$) and in patients infected with A/H3N2 virus compared with seasonal A/H1N1 or A/H1N1pdm09 viruses ($P < .001$ for both comparisons). In patients infected with A/H1N1pdm09 viruses, viral loads increased slightly over time ($P = .008$). With the exception of B/Yamagata virus infections, high viral load was associated with febricity ($P < .05$). In A/H1N1pdm09 and A/H3N2 virus infections, high viral load was associated with shorter time between onset of symptoms and baseline swabs ($P < .01$). Viral load was not associated with patient age, vaccination status, or baseline influenza disease severity score ($P > .1$ in all cases). The main risk factor for high baseline influenza disease severity score was patient age. Baseline influenza disease severity score was higher among young and middle-aged adults compared with children aged < 6 years and elderly patients. Baseline influenza disease severity scores were independent of virus type, subtype ($P = .13–.60$), or vaccination status ($P = .57$). Conclusions: Similar influenza disease severity scores were noted for influenza A and B virus infections at baseline. No NAI resistance was detected at baseline after 21 September 2009. Ongoing analyses aim to identify subtype-specific traits associated with influenza viral titer, baseline influenza disease severity score, and emergence of NAI resistance.

P2-612

Establishment of a scoring system for identifying severe cases of influenza-like illness by comorbidity and age—a nationwide cohort analysis

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Background: Influenza-like illness (ILI) is often the first presentation to clinicians in daily clinical practice. With limited healthcare resources and finite medical attention, how to identify high-risk ILI cases by considering the underlying comorbidity attributes becomes an important issue for appropriate case management at clinical frontline.Materials and Methods: Retrieved from the one-

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million-per-year systematic sampling patients of Taiwanese National Health Insurance Database from 2008 to 2010, our cohort composed of all inpatient encounters (admissions) with the diagnostic ICD-9 (International Classification of Diseases, Ninth Revision) that matched the ILI definition proposed by Marsden-Haug's Code-based syndromic surveillance in the defined scenarios. The defined scenarios were: within the same hospitalization, or with a prior ambulatory visit (outpatient clinic or emergency room) 1 or 2 days earlier. Comorbidity attributes were defined by appropriate ICD-9 codes that included most chronic diseases in several categories: immune-related, pulmonary, metabolic, cardiovascular, and others. Four outcome measures represented severe ILI cases: hospitalization cost, length of stay (LOS), death, and intensive care unit (ICU) entry. Univariate and multivariate analyses were done with the dependent variables as worse outcome, and the comorbidity attributes as covariates with the adjustment for gender in predefined age strata. The selected comorbidity attributes formulated comorbidity vector and the corresponding age strata formulated age vector. Comorbidity score was defined as the dot product of comorbidity vector and age vector. Its performance was assessed by Spearman correlation and by receiver operating characteristic (ROC) curves. Results: Our cohort had 319,775 ILI inpatient cases, of which 8.82% entered ICU and 3.83% died at hospital discharge. The significant comorbidity attributes varied in each age stratum: congenital anomaly and heart failure in the age from 0 to 5 years; congenital anomaly, cancer, heart failure, nondialyzed renal insufficiency, and transplant in the age from 6 to 17 years; cancer, diabetes, heart failure, stroke, chronic obstructive pulmonary disease (COPD), liver cirrhosis, nondialyzed renal insufficiency, and human immunodeficiency virus (HIV) in the age from 18 to 44 years; heart failure, stroke, and nondialyzed renal insufficiency in the age from 45 to 64 years; heart failure, COPD, and nondialyzed renal insufficiency in the age from 65 to 74 years; tuberculosis, heart failure, and nondialyzed renal insufficiency in the age from 75 years and above. Comorbidity vector was (heart failure, nondialyzed renal insufficiency, cancer, tuberculosis, stroke, congenital anomaly, transplant, HIV). Age vector was (1, 1, 6<=age<45, 75<=age, 18<=age<65, 0<age<=18, 6<=age<18, 18<=age<45). Comorbidity score, the dot product of comorbidity vector and age vector, showed significant correlation with hospitalization cost (Spearman rho = 0.1885, P < .0001), and with LOS (Spearman rho = 0.1717, P < .0001). Its ROC area-under-curves (AUC) were 0.7454 with death and 0.6840 with ICU. Conclusions: Higher clinical alertness should be set once an ILI case matches the following attributes: heart failure in any age, nondialyzed renal insufficiency in any age, cancer in school-age children up to mid-age adults, tuberculosis in the elderly, stroke in adults, congenital anomaly in children and adolescents, transplant in school-age up to adolescents, or HIV in young adults. The risk estimation would facilitate us to address population measures for upcoming severe influenza epidemics, and further allocate resources optimally for prevention and control in public health decision-making.

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Use of antivirals and antibiotics in pandemic influenza: findings from the UK MOSAIC Study

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Background: Influenza outbreaks are unpredictable and clinical strategies that are appropriate for seasonal influenza are not necessarily those applicable during pandemics. Although the 2009-2010 influenza pandemic strain (pH1N1) generally caused mild disease, many severe cases occurred in relatively young people; pH1N1 is estimated to have caused 250,000 deaths worldwide during June 2009-August 2010. The large number of patients admitted to hospital with influenza during successive waves of disease provided a unique opportunity to study the immunopathogenesis and determinants of severity in a relatively uniform outbreak. The Mechanisms of Severe Acute Influenza Consortium (MOSAIC) was established in 2009 to conduct an integrated study of host, pathogen, and copathogen in hospitalised cases. Materials and Methods: We recruited 255 adults and children with influenza-like illness (ILI) from 10 hospitals in London and Liverpool during the second (2009/10) and third postpandemic (2010/11) waves. We developed detailed and specific protocols, standardised sampling

regimens, and an archiving strategy within a few weeks of launch. Sequential samples were obtained from the respiratory tract and blood throughout illness and recovery. Patients with ILI caused by other viral and bacterial pathogens were also recruited along with asymptomatic healthy controls. We accumulated an 8000 sample biobank and a highly detailed database of clinical information with approximately 4000 data fields per patient. Results: In total, 170 patients had confirmed influenza (87% pH1N1); the cause of ILI was determined in noninfluenza cases. We will describe the clinical characteristics, presentation, severity, and outcomes of patients in the MOSAIC cohort, focusing on the incidence and significance of viral and bacteriological detection and anti-infectives used prior to and during hospitalisation, comparing key clinical findings from analysis of patients recruited during 2 successive epidemic waves. Previously, it has been shown that the UK's third wave was surprisingly severe, with many hospitalisations and fatalities. Possible explanations include virus evolution, cocirculating pathogens, and changes in prescribing patterns in primary and secondary care. In the MOSAIC cohort of 170 third-wave patients, only 5 (3%) received preadmission antivirals despite many being in known to be at high risk of complicated influenza, compared with a reported rate of prehospital antiviral use of 10%-12% in the UK. By contrast, 38 (23%) of 164 third-wave patients (with data available) had received antibiotics. Conclusions: Launching an intensive and complex study with 45 principal coinvestigators during the pandemic was challenging but achievable. We believe that earlier administration of antivirals (rather than antibiotics) might have prevented some hospitalisations in our cohort.

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P2-614 SCHOLARSHIP International Research Scholarship

Predicting mortality among hospitalized children with respiratory illness in Western Kenya, 2009-2012

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Background: Pediatric respiratory disease is a major cause of morbidity and mortality in the developing world; 70% of global deaths from respiratory disease in children occur in Africa and South East Asia. In Kenya, approximately 16% of annual deaths in children < 5 years of age are attributed to acute respiratory infections (ARI). In most health facilities in Kenya, resources are limited; having a better understanding of which children hospitalized with respiratory illness are at greatest risk of severe outcomes could improve patient triage and inform resource allocation. We developed a modified Respiratory Index of Severity in Children (mRISC) scoring system using easy to monitor syndrome-based risk factors for in-hospital mortality in children aged less than 5 years that were hospitalized with a respiratory illness in Siaya District Hospital (SDH) in Western Kenya. Materials and Methods: Since August 2009, the Kenya Medical Research Institute and the US Centers for Disease Control (KEMRI/CDC) have conducted hospital-based surveillance for severe respiratory illness at Siaya District Hospital (SDH) in rural Western Kenya. Surveillance officers, mostly nurses, enrolled all consenting patients who were admitted to SDH with severe respiratory illness (defined as acute onset of cough or difficulty breathing within the last 14 days requiring hospital admission). We analyzed data from children < 5 years old who were hospitalized at SDH between August 2009 and July 2012. We developed a multivariable logistic regression model for in-hospital mortality using patient characteristics identified at admission, including demographics, co-morbidities, clinical signs and symptoms, and growth standards. Points were assigned to risk factors based on their β coefficients (rounded off to the nearest integer) in the multivariable model. The mRISC score for each child was developed by adding up the points assigned to each risk factor present, with a higher score corresponding to a greater number of possible risk factors for mortality. Ability of the model to discrimate between children who died and those who did not was evaluated using the concordance (or *c*) statistic. Using bootstrap samples, with 500 replications we re-estimated the coefficients and

their corresponding 95% confidence intervals (CIs) as well as the optimism of the model. Results: We analyzed data from 3,581 children hospitalized with respiratory illness; including 218 (6%) respiratory deaths. The median age was 12 months (10 months among those who died), and 54% (52% among those who died) were male. Caretaker-reported history of unconsciousness (aOR = 2.4; 95% CI 1.6-3.5), inability to drink or breastfeed (aOR = 2.0; 95% CI 1.3-3.0), chest wall in-drawing (aOR = 2.1; 95% CI 1.5-3.1), not being alert on physical exam (aOR = 8.6; 95% CI 5.5-13.4), low weight-for-age (aOR = 2.1; 95% CI 1.4-3.2), very low weight-for-age (aOR = 3.9; 95% CI 2.8-5.5) and admission diagnosis of dehydration (aOR = 2.0; 95% CI 1.4-2.8) were positively associated with in-hospital mortality. The mRISC score ranged from -2 to 7. The positive predictive value for mortality increased with increasing mRISC scores from 6% at a cut-off score of ≥ -2 to 80% for patients with a cut-off score of ≥ 6. The prediction model also showed good discriminating power, as measured by c-statistics of 0.852 in the original dataset and 0.854 in 500 bootstrap samples. Conclusions: A simple modified RISC scoring system based on a set of easily measurable clinical features at admission was able to identify children at greater risk of death from respiratory illness in Kenya.

P2-615 SCHOLARSHIP International Research Scholarship

Physician's knowledge, attitudes, and practices for pandemic, seasonal, and avian influenza in Indonesia

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Background: Indonesia has reported the most confirmed human cases of highly pathogenic avian influenza (HPAI) A(H5N1) virus infection worldwide with 192 cases and 83% mortality since 2005. Most human cases of HPAI H5N1 have occured in the western part of the main Indonesia island of Java. We conducted a survey among primary care and hospital-based physicians in 2 districts in Western Java, East Jakarta, and Bogor, to determine the knowledge, attitudes and practices (KAP) of physicians regarding recognition, influenza testing, treatment, and prevention recommendations for seasonal influenza, influenza A(H1N1)pdm09, and HPAI H5N1. This study reports on the findings of the cross-sectional KAP survey conducted during March to June 2012. Materials and Methods: The study population was comprised of practicing physicians providing medical care for adult and/or pediatric patients at government or private sector health facilities in East Jakarta and Bogor Districts. The study utilized a list of all registered physicians in the 2 districts, and used purposive sampling to select general primary care clinicians and subspecialists. Study personnel were trained in the use of a standardized data collection instrument, and face-to-face interviews with participants were conducted in Bahasa Indonesia language. Among 1130 registered physicians recruited in the 2 districts, 576 declined to participate and 554 were enrolled in the survey. Results were analyzed using descriptive statistics. Results: Of the 554 physicians enrolled, 43% were from East Jakarta and 57% from Bogor; 87% were general practitioners and 13% were subspecialists. Overall, 71% of physicians reported providing outpatient care only, 2% provided inpatient care only, and 27% provided both outpatient and inpatient care. Clinical signs and symptoms believed by enrolled physicians to be associated with influenza in outpatients and hospitalized patients were fever (96%), cough (83%), and runny nose (72%) for seasonal influenza; fever (86%), cough (65%), nasal congestion (42%), and sore throat (40%) for A(H1N1)pdm09; and fever (91%), cough (73%), runny nose (53%), and sore throat (43%) for HPAI H5N1. Hospital-based physicians reported shortness of breath as a symptom in at least 50% of seasonal influenza, A(H1N1)pdm09, and HPAI H5N1 patients admitted to hospital. For outpatients, over 90% of physicians reported ever making a clinical diagnosis of seasonal influenza, only 2% diagnosed A(H1N1)pdm09, and 4% had diagnosed a case of suspected HPAI H5N1. For hospitalized patients, 10% of physicians reported ever making a clinical diagnosis of seasonal influenza, 0.4% diagnosed A(H1N1)pdm09, and 1% had diagnosed a suspected case of HPAI H5N1. Only 2% of physicians ordered any kind of seasonal influenza testing for outpatients and 1% for hospitalized patients. Very few physicians ordered A(H1N1)pdm09 testing, only 1% for outpatients and 0.2% for hospitalized patients. For H5N1 testing, 1% of physicians ordered testing for outpatients and 1%

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ordered testing for hopitalized patients. Fifty-one percent of physicians prescribed antibiotics and only 2% prescribed antiviral treatment for influenza patients. For clinically diagnosed seasonal influenza patients, physicians were most likely not to order influenza testing because they thought it was unnecessary (37%), too expensive (33%), or unavailable at hospitals (28%). Conclusions: The findings indicate that most physician respondents were familiar with common signs and symptoms of seasonal, pandemic, and HPAI H5N1 influenza. However, while most physicians reported making a clinical diagnosis of seasonal influenza in outpatients, few had ever diagnosed A(H1N1)pdm09 or HPAI H5N1 in outpatients. Overall, a very low percentage of participants had ever diagnosed seasonal, A(H1N1)pdm09, or HPAI H5N1 in hospitalized patients, suggesting that physician awareness that influenza can result in severe disease is limited. Use of influenza testing was low in outpatients and hospitalized patients, and use of antiviral treatment was very low for clinically-diagnosed influenza patients. These findings suggest that much more education of physicians is needed about testing, severity, and antiviral treatment of influenza in Western Java. Further research is also needed to explore health system barriers for influenza diagnostic testing and availability of antivirals for treatment of influenza.

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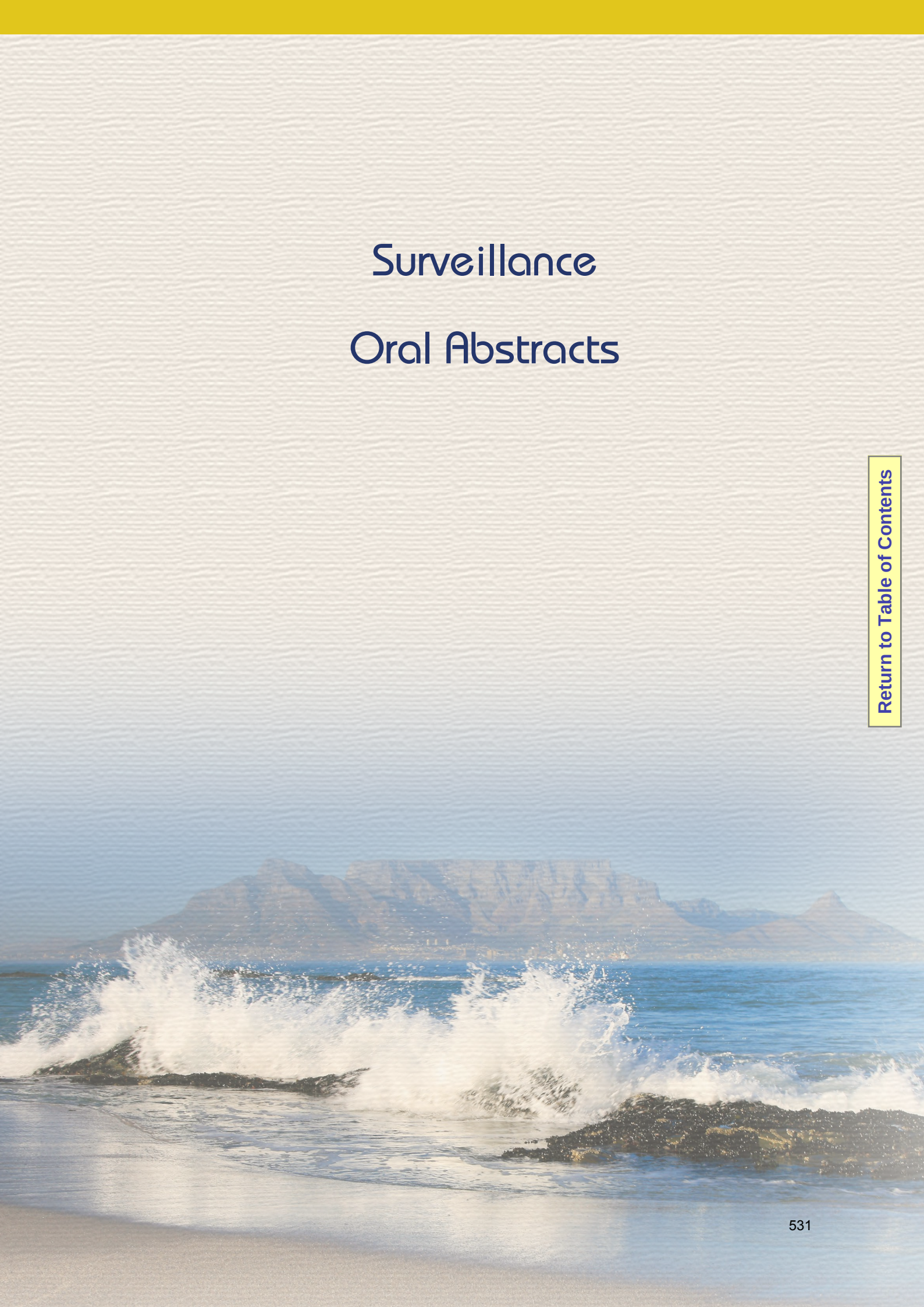

O-894

Southern Hemisphere Influenza and Vaccine Effectiveness Research and Surveillance (SHIVERS) in New Zealand

QS Huang[1], S Roberts[2], C McArthur[2], M Baker[3], C Grant[5], D Williamson[2], A Trenholme[4], C Wong[4], S Taylor[4], T Wood[1], A Bissielo[1], G Mackereth[1], D Bandaranayake[1], R Hall[1], N Turner[5], N Pierse[3], P Thomas[6], R Webby[6], D Gross[7], J Duque[7], and MA Widdowson[7], on behalf of the SHIVERS investigation team*

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Background: The Southern Hemisphere Influenza and Vaccine Effectiveness Research and Surveillance (SHIVERS) project was established in October 2011. It is a multicentre and multidisciplinary collaboration between the Institute of Environmental Science and Research, Auckland District Health Board (ADHB), Counties Manukau District Health Board (CMDHB), University of Otago, University of Auckland, WHO Collaborating Centre at St Jude Children's Hospital and the US Centers for Disease Control and Prevention. The SHIVERS project has 9 objectives: 1) determine the incidence and prevalence of severe acute respiratory infections; 2) assess influenza vaccine effectiveness; 3) study the interaction between influenza virus and other pathogens; 4) ascertain the causes of respiratory mortality; 5) determine the incidence and prevalence of nonsevere respiratory illness; 6) conduct an influenza seroprevalence study; 7) determine influenza risk factors; 8) study the immune response to influenza; 9) determine the healthcare and societal economic burden and vaccine cost-effectiveness of influenza. Materials and Methods: To address the 9 objectives, we have proposed *2 surveillance systems for the 838,000 residents living in Auckland's ADHB and CMDHB regions:* 1) hospital-based surveillance: enhanced, active, year-round surveillance for hospitalized severe acute respiratory infection (SARI) cases and a proportion of non-SARI respiratory cases, ICU admissions and deaths with laboratory confirmation for influenza and other respiratory infections; 2) community-based surveillance: enhanced, active, year-round sentinel general practice surveillance for illness caused by influenza and other respiratory pathogens. Results: Since 30 April 2012, four hospitals serving ADHB and CMDHB have been enrolling SARI cases. As of 30 September 2012, a total of 4417 patients hospitalised with suspected respiratory infections were assessed, and 2033 (46%) patients met the SARI case definition. Of 1430 SARI cases with specimens taken, 324 (23%) tested positive for influenza. Infants aged under 1 year experienced the highest rate of influenza hospitalization (282/100,000) followed by persons 80 years and older (144/100,000). Rates among Pacific People and Maori were 93/100,000 and 46/100,000, respectively, compared with 21/100,000 among those of European descent. The lower socioeconomic status (SES) patients had the highest rate of influenza hospitalization (32/100,000) followed by middle SES patients (20/100,000) and upper SES patients (12/100,000). Conclusions: Results from the first season of hospital-based surveillance by SHIVERS suggest a high burden of severe influenza among the young, elderly, ethnic minorities and lower SES status patients. Community-based surveillance will commence in 2013 and will document the burden of less severe disease. The hospital and community-based surveillance systems will provide a research platform over the next 4 years to enable focus on the other SHIVERS objectives and therefore comprehensively investigate influenza epidemiology, aetiology, immunology and vaccine effectiveness. The outcomes of the SHIVERS project are to enable enhanced disease surveillance, assist with early detection and prediction, guide targeted vaccination strategies for the population and subgroups and inform better vaccine design, optimize clinical management and laboratory diagnosis, identify risk factors, understand host immune responses and identify better immune diagnostic markers.

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Applications of pyrosequencing in the molecular surveillance of influenza

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Background: Surveillance plays an important role in controlling influenza globally. It provides information about the predominant circulating influenza viruses for the WHO biannual recommendations on vaccine viruses. Currently, there are 2 subtypes of influenza A (H3N2 and H1N1pdm09) and 2 antigenically distinct lineages of influenza B (Yamagata and Victoria lineages) viruses co-circulating in the human population. It is important to monitor the changes in these viruses in order to select the best representative viruses as vaccine candidates. Pyrosequencing is a DNA sequencing method based on the sequencing-by-synthesis principle. In recent years, pyrosequencing has become increasingly important in several surveillance areas because it offers rapid high-throughput screening. Pyrosequencing can measure a short sequence of up to 50 nucleotides in real time from 96 samples simultaneously within 1 hour and has the capacity to quantify a target virus in a mixed viral population based on single nucleotide polymorphism sites. Materials and Methods: Three pyrosequencing-based methods were developed in our laboratory, designed to (i) screen for the antiviral oseltamivir resistance marker H275Y in the neuraminidase of both seasonal and pandemic 2009 H1N1 clinical samples and isolates, (ii) detect and subtype potential human reassortant influenza A viruses based on signature sequences of all 8 individual influenza genes, and (iii) differentiate both the influenza B lineage and its sublineages based on the hemagglutinin gene sequence. Results: Over 1000 samples containing either seasonal H1N1 or H1N1pdm09 virus were screened by pyrosequencing for the H275Y mutation, known to cause a high level of resistance to the antiviral drug oseltamivir. An accuracy of 100% was achieved when cross-checked with the conventional Sanger sequencing and/or neuraminidase inhibition assay. In addition, the assay was able to accurately quantify the H275Y mutant in 5%-95% mixtures with its wild-type virus in the clinical samples. A pyrosequencing-based assay was also developed to detect potential influenza A reassortant viruses. When applied to over 120 original specimens and laboratory isolates containing H1N1, H3N2, H1N1pdm09, or H1N2 viruses, the assay was able to correctly identify each virus subtype in all cases. A third pyrosequencing assay was designed for influenza B virus lineage differentiation. When tested on more than 300 samples, this assay was able to identify both the Yamagata and Victoria lineages in all samples. Further, this assay could differentiate the Yamagata viruses into 2 currently co-circulating sublineages. Conclusions: The pyrosequencing-based assays described in this report provide additional molecular tools that enable the detection of viruses with known mutations that cause antiviral drug resistance, the determination of the seasonal influenza A virus subtypes as well as the identification of possible influenza A reassortants, and the differentiation of the lineages and sublineages of influenza B viruses. Overall, our results demonstrate that these assays are robust with a high-throughput capacity that will make it possible to perform large-scale screening in a timely manner. In conclusion, pyrosequencing represents a valuable cost-effective addition to the current molecular assays for influenza surveillance.

O-896

The “Global Influenza Hospital Surveillance Network” (GIHSN): a step forward

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Background: Because the circulating strains, the intensity and severity of the influenza epidemic vary between years and between countries, networks of high-quality surveillance systems are very relevant

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tools to get a better understanding of influenza and of the effect of influenza vaccination programs. While several surveillance systems already exist, very few focus on very specific outcomes (laboratory-confirmed influenza) and on serious disease requiring hospitalization. Moreover, the low level of standardization across sites usually does not allow for a global analysis of the results. In order to fill this public health knowledge gap, a public-private partnership was initiated in 2012 between Sanofi Pasteur, a Centre of Excellence experienced in hospital influenza surveillance from Spain (Centro Superior de Investigación en Salud Pública – CSISP), Fondation Merieux and several country sites. The pooled analysis of the results was presented in June 2013 at the first annual meeting of this network. Materials and Methods: The network is composed of country sites (mainly affiliated with health authorities) supervising a group of 1-5 hospitals each. Each site follows a standard core protocol with an option to add site-specific questions of local research interest. In each hospital, all consecutive patients admitted to the hospital during the influenza season with any antecedent of influenza-like illness (ILI) in the preceding 7 days are swabbed. All samples are analyzed by RT-PCR for confirmation. Demographic characteristics, influenza-related risk factors and influenza vaccination status are collected for all swabbed participants. In some sites, presence of other respiratory viruses is also assessed. Influenza cases were compared with negative controls to assess vaccine effectiveness. Technical assistance and support are provided by CSISP to ensure and facilitate the standardization of data collection and standard operating procedures across all sites. The pooled analyses describe the burden of severe influenza disease by strain and age group as well as vaccine effectiveness when vaccine coverage is sufficient. During the 2012-2013 influenza season, the network included 21 large hospitals (5 in Spain, 5 in France, 4 in the Russian Federation and 7 in Turkey). A pooled analysis was performed by the CSISP and discussed at the annual meeting between sites and external experts. Results: During the 2012-2013 influenza season, 8078 patients were screened and 4922 patients were admitted to the participating hospitals with an ILI in the last 7 days. More than 1000 laboratory-confirmed influenza cases were identified. These data allowed computing for some hospital the rate of hospitalization related to influenza for some age group. The main circulating strain was H1N1 (55%) followed by B and H3N2. Vaccine effectiveness was estimated by strain and age group. For example, the vaccine effectiveness in adults against hospitalizations from influenza and related complications against the B/Yamagata strain in Spain was 45% (95%CI: 17%-63%) this year. Conclusions: The first year of this network already produced relevant, useful and timely results (before the next season). Additional countries already agreed to join the network next season. This will add geographic representation including the southern hemisphere. The GIHSN, with its broad regional representativeness and sustainable framework over sequential influenza seasons should contribute significantly to our knowledge of influenza epidemiology. Such international public-private partnerships can also provide a strong platform for further research and public health activities. Additional components could be added to respond to other scientific questions. The experience so far has been very rewarding. However, additional partners and contributors will be needed to strengthen this initiative and ensure its sustainability for the benefit of the influenza scientific knowledge and global public health. This network activity is partly funded by Sanofi Pasteur.

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Etiology severe acute respiratory infection during 2 years of hospital-based surveillance in Madagascar

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Background: The burden of acute respiratory infection (ARI) in outpatients is well documented, but little is known on the impact of ARI in hospital care. In Madagascar, hospital-based surveillance of

severe ARI (SARI) has been in effect since November 2010 in 2 hospitals in Antananarivo and Moramanga. Here we report the distribution of virus and bacteria associated with SARI patients during our first 2 years of surveillance. We also present potential risk factors in relation to the evolution and outcome of severe respiratory illnesses. Materials and Methods: Patients of all ages hospitalized for acute respiratory infections were enrolled following admission in either of the 2 surveillance hospitals. The SARI case definition was adapted from the WHO. Demographic, epidemiological, clinical information as well as clinical specimens were collected from consenting patients. Nasopharyngeal samples were tested for 14 respiratory viruses whereas sera samples were screened for a large panel of bacteria. Deaths, clinical aggravation, and hospitalization exceeding 10 days were considered as aggravation outcomes. Results: From November 2010 to October 2012, 565 patients meeting the SARI case definition were recruited. Twenty-seven cases (4.8%) of worsening condition and 32 deaths (5.7%) were registered. Ages ranged from 0 to 81 years. Children younger than 5 years were the most represented group among SARI inpatients (75.8%; 428/565). Bronchiolitis (39.8%) was the most reported diagnosis at admission followed by bronchopneumonia (15.2%) and pneumonia (9.7%). Other lower respiratory tract infections were diagnosed in 19.1% of the included patients. At least 1 pathogen was detected in 86% (486/565) of inpatients, of which 45.5% (221/486) were positive only for viruses and 12.6% (61/486) were positive only for bacteria. Co-infection between viruses and bacteria occurred in 42 % (204/486) of infected patients. The highest percentage of co-infection—87% (177/204)—was reported in children younger than 5 years. Among viral infections, RSV (33.4%), Influenza A (22.6%), and rhinovirus (14.5%) were the most detected, whereas *S. pneumoniae* (45%) and *H influenzae* (17.9%) were the most common bacterial infections. In children younger than 5 years, comorbidities and low household incomes were significantly associated with worsening condition during hospitalization. Conclusions: This study showed that SARI associated with viral and bacterial infections are an important cause of hospitalization in Madagascar. Among SARI cases, children younger than 5 years are the most represented. Interestingly, the most prevalent viruses detected in SARI cases are the same found in our previous study focusing on ARI within the community. These findings expand our knowledge about the epidemiological features of respiratory pathogens in hospitalized patients with clinical evidences of ARI in Madagascar. It will be helpful to assess vaccine impact but also to recommend appropriate control measures. However, increasing surveillance in hospitals will lead to more representative data and better guidance.

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Establishing a web-based application for respiratory pathogen biosurveillance within a diverse global partner network

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Background: Lessons learned from the 2009 influenza pandemic have driven many changes in the standards and practices of respiratory disease surveillance worldwide. The US Department of Defense (DoD) Armed Forces Health Surveillance Center (AFHSC) and the Johns Hopkins University Applied Physics Lab (JHU/APL) have partnered to develop an Internet-based data management system known as the Respiratory Disease Dashboard (RDD). The goal of the RDD is to provide AFHSC partners with a centralized, standardized, user-friendly system for the monitoring and tracking of lab-confirmed respiratory pathogens. Objective: The objective of this abstract is to communicate the development of a DoD respiratory pathogen surveillance system used in a multitude of settings worldwide. Methods: The RDD is based on the OpenESSENCE platform and developed as a web-based application. It is highly flexible and can be modified by a public health administrator. Currently 10 DoD partner laboratories are actively entering respiratory pathogen data into the RDD, encompassing specimens from sentinel sites in 26 countries. Users have been individually trained on data entry and visualization within the application. Most laboratories currently only test respiratory specimens for influenza, but select laboratories have the ability to routinely test for an expanded respiratory panel, including RSV, adenovirus, coronavirus, *Haemophilus influenzae*, and other pathogens. Results: To date, 26,372 respiratory pathogens have been isolated from 110,466 specimens and input into the RDD. Of the isolated pathogens, 23,215 were influenza, 272 were

Haemophilus influenzae, 588 were rhinovirus, 131 were coxsackie/echoviruses, and the remaining 2166 were other respiratory pathogens. Conclusions: The RDD is a user-accepted platform for data entry and visualization and provides a central database from which to pull global DoD respiratory information. Four additional DoD partner laboratories will be contributing to the RDD in the first quarter of 2013. The RDD application is being explored for use in enteric pathogen data reporting and visualization.

O-899

Patterns of influenza circulation—Latin America and the Caribbean, 2010-2012

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Background: Patterns of influenza circulation, including the co-circulation of different virus lineages, are important to determine vaccine composition and timing of application, to ensure maximum vaccine effectiveness. These patterns, however, have not been well described in all Latin American subregions. We analyzed influenza virologic surveillance data from Latin America and the Caribbean (LAC) to better understand the patterns of influenza circulation and to inform decision-making about influenza vaccine timing and composition, as both Northern (NH) and Southern Hemisphere (SH) vaccines are used in the region. Materials and Methods: In LAC, there are a total of 23 National Influenza Centers (NICs) (22 countries) and an additional four national laboratories that conduct virologic surveillance, determining types and subtypes of influenza viruses, using real-time RT-PCR techniques. These laboratories send, on a routine basis, samples to a WHO Collaborating Center (CC) for additional viral characterization. We analyzed viral influenza data provided to WHO from all 23 NICs and four national laboratories for the period January 2010-December 2012. Grouping the laboratories into four subregions (Mexico/Central American, Caribbean, Andean, and Southern Cone), we analyzed peak influenza percent positivity, predominant virus type/subtype, as well as virus lineages over the study period. Results: During 2010-2012, > 550,000 samples were tested for influenza in LAC. By subregion, 17.5% were from Mexico/Central America, 5% from the Caribbean, 14.7% from the Andean countries, and 62.8% from the Southern Cone. Among the samples tested (n = 586,317), 9.9% were positive for influenza (78.8% influenza A [27.5% influenza A(H1N1)pdm09, 43.3% A(H3N2), 28.9% influenza A not subtyped] and 21.2% influenza B). In Mexico/Central America, during the study period, influenza activity peaked in epidemiologic week (EW) 35 [2010, predominant virus A(H3N2), EW 42 [2011, predominant influenza A(H1N1)pdm09], and EW 02 [2012, predominant influenza A(H1N1)pdm09]. In the Caribbean, influenza activity peaked in EW 37 [2010, predominant influenza A(H3N2) and influenza A(H1N1)pdm09 and 2011, predominant influenza A(H3N2)] and EW 23 [2012, predominant influenza B and influenza A(H3N2)]. In the Andean countries, influenza activity peaked in EW 31 [2010, predominant influenza A(H1N1)pdm09], EW 39 [2011, predominant influenza A(H1N1)pdm09], and EW 23 [2012, predominant influenza A(H1N1)pdm09]. In the Southern Cone, influenza activity peaked in EW 38 [2010 and 2012, predominant influenza A(H3N2)] and EW 32 [2011, predominant influenza A(H1N1)pdm09 and influenza A(H3N2)]. During 2010-2012, 6.9% (n = 4070) of the influenza-positive samples were sent to a WHO-CC for additional characterization. During 2010 and 2011, 100% of the influenza A(H3N2) viruses antigenically characterized were A/Perth/16/2009-like virus (WHO vaccine-recommended strain NH 2011/12 and SH 2012 vaccine component). In April 2012, A/Victoria/361/2011-like H3N2 virus emerged and represented 23.8% of the influenza A(H3N2) viruses analyzed—first detected in the Andean countries (April 2012), followed by the Southern Cone (June 2012) and then Mexico/Central America (July 2012). No A/Victoria/361/2011-like viruses were detected in the Caribbean samples analyzed. During 2010 and 2011, influenza B-Yamagata lineage viruses were detected sporadically. However, in December 2011, more sustained circulation of Yamagata viruses (NH 2012/13 and SH 2013 vaccine component) was detected—first in the Caribbean, followed by the Southern Cone (February 2012), Mexico/Central America (May 2012) and then the Andean countries (June 2012). Overall, in 2012, B-Yamagata lineage viruses represented 30.7% (n = 70) of the B viruses characterized. Conclusions: The timing of influenza virus peak circulation was similar within most LAC subregions during the study period, but predominant types/subtypes of viruses varied from year to year. The inclusion of A/Victoria/361/2011-

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like H3N2 virus in the vaccine for the NH 2012/13 and SH 2013 influenza seasons was well matched to the circulating H3N2 viruses observed in LAC in 2012; however, the inclusion of an influenza B-Yamagata lineage virus in the NH 2012/13 and SH 2013 vaccines might not well match currently circulating viruses, as this lineage represented less than one-third of the B viruses characterized during 2012. These data are useful to inform public health policy decisions such as the timing of vaccine application and the use of quadrivalent influenza vaccines that include both B-Yamagata and Victoria lineages.

O-906

Incursion of clade 2.3.2.1 avian influenza viruses into the Mekong Delta of Vietnam

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Background: The geographic distribution of influenza A/H5 variants in Vietnam is closely monitored through both active and passive poultry surveillance programs. Clade 1 H5N1 viruses have been detected in Vietnam continuously since 2003; however, since 2004, clade 1 descendants have been almost exclusively restricted to southern provinces of the Mekong delta. In contrast, influenza A/H5 transmission patterns in northern Vietnam have been characterized by repeated incursions from southern China and frequent lineage replacements, with predominance of clade 2 variants (first clade 2.3.2 from 2005 to 2007; then clade 2.3.4 from 2007 to 2009; and since 2010, the emergent 2.3.2.1 A, B, and C subclades). Material and Methods: Active surveillance to monitor silent circulation of avian influenza through live bird markets (LBMs) was established in September 2011, and thus far sampling has involved 268 markets distributed over 30 provinces. Results: H5N1 is widespread in all regions of the country, with overall prevalence of approximately 4.15% in ducks sampled at LBM. In 268 LBMs visited between September 2011 to February 2012, 187 markets were positive with influenza A (69.8%), 79 markets were positive with H5 (29.5%), and 69 markets were positive with H5N1 (25.8%). During the first months of 2013, cocirculation of both clade 1.1 and clade 2.3.2.1 variants were detected from poultry outbreaks and in live bird markets of southern Vietnam. Conclusions: Implications for risk assessment and vaccination control programs will be discussed.

O-907

Improving the representativeness of influenza viruses to inform seasonal influenza vaccine strain selection

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Background: The production of seasonal influenza vaccines requires continuous global monitoring of influenza viruses for the emergence and circulation of new antigenic drift variants. Sharing of viruses by National Influenza Centres (NICs) within GISRS is the cornerstone of this process. WHO 2010 guidance recommends that viruses shared for vaccine strain selection are representative of age groups, clinical settings, geographical location, and phase of influenza activity. This paper describes the first analysis of timeliness and geographic and epidemiologic representativeness of influenza

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viruses shared by NICs in the WHO European Region (WHO/Europe) during the 2010-2011 and 2011-2012 northern hemisphere influenza seasons. The results can be used to improve virus sharing within GISRS. **Materials and Methods:** The following variables provided alongside influenza viruses shared during 2 seasons were considered: completeness of forms, timeliness of virus collection by NICs and submission to WHO CC, geographic location, virus type/subtype, patient age and clinical setting. Timeliness was evaluated based on the date of shipment receipt by WHO CC compared with the deadline of viruses being characterised by the fifth week of the year for inclusion in the February Vaccine Composition Meeting (VCM). Geographic representativeness was analysed by grouping the WHO/Europe countries into 5 subregions according to UN definitions (Southern, Northern, Eastern, and Western Europe, Central and Western Asia; http://unstats.un.org/unsd/methods/m49/m49regin.htm). **Results:** During 2 influenza seasons, NICs representing 40 countries shared 2954 influenza viruses with WHO CC London. For both seasons, forms were 100% complete for Centre ID, specimen type, and virus type/subtype. Data was available on 99% of collection dates, 84% of patient ages and only 45% clinical setting. During 2010-2011, 1204 out of 1837 (66%) viruses received from NICs from 38 countries were collected before week 5/2011, and 606 (50%) were shipped within the characterisation deadline. During 2011-2012, 537 out of 1117 (50%) viruses received from NICs in 35 countries were collected before week 5/2012, and 340 (63%) were shipped within the characterisation deadline. For both seasons combined, 795 viruses were collected but not shipped by the characterisation deadline. The average time between specimen collection date and shipment was 90 days and 54 days for the first and second seasons, respectively, but varied significantly by subregion. Most viruses were shared from Eastern, Northern, and Southern Europe, while Western Europe, West Asia and in particular Central Asia submitted fewer viruses. During both seasons, viruses were shared from both mild and severe cases and from several age groups**. Conclusions:** NICs in WHO/Europe during 2 influenza seasons shared 2954 influenza viruses with GISRS, representing a significant contribution to the VCM. Viruses shared in time for the February VCM represented different geographic subregions, age groups and severity of disease. However, approximately half of viruses whose specimen collection date was before the VCM deadline were not shared in time for the February VCM, there was on average a 72 days delay between specimen collection date and shipment, a number of sub-regions were underrepresented, and due to missing data, the true extent of demographic and epidemiologic representativeness could not be determined. Late receipt of specimens by the WHO CC may largely be explained by the fact that influenza seasons peak concomitantly with or after the virus characterization deadline, notably for the eastern versus the western part of the region. Nevertheless, these samples are highly valued for the Southern Hemisphere VCM held in September each year, with the focus of the analysis being on viruses with collection dates after 1 February of the corresponding year. Greater insight into virus selection criteria by countries, the reasons for viruses not being shipped in time for the February VCM as well as more complete data on shared viruses is needed to fully understand the representativeness of viruses being shared within GISRS, to assess the importance of this for vaccine strain selection and whether WHO guidance should be adjusted. The results will guide enhancement of virus sharing in WHO/Europe and globally.

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O-908

Influenza epidemiology in Bangladesh: a pre- and post-pandemic comparison of the national hospital-based influenza surveillance findings (2007-2013)

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Background: In 2007, the Institute of Epidemiology, Disease Control and Research (IEDCR) and the International Centre for Diarrhoeal Disease Research, Bangladesh (icddr,b), supported by the Centers for Disease Control and Prevention (CDC), established national hospital-based influenza surveillance in 12 hospitals in Bangladesh to characterize circulating influenza strains and the

seasonal distribution of influenza infections. Here we present surveillance data focusing on how the introduction of the 2009 pandemic strain impacted the seasonality, strain dominance and age distribution of influenza infections in the country. Material and Methods: In each surveillance hospital, physicians systematically identified patients aged ≥ 5 years with severe acute respiratory illness (SARI; hospitalized with history of measured/subjective fever and cough/sore throat in the previous 7 days) or patients aged < 5 years with severe pneumonia (SP; hospitalized with cough/difficulty breathing with ≥ 1 of 6 danger signs) or presenting as outpatients with influenza-like illness (ILI; history of subjective/measured fever and cough/sore throat within 7 days of onset). Staff collected demographics and clinical history data using a structured questionnaire and obtained nasopharyngeal and throat swabs for real-time reverse transcription polymerase chain reaction (rRT-PCR) influenza typing (A and B) and subtyping of positives (A/H1, A/H3, A/H5 and A/H1N1pdm09). We defined influenza epidemiological periods as pre-pandemic (May 2007-May 2009), pandemic (June 2009-July 2010) and post-pandemic (August 2010-February 2013) for comparisons. We defined months of influenza peak activity as months with > 20% influenza positivity among samples tested. Results: A total of 18,311 case-patients' samples were tested for influenza virus; 4902 were during pre-pandemic, 4388 during pandemic and 9021 during post-pandemic periods. Overall, 2486 (14%) samples (SARI 1002/17%, SP 145/5% and ILI 1339/17%) were influenza positive, with influenza A being the dominant type (9%). Among all the hospitalized inpatients, A/H1N1pdm09 was the dominant strain (466/5%). Influenza infection among all case-patients was higher during two post-pandemic as opposed to two pre-pandemic years (15% during 2011-2012 vs. 10% during 2007-2008; $P < .001$). During the pre-pandemic period, there was a single annual peak of influenza activity followed by a 3- to 6-month period with no influenza circulation detected. In contrast, influenza circulation was detected every month from March 2011 to February 2013 in the post-pandemic period. In 2012, there were 2 discrete peaks of influenza activity, the first one during March-May, almost exclusively due to A/H1N1pdm09, and the second one during August-October, predominantly due to influenza B. We observed an increase in influenza B infection (1.4% to 36%) among influenza-positive SARI case-patients in the post-pandemic compared to the pre-pandemic period. Among influenza A-positive SARI case-patients, the pandemic virus A(H1N1)pdm09 was detected by this surveillance platform in August 2009 and has become the dominant influenza strain detected since January 2012, displacing seasonal influenza A/H1 strain completely. Influenza affected all age groups, but the proportion of laboratory-confirmed influenza infections was highest among patients aged 5-19 years old (21%) during the study period. The median age of all influenza positive cases was higher during the pandemic and post-pandemic periods compared to the pre-pandemic period (19 and 20 years vs. 13 years; $P < .001$). The median age of A/H1N1pdm09-positive case-patients was significantly higher than that of case-patients affected by non-pandemic strains (20 years vs 17 years; $P < .001$). Conclusions: The introduction of pandemic virus A(H1N1)pdm09 affected the age distribution of influenza infections and influenza seasonality in Bangladesh, with year-round detection of influenza since March 2011 and increased frequency of peak influenza activity in the post- as compared to the pre-pandemic period. The irregular seasonality patterns may hinder the deployment of effective public health interventions, in particular scheduling an immunization campaign so as to optimize protection before vaccine-induced immunity wanes. Additionally, this increases the amount of time that avian and human influenza are co-circulating in Bangladesh and so increases the risk for re-assortment and emergence of new strains. Support for influenza surveillance should be continued.

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Surveillance and characterization of Influenza viruses circulating in the central region of Chile

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Background: Waterfowl and shorebirds are the main reservoirs of avian influenza viruses (AIVs) in nature, particularly for low pathogenicity (LPAI) strains. Bird migration is believed to be one of the main routes in which the virus is able to spread throughout large geographic areas. Chile forms part of the Pacific migratory flyway, a bird migration route that stretches from the furthermost tip of Alaska to the South American Patagonia. However, influenza surveillance along the southern hemisphere section of this flyway has been limited, even though the only reported highly pathogenic avian influenza (HPAI) outbreak in South America occurred in commercial poultry in Chile in 2002. No other reports of AIV have been made since then in Chile, but it is believed that AIV could still be present. To address this question, several sample effort were undertaken. Material and Methods: During autumn and spring 2012, 509 blood samples were gathered from poultry and 127 from domestic swine. Furthermore, during winter 2012 and summer 2013, 3172 environmental fecal samples were collected, of which 942 corresponded to wild birds, 2072 to domestic poultry and 340 to wild captive birds from zoos and bird rescue centers. Moreover, a total of 68 swine and 7 equine nasal swabs were collected during this period as well. All domestic animal samples, both swabs and sera, were collected from backyard production systems located within the central LGB O´Higgins region of Chile. Wild bird samples were collected in wetlands within this region and from the adjacent Santiago metropolitan area. Blood samples were collected from the brachial vein in birds and the marginal ear vein in swine. Sera samples were subsequently screened by influenza A–specific ELISA kits. Environmental feces and nasal swab samples for real-time PCR screening were collected with single-use sterile swabs and stored separately in cryovials containing PBS-glycerol media and antibiotics in addition to being kept at –70°C until analysis. Samples were screened by real-time RT-PCR for the influenza M gene, and a cycle threshold value of less than 40 was considered positive. Virus isolation was attempted either by cell culture or embryonated chicken eggs, and virus titers were determined by $TCID_{50}$ in MDCK cells. Gene segments were sequenced and then phylogenetically analyzed with the neighbor-joining method. Results: Twenty sera samples came up positive by ELISA and yielded 3.5% influenza A–specific antibody prevalence for poultry and 1.6% for swine. Of the 41 positive samples tested by real-time RT-PCR, the positives were 3.4% in wild birds and 0.9% in poultry. No wild captive bird swabs delivered positive results during screening. The percentage of influenza-positive swine swabs was 1.5%, whereas horse swabs did not yield any positive results. Viral isolation was successful on one of the wild bird samples (*Anas georgica spinicauda*) and genetic analysis revealed a nonpandemic H1N1 virus. Interestingly, even though phylogenetic analysis of the hemagglutinin, neuraminidase and most other internal genes (PB2, PA, M) links this virus closely to other South American AIV isolates, some internal genes (PB1, NP) match closely to North American AIV isolates. Furthermore, a pathogenicity study in BALB/c mice showed no weight loss or disease, although viral particles could be detected in lung homogenates until day six post infection. Conclusions: This is the first report of influenza-positive waterfowl, swine and backyard poultry in Chile, either serologically or by PCR. Even though the one isolate obtained is a LPAI virus, its genetic similarities to other influenza A viruses throughout the continent verify the hypothesis that flyways provide a route in which recombinant events between geographically distant viral strains do occur. Additionally, its capability to successfully replicate in a mammalian host highlights the importance of this study. To fully understand the spread and impact of AIV on wildlife, poultry production and human health in Chile and other South American countries, it is necessary to continue active influenza virus surveillance in the region.

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WHO efforts on global capacity of influenza virus detection and antiviral susceptibility monitoring

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Background: The WHO Global Influenza Surveillance and Response System (GISRS), comprising a vast network of laboratories, has been conducting surveillance to detect and monitor the circulation and the evolution of influenza viruses infecting humans for more than sixty years. Robust laboratory diagnostic measures for virus detection is a key requirement. Recognizing this need, the WHO external quality assessment programme (EQAP) for detection of influenza viruses by RT-PCR was

initiated in 2007. The project, coordinated by WHO's Global Influenza Programme (GIP), has been implemented by the H5 Reference Laboratory and National Influenza Centre at the Centre for Health Protection, Hong Kong Special Administration Region, China, and supported by WHO Collaborating Centres (CCs) for Reference and Research on Influenza, other H5 reference laboratories and WHO regional offices. Monitoring the susceptibility of antiviral drugs is key in terms of updating clinical management guidelines and adjusting the relevant components of national preparedness plans. In the past few years, the need for guidance for laboratories to establish standardized antiviral susceptibility monitoring and results has been identified. Although capacity currently exists in all WHO regions, laboratories use a wide range of protocols, and there is a lack of standards for interpreting and reporting test results. The role of WHO in addressing these aspects is described here. Methods: Laboratory response to avian influenza A(H7N9): Immediately after notification of the emergence of influenza A(H7N9) in China, WHO worked closely with the GISRS CCs to make RT-PCR protocols and reagents available for the detection of the new virus. EQAP: Since 2007 EQAP panels of RNA and/or viruses for RT-PCR detection were sent to participating laboratories once or twice a year. The scope was expanded from initially focusing on influenza A(H5N1) to include other influenza A viruses of pandemic potential as well as influenza B. Laboratory capacity was assessed by including a Good Laboratory Practices questionnaire-based survey with some panels. Antiviral susceptibility monitoring: The results of a global survey conducted in 2010 by the WHO's GIP identified major gaps, and a WHO Expert Working Group on Antiviral Susceptibility was formed in 2011 to review the scope of antiviral surveillance performed by the GISRS, antiviral testing capacity and the laboratory methodologies available. Interpretation of antiviral susceptibility data was also assessed to provide practical guidance for laboratories seeking to implement and monitor the quality of antiviral testing surveillance. Results: A number of RT-PCR protocols for avian influenza A(H7N9) detection were made available on the WHO Web site in a timely manner. Since 2007 the number of laboratories participating in the WHO EQAP has increased from 54 to more than 160. Improvement in the overall accuracy for the detection of all the influenza virus subtypes was observed. Similarly, the number of laboratories that were able to detect A(H5N1) increased. Antiviral susceptibility surveillance strategies, laboratory methodologies, data interpretation and reporting have been examined, and guidance is currently being compiled for GISRS laboratories for publication on the WHO Web site. Conclusions: The WHO EQAP has become a channel to monitor the quality and comparability of the performance of participating laboratories in routine diagnosis and surveillance of seasonal and other emerging influenza viruses by RT-PCR. The programme will continue to be updated and expanded to adapt to changing needs over time, such as the inclusion of avian influenza A(H7N9) viruses in the next panel. Next to vaccines, antiviral drugs are an essential intervention of epidemic and pandemic influenza infection and diseases. The provision of updated technical guidance for establishing antiviral susceptibility monitoring by GISRS and other reference influenza laboratories will help to improve the quality of antiviral resistance testing and to provide global updates of incidence and distribution of resistant influenza viruses of public health significance.

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Circulating seasonal and pandemic influenza A and influenza B virus genotypes from 2009-2012: vaccine strain match and breakthrough infections in South Africa

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Background: Vaccination against seasonal influenza viruses is important to reduce morbidity and mortality annually in the most vulnerable of the population. The Viral Watch surveillance program for influenza-like illness and enhanced surveillance (Enhanced Viral Watch) for severe disease provide epidemiologic and virologic data to describe the annual influenza seasons in South Africa. Materials and Methods: Reverse transcription followed by real-time polymerase chain reaction (RT-PCR) was used to detect influenza A and B infection in: n = 3475 (2009), n = 2071 (2010), n = 2680 (2011) and n = 1518 (2012) respiratory samples followed by subtyping of influenza A positives using the CDC influenza A subtyping kit and protocol. The HA1 region of the influenza hemagglutinin gene was

amplified by strain-specific nested RT-PCR followed by direct sequencing of amplicons. Sequence alignment and phylogenetic analysis were performed using BioEdit version 7.1.7 and MEGA5 programs. The ATIVS tool was used to predict antigenic distances between A(H3N2) vaccine and seasonal strains where antigenic distances ≥ 3.25 predict low reactivity. Results: In 2009 from 1385 influenza positives, the majority of infections were caused by influenza A(H3N2) (720/1385 or 52%) and the pandemic strain, A(H1N1)pdm09 (529/1385 or 38%). Influenza B was a major contributor (425/826 or 51%) followed by influenza A(H3N2) (25%, 209/826) and A(H1N1)pdm09 (21%, 170/826) for the total of 826 positives in 2010. During the 2011 season from the total of 986 positives, influenza A(H1N1)pdm09 contributed to 79% (774/986) of positives, influenza B to 11% (105/986) and A(H3N2) to 10% (101/986). In 2012 a total of 576 positives were detected, with influenza A(H3N2) identified in 58% (332/576) and influenza B in 41% (236/576). Influenza virus breakthrough infections were identified in 44/113 of vaccine recipients in 2009, in 25/81 in 2010, in 14/90 in 2011 and in 8/35 in 2012. Vaccine breakthrough infections associated with influenza A(H1N1) were primarily as a result of infection with the new pandemic strain, A(H1N1)pdm09 in 2009. The mean amino acid distance between A(H1N1)pdm09 strains from 2009 (n = 14) and the seasonal vaccine strain (A/Brisbane /59/2007) was 35.7% (range 35.9%-37.2%). The mean amino acid distance between breakthrough infection strains from 2010-2011 and the A(H1N1)pdm09-like vaccine strain (A/California/07/2009) was < 1%. The majority of vaccine failures associated with influenza A(H3N2) were identified in 2009 and are characterised by antigenic distances ≥ 3.25 compared with the vaccine strain, A/Brisbane/10/2007. Vaccine update in 2010 to match circulating strains resulted in reduction of antigenic distances to < 3.25 for all except 1 strain from a vaccine breakthrough infection. In 2011 lineage 7 was identified as an emerging lineage in South Africa, and circulating strains from 2012 mainly belonged to lineages 7 and 3A. Antigenic distances ≥ 3.25% were observed when circulating and 4 breakthrough infection strains from 2012 were compared with the 2012 and 2013 Southern Hemisphere vaccine strains. Circulating strains from 2009-2012 grouped with both the B/Victoria and B/Yamagata lineages. Eight vaccine breakthrough infection strains from 2010, 1 from 2011 and 1 from 2012 were B/Brisbane-like. Another 2 from 2011-2012 were B/Yamagata-like with mean amino acid distance of 13% compared with the vaccine strain, B/Brisbane/60/2008. The B/Brisbane-like breakthrough infection strains carried the I146V mutation and an additional L58P mutation in 2. Conclusions: The mechanism for vaccine escape needs to be explored further in cases where the antigenic distance between the vaccine and seasonal viruses is < 3%. The emergence of the pandemic influenza A/H1N1 strain in 2009 demonstrates vaccine escape as a result of antigenic shift. Antigenic drift overtime earmarked vaccine escape for influenza A(H3N2) strains in 2009, which coalesced with the introduction of a vaccine strain matched to circulating strains. Vaccine escape is also predicted for influenza A(H3N2) if lineages 7 and 3A viruses would circulate in the 2013 season.

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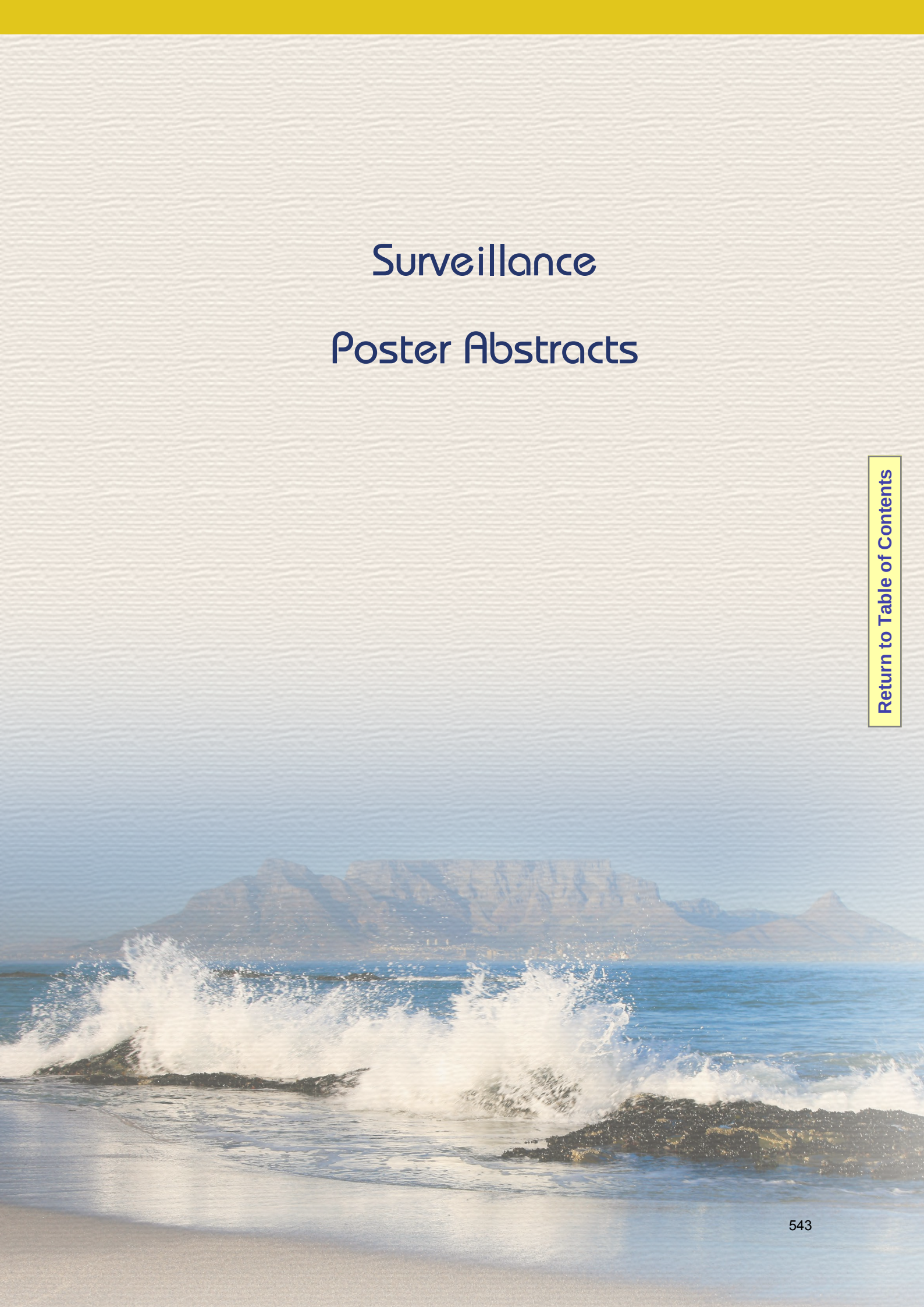
Surveillance

Poster Abstracts



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Use of Hangeul Twitter to track and predict human influenza

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Influenza epidemics arise through the accumulation of viral genetic changes. The emergence of new virus strains coincides with a higher level of influenza-like illness (ILI), which is seen as a peak of a normal season. Monitoring the spread of an epidemic influenza in populations is a difficult and important task. Twitter is a free social networking service whose messages can improve the accuracy of forecasting models by providing early warnings of influenza outbreaks. In this study, we have examined the use of information embedded in the Hangeul Twitter stream to detect rapidly evolving public awareness or concern with respect to influenza transmission and developed regression models that can track levels of actual disease activity and predict the influenza epidemic situation in the real world. Our prediction model using a delay mode provides not only a real-time assessment of the current influenza epidemic activity but also a significant improvement in prediction performance at the initial phase of ILI peak when prediction is of most importance. This work was supported by grants from Mid-career Researcher Program (2010-0029242) through NRF funded by the MEST and from TEPIK (A103001) funded by the Ministry for Health, Welfare & Family Affairs, Korea.

P2-618

Potential use of rat polyclonal antisera for antigenic analysis of human influenza viruses

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Background: Hemagglutination inhibition (HI) test is the basic tool for evaluation of antigenic drift in influenza viruses. Traditionally, ferret polyclonal antisera against currently circulating influenza viruses are used to perform the HI test because ferrets are considered as the most suitable model to mimic human influenza infection in animals. Considering the substantial cost of these animals and the need for special housing conditions, only a few laboratories have the capacity to work with ferrets on a regular basis. However, no suitable alternative to ferrets has been proposed so far. Nevertheless, it should be noted that in the Russian Federation, rat antisera have been used for typing, subtyping and antigenic analysis of human influenza viruses for many years. White rats are easily available and do not naturally suffer from influenza. Rats do not have influenza antibodies in normal blood serum, a fact that confirms their insusceptibility to influenza infection. Thus rats can be used for immunization directly from the nursery right after the quarantine. The main aim of this study was the comparative investigation of rat and ferret antisera in antigenic analysis of influenza A and B viruses in the HI test. Materials and Methods: White outbred rats 12-15 weeks old were used to obtain rat polyclonal antisera. Ferret polyclonal antisera were kindly provided by Dr J. McCauley (WHO CC for Reference and Research on Influenza, London, United Kingdom). The HI test was performed in full compliance with the WHO manual. Results: We present data on the potential use of rat polyclonal antisera in the HI test as a cheap and easy-to-operate alternative to ferret antisera. Parallel experiments with rat and ferret antisera indicated good comparability of the results. High indexes of correlation calculated to compare the results clearly demonstrated the validity of the data. Rat antisera permitted us to perform detailed antigenic analysis of human influenza isolates that circulated in the Russian Federation in 2007-2012. In general, ferret and rat antisera gave comparable results in the HI test. However, in some cases, rat antisera appeared to be more sensitive at disclosing antigenic drift of contemporary human influenza viruses; in the other cases, ferret antisera revealed antigenic differences more clearly. To provide additional proof for the potential use of rat antisera, we created antigenic maps

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based on the results of the HI test for influenza A(H1N1)pdm09 strains for 72 antigens and 4 rat antisera, for influenza A(H3N2) viruses for 154 antigens and 12 rat antisera, for influenza B (Victoria lineage) for 101 antigens and 12 rat antisera and for influenza B (Yamagata lineage) for 93 antigens and 18 rat antisera, respectively. Antigenic cartography with the application of rat antisera demonstrated the adequate evaluation of the evolutionary trends of contemporary human influenza viruses, and the obtained results were similar to those obtained by other methods. Conclusions: The data presented indicate the applicability of rat antisera in antigenic analysis of human influenza A and B viruses that belong to different subtypes or lineages and for influenza surveillance; the obtained results are comparable with those obtained in HI tests with ferret antisera. These results could add to the understanding of peculiarities of natural influenza virus antigenic drift.

P2-619 SCHOLARSHIP International Research Scholarship

Influenza viruses in Cameroon: 5 years of sentinel surveillance data, 2007-2012

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Background: Detection and characterization of the local circulating strains of influenza viruses are essential to prevent and control epidemics and pandemics. Little information is available in Cameroon with regard to the virus. This report presents the epidemiology of influenza viruses circulating in Cameroon based on a sentinel surveillance system for influenza-like illness (ILI) program in the 5 years since November 2007. Materials and Methods: All ILI cases were considered eligible for enrollment. A standardized questionnaire to record patients' demographic characteristics and medical history was used. Nasopharyngeal swabs were collected from all enrolled cases in different surveillance sentinel sites and then sent to the laboratory for biological confirmation. Detection and subtyping of influenza viruses were performed by real-time RT-PCR using the CDC protocol. Patient information and laboratory results were recorded in a central database (MS Access®) situated at the CPC. Statistical significance was assessed at $P < .05$ for all parameters. Results: We collected and tested a total of 9829 respiratory specimens from November 2007 to December 2012. Of these, 1751 (17.8%) tested positive. Overall, influenza viruses were detected in 34.8%, 29.1%, 26.0%, 16.6%, 18.2%, and 15.4% of ILI cases in late 2007, 2008, 2009, 2010, 2011, and 2012, respectively. During this period, influenza infections were observed mainly during the rainy season from September to November. However, in 2008, influenza activity was observed throughout the year, and in 2009-2010, some influenza activity was observed outside of this period due to the pandemic context. In late 2007, all of the isolates were A(H3N2) viruses, while A(H1N1) viruses were the most detected in 2008 (85.7%). In 2009, the majority of isolates (90.7%) were A(H3N2) viruses, and the first 6 cases of influenza A(H1N1)v virus were confirmed in Cameroon on August 5, 2009. In 2010 and 2011, the majority of isolates were influenza B viruses (54.9.8% and 53.6%, respectively), and a co-circulation of influenza A(H1N1)v (34.6% and 26.7%, respectively) and A(H3N2) (10.4% and 19.8%, respectively) was also observed. Finally in 2012, influenza A(H3N2) became the virus most detected (76.9%), with only 22.6% of isolates being influenza B (50%) viruses. and viruses. Conclusion: These data suggest that July-August may be most appropriate months for seasonal influenza vaccination in Cameroon. This system provides a robust profile of the epidemiology of influenza viruses in Cameroon and has proven to be useful for public health planning. These results highlight the importance of continuing surveillance and characterization of emerging influenza drift variants in sub-Saharan Africa.

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Molecular characterization of pandemic strains of influenza A virus circulating during the postpandemic period in India

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Background: A major pandemic of influenza was caused by novel strains of influenza A/pandemic H1N1 in 2009. Since then, virus has been circulating and leading to outbreaks on both local and global scales. The phenomenon of persistence of the same influenza A strain during such a long time interval is not well understood. Therefore, molecular characterization of the strain circulating in 2012 is required to find any antigenic or phylogenic changes. Hemagglutinin (HA) is a major surface protein of the influenza virus envelope which is hyper-variable and act as an epitope. HA1 domain of it acts as a receptor binding site to the host and generates an immune response in humans. Changes in this region directly affect transmission and pathogenecity of influenza virus. Hence, the molecular characterization of this region of HA gene is important to notify any significant changes. The present study was planned to study the molecular epidemiology of influenza virus and to determine the variation in HA gene (HA1 domain) sequences of influenza A/pandemic H1N1 strains circulating in 2012. Material and Methods: A total of 2669 cases presenting as influenza-like illness were enrolled during 2010-2012. Nasopharyngeal aspirate from every case was tested for influenza A virus and its subtypes by real-time RT-PCR according to WHO protocol. A total of 13 samples from influenza A/pandemic H1N1–positive strains were randomly selected for sequencing study. The HA gene was directly amplified from clinical samples by 2-step conventional RT-PCR assay specific for 447 bp fragment (nucleotide position 434-905) in the HA1 domain. Purified products were sequenced using BigDye Terminator Cycle-Sequencing Kit (Applied Biosystems, Foster City, California, United States) and ABI Prism 3130xl DNA-sequencer (Applied Biosystems). All sequences were aligned by Clustal W. Sequences of study strains of influenza A/pdmH1N1 strains were analyzed and edited with Bio-edit software. A phylogenetic tree was constructed by the neighbour-joining (NJ) method using MEGA 5.05 software. Evolutionary divergence analysis between HA sequences was performed using a Kimura 2-parameter model with 1000 bootstrap replicates in MEGA 5.0.5 version. The sequences were deposited in Gene Bank, National Centre for Biotechnology Information (NCBI) [http://www.ncbi.nlm.nih.gov]. Results: A total of 192/2669 cases were positive for Influenza A virus, of which 129 were influenza A/ pandemic H1N1. After 2010, influenza A/pandemic H1N1activity was not observed in the population until August 2012, when it reappeared. Phylogenetic analysis revealed that 11/13 isolates showed 98% similarity with reference strains of influenza A/pdmH1N1 clade 7 and the remaining 2 were 99% similar to reference strains of influenza A/pdmH1N1 clade 1. Clade 7 viruses are known to be characterized by S203T mutations in the HA1 gene of influenza A/pdmH1N1 as signature sequences which were present in all clade 7 strains of this study also. Comparison of amino acid sequences of these strains with respect to 2009 prototype strain of influenza H1N1 (A/California/04/2009) revealed 2 nonsynonymous changes, including HA S220T and S203T, which were located within or adjacent to HA antigenic sites. Conclusion: This study revealed that more than 1 clade of influenza A/pdmH1N1 strain circulated in this region. The unique finding of this study is the presence of Clade 1 strains in the population along with Clade 7 in the postpandemic period, as Clade 1 was reported in the beginning of pandemic only. No major changes had occurred in influenza A/pdmH1N1 strains since 2009, which has been found in other studies, too.

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Influenza infection patterns among cases with influenza-like illness (ILI) and severe acute respiratory infection (SARI) in Nigeria (2009-2012)

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Background: The introduction of the highly pathogenic avian influenza (A/H5N1) in Nigeria in 2006 resulted in substantial economic losses to the poultry industry and claimed 1 human casualty. This event, coupled with the fears of further human-to-human spread of the disease, necessitated a nationwide study of the human epidemiology of influenza, which hitherto was lacking in the country. Toward this, a National Influenza Sentinel Surveillance (NISS) system was established by the Federal Government of Nigeria between March 2008 and April 2009. The NISS comprised 4 sentinel sites in 4 of the nation's 6 geopolitical zones and a national influenza reference laboratory (NIRL), established in 2007, with molecular diagnostic capacity. We documented the spatial and temporal distribution of influenza virus types and subtypes in populations of enrolled ILI and SARI cases between April 2009 and December 2012. Materials and Methods: In each of the 4 sentinel sites, the first 4 ILI cases were enrolled daily from the outpatient department from Monday to Thursday of each week. All inpatient SARI cases were recruited. Demographic and clinical information for the cases, as well as their oro- and nasopharyngeal swabs, were taken and transported, under appropriate conditions, within 48 hours to NIRL. The specimens were diagnosed for influenza A, B and A subtypes using real-time reverse transcriptase-polymerase chain reaction (RT-PCR). Data were analysed using Epi-info 3.5.1. Results: Between April 2009 and December 2012, a total of 6324 ILI and 1566 SARI cases were tested, of which 597 (9.4%) and 79 (5.0%) were positive for influenza, respectively. Influenza positivity was highest (10.1%) among children 0-4 years of age with ILI and the elderly, 65 years of age and above, with SARI (6.3%). Influenza A viruses infection was most common among ILI (6.4%) cases, whereas infection with influenza B virus was slightly more common among SARI cases. Among subtypes of influenza A, A/H3N2 was slightly dominant over A(H1N1)pdm09 for both ILI (48.8% vs 46.3%) and SARI (48.6% vs 43.2%) cases. Temporal distribution showed the dominance of A/H3N2 in the prepandemic months of April to September 2009 and a short displacement by influenza B during November and December 2009. A(H1N1)pdm09 was first detected in December 2009 and assumed dominance during January to April 2010. Influenza B co-circulated at low levels during the period but became the dominant subtype from May 2010 through December 2012. Influenza A(H1N1)pdm09 and H3N2 were detected sporadically during the period. Dual peak viral activities were manifest in January/February and September/November of each year, with minor peaks occurring year-round. Conclusion: Influenza affected more young children with ILI and more elderly people with SARI during the investigation period. Influenza A viruses were major causes of infection among ILI cases, while both influenza A and B viruses were found to be equally responsible for SARI. Following the 5 months (December 2009-April 2010) dominance by A(H1N1)pdm09, the seasonal influenza viruses (A/H3N2 and Flu B) were re-established. Influenza activities occurred throughout the year in Nigeria, with 2 seasonal peaks observed toward the end of the rainy season (September-November) and during the dry season (January-February). The appearance of pandemic influenza in 2010 did not affect the seasonality pattern. Continuous influenza surveillance is needed to better understand the epidemiology and burden of seasonal influenza in the country in order to guide the institution of appropriate prevention, control and mitigation interventions.

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Use of deep-sequencing to evaluate the intrinsic heterogeneity of human influenza type A viruses directly in nasal swabs

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Background: In 2009, a new influenza type A virus of H1N1 subtype (H1N1pdm09) entered the human population and caused the first pandemic of the 21st century. After this pandemic wave, it replaced the previously circulating H1N1 virus and, along with the H3N2 subtype, is now responsible for the seasonal influenza epidemics. The genome of influenza type A viruses is composed of 8 single-stranded RNA segments of negative polarity. So far, the evolutionary potential of influenza viruses has been mainly documented by consensus sequencing data. However, RNA virus polymerases are considered to have low fidelity, and a virus thus exists as a cloud of closely related sequences (referred to as a quasispecies), which could influence its fitness and its adaptability. Interest in the quasispecies nature of influenza viruses has only recently increased with the development of next-generation sequencing (NGS) technologies that allow a wider study of the genetic variability. Materials and Methods: NGS deep-sequencing methodologies were developed to determine the whole-genome genetic heterogeneity of the 3 subtypes of influenza type A viruses that circulated in humans between 2007 and 2012 in France. For each subtype, between 20 and 30 nasal swab samples, collected from mild and severe human cases of influenza, were selected to cover the different epidemic seasons. After RNA extraction and specific reverse transcription, the viral genome was amplified by PCR. Library preparations and Illumina GAII runs were performed by multiplexing 6 samples per lane (2 tags per sample). Virus-specific bioinformatics tools were developed to process the obtained raw data. Short sequences generated by Illumina technology were aligned to the respective reference sequences and underwent different quality controls. The outputs giving the proportions of each base at each nucleotide position were then used for analysis and comparison. Results: We are presenting here the results of the comparison performed to identify subtype/severity signatures focusing on mutation frequencies and specific nucleotide polymorphisms. We first looked at the overall mutation frequency and at the gene-specific mutation frequencies to try to identify trends for either each subtype (pandemic H1N1, seasonal H3N2, seasonal H1N1) or each severity group (mild vs severe within each subtype). We then looked at nucleotide polymorphisms. We thus identified subtype-specific positions where subpopulations in apparently similar proportion exist for all the samples of the given subtype we tested. Finally, we searched for common or divergent positions between viruses of the 2 severity groups to try to identify subpopulations that could be landmarks of the severity. Conclusions: This study using a significant number of samples for 3 influenza type A viruses that have been circulating in the human population for the past 6 years provides us with a clearer view of the intrinsic heterogeneity of influenza viruses. It will be helpful to better understand the role that this variability might have in the virus's biology. This will lead to further studies aiming at understanding more precisely how the viral quasispecies nature is involved in the pathogenicity, evolution, and adaptability of influenza viruses.

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Etiology of community-acquired pneumonia among hospitalized adults in the United States: preliminary data from the CDC Etiology of Pneumonia in the Community (EPIC) study

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Background: Pneumonia is a common cause of hospitalization. Over the last 2 decades, molecular methods have improved diagnostic capability and community-acquired pneumonia (CAP) etiology has likely changed due to antimicrobial use and expanded vaccine use. Using traditional and innovative methods, the Centers for Disease Control and Prevention (CDC) Etiology of Pneumonia in the Community (EPIC) study aims to determine CAP etiology among hospitalized adults. Methods: CAP patients ≥ 18 years old were enrolled in 5 hospitals in Chicago and Nashville. CAP was defined as an acute respiratory infection and a new radiographic pneumonia in an adult from and hospitalized in the catchment area without recent hospitalization or severe immunosuppression. Blood for culture, high-quality sputum for routine culture, sputum for *Legionella* PCR, urine for pneumococcal and *Legionella* antigen testing, and naso/oropharyngeal swabs (NP/OP) for PCR of respiratory viruses and atypical bacteria were collected as soon as possible after admission. If therapeutic thoracentesis was required, pleural fluid samples were tested by culture and PCR methods. Results: From January 1, 2010, to June 30 2012, we enrolled 2483 (68%) of 3643 eligible adults with CAP aged 18-49 (30%), 50-64 (34%), and ≥ 65 years old (36%). The majority were white (47%), black (39%), or Hispanic (10%). Twenty-one percent of adults were admitted to intensive care, 5% required invasive mechanical ventilation, and 2% died. Antibiotic use before hospitalization was reported by 20% of adults. Eighty-one percent of blood cultures were collected before inpatient antibiotic administration. A viral (25%) or bacterial (13%) pathogen was detected in a total of 843 of 2410 adults (35%) with blood culture, high-quality sputum, sputum for *Legionella*, urine antigen testing, or pleural fluid, and NP/OP results available. More than 1 pathogen was detected in 4% of adults (57 had bacteria and viruses, 22 had ≥ 1 virus, 7 had ≥ 1 bacteria). No pathogen was detected in 65% of adults. The following pathogens were more commonly detected: rhinovirus (9%), influenza (5%), *Streptococcus pneumoniae* (5%), human metapneumovirus (3%), respiratory syncytial virus (3%), parainfluenza viruses 1,2,3 (2%), coronaviruses (2%), *Mycoplasma pneumoniae* (2%), and *Staphylococcus aureus.* Conclusion: Preliminary results from this multisite prospective study conducted in the United States indicate that viruses were more commonly detected in adults with CAP requiring hospitalization, although a large proportion was of unknown etiology. Bacteria may have been underdetected due to limited sensitivity of available tests and prior antibiotic use. Additional testing, including serology in cases and NP/OP PCR in healthy controls, is underway to help further elucidate etiology and clinical relevance. Clinicians should test for viruses and bacteria in patients with suspected pneumonia to optimize antimicrobial use. The CDC EPIC study is ongoing and will continue to provide insights into adult CAP etiology and incidence.

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Phylogenetic analysis of influenza viruses of H5 subtype isolated in Kazakhstan

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Background: A highly pathogenic H5N1 strain of avian influenza caused huge mortality in wild birds all over the world. Millions of domestic poultry have either died or been culled in Asia. Annual monitoring among wild birds has been conducted in Kazakhstan to track the situation in this country. In 2006-2009, influenza viruses A/mute swan/Aktau/1460/06 (H5N1) from dead swan and A/ruddy shelduck/South Kazakhstan/kz-0783/07 (H5N2), A/white-fronted goose/Central Kazakhstan/3733/09 (H5N3) and A/Greylag Goose/Central Kazakhstan/3735/09 (H5N3) from live birds were isolated in Kazakhstan and further genetically characterized. Materials and Methods: Tracheal and cloacal samples were collected using sterile cotton swabs, stored in viral transport medium and maintained in liquid nitrogen before analyses. Genomic RNA was extracted from swab materials using the QIAamp Viral RNA Mini kit (Qiagen). Multiplex RT-PCR assays were performed using AccessQuick RT-PCR Kit (Promega) with primers targeting fragments of H5 and M genes. After purification, the PCR products were sequenced on an ABI 3730xl DNA analyser (Applied Biosystems). Phylogenetic trees were generated using the Neighbour-joining method, and the Tamura-Nei model with 500 bootstrap replicates in MEGA 5.0 software. Results: Before molecular research, intravenous pathogenicity indexes (IVPI) of isolated Kazakhstan viruses were determined. Viruses A/white-fronted goose/Central Kazakhstan/3733/09 (H5N3) and A/Greylag Goose/Central Kazakhstan/3735/09 (H5N3) had zero index, which is considered nonpathogenic. Virus A/mute swan/Aktau/1460/06 (H5N1) had index 1.77, which is highly pathogenic.
HPAI H5 strains harbor insertions of positively charged amino acids Arg and Lys in the HA cleavage site: PQGE**RRKKR**GLFG. Kazakhstan viruses A/mute swan/Aktau/1460/06 (H5N1) and A/ruddy shelduck/South Kazakhstan/kz-0783/07 (H5N2) were identical and had PQGETGLFG amino acids at the cleavage site, typical for LPAI. Two other Kazakhstan viruses A/white-fronted goose/Central Kazakhstan/3733/09 (H5N3) and A/Greylag Goose/Central Kazakhstan/3735/09 (H5N3) were different from previous viruses and had an insertion of Arg in the HA cleavage site (PQGET**R**GLFG) and counted as LPAI as well. Substitution of glutamic acid (E) for lysine (K) at position 627 in PB2 protein is a molecular determinant of high virulence. A/mute swan/Aktau/1460/06 virus contained E, unlike the highly pathogenic Kazakhstan virus A/domestic goose/Pavlodar/1/2005 that harboured lysin.Substitution of E for K in PDZ-binding consensus ESKV at the C-terminus of the protein NS1 influences the virulence of HPAI Asian viruses, such as the A/bar-headed goose/Qinghai/0510/05 (H5N1) in chicken. The virus under study, A/mute swan/Aktau/1460/06 (H5N1), did not have the E→K substitution. Another factor of increased virulence is deletion in NS1 gene in position 263-273, and virus A/mute swan/Aktau/1460/06 (H5N1) also did not harbor this deletion. So, the Kazakhstan isolate A/mute swan/Aktau/1460/06 (H5N1) did not harbor any molecular determinants of highly pathogenic influenza virus, although it has high IVP index. One of the possible explanations of that is the presence of PB1-F2 protein in the structure of A/mute swan/Aktau/1460/06 (H5N1) virion. Expression of the PB1-F2 gene and accumulation of appropriate protein in mitochondria leads to induction of apoptosis in affected monocytes and macrophages, with subsequent leucopenia and increased susceptibility to bacterial infections. Investigations of phylogenetic relationships of the HA gene of H5 subtype Kazakhstan isolates showed highest (99,1%) identity of the virus A/mute swan/Aktau/1460/06 (H5N1) with A/ruddy shelduck/South Kazakhstan/kz-0783/07 (H5N2) virus also isolated in Kazakhstan. Together they form a separate cluster of Asian viruses. The other Kazakhstan isolates, A/white-fronted goose/Central Kazakhstan/3733/09 (H5N3) and A/Greylag Goose/Central Kazakhstan/3735/09 (H5N3), appeared to be close to western European isolates of 1999-2005. The highly pathogenic Kazakhstan isolate A/domestic goose/Pavlodar/1/2005 (H5N1) formed a separate cluster (genotype Z) with viruses A/bar-headed goose/Qinhai/051/05 (H5N2) and A/Azerbaijan/001-161/2006 (H5N1) ascending to A/goose/Guangdong/1/96 (H5N1). **Conclusions:** Kazakhstan viruses of H5 subtype were determined as nonpathogenic by IVPI with the exception of A/mute swan/Aktau/1460/06 (H5N1), whose index was 1.77, which is highly pathogenic, but no molecular determinants typical for highly pathogenic virus were found. Phylogenetic studies showed 99,1% identity of the virus A/mute swan/Aktau/1460/06 (H5N1) with A/ruddy shelduck/South Kazakhstan/kz-

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0783/07 (H5N2) virus also isolated in Kazakhstan. Viruses A/white-fronted goose/Central Kazakhstan/3733/09 (H5N3) and A/Greylag Goose/Central Kazakhstan/3735/09 (H5N3) appeared to be close to western European isolates of 1999-2005.

P2-625 FELLOWSHIP GATES Fellowship

Influenza surveillance among cases with influenza-like-illness and severe acute respiratory illness in Niger, 2009-2012

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Background: Influenza virus infection is responsible for substantial global morbidity and mortality every year, in particular among individuals < 5 and > 65 years of age. However, there is little information about the burden associated with influenza infection in Africa. In 2009, the government of Niger established a national influenza surveillance system aiming to monitor seasonal influenza as well as detect the emergence and spread of novel influenza strains with pandemic potential. The system comprised height sentinel sites, located in 5 of the height regions of the country and a national influenza reference laboratory (NIRL) with the capacity for molecular diagnosis of influenza virus. We documented the epidemiology of seasonal and pandemic influenza among outpatients with influenza-like-illness (ILI) and inpatients with severe acute respiratory illness (SARI) presenting at selected sentinel sites between April 2009 and April 2013. Materials and Methods: Up to 5 patients per week meeting the ILI or the SARI case definitions and presenting at the outpatient or inpatient department of the selected sentinel sites were enrolled from Monday to Friday. An ILI case was defined as an outpatient presenting with fever (≥ 38°C) and cough or sore throat. A SARI case in children < 5 years of age was defined as a hospitalized patient with cough or difficulty breathing and at least 1 of the following danger signs: unable to drink or breastfeed, lethargic, vomits everything, convulsions, nasal flaring, chest indrawing, stridor in a calm child or tachypnea. For patients ≥ 5 years of age, a SARI case was defined as any inpatient with fever (≥ 38°C), cough and shortness of breath or difficulty breathing. Epidemiological data as well as nasopharyngeal swabs were collected from each enrolled patient. The respiratory samples were tested by real-time reverse transcriptase polymerase chain reaction (rtPCR) for the detection of influenza A and B viruses. Samples positive for influenza A virus were further subtyped. The analysis was implemented using STATA 12 (StataCorp, College Station, Texas, USA). Results: During the study period, laboratory results were obtained from 1179 ILI and 952 SARI cases, of which 142 (12%) and 53 (6%) tested positive for influenza virus, respectively. The influenza positivity rate was highest in the 5-14 years age group (28/115; 24% among ILI patients and 6/61; 10% among SARI patients) followed by the 1-4 years age group (56/381; 15% among ILI patients and 31/333; 9% among SARI patients). Conversely, the influenza infection rate was lowest in the < 1 year age group (17/343; 5% among ILI cases and 11/455; 2% among SARI cases). Of the 195 influenza positive cases 102 (52%) were A(H1N1)pdm09, 61 (31%) were A(H3N2) and 32 (17%) were B. Influenza virus was detected predominantly from November to April, with peak viral activity observed in February (25% detection rate). Influenza A(H3N2) was the dominant circulating subtype from November to December 2009. Influenza A(H1N1)pdm09 was first detected in Niger in January 2010 and became the dominant subtype during the 2009-2010 and 2010-2011 influenza seasons. Conversely, influenza A(H3N2) and B co-circulated during the 2011-2012 and 2012-2013 influenza seasons. Conclusion: The Niger sentinel surveillance system allowed monitoring of the circulation of seasonal influenza as well as the introduction and spread of influenza A(H1N1)pdm09 in the country. Influenza virus was detected in both ILI and SARI cases, and the burden was highest among young and older children. Influenza virus circulation was detected predominantly from November to April, which corresponds to the cold-dry season in the country. After its introduction and spread in 2010, influenza A(H1N1)pdm09 became the predominant subtype for 2 consecutive seasons and reappeared in 2013. Continuous influenza surveillance is needed to better understand the epidemiology and burden of seasonal influenza in the country and monitor the emergence of influenza strains with pandemic potential.

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Phylogenetic characterization of influenza H1N2 subtype viruses isolated from wild birds in Kazakhstan

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Background: Among animals, the largest number of antigenic variants of influenza A viruses were isolated from birds. The aim of the present work was to study the phylogenetic relationships of influenza A virus isolates with hemagglutinin H1 circulating in populations of different species of wild birds in several regions of Kazakhstan during the pandemic of "swine flu" in 2010. Circulation of influenza virus of this subtype among wild birds in the territory of Kazakhstan was not seen in the last 10 years, and their appearance in the populations of wild waterfowl in the region coincided with the pandemic of "swine flu H1N1." Materials and Methods: Virus isolation was performed by inoculating each sample of the material in the allantoic cavity of 10-11-day-old chick embryos. To establish membership of hemagglutinating agents to influenza A viruses, RT-PCR was used with primers to the M gene. Viral RNA was extracted from 140 uL samples using QIAamp Viral RNA Mini Kit. Reverse transcription was performed with reverse transcriptase AMV reverse transcriptase with primers Uni 12 (agcaaaagcagg). Amplification of cDNA was performed using primers c recommended for sequencing the genome segments of influenza A viruses. After purification, the PCR products were sequenced on an ABI 3730xl DNA analyser (Applied Biosystems). Phylogenetic trees were generated using the Neighbour-joining method, and the Tamura-Nei model with 500 bootstrap replicates in MEGA 5.0 software. Results: We performed sequencing of the coding regions of HA, M and NS genes of influenza virus subtype H1, isolated in 2010 from wild birds in different regions of Kazakhstan compared their nucleotide sequences with those of the strains from GenBank. The cluster system was dedicated as described Liu et al (2009). The HA genes of all Kazakh influenza viruses of subtype H1 exhibit similarity to each other and are included in the cluster H1.1.2 formed mainly by Eurasian avian viruses. The HA genes of the Kazakhstan H1N2 influenza viruses are not phylogenetically related to the pandemic strain of "swine flu" A/Almaty/347/2009 (H1N1) and porcine virus A/swine/Guandong/1/2009 (H1N2), which represent a separate lineage of genetic cluster H1.3. The HA genes of the isolates A/black-headed gull/Atyrau/4378/2010 and A /red-crested pochard/Balkhash/4529/2010 isolated in different regions were completely identical to each other and difference from the A/red-crested pochard/Balkhash/4523/2010 was only one nucleotide as a result of transition A → G, resulting in nonsynonymous encoded amino acid substitution at position 39, Lys for Arg. Unlike other viruses studied, the HA gene of Kazakhstan H1N2 isolates has unique amino acid substitutions: Ala15 → Ile, Thr44 → Ser, Thr99 → Asn. In contrast to the HA gene, the M gene phylogeny of Kazakhstan H1N2 virus isolates is represented by different evolutionary lineages. The M gene of strain A/red-crested pochard/Balkhash/4523/2010 refers to the Eurasian avian viruses cluster in line with HA H1, H8, and H11 isolated from wild ducks in South Korea and China in 2003-2007. In turn, the M gene of the Caspian isolate A/black-headed gull/Atyrau/4379/2010 (H1N2) belongs to the gull lineage of Eurasian viruses. The NS gene of the isolate A/black-headed gull/Atyrau/4379/2010 (H1N2) is similar to Siberian strain A/common gull/Ust-Ilimsk/121/2008. Both of these isolates with viruses H13N9 from Alaska form a separate group that is part of a sublineage allele A unifying viruses H3N8, H4N6, H5N3, and H6N5 from Primorye, Korea, Mongolia, and Malaysia, as well as epizootic strain of 2003 H7N7 from The Netherlands and Germany. Kazakhstan isolate A/red-crested pochard/Balkhash/4523/2010 (H1N2) by the nucleotide sequence of the NS gene was most similar to the Caspian strain A/tufted duck/Aktau/1456/2006 (H4N6), and formed with him and viruses of different geographic origin separate sublineage allele A. Conclusions: The study revealed that the gene pool of Kazakh isolates of H1N2 influenza virus from wild birds is similar to that of the viruses of Eurasian avian lineages, but not identical to them. In their structure, genes of influenza viruses of pandemic or swine origin are absent, and the exchange of genetic material involved only influenza viruses within this evolutionary line.

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P2-627 SCHOLARSHIP International Research Scholarship

Etiology of severe acute respiratory infections among hospitalized children in Lusaka, Zambia, 2011-2012

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Background: A limited number of studies have been conducted in resource-limited settings to determine the etiology of severe acute respiratory infections. Influenza surveillance studies in Zambia from 2008-2012 have shown that influenza is detected in less than 5% of severe acute respiratory infections (SARI). The objective of this study was to identify pathogens associated with SARI in hospitalized children under 5 years of age using singleplex and multiplex polymerase chain reaction (PCR). An understanding of the viral and bacterial etiologies of SARI could lead to better clinical management of patients and will identify which pathogens constitute the largest disease burden and should be targets of vaccines and pharmaceutical development. Methods: From January 2011 to December 2012, as part of an ongoing influenza surveillance program in Zambia, we approached all hospitalized under 5 years SARI patients presenting to the University Teaching Hospital (UTH) in Lusaka, Zambia. For those who were eligible and consented to participate, we used a structured questionnaire to collect clinical data, including information on case history and demographics, and obtained combined nasopharyngeal and throat swabs. For the current study, we tested respiratory swab specimens that were laboratory confirmed as influenza negative. Specimens were examined for 7 viruses using singleplex real-time reverse transcription PCR (rRT-PCR) and for 18 viruses and 12 bacteria using multiplex rRT-PCR. Results**:** A total of 496 influenza-negative specimens from SARI cases were tested; a set of 297 were screened by singleplex rRT-PCR, and a different set of 199 were screened by multiplex rRT-PCR. The average duration of hospitalization was 3.78 days. The most common diagnosis was pneumonia (384/496; 77% of specimens), and the second most common was bronchiolitis (56/496; 11%). Using the singleplex PCR, 184/297 (62%) specimens were positive for at least 1 virus; of these, 34/184 (18%) were co-infections. We detected all 7 viruses included in the singleplex panel; in decreasing order of prevalence, they were human rhinovirus (HRV; 54/297; 18%), human adenovirus (HADV; 50/297; 17%), respiratory syncytial virus (RSV; 45/297; 15%), human metapneumovirus (hMPV; 14/297; 5%), and parainfluenza virus 1-3 (PIV; 21/297; 11%). Among coinfections, the most commonly identified viruses were HADV (20/34; 59%), HRV (19/34; 56%), and RSV (15/34; 44%). Using multiplex PCR, at least 1 virus was isolated from 167/199 (85%) specimens, and at least 1 bacteria was isolated from 197/199 (99%) specimens. Cytomegalovirus (CMV) was the most commonly detected virus (116/199; 69%), followed by RSV (20/199; 12%). The most commonly detected bacterial infections were *Streptococcus pneumonia* (109/199; 55%) and *Moraxella catarrhalis* (92/199; 47%). Among the specimens with multiple infections, the most commonly detected virus was CMV (106/116; 63%), followed by RSV (15/116; 9%), HBov (14/116; 8%), and HMPV (11/116; 8%). CMV and HBov were found in all specimens with multiple infections that included at least 1 bacterium, and *Streptococcus pneumonia* was found in all specimens with multiple infections that included at least 1 virus. While rates of SARI hospitalization were highest between March and August, no distinct seasonality was observed in Lusaka. Conclusion: Many bacterial and viral pathogens were isolated from SARI cases among hospitalized children in Zambia. These results call for further investigation of respiratory infections to determine disease burden,and inform drug and vaccine development, and emphasize the need to use different testing methods to determine the cause of SARI in Zambian children.

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Influenza in Burkina Faso: identification of key vulnerable groups in sentinel sites

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Background: Although influenza surveillance has increased in some sub-Saharan African countries in recent years, no information is yet available from Burkina Faso. Our study was the first to determine the prevalence of influenza viruses circulating in Burkina Faso and to identify key vulnerable groups in sentinel sites. Materials and Methods: We conducted sentinel surveillance with oropharyngeal swabs collected from outpatients (aged 1 month to 83 years) from 6 sites in Bobo-Dioulasso and Ouagadougou, according to WHO/CDC case definition for ILI (fever ≥ 38ºC AND cough and/or sore throat [in the absence of other diagnosis]) from July 2010 to May 2012. Influenza viruses were detected by real time RT-PCR using CDC primers and protocols. Results: A total of 854 outpatients with ILI were sampled, with 58 (6.8%) being positive for influenza (29 influenza A and 29 influenza B). After subtyping the influenza A viruses, 55.2% (16/29) cases were influenza A (H1N1) pdm 09 and 44.8% (13/29) cases were seasonal A/H3N2. No cases of seasonal A/H1N1 were detected. Children of preschool and school age (0-5 years and 6-14 years) were most affected, 35.2% and 27.8% with laboratory-confirmed influenza, respectively. Although adults 35-50 years old were unaffected, older adults > 50 years of age were positive, 5%. Influenza peak occurred during the dry, dustry Harmattan months from November to March. Conclusion: This study was the first confirming the circulation of influenza A (H1N1) pdm 09, A (H3N2) and influenza B viruses in humans in Burkina Faso, with infection occurring in all ages, except the 35-50 years age group.

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Evolution of diagnosis and characterization of influenza viruses by the National Network of Influenza and Respiratory Viruses in Argentina

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Background: Surveillance of influenza viruses is essential for updating vaccines and also gives important insights into the mechanisms of virus evolution; for that purpose, it is essential to have a critical mass of trained personnel with skills to detect and characterize virus. In the early 1990s, at the National Institute of Microbiology "Carlos G. Malbrán," now the National Institute of Infectious Diseases (INEI), ANLIS "Carlos G. Malbrán," virological diagnostic courses were given, which addressed the issue of acute respiratory infections (ARI). Each year, participants in those courses remained in contact with the Respiratory Viruses Service (RVS) in trying to implement in their hometowns rapid techniques applied to the diagnosis of ARI The contact with those laboratories was continuous, and gradually, the networking format emerged. In 1997, the National Network of Influenza and Respiratory Viruses (Network) was started with its head at RVS, the actual National Reference Laboratory for Influenza (NRLI) and WHO National Influenza Center (NIC) and the other 2 Argentinean NICs are located in Mar del Plata and Cordoba cities. Material and Methods: Initially, 10 provincial laboratories (LP) of the network started performing the diagnosis of influenza through the detection of viral antigens by IFA. NRLI performed viral isolation in MDCK cells, subtyping and

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antigenic characterization by HI using sera from sheep and ferret, respectively. In 1999, the NRLI incorporated the detection and subtyping by RT-PCR and sequencing as tools for genomic characterization. In 2007, the NRLI began to study antiviral susceptibility; in 2008, the shift the diagnostic and subtyping to real time PCRs techniques occurred. Results: That first year, 1997, 7246 samples were processed by the network, 232 influenza A (FluA) were detected and 94 influenza B (Flu B); 52% of viruses could be isolated and characterized, and 35 strains were sent to the CDC. The network has 65 laboratories that continue the diagnosis by IFA, 35 of them have implemented real-time FluA/FluB RT-PCR. In 2012, the network processed 62,200 samples; 2200 influenza viruses were detected, 780 FluB and 1420 FluA. The NRLI subtyped all the FluA clinical samples detected, and the B lineages were determined by real-time RT-PCR. Sequencing of several genomic segments (HA, NA, M) of these viruses was performed. Almost 80 viruses were sent to the CDC. Conclusions: The diagnostic capacity of the network increased more than 100 times and the ability to characterize the virus at the NRLI increased 805 times using techniques recommended by international reference centers. The rapid detection and characterization of circulating viruses are important tools for health authorities to take preventive measures and control, and contribute to global surveillance of influenza viruses.

P2-630

Monitoring of avian influenza viruses subtypes H5 and H7 in wild birds in the Azov-Black Sea region

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Background: To date, influenza remains an unpredictable infection for animals, birds and people. The constant emergence of new strains and variants with new properties and pathogenicity for new hosts requires constant monitoring and careful research of new viruses. Since the main and primary reservoir of influenza viruses in nature is wild birds, especially waterfowl and shorebirds, constant epizootiological monitoring in populations of these birds is necessary. The National Scientific Center "Institute of Experimental and Clinical Veterinary Medicine" in Ukraine, since 2000 conducts monitoring for influenza viruses in in wild waterfowl and shorebirds in the Azov-Black Sea region of Ukraine. Particular attention was paid to the circulation of avian influenza viruses of subtypes H5 and H7, which can potentially be dangerous to poultry. Materials and Methods: Sampling of wild birds was conducted from 2000 to 2011 in the Azov-Black Sea region of Ukraine. This region is the most important region of Eastern Europe concerning ornithology, a place of passage, stops during migration, and nesting of migratory and many other bird species with a very high level of biodiversity. This region is in the cross of lines of flight of birds from the Baltic and Caspian seas to the Black and Mediterranean Seas, from Western Siberia and Kazakhstan to Western Europe and North Africa. During the period of 2000 to 2011, biological material (cloacal, tracheal swabs, samples of faeces) was collected from more than 6000 wild birds of 66 species, orders *Anseriformes* and *Charadriiformes*. Virological investigations were carried out by standard methods recommended by the OIE. Results. During the period of 2005-2008, highly pathogenic avian influenza (HPAI) virus subtype H5N1 was detected in wild birds. Five viruses were isolated from great cormorants in 2006, and 3 viruses were isolated from a Great Grebe in 2008. These viruses belong to HA clades 2.2 and 2.2.3., originating from Asia, and Western Europe respectively. For the period from 2010 to 2012, during the large-scale monitoring studies of wild birds of different ecological groups, 59 influenza viruses of different subtypes were isolated, including a low pathogenic avian influenza (LPAI) virus subtype H5N2, which had a sequence similar to this subtype viruses that circulated in Europe in 2002. With regard to avian influenza virus subtype H7, no viruses were isolated during the 2000-2003 period, although antibodies to influenza virus H7 were detected in serum and egg yolks from some wild ducks. In the period 2010-2012, seven viruses H7 subtype with different neuraminidase (H7N3, H7N6, H7N7) were isolated, representing 11.86% of the total number of influenza viruses collected during this period. All viruses of subtypes H5 and H7 were isolated from the wild Mallards during their autumn migration and wintering in the Azov-Black Sea region. During virological studies, it was found that viruses do not cause disease in chickens after intranasal and intramuscular

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inoculation. Conclusions: The results indicate circulation of low pathogenic avian influenza viruses subtype H5 and H7 in wild waterfowl populations in the Azov-Black Sea region. These findings support the need for ongoing monitoring of avian influenza for early prevention of highly pathogenic variants of viruses that may pose a threat to poultry.

P2-631 FELLOWSHIP GATES Fellowship

Implementation of influenza-like-illness sentinel surveillance in Togo

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Background: In tropical and subtropical regions where viral transmission occurs throughout the year, data on the burden and impact of influenza are limited. The emergence of avian A/H5N1 in 2003 as well as the pandemic A/H1N1 in 2009 highlighted the need to establish influenza sentinel surveillance in Togo. The Ministry of Health in collaboration with the Institut National d'Hygiène (INH) decided to introduce influenza-like-illness (ILI) to the list of Diseases with Epidemic Potential and began monitoring through the Integrated Disease Surveillance and Response (IDSR) Program. By April 2010, Togo was actively involved in influenza surveillance through its new sentinel network. The objective of this study is to describe the implementation of ILI surveillance and results obtained during April 2010 to December 2012 activities. Materials and Methods: The Division of Epidemiology within the Ministry of Health coordinated the sentinel surveillance system. The first site (Hôpital de Bè) was chosen for its geographical situation in an area of high population density and high consultation rate. In December 2011, a second site, Service de Santé des Armées de Lomé, was added based on the essential role of the Army in case of a pandemic and the ability to reach both military and civilian personnel. Patients presenting at sentinel sites with ILI were recruited by trained medical staff using World Health Organization (WHO) case definitions. Epidemiological and clinical data as well as samples were collected and sent to the Influenza National Reference Laboratory located within the INH. Samples were processed using real-time RT-PCR. Laboratory results and epidemiological data were entered in a single database, and a weekly report of samples received and confirmed cases of influenza A and B were produced and shared between sentinel sites, Ministry of Health, Division of Epidemiology, WHO and CDC/NAMRU-3. Results: Two sentinel sites were chosen, their staff trained on WHO case definitions for ILI and sample collection began in April 2010. During the study period, the yearly distribution of samples collected was 87 (2010), 335 (2011) and 533 (2012), for a total of 955 samples. The median age of patients was 6 years, with 51% of the study population falling between age 0 and 4 years. Of the 955 samples, 236 (24.7%) tested positive for influenza; with 136 (57.6%) positive for influenza A and 100 (42.4%) positive for influenza B. Among influenza A, 71 (52.2%) tested positive for seasonal H3N2; 62 (45.6%) for pandemic influenza A/H1N1 (2009) and 3 (2.2%) were unsubtypeable. Though the first case of pandemic influenza A/H1N1 (2009) was confirmed in May 2010, it only became predominant from October 2010 to March 2011. Influenza B became predominant in 2011 between April and August. From September 2011, there was a co-circulation of seasonal influenza A/H3N2, pandemic influenza A/H1N1 (2009) and influenza B. Conclusions: Togo successfully established an influenza sentinel surveillance system consisting of 2 sites selected based on their predicted representativeness. Study results identified the median age of patients as 6 years of age. Theories are that initially adult patients self-medicate and only seek treatment if their condition worsens. Further investigation and confirmation of the age distribution is needed. Samples taken from these sites were used to confirm the circulation of influenza viruses and particularly the pandemic influenza A/H1N1 (2009) in the country.

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Patterns in the viral etiology of acute respiratory illnesses in children presenting at outpatient clinics in Hong Kong

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Background: Respiratory viruses cause acute respiratory diseases in all age groups and are an important cause of hospitalizations and deaths every year in Hong Kong. These viruses often result in a broad and overlapping spectrum of symptoms. Co-infection of different respiratory viruses is well documented, but the implication has not been clarified to date. The aim of this study is to characterize the clinical symptoms associated with important respiratory virus infections and to identify the patterns in circulation of various respiratory viral infections in children in Hong Kong. Materials and Methods: Children presenting to an outpatient clinic with acute upper respiratory tract infection were recruited, their clinical signs and symptoms were recorded and a nose swab and throat swab were pooled for testing. Swabs were tested using the xTAG multiplex assay, which can detect 17 respiratory viruses. The differences in clinical symptoms of various viral infections was compared and tested by Chi-square test, while seasonal patterns were explored with descriptive analysis. Results: Among 2090 specimens collected from children presenting from 2007 through 2010, 1343 (64.2%) were positive for any respiratory virus, and 815.6%) specimens were positive for co-infection (with 2 specimens positive for 3 kind of viruses). There were 491 (23.5%) specimens positive for enterovirus/rhinovirus; 408 (19.5%) were positive for influenza A virus. Entero/rhinovirus and influenza A were most frequently detected with other respiratory viruses. However, para-influenza and RSV have a greater percentage of their detection as co-detection. The most commonly detected viruses among children 6-15 years old were influenza (A, B) and human coronavirus, while many different viruses were detected in children younger than 6 years. Except for influenza (A, B) and adenovirus, which were associated with higher mean body temperature, all the other respiratory viral infections were associated with similar patterns in symptoms. Influenza epidemics occurred twice per year, while there were no clear seasonal patterns in the circulation of other respiratory viruses. Conclusions
Influenza A and B and human coronavirus were more commonly detected in older children with acute URTI. Except for influenza (A and B) and adenovirus infection, which were associated with a higher chance of febrile illness, the other viral infections in children were associated with similar clinical symptoms. Moreover, many of the non-influenza viruses let to illness meeting the ILI surveillance definition, potentially affecting the interpretation of ILI data for influenza surveillance.

P2-634

Influenza as a cause of reported severe viral pneumonia cases in Vietnam, 2006-2013

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Background: In 2005, Vietnam had the highest number worldwide of human cases and deaths from avian influenza A/H5N1. Concern for respiratory viruses, such as influenza A/H5N1 and other pathogens with pandemic potential, led the Ministry of Health to institute a national surveillance system for severe viral pneumonia (SVP). This surveillance has been in place since 2006 in hospitals of 63 provinces and cities.

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Materials and Methods: We use the following case definition for SVP: sudden onset of fever > 38°C (measured or reported), difficulty breathing, chest radiograph consistent with viral pneumonia, and no other diagnosis. For patients meeting inclusion criteria, we collected epidemiological and outcome data, as well as throat swabs which were tested for influenza using RT-PCR with WHO standardized procedures. Results: From January 2, 2006, to May 24, 2013, 1160 SVP cases were reported, 708 male (61%), with a median age of 38 years (IQR 23-53 years), with a range of 1 month to 96 years. Influenza viruses were identified in 170 (14.6%). Pandemic A/H1N1/2009 (pH1N1) virus was detected in 75 patients (44%), A/H5N1 in 31 (18%), A/H3N2 in 28 (16.5%), influenza B in 26 (15.3%), and seasonal A/H1N1 in 9 (5.3%). Through SVP surveillance we detected 31 of 32 reported cases of human influenza A/H5N1 in Vietnam since the system's initiation in 2006. Among SVP cases, the case fatality rate for patients testing negative for influenza was 244/798 (30.5%), for patients with A/H5N1 was 20/31 (64.5%), and for patients with other influenza viruses was 33/107 (30.8%). For SVP case-patients with influenza, median time from onset date to admission date into hospital was 5 days (IQR 2-7 days) for 51 fatal cases, and 2 days (IQR 1-4 days) for 63 nonfatal cases ($P < .01$ by Wilcoxon rank-sum test). Conclusions:
Although SVP surveillance met its primary goal of detecting influenza A/H5N1, most of the influenza cases detected were pandemic A/H1N1 and seasonal strains, which had a surprisingly high case fatality rate. These influenza deaths were significantly associated with longer duration from symptom onset to hospitalization. Additional testing is planned on the samples to determine the other etiologies of SVP cases. For the future of SVP surveillance, increased testing with a more standardized and sensitive case definition is being considered.

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Prevalence and diversity of respiratory viruses in patients over 50 years old with ILI in Senegal

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Background: A limited number of pediatric studies have described the etiology of influenza-like illness (ILI) due to viruses, including non-influenza viruses. No serious study has been conducted to describe the prevalence and diversity of respiratory viruses in West African elderly. It is well established by studies conducted in other regions that older people are at greater risk of death from complications arising from influenza compared with younger adults. In addition, the specificity of the West Africa climate combined with some habits such as self-medication and the use of traditional medicine to treat ILI largely may cause epidemiological differences. Therefore, the goal of this study was to determine the prevalence and the diversity of respiratory viruses associated with ILI cases in adults over 50 years. Materials and Methods: The recruitment period of this prospective observational study was from January 2009 to December 2011. The study population consisted of 231 patients, over 50 years old, presenting with ILI. It should be noted that samples were collected in the context of the flu monitoring. Inclusion criteria for ILI were a sudden onset of fever (≥ 38°C) and cough or sore throat fewer than 3 days in duration, whether or not accompanied by general symptoms, such as myalgia, prostration, headache or malaise. Nasal-pharyngeal and/or oral-pharyngeal swabs were collected, as well as a form collecting demographic and clinical data. RNA extraction was performed using the QIAamp Viral RNA kit. A 2-step real-time RT-PCR was performed using the CFX96 Real-Time PCR Detection System (Bio-Rad). Synthesis of cDNA with the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific) and the Anyplex™ II RV16 Detection kit (Seegene) for viral PCR detection were performed. Results: A total of 231 patients over 50 years old were enrolled into the study; 129 (55.8 %) are women and 102 (44.2 %) are men. Subject ages ranged from 51 to 97 years old with a mean age of 66 years. Fever is the most frequently reported clinical sign (reported by 92.2 %; 213/231) of patients, followed by cough (78.3 %; 181/231), rhinitis (74 %; 171/231) and myalgia (54.1 %; 125/231). In all, 131 (57 %) of the 231 study participants were found to be infected with at least 1 of targeted viruses. In total, 149 viruses were detected, of which influenza viruses (44 %; 66/149) and rhinoviruses (27 %; 40/149) are the most prevalent. We detected 13 human Parainfluenza viruses (9%), 7 human respiratory syncytial viruses (4.7%), 6 coronaviruses (4%), 5 human Metapneumovirus (3.5 %), five human adenovirus (3.5 %) and one human bocavirus (0.7%). A total of 16 cases (6.9%;

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16/231) of dual virus infections and 1 triple viral detection case were encountered. Influenza viruses (13) and rhinoviruses (8) were the most commonly included dual infections. If we consider different age groups, viruses were detected in 54 of the 98 patients (59.2 %) in the 50- to 64-year-old age group, 56 of the 77 patients (72.7%) in the 65- to 74-year-old age group, and 35 of the 56 patients (62.5 %) whose age was ≥ 75 years. The viral co-infections are more frequent in the 65- to 74-year-old age group (9/15) followed by the ≥ 75-year-old age group (4/15). Conclusions: This pilot study demonstrates the presence of a variety of respiratory viruses in the elderly. It also shows a high prevalence of these viruses in this cohort. Viral prevalence and co-detections seem to be more frequent in adults aged 65 years and over. In light of these results, a more exhaustive study seems necessary in order to provide a more complete picture of the influence of respiratory viruses on the morbidity among adults over 50 years in the sub-Saharan context.

P2-638

MOSAIC: mobile surveillance for ARI and ILI in the community

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Background: Acute respiratory infections (ARI), including influenza-like illnesses (ILI), can result in significant morbidity and mortality, increased outpatient visits and hospitalizations, and indirect costs from loss of school and workdays. Previous population-based studies assessing the epidemiology of ARI/ILI were conducted decades ago when a viral pathogen could be identified in < 25% of samples. While modern molecular diagnostic techniques expand our ability to identify viral pathogens, this technology has not been used in a US community-based sample. This study's purpose is to follow a sample of households from an urban, underserved community and identify the incidence, etiology and risk factors associated with ARI/ILI using molecular diagnostic strategies to detect a wide variety of respiratory pathogens. We also explore the use of text messaging as a novel method of disease surveillance. Materials and Methods: Approximately 250 households will be followed for 1-4 years, with replacement as necessary, to maintain a surveillance population of ≈ 1000 individuals. Potential households are identified by randomly sampling of a cohort of households (≥ 3 members and ≥ 1 child) currently enrolled in a community-based survey study located in northern Manhattan in New York City. At enrollment, household and individual demographic information are collected. Once enrolled, households receive text messages twice weekly to identify members with symptoms meeting study case definitions for ARI/ILI, and a nasal swab is obtained from individuals in their homes by trained research assistants. Swabs are analyzed via multiplex RT-PCR using the FDA-approved FilmArray Respiratory panel that identifies 19 respiratory pathogens (BioFire Diagnostics, Inc). Information regarding illness duration, medical care and antibiotic utilization, and loss of school and work, is documented. Monthly home visits are performed. Results: Between December 2012-February 2013, 85% of 187 eligible households were recruited. There are an average of 4.8 members per household, average household density is 2.3 people/bedroom (range of 1-7). Of the 440 adults and 323 children, 10.9% are < 5 years old, 31.4% 5-17 years, 54.3% 18-64 years and 3.4% 65 years and older. Nearly two-thirds are female (61.6%). The majority are Latino (99.2%), 43.1% have limited English proficiency, and 54.3% are foreign born. Less than half of all adults do not have a high school degree (45.2%); 25.4% have only a high school education (or equivalent). Most participants are publicly insured (77.8%); 13.2% are uninsured. Most self-report excellent or good health status (65.5%); 23.0% have an underlying high-risk condition. Nearly a quarter (24.1%) of households include someone who smokes. Most (87.6%) children are in daycare or school. Most adults and children spend more than 20 hours a week outside the home (67.3%). Over the first 24 weeks of the study, 4963 text message queries were sent; 75.4% received a response with 88.4% of those responses received within 4 hours of the initial message. To date, 247 ARI/ILI episodes have been reported (54.5% observed in children). The median time from onset of symptoms to a nasal swab sample was 2 days. Nearly half (42%) were obtained within 1 day, 68% within 2 days, 85% within 3

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days, and 98% within 5 days of symptom onset. More (64.2%) were for an index case. Overall, 66.7% of samples were positive for a respiratory pathogen, 26.8% of those were positive for influenza A/H3N2 or B; 4.3% showed coinfection. Eleven noninfluenza respiratory pathogens were detected: subspecies of coronavirus (22.6%), parainfluenza (3.7%), rhinovirus/enterovirus (40.2%), RSV (5.5%), adenovirus (0.6%), metapneumovirus (4.9%), and Chlamydophila pneumonia (0.6%). We plan to assess ARI/ILI incidence and factors associated with infection and transmission. We will also assess antibiotic utilization for ARI/ILI, days lost from school and work, and influenza vaccine effectiveness. Conclusions: This study will provide important information about ARI/ILI incidence, transmission and etiology in a community-based sample. In addition, the high response rate and rapidness of replies to the text messages has led to collection of the vast majority of specimens within 72 hours of onset of symptoms. This use of text messaging for ARI/ILI reporting is a promising novel approach to real-time surveillance.

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Non-influenza respiratory pathogens in Mongolia: results of multiplex real-time polymerase chain reaction (mx-rt-PCR) surveillance in 2008-2013

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Background: Acute respiratory infections (ARI) are the leading cause of human morbidity and mortality globally. The control activities of ARI are complicated with the multiple pathogens causing a disease with similar clinical expressions. A screening of respiratory samples collected from ARI patients in Mongolia in 2003-2007 with the “classical” MDCK cell culture yielded only 9.8% (691/7,073) influenza viruses (Darma B, et al. 2009), leaving the vast majority of ARI cases with unknown etiology. The aim of this study is to test a current nucleic acid based multiplex technology to study the etiology of ARI in Mongolia. Materials and Methods: We studied 2516 archival samples that tested negative for influenza viruses by rt-PCR, kept in the National Influenza Center, National Center of Communicable Diseases, Mongolia (NIC), collected from patients with ARI by sentinel surveillance sites all over the country from November 2008 through March 2013. The nucleic acids were isolated from the respiratory samples by NucliSENS Minimag (bioMerieux, France) and specific pathogens were detected by ABI Fast Real Time PCR System 7500 (Applied Biosystems, USA) with an mx-rt-PCR assay for simultaneous detection and identification of 19 respiratory pathogens: respiratory syncytial virus (RSV), human rhinovirus (HuRhV), parainfluenza viruses (PIV: types I, II, III, and IV), influenza viruses (IV: types A and B, A/H1N1pdm), human metapneumovirus (HuMpV), enterovirus (EV), parechovirus (PEV) human bocavirus (HuBV), human coronaviruses (HuCoV: strains HCoV-43, HCoV-63, HCoV-229, and HCoV-HKU), adenovirus (AdV), *Mycoplasma pneumonia* (MyP) using “FTD Respiratory Test Kit” (Fast-Track Diagnostics, Luxembourg). Results: We have detected in total 1485 pathogens from 1274 (50.6%) tested samples, among them 263 (17.7%) HuRhV, 260 (17.5%) RSV, 196 (13.2%) PIV, 195 (13.1%) HuCoV, 142 (9.6%) HuBV, 114 (7.7%) HuMV, 111 (7.5%) IV-A, 104 (7.0%) AdV, 55 (3.7%) EV, 22 (1.5%) MyP, 18 (1.2%) IV-B and 5 (0.3%) PEV. 204 (8.1%) of samples yielded more than 2 pathogens (co-infection). The virus detection was significantly higher ($P \leq .001$) in young age groups: 0-5 (56% = 958/1709) and 6-10 (52.3% = 127/243) years in comparison to the older age groups (33.5% = 189/564). 1093 (43.4%) samples were from the outpatient population and 1423 (56.6%) were from the hospitalized patients, yielding slightly higher virus detection in the outpatient samples (51.7% vs 49.7%). The dominating viruses detected were: HuRhV (21.1%), HuCoV (15.0%) and PIV (12.1%) among 654 pathogens from outpatient samples, whereas RSV (22.4%), HuRhV (15.0%) and PIV (14.1%) were dominant among 831 pathogens from inpatient samples. Analysis of monthly distribution of average detection rates for individual pathogens revealed different patterns: PIV, RSV, HuCoV, HuBV caused epidemics 2 times a year in October-December and in March-May; IV prevailed in December-February; PEV was detected mostly in July-September; whereas HuRhV, AdV, EV and MyP were detected year-round. Conclusions: The detection rate of respiratory pathogens in respiratory samples from ARI patients can be significantly improved with mx-rt-PCR method and it is recommendable to introduce it into the routine virological surveillance for ARI.

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Monitoring of influenza viruses in Moscow region, 2009-2013

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Background: The influenza viruses have uncontrolled spread. New subtypes of influenza A virus that transmit human to human appear from time to time. The Institute of Virology is 1 of 4 Reference Laboratories involved in influenza virus monitoring in Russia. The influenza virus seasonal activity was studied over 2009-2013 in Moscow. Materials and Methods: Nasal swabs from patients with ILI and tissues from dead individuals were analyzed to detect influenza A and B viruses by rt-RT-PCR (Amplisens, Russia). Virus isolation in MDCK and embryonated chicken eggs, HA, and HI tests were used to study influenza virus strains properties. Results: After the first case of influenza A(H1N1)pdm09 virus brought into Moscow in May 2009, no other cases of the infection being transmitted followed. The period of October-December was characterized by the wide spread of pandemic virus with the peak of its activity in November. During this period the number of A(H1N1)pdm09-positive cases exceeded 50% every week. The period of February-April 2010 was the second wave of epidemic caused by influenza B virus activity. Meantime, the activity of seasonal influenza viruses was low: A(H1N1)—0.2%; A(H3N2)—0.1%. Antigenic analysis of 96 pandemic strains showed that all of them were A/California/7/2009-like; 70 strains belonged to reference strain B/Brisbane/60/2008 (B/Victoria-lineage) and only 1 of these to the B/Yamagata lineage. The pandemic virus continued circulating in Moscow in 2010-2011 season with its highest activity in February-March. A(H1N1)pdm09 was found in 699 samples (39.7%) of 1761 specimens, B—51 (2.9%), A(H3N2)—4 (0.2%). Out of 54 fatal cases, A(H1N1)pdm09 was confirmed in 49 and influenza B in 5 cases. All 256 isolates were related to reference vaccine strains. However, 8.3% of A(H1N1)pdm09 showed reduced titers with antiserum produced against A/California/7/2009, and we found 9 influenza viruses B/Yamagata-like that the vaccine didn't include. The 2011-2012 epidemic, unlike 2 previous seasons, was caused by influenza A(H3N2) and B circulation. The first cases of influenza infection were registered at the end of January with peak of their activity in March 2012 (30.8%). The A(H1N1)pdm09 viruses had sporadic activity. 1142 swabs were tested by PCR, 373 (33%) of them were found positive. Isolated influenza viruses accounted for 340: A(H1N1)pdm09—8 (2.3%), A(H3N2)—192 (56%) and B—140 (41%). Antigenic analysis showed that the isolated strains possessed properties of vaccine strains used in that period. However, 5 influenza B belonged to B/Yamagata-lineage different from the vaccine strains. The A(H1N1)pdm09 viruses have been found out to be back in circulation in the current 2012-2013 season with the highest activity in January-March 2013. The period of April-May was characterized by the increased spread of influenza A(H3N2) and B viruses. The study of 1990 specimens have shown that 288 (8.34%) were A(H1N1)pdm09 positive, 184 (4,24%)—influenza B and 95 (6.97%)—A(H3N2). According to the antigenic analysis of 342 isolates 128 influenza A viruses belonged to A/California/01/2009pdm09, 121 were influenza A(H3N2) A/Victoria/361/2011-like, 84 influenza B were similar to the current vaccine reference strain B/Wisconsin/01/09 (B/Yamagata lineage) and 25 were B/Brisbane/60/2008 like. Over 2012-2013 period 56 lethal cases have been registered. Influenza A(H1N1)pdm09 has been confirmed in 54 cases, influenza A(H3N2) and B—in one cases, correspondingly. Conclusions: A(H1N1)pdm09 viruses were the predominant circulating subtype at high levels in last years in Moscow and replaced seasonal influenza A(H1N1). Influenza A(H1N1)pdm09 cocirculated with influenza A(H3N2) and B with different activity in different periods. Funding: The study was supported by the Centers for Disease Control and Prevention, Atlanta, Georgia, United States, CoAg: U51IPOOO527-02.

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Characterization of viral agents of acute respiratory infections during influenza season 2011-12

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Background: Acute respiratory illnesses (ARIs) are a leading cause of infectious disease-related morbidity and mortality, particularly in developing countries. Regardless of geographic location, the most common etiologic agents of ARIs are influenza A and B, respiratory syncytial virus (RSV), human metapneumovirus (hMPV), adenoviruses (AdV) and parainfluenza viruses. However, the dominant viral etiologies responsible for acute respiratory infections (ARIs) are poorly understood in resource-limited countries like Pakistan, mainly due to inadequate available data and inadequate integrated disease surveillance systems. The development of better, sensitive and specific molecular detection methods has facilitated rapid and simultaneous detection of a variety of respiratory viruses. This descriptive study retrospectively analyzed the viral aetiologies, clinical and epidemiological features of viral pathogens contributing to presentation with acute respiratory infections at a tertiary care public hospital from October 2011 to April 2012. Materials and Methods:Nasopharyngeal and throat swabs were collected from patients of all age groups admitted with WHO case definitions of influenza-like illness (ILI) or severe acute respiratory syndrome (SARI). Detailed epidemiological information, including underlying risk factors/disease conditions, was collected using a standardized ARI form following informed consent. Samples were tested for viral respiratory pathogens, including seasonal influenza A or B and non-influenza respiratory viruses (NIRV) like RSV, hMPV and AdV using the CDC real-time RT-PCR (RRT-PCR) protocol. The epidemiological data was analyzed using SPSS version 17.0. Results: There were 837 participants in the study, with 55% females. The mean age was 17+/-18 years, ranging from 1 month-87 years. Viruses were identified in 367 (44%) subjects, and there were 8 cases of co-infection with more than 1 virus. Influenza viruses, including pandemic H1N1 2009, were the most frequently detected pathogens, accounting for 37% (n = 312) of all detected viruses. RSV 4.8% (n = 37), adenovirus 1% (n = 8) and HmPV 0.4% (n = 3) were other causative agents of ARI. There were 3 cases each of co-infection with RSV/influenza, and HmPV/influenza and 1 case each of RSV/HmPV and adenovirus/influenza. The relationship between identified pathogen and age of the patients was statistically significant by analysis of variance using Anova (*P* = .003). Analysis of clinical features and disease-associated morbidity showed only sore throat as a significant factor, while presentation with fever and cough was equivocal. However, frequency of fever and cough were also highest amongst cases with influenza A or B. Amongst the cases with positive detection of a viral etiology, 33 (9%) were admitted with severe respiratory disease, including pneumonia, and this association was statistically significant by Chi-square test. Presence of underlying clinical illness, such as cardiac (1%), respiratory (2%), hepatic/metabolic conditions (3%) and habits such as smoking (4%) had no impact on the viral etiology. Only history of allergy was a significant risk factor for infections with viral pathogens (*P* = .001). Conclusions: This study contributes baseline epidemiological and virological data on ARI in Pakistan and highlights the importance of influenza and RSV as dominant viral etiologies. Applying molecular diagnostic testing enabled identification of a viral agent in over 40% of cases who presented for evaluation of ARI. Our data demonstrates a high frequency of viral respiratory infections, emphasizing the need for a more accurate diagnosis, particularly for the emerging respiratory viruses. The percentages of detected viral pathogens may increase if samples are also tested for rhinoviruses and coronaviruses. For better understanding of the full spectrum of respiratory viruses causing ARIs, there is a dire need to enhance laboratory capacity in order to improve preventive and therapeutic strategies. Furthermore, enhancement of existing surveillance systems will be an important step to better understand disease burden of respiratory pathogens.

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Circulating influenza A (H3N2) viruses in Argentina in the post-pandemic period

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Background: Influenza is a public health problem that affects 5%-20% of the world population annually, causing high morbidity and mortality, especially in groups at risk, and at the same time posing a potential pandemic threat. Currently, 2 influenza A subtypes, A(H3N2) and A(H1N1)pdm09, are circulating in the human population. Acquisition of point mutations in the haemagglutinin (HA) glycoprotein leads to continuous antigenic change, a process called “antigenic drift.” Every 3 to 8 years, predominant A(H3N2) viruses are replaced by a novel antigenic variant, prompting an update of the recommended influenza vaccine strain. Before the A(H1N1) 2009 pandemic, the Argentinean Ministry of Health recommended the influenza vaccination annually for people from 6 months to 65 years of age with risk factors and for those aged over 65 years. Since 2011, all children between 6 months and 2 years of age have been included. Surveillance of influenza viruses circulating in Argentina is an essential tool that enables early detection of new viruses and facilitates the decision about the annual vaccine formulation. Materials and Methods: Influenza-positive clinical samples are routinely submitted to the National Influenza Centre (NIC) through the National Influenza and Respiratory Viruses Laboratory Network for further characterization. Subtyping of positive influenza A clinical samples is carried out by a 1-step real-time RT-PCR assay using specific primer/probe sets for the HA gene developed by CDC. Since 2010, influenza A(H3N2) clinical samples have been inoculated in MDCK-SIAT1 for viral isolation. The presence of virus in the supernatant is confirmed by direct IF and/or hemagglutination assay. A subset of isolates (54 from 2011 and 9 from 2012) was selected for antigenic characterization by haemagglutination inhibition (HI) assay. 80 isolates were selected for phylogenetic analysis based on the sequences of the HA1 region of the HA gene. The sequences obtained were edited using the BioEdit program; phylogenetic trees were constructed using MEGA 4 and MEGA 5 programs with Neighbor Joining distance method and a bootstrap of 1000. Results: During the period 2010-2013, 4567 influenza-positive clinical samples were received at the NIC: 1147 in 2010, 1118 in 2011, 2212 in 2012 and so far 90 in 2013. From this, 2061 samples resulted in A(H3) by real time RT-PCR, 506 in 2010, 844 in 2011, 677 en 2012 and 34 in 2013. From this, 472 viruses were recovered in tissue cultures. Based on HI assay results, the majority of A(H3N2) viruses that circulated in 2011 all over the country were antigenically closely related to the A/Perth/16/2009 vaccine virus. However, viruses isolated in 2012 were closely related antigenically to A/Victoria/361/2011, the A(H3N2) vaccine component for the 2013 Southern Hemisphere season. No isolate was characterized by IHA in 2010; complementary results from 2013 strains will be presented on posters. Phylogenetic tree for the HA1 region shows that the majority of A(H3N2) isolates analyzed up to now in this report belong to the A/Victoria/208/2009 (78/80, 97.5%) genetic clade (subgropus 3C, 4, 5 and 6), although until 2011 the majority of the circulating strains remained antigenically related to A/Perth/16/2009. Conclusions: Surveillance of influenza viruses is essential for updating vaccines and also gives important insights into the mechanisms of virus evolution. Based on results of both HI and phylogenetic analysis, most influenza A(H3N2) strains characterized up to 2011 influenza season were antigenically related to the recommended vaccine strain. In 2012, the majority of representative A(H3N2) isolates were antigenically similar to the strain A/Victoria/361/2011, an antigenic variant of the A(H3N2) vaccine component A/Perth/16/09. It should be considered that several of the substitutions accumulated in the HA could affect the antigenicity of the virus. The strain A/Victoria/361/2011 was recommended by WHO as the H3 component for the 2012-2013 Northern Hemisphere and 2013 Southern Hemisphere formulation.

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A review of the surveillance systems of influenza in selected countries in the tropical region

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Background: Influenza viruses cause annual epidemics of respiratory tract disease that affect all age groups. Many developing countries do not have an influenza surveillance system or adequate laboratory capacity for virus detection. The objective of this study was to describe the influenza surveillance systems in the different countries in the tropics and to identify outstanding research needs. Methods: A questionnaire was designed to gather information specifically on the surveillance system, sentinel sites, specimen and data collection, and laboratory testing. The questionnaire was sent to the different national influenza centers (NICs) and/or ministries of health (MOHs). Additional information was obtained either online from the MOH Web sites of the different countries. Annual reports and presentations made by the NICs or MOHs to international conferences (eg, the Annual African Network for Influenza Surveillance and Epidemiology) were also included. Results: Questionnaires were sent to 52 NICs and MOHs in the different countries in tropical Asia and Africa. Replies were received from 32 NICs and MOHs (61.5% response). In the study, 32 countries were included among which 17 were located in tropical Asia. For Africa, only 15 countries were included. These were the countries whose NICs responded to the questionnaires sent or responded by providing presentations or publications from the country. Human Influenza surveillance comprises 2 components: influenza-like surveillance (ILI) and severe acute respiratory infection (SARI). ILI and SARI surveillance have been initiated in all the countries in tropical Asia except for Malaysia and Cambodia. There are 20 WHO recognized NICs in tropical Asia and 14 in tropical Africa, all with virus isolation and polymerase chain reaction (PCR) testing capacity. In terms of surveillance sites, for Asia, only Hong Kong and Singapore reported that the patient population from the sites represents the broader community. In tropical Africa, only Senegal has sentinel sites distributed all over the country contributing to the geographic representativeness of the surveillance system. The rest of the countries in Africa have just established their influenza surveillance system in the past decade and are working toward geographic expansion of the ILI and SARI sites. In most of the NICs, epidemiologists are full-time employees who are in charge of outbreak investigation and response, risk communication and analysis of ILI and SARI data. Relevant technical personnel have been trained by staff from the WHO or US Centers for Disease Control and Prevention (CDC) in Atlanta, United States, or Institut Pasteur. All NICs and MOHs in tropical Asia and Africa collaborate with the WHO. Initiatives for building national capacity in the countries in tropical Asia and Africa include cooperative agreements with the US CDC, the US Naval Medical Research Unit-3 (NAMRU-3) or Institut Pasteur. Conclusions: There are existing influenza surveillance systems in several countries in Asia and Africa. WHO and different organizations such as the US CDC, NAMRU, and Institut Pasteur lend support to NICs and laboratories in Asia and Africa. Surveillance will allow determination of the rates of influenza and a description of the clinical characteristics of the disease. With such epidemiologic data, public health officials will have better data to formulate influenza control strategies for either seasonal or pandemic influenza. Most of the countries are working toward geographic expansion of the ILI and SARI sites. Very few countries in tropical Asia and Africa have the capacity to measure the diseases burden of influenza. About half of the countries in Africa still do not have the laboratory capacity or infrastructure to perform influenza surveillance. This makes it difficult to justify the importance of influenza vaccine or other influenza control measures as a strategy for improving population health. Collaborations between countries should be strengthened for efficient utilization of laboratory services and capacities.

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Epidemiology of influenza B lineages prevalent from 2011 to 2013 in Pakistan; the 2011-12 vaccine was a mismatch

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Background: Influenza virus type B is a major epidemic strain responsible for considerable mortality and morbidity. Since the mid-1980s, the HA gene of influenza B viruses has diverged into 2 antigenically and genetically distinct lineages represented by B/Yamagata/16/88(B/Yam) and B/Victoria/2/87(B/Vic)-like viruses that co-circulate among humans with seasonal/periodic variation in the dominant lineage. Knowledge of prevalent influenza B lineages year after year is important to assess whether the vaccine strain will match closely enough to protect the recipient population. This study determined the circulation of influenza B lineages in Pakistan during January 2011 to April 2013. Methods: Respiratory samples, including nasopharyngeal and throat swabs, were collected with epidemiological information from 7 influenza sentinel sites. WHO case definitions for influenza-like illness (ILI) or severe acute respiratory illness (SARI) were used for patient selection. Representative viruses were grown on Madin Darby Canine Kidney (MDCK) cell line and subtype identification for Influenza B was carried out using WHO Influenza reagent kit. Samples were confirmed for type and subtypes of seasonal influenza A or B using the WHO and CDC Real time RT-PCR (rRT-PCR) Protocol for detection and characterization of influenza viruses. The epidemiological data was analyzed using SPSS version 17.0. Results: A total of 3642 nasal/throat swabs were processed from January 2011 to April 2013. Out of these, 26% (n = 932) samples were identified as influenza A or B. Detection of influenza A viruses was dominant, with positive detection in 78% (n = 731) of total sample, while influenza B accounted for 22% (n = 201) of positive cases. Among the influenza B viruses, 54% (108/201) were typed as B/Victoria lineage, while 46% (n = 93) were identified as B/Yamagata lineage. Influenza B circulation was observed throughout the year, but there was a surge in cases during the influenza season from October-March. Detection frequencies for influenza B during 2011, 2012 and 2013 were 15%, 29% and 74% respectively. The 2 influenza B lineages co-circulated during the entire study period, with increased prevalence of B/Victoria than B/Yamagata. Only during 2011, B/Yamagata was the dominant circulating lineage, making up to 75% of all detected influenza B cases, while during 2012 and the early part of 2013, B/Victoria lineage prevailed, with frequencies of 76% and 64% respectively. Cross-sectional analysis showed that influenza B subtypes variation between geographic regions was statistically significant ($P < .001$) for 3 consecutive years in this study. Both B/Yam and B/Vic lineages were seen at a higher proportion among children ≤ 5 years of age (31%-32%) followed by adults between 21-30 years of age (16%-21%). Mean age was higher for influenza B/Yam; 24 ± 22 years as compared to B/Vic at 19 ± 16 years. However, no significant differences were observed with respect to gender and hospitalization status between the 2 lineages. Conclusion: This study showed that influenza B viruses are responsible for considerable morbidity in the Pakistani population. The composition of the 2011-12 northern hemisphere influenza vaccine recommended by the WHO included a B/Brisbane/60/2008-like virus from the B/Victoria lineage, which did not match with the prevalent B/Yamagata strains. In time characterization of lineage of influenza B viruses and submission of representative isolates to GISRS (Global Influenza Surveillance and Response System) is therefore pivotal towards choice of relevant strains to be included in the subsequent annual vaccine. Since the epidemiology of the 2 influenza B lineages keeps changing, considerable morbidity associated with influenza B stresses the need for careful consideration of a quadrivalent influenza vaccine that includes viruses of both circulating lineages.

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Analysis of 5-year influenza surveillance and laboratory data from Pakistan (2008-13): informed disease prevention strategies

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Background: Through centuries, influenza viruses have caused infections in a plethora of hosts. Their segmented genome permits rapid evolution through antigenic drift and shift, leading to emergence of novel subtypes that cause epidemics and pandemics. Human cases of avian influenza H5N1 since 1997, human H7N3 infections in The Netherlands in 2003, emergence of a re-assortant pandemic A/H1N1 virus in 2009 and the currently ongoing pandemic threat of H7N9 influenza exemplify this potential. In Pakistan, prior to 2007, influenza burden of disease was largely undetermined as no formal surveillance system was in place. This report describes the establishment of a lab-based influenza surveillance network in the country since then and the first 5 years' surveillance data. Methods: Nasopharyngeal or throat swabs were collected from influenza-like illness (ILI) and severe acute respiratory illness (SARI) cases as per WHO case definitions from 7 sentinel sites at tertiary care hospitals and various districts along with relevant epidemiological information. The samples were tested using CDC Real time RT-PCR (rRT-PCR) Protocol for detection and characterization of Influenza viruses. All epidemiological and laboratory data was analyzed using SPSS (version 18.0). Results: From Jan 2008-April 2013, 8565 respiratory samples were received for influenza testing. On analysis by geographical distribution, the highest number of samples were positive for influenza in Federal capital-71% followed by Punjab-14%, Sindh-8%, Kyber-Pakhtunkhwa- 2%, Balochistan-4% and from AJK-1% (Jan-April 2013). Over all, the frequency of influenza detection was 16% (n = 1485), and influenza A viruses constituted 73% (n = 1088) of positive cases while 27% were influenza B. The influenza A (Inf. A) subtype distribution was; Inf. A (H1N1) pdm 2009-65 % (n = 704), Inf. A/H3-31% (n = 336) and A/Seasonal H1-4% (n = 48).The ratio of cases reported was slightly higher in males (57%) as compared to females. The overall mean age was 20 ± 19 years ranging from 1 month-90 years. The highest number of positive cases (32%) was reported amongst patients under 10 years (95% CI: 3-9). The statistical relationship between age groups and presence of influenza infection was found significant (*P* = .001). The mean age of ILI cases was 28 ± 17 and 41 ± 20 for SARI cases. The significant relationship was found in ILI and SARI-positive cases with age by Fisher's chi-square test (*P* = .002). Hospitalization rates were highest for Inf. A (H1N1) pdm 2009 at 32% followed by 21% for Inf. B and 9% for other seasonal influenza. Positivity rate was higher among SARI cases 22 % (n = 450) than ILI-18% (n = 955).The frequency of underlying risk factors among influenza-positive hospitalized cases was highest for respiratory conditions (9%) and asthma (25%) followed by smoking (12%) diabetes (13%) and cardiac disease (3%). Conclusion: During 2008-2013, the lab-based influenza sentinel surveillance system has generated important baseline data on influenza related respiratory illness which can be used to strengthen future preparedness and prevention plans. The influenza surveillance system has proved its worth by thoroughly supporting the pandemic preparedness and response through provision of laboratory diagnostic support for seasonal, pdm H1N1 and human cases of H5N1 avian influenza at the country level. The network provided critical technical and capacity building support for national and provincial public health personnel in case detection, response and control measures. We have established that influenza is a significant public health problem with evidence of virus circulation throughout the year and seasonal peaks in winter and early spring. Our 5-year data shows that Influenza accounts for a large proportion of all hospitalizations. Therefore, provision of viral testing for SARI cases at tertiary care facilities would be helpful towards more succinct diagnosis and treatment. We found that only < 1% of total cases

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reported vaccination against influenza, indicating limited awareness, which highlights the need for promoting public awareness about influenza and availability of vaccine.

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Molecular epidemiology of influenza A/H3N2 viruses circulating in Pakistan from 2008-2012

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Background: Influenza viruses are negative sense RNA viruses that infect a wide variety of birds and mammals. Human viruses continually circulate in yearly epidemics, and novel strains emerge intermittently/sporadically as pandemic viruses. Pandemic influenza A viruses have emerged 4 times during the last century: in 1918 (H1N1 subtype), in 1957 (H2N2), and in 1968 (H3N2) and 2009(H1N1 Pdm). This study retrospectively analyzed the epidemiological linkages of human H3N2 viruses in Pakistan by partial genome sequence analysis. Materials and Methods: Respiratory samples, including nasopharyngeal and throat swabs, were collected from all age groups presenting with influenza-like illness (ILI) or severe acute respiratory illness (SARI) based on WHO case definitions. Samples were tested for influenza A/H1, A/H3N2 or A/H1N1 (Pdm09) and influenza B viruses using the CDC Real time RT-PCR Protocol for detection and characterization of Influenza. Haemagglutinin (HA), neuraminidase (NA) and matrix (M) genes of representative H3N2 isolates were amplified using RT-PCR and CDC primer sets. Sequences were aligned using the Bioedit program. Nucleotide and amino acid sequences were compared using the MEGA program version 5.0 for tree building by the neighbor-joining method. The epidemiological data was analyzed using SPSS version 17.0. Results: During November 2008-December 2012, a total of 6462 respiratory samples were collected from patients at Federal Government General Hospital Islamabad. Out of these 969 (15.3%) tested positive for influenza A or B. Influenza A/H3N2 accounted for 39% (n = 289) of influenza A strains. The detection frequency for A/H3N2 viruses was highest during 2012; 37% and 2011 (31%). The majority of H3N2 cases (89%) were outpatients reporting with ILI. The mean age of H3N2 positive cases was 22.59 +/-20 SD, and 38% of patients were < 10 years of age followed by the 20-30 years of age group (18%) On phylogenetic analysis, both H3 and N2 sequences of Pakistan viruses formed distinct clusters with regional viruses and some swine origin human H3N2 strains. Kimura genetic distances between the 2008 isolates and the 2009 isolates ranged from 0.2% to 1.8% at the nucleotide level and from 0.9% to 3.1% at the amino acid level for the HA gene. All of the isolates had the classical receptor binding site motif with 136S, 153W, 183H, 195Y, and 225–228 NIPS in the HA protein, except position 19Y. Major differences between the 2009 and 2012 isolates were at antigenic sites A, B, and D. Analysis of the HA sequences indicated that the 2008-09 Pakistan isolates were similar to A/Brisbane/10/07-like viruses; all carried the signature genetic marker K173Q, while some 2009 strains had diverged from the A/Brisbane/10/07-like viruses. The 2009 viruses carried substitutions N189K and K158N, similar to vaccine strains A/Victoria/208/09 and A/Perth/16/09-like viruses. Furthermore, none of the analyzed isolates clustered with the A/Perth/16/09-like strains, defined by additional substitutions E62K and N144K, but some 2009 and later 2012 isolates had the T212A substitution characteristic of the A/Victoria/208/09 and later A/Victoria/361/2011 (2012-13 H3N2 vaccine strain for northern hemisphere). Phylogenetic analysis of the NA genes of the Pakistan isolates indicated that most of these viruses were related to A/Brisbane/10/2007-like viruses, with D147N and I215V genetic substitutions. All the viruses were susceptible to oseltamivir and did not carry resistance conferring E119V substitution. Similarly analysis of M gene showed that all isolates had the S31N genetic marker for adamantine resistance in M2 sequence. Conclusion: This is the first report on molecular epidemiology of H3N2 viruses from Pakistan. Our analysis shows that both HA

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and NA genes of our isolates share up to 99% homology not only with India, Nepal and Iran, they also show 97%-98% similarity with the human H3N2 variants of swine origin. In view of endemic H9N2 viruses and previous history of H5N1 and H7N3 avian influenza outbreaks in poultry in Pakistan, it is vital to track these evolutionary changes in human viruses for early detection of re-assortment events or emergence of antiviral resistant subtypes. Therefore, the importance of continued molecular surveillance of human influenza viruses towards best matched annual vaccine development cannot be overemphasized.

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Influenza virus strain data from Thailand, 2005-2012: how well does the vaccine match?

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Background: Thailand's annual public influenza vaccination campaign begins in June and uses the Southern Hemisphere formulation. Both Northern (NH) and Southern (SH) Hemisphere formulations are sold in the private sector. A national sentinel surveillance network for influenza-like illness and severe acute respiratory illness is used to monitor weekly influenza virus activity and herald an increase in influenza activity. Here we use these data to evaluate the match and timing between strains circulating in people and those in the trivalent vaccine. Methods: Respiratory samples positive by real-time reverse transcription polymerase chain reaction were cultured on MDCK cells. Fifty percent of high HA titer (≥1:64) influenza isolates were randomly sampled each month and antigenically characterized at the Thai National Influenza Center using the hemaglutination inhibition assay (HI assay) with standard reagents provided by WHO. In addition, every year some uncharacterized isolates and known strain samples were selected and sent twice a year to the WHO Collaborating Centers in Melbourne, Australia and US CDC, Atlanta, Georgia, for confirmation (about 50 isolates and original samples per shipment). If results were discrepant, we used the WHO CC results. Our data were analyzed by using the timing of new virus introduction relative to vaccine availability from 2005-2012. Northern Hemisphere vaccine (NH-vaccine) and Southern Hemisphere vaccine (SH-vaccine) strains were compiled from the WHO website. Results: From 2005-2012, we characterized 1594 influenza viruses (range, 108-353 isolates/year); 973 (61%) were influenza A viruses (479 H1, 494 H3) and 621 (39%) were influenza B. The predominant type by year was H3 (2005, 2007, 2008), H1 (2006, 2009, 2010) and B (2011-12). The circulating strain to SH-vaccine match by year was as follows: 2005 (100% H1, 34% H3, 60% B), 2006 (94% H1, 0% H3, 33% B), 2007 (0% H1, 54% H3, 23% B), 2008 (6% H1, 100% H3, 60% B), 2009 (18% H1, 69% H3, 0% B), 2010 (100% H1, 100% H3, 89% B), 2011 (100% H1, 100% H3, 95% B), 2012 (100% H1, 26% H3, 58% B). For most of the 8 years, the 2 B strains (Victoria and Yamagata lineages) circulated in Thailand with a ratio of 1:1 to 2:1 or 1:2, except in 2010 and 2011, when around 95% of influenza B belonged to Victoria and matched well with both NH and SH-vaccines. In years with a poor H3 match (2005-7, 2009, 2012), we found co-circulation of the H3 vaccine strain and a new H3 strain that was detected in our surveillance system in August-September each year. This suggests that the essential period for monitoring for the appearance of new strains should be concentrated during August to December. Conclusions: Our study of Thai local influenza strains from 2005-2012 revealed that predominant influenza strains changed year to year and influenza A viruses were almost always the predominant type. For most years, the majority of all strains matched the SH-vaccine. Close monitoring of strains in the second half of the year is likely to pick up emerging strains. The common co-circulation of the 2 B lineages made it difficult to predict the upcoming predominant B strain and suggests that the use of the new quadrivalent influenza vaccine, not yet licensed in Thailand, may improve protection.

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Association of influenza and other respiratory viruses with severe acute respiratory infection and influenza-like illness, relative to healthy controls in South Africa

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Background: Several viruses are detected in patients with acute respiratory infections that may be the cause of severe acute respiratory diseases, influenza-like illness (ILI). The relative contribution of many of these respiratory viruses to severe acute respiratory disease in sub-Saharan African settings with a high prevalence of HIV may differ from that in the developed world. We compared the prevalence of 10 common respiratory viruses in a systematic surveillance programme for patients hospitalized with severe acute respiratory illness (SARI) as well as outpatients with ILI and with control subjects who did not report any respiratory or gastrointestinal illness attending an outpatient clinic in the same region to describe the viral etiology of respiratory disease in South Africa and assess the role of respiratory viruses in severe disease. Methods: We conducted active, prospective SARI surveillance at 2 hospitals and conducted ILI surveillance and enrolled control patients at 2 affiliated outpatient clinics in South Africa: Edendale Hospital and Gateway Clinic (Pietermaritzburg, KwaZulu-Natal Province) and Tshepong-Klerksdorp Hospital Complex and Jouberton Clinic (Klerksdorp, Northwest Province). Patients meeting the case definitions were systematically enrolled during May 2012 to April 2013. SARI case definition: patients ≥ 5 years old hospitalized with onset of illness within 7 days of admission, children 2 days through 2 months old as physician-diagnosed sepsis or lower respiratory tract infection (LRTI) and children 3 months through 5 years old as physician-diagnosed acute LRTI; ILI case definition: acute fever > 38°C and/or self-reported fever and cough within the last 7 days. Controls had no history of respiratory illness, diarrheal illness or fever in the preceding 14 days and were frequency matched to SARI cases by age and time. Nasopharyngeal aspirates were collected from children < 5 years of age and nasopharyngeal and oropharyngeal swabs were collected from individuals ≥ 5 years of age. The respiratory samples were tested by multiplex real-time polymerase chain reaction (PCR) assay for 10 respiratory viruses: influenza A & B, respiratory syncytial virus (RSV), adenovirus, enterovirus, human metapneumovirus (hMPV), rhinovirus and parainfluenza virus 1-3. We used multinomial logistic regression analysis to compare SARI and ILI patients to controls. The analysis was performed using STATA 12 (Stata Corporation, Texas, United States). Results: Of the 3907 patients enrolled, 2125 (54.39%) were SARI cases, 1325 (22.91%) were ILI cases and 457 (11.70%) were controls. Of the SARI cases, 730 (34.35%) were younger than 4 years old, compared to 298 (26.07%) and 115 (25.16%), (P = .226) of the ILI cases and controls respectively. The most common virus identified among SARI, ILI and controls were: rhinovirus [(515/962, 26.59%), (356/962, 28.30%) and (91/962, 22.03], followed by adenovirus [(338/581, 17.44%), (173/581, 13.75%) and (70/581, 16.95%)], influenza A and B [(91/282, 4.70%), (188/282, 14.94%) and (3/282, 0.73%)] and RSV [(259/401, 13.37%), (117/401, 9.30%) and (25/401, 6.05%)]. Enterovirus, hMPV and parainfluenza viruses were each found in less than 3% of SARI, ILI and controls. In multinomial regression model analyzing the association of the different respiratory

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viruses with disease severity controlling for HIV infection and age, influenza virus [(adjusted relative risk ratio (aRRR) = 23.6; *P* = .001 for ILI), (aRRR = 7.2; *P* = .001 for SARI)], RSV [(aRRR = 1.9; *P* = .013 for ILI), (aRRR = 2.2; *P* = .001 for SARI)], hMPV [(aRRR = 16.5; *P* = .006 for ILI), (aRRR = 9.2; *P* = .030 for SARI)], and rhinovirus [(aRRR = 1.9; *P* = .001 for ILI), (aRRR = 1.6; *P* = .001 for SARI)], infection was associated with both ILI and SARI. Similar prevalence of other respiratory viruses was found among SARI, ILI and controls resulting in lack of association with clinical syndromes. Conclusions: These preliminary results indicate that influenza, RSV, hMPV and rhinovirus infection is associated with both mild and severe respiratory disease in South Africa relative to healthy controls. Other viruses were not associated with severe or mild respiratory illness in this study. This study should be continued to include an entire year of ILI and control surveillance to account for seasonality and to allow for age-stratified analysis, this will provide useful information on how each pathogen impacts disease severity to help prioritize vaccine development.

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Trends of circulating influenza viruses in a rural Indian community under active surveillance: impact on vaccination timing

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Background: Improved understanding of temporal and geographic circulation of influenza viruses is essential for development of effective prevention and control strategies. The efficacy of influenza vaccine not only depends on the antigenic match between the circulating strain with vaccine composition, but also timing of vaccination as vaccine-induced immunity likely wanes with time. Active virologic surveillance over a three-year period was assessed to identify the peak of influenza circulation and reassess the vaccination timing in and around Delhi region. Methods: From November 2009 to November 2012, three villages in Ballabgarh, Haryana state, north India, were under prospective weekly active surveillance for febrile acute respiratory illness (FARI) under the Comprehensive Rural Health Services Project, of All India Institute of Medical Sciences as part of a trial of seasonal inactivated trivalent influenza vaccine in children. Weekly house-to-house surveillance for FARI was carried out by trained field workers in this dynamic cohort of > 18,000 individuals. Nasal and oropharyngeal swabs were collected from reported FARI cases and were tested for influenza types and subtypes by real-time RT-PCR. Results: A total of 3251 (11.2%) of 28,955 samples from FARI cases were influenza positive, with consistent peaks of influenza circulation between July and September over the three-year period. Of the 3251 influenza-positive cases, 955 (29.4%) were identified as A/H1N1pdm09, 566 (17.4%) as seasonal influenza A/H3, and 1733 (53.3%) as influenza B viruses. The prevalence of influenza types and subtypes varied significantly over the study period; a distinct peak of A/H1N1pdm09 was observed in November-December 2009, with a second wave during August-September 2010, followed by the distinct appearance of seasonal influenza A/H3 viruses in July-August 2011 and then reemergence of A/H1N1 pdm09 in August-September of 2012. Influenza B viruses co-circulated throughout the study period at low levels, however, higher positivity was observed during the influenza peak period from August-September. In India, influenza vaccine typically follows the northern hemisphere (NH) vaccination regimen (due to physical location of India in NH), with vaccination occurring prior to the winter season. Conclusions: The data show a complex pattern of circulating influenza viruses in a large surveillance setting in this rural Indian community. Given that cumulative data over a three-year study period demonstrates peaks of influenza circulation from July-September, we suggest appropriate timing for influenza vaccination should precede the peak of influenza in July-September. Both northern and southern formulations should be available in the months before July, so assuming that the composition is the same, then either NH or SH vaccine formulations can be used. We believe that well-timed and matched vaccination may induce better immunity and hence confer better protection. Studies are under way to assess if vaccination with SH Influenza vaccine in April-May in Delhi regions results in better efficacy for direct protection against Influenza infection. These studies

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The Global Influenza Hospital Surveillance Network (GIHSN): assessment of statistical heterogeneity between study sites

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Background: The Global Influenza Hospital Surveillance Network (GIHSN) aims to provide timely data on population-based, laboratory-confirmed annual estimates of influenza-related hospitalizations from diverse geographic areas in the world. The ultimate goal is to guide local and global control strategies against influenza severe outcomes. A common core protocol for the 2012-13 influenza season was developed, including a pooled analysis, to facilitate, when possible 1) more precise influenza-hospitalization estimates at the global level and 2) more power to assess groups that are hard to study without combined data. In this work, we report statistical heterogeneity across study sites and the possible reasons for statistically heterogeneous results. Materials and Methods: We estimated influenza and non-influenza hospitalizations in all ages with data for 5 countries: Spain (5 hospitals), Russia (4 hospitals), Turkey (7 hospitals) and France (5 hospitals) during the 2012-2013 season by using a hospital-based test negative design. Individual data from each study was pooled and the presence of heterogeneity between individual studies was assessed by using the Cochran's Q test and the I^2 statistics in a series of a priori defined subgroup analysis. All statistical analyses were performed by using Stata v.12. Results: A total of 4630 records were analysed for the pooled GIHSN data set (Spain N = 2335, Russia N = 1837, Turkey N = 54, France N = 404). Overall, statistical heterogeneity across study sites for estimates of influenza hospitalizations by patient characteristics was not significant (I^2 < 40% and P > .10), but sample size (in strata) remained low to adequately address heterogeneity. Subgroup analysis identified age, influenza strain and presence/absence of high-risk conditions as important characteristics associated to severe disease burden. Conclusions: The insignificant statistical heterogeneity seen between study sites suggests that pooled analysis of the GIHSN network of hospitals is feasible. The assessment of statistical heterogeneity between GIHSN study sites has informed us about how to deal with clinical heterogeneity in analysis. Increased sample size within this network should allow stratified/subgroup IVE estimates. This network activity is partly funded by Sanofi Pasteur.

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Pooled analysis from a worldwide network of hospitals - "Global Influenza Hospital Surveillance Network" (GIHSN)

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Background: The predominant circulating strains and the level of severity of the influenza epidemic vary between years and between countries. In order to control and have an overview of influenza epidemics, high-quality surveillance networks should be put in place. The GIHSN focuses on estimating the incidence of severe influenza diseases leading to hospitalization, documenting influenza strains that are circulating and the burden of disease attributable to each strain. If possible, it also provides the framework for estimating effectiveness of the seasonal influenza vaccines in prevention of severe cases in various age and risk groups. During the influenza season of 2012-2013, the network was composed of 5 hospitals in Spain, 5 in France, 4 in the Russian Federation and 7 in Turkey. With broad regional representation and a platform of international public-private partnerships, it will lead to a better understanding and knowledge of influenza epidemiology. The aim for pooling data from the 21 participating sites is to observe and identify patterns of circulating influenza viruses for different regions of the world (including likelihood of B-strain vaccine mismatch, co-circulation) and possibly informing the World Health Organization (WHO) of strain selection from the pooled epidemiological data. Materials and Methods: A core protocol, questionnaire, spreadsheet was created to compile the essential elements of the influenza epidemiological research project. All participating sites followed homogenously these core documents in terms of the purpose of the study, design, target population, data and variables needed, in order to have homogenous data for the pooled analysis. All sites' spreadsheets were merged together into a pooled database. Summary tables, frequency tables and visual representations of representative values of the pooled database were used to detect irregular, absent and questionable values. All data management and analysis was performed with STATA version 12. Results: Descriptive qualitative characteristics of each site were gathered such to assess heterogeneity, and explore potential sources of heterogeneity like vaccination coverage, age groups, viral isolates, or core protocol deviations. Study participants were described by basic sociodemographic characteristics. Patient characteristics of positives and negatives for influenza were compared using chi-squared tests. A *P* value < .05 was considered as statistically significant. Confounding factors were assessed by comparing crude and adjusted odds ratio (OR) for each baseline characteristics in univariate analyses. A *P* value of <0.1 was considered as significant for inclusion in multivariate models. Tabulations and simple graphical methods were employed to visualize results of the incidence of influenza-associated hospitalizations across age groups, the incidence of the disease attributable to each strain of the influenza virus, and the distribution of different influenza strains among cases. Vaccine effectiveness for preventing influenza related admissions in the population targeted for vaccination will be estimated at site level. Heterogeneity of each site estimates will be evaluated and explained. According to the data available, and taking into account heterogeneity, pooled estimates will be provided. Conclusions:The study allowed for the collection of good quality data, however for complete assessment of all pooled data, further investigation and consistent results are needed. In order to better quantitatively asses the vaccine effectiveness, larger samples are needed. A multicentre, hospital-based study is feasible, and maintaining a homogenous practice across sites through strong coordination and support ensures quality of pooling of data. This network activity is partly funded by Sanofi Pasteur.

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Impact of social and economical constraints on the performances of highly pathogenic avian influenza surveillance systems in Vietnam and Thailand

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Background: Effectiveness of HPAI control strategies depends strongly on passive surveillance, also named reactive surveillance, especially in developing countries. This type of surveillance relies on spontaneous reports of disease suspicions by actors of the animal production sector to sanitary authorities. This system, if implemented properly, is considered as the most cost-effective way for early outbreak detection and to gather information on the disease situation for decision making on control strategies. However, in practice, sensitivity and timeliness of passive surveillance are often not optimal and under-reporting of suspicious cases is considered as a major source of failure to control HPAI epizootics. The objective of our work was to better understand the factors for under-reporting at the local level and to provide recommendations for improvement of the systems. We conducted case studies in Vietnam and Thailand to assess the socioeconomic constraints impacting the surveillance of avian diseases. Materials and Methods: Four case studies were conducted in rural communes from 4 provinces: Hải Dương in north Vietnam, Tiền Giang and Đồng Nai in south Vietnam and Sukhothai in north Thailand. Samples of 80 to 120 participants representing all the actors involved in poultry health and poultry production were interviewed in each location. Semi-structured interviews were applied, along with participatory epidemiology tools (eg, proportional piling, disease impact matrix scoring). Social network analysis was used, based on data collected from these interviews, in order to describe and analyze the pathways of information sharing on poultry mortality events. Qualitative indicators of the socioeconomic constraints influencing the reporting of mortality events were identified using participatory approaches and economic tools of decision-making analysis. Results: Socioeconomic factors impacting the reporting of mortality events are closely related to the characteristics of the production systems. Many actors of poultry production relied on commercial stakeholders, such as local input suppliers or the agro-industry, or on recreational communities (eg, networks of cockfighting players) in their management of animal health issues. These private sector networks represent the main pathways for poultry health information sharing and the integration of producers in such networks tends to go with a lower inclination to deliver information to authorities. The possibility of mitigating the impact of diseases by selling unhealthy animals is a strong disincentive for reporting diseases to authorities, and this practice is directly linked with the level of tolerance of some consumers toward sanitary risks. Finally, the decentralization of the administrative responsibilities regarding the management of animal health also represents a critical impediment to effective outbreak reporting from the local to central level. Discussion: Official surveillance networks interfere with private sector information networks that specifically aim at protecting their own economic or cultural interests. The exchange of sanitary information between the different actors is linked to the different economic and social issues that are highly specific for each production systems. This leads to a difficult question: Could one size fit all? A potential method of improvement would consist in trying to adapt surveillance and control policies to each of these particular production sectors. Moreover, the improvement of passive surveillance needs to be linked to global improvement of sanitary controls and quality assurance of distributed poultry products. Finding ways to improve public-partnership collaboration at the local level would represent a critical challenge for the improvement of surveillance systems' performances.

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Cellular-linked (cloud-based) system for near-real time surveillance of influenza viruses A and B using the Sofia® fluorescence immunoassay platform

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Background: The Sofia Analyzer is an immunofluorescence, point-of-care instrument for use with assays developed for the rapid detection of viral and bacterial pathogens, including the Sofia Influenza A+B Fluorescence Immunoassay (FIA). The system was waived by the Clinical Laboratory Improvement Amendments (CLIA) and available for clinical use in October 2011. In November 2012, a cellular wireless capability was added to the Sofia Analyzer with the primary purpose of providing near real-time, patient de-identified influenza surveillance data to public health laboratories and other organizations. Materials and Methods: Outpatients presenting at 2 small clinics in southern Wisconsin with at least 2 respiratory symptoms (fever, cough, sore throat, rhinitis, and congestion) had paired nasal (NS) and nasopharyngeal (NP) swab specimens collected. The Sofia Analyzer and Sofia Influenza A+B FIA kits were provided by Quidel (San Diego, CA), and the wireless router was obtained from Digi International (Minnetonka, MN). The NS was tested using Sofia, and the NP specimen was promptly transported to the Wisconsin State Laboratory of Hygiene for testing by PCR using the Centers for Disease Control and Prevention (CDC) human influenza virus real-time RT-PCR diagnostic panel. Sensitivity, specificity, and overall accuracy were calculated. The Sofia Analyzer and router enabled transmission of the doubly encrypted, patient de-identified Sofia test result within 1 minute of testing to a cloud-based server. The data were received wirelessly at Quidel and uploaded to a secure FTP site supported by the CDC each night between midnight and 1:00 AM Pacific time. The Sofia Analyzer data (hard copy printout) were compared to the data received wirelessly by the CDC for data transmission accuracy, and assay results from Sofia were compared to those obtained from the PCR assay. Results: Of the 100 patients tested, all test results were shown to have been transmitted and received correctly. Software validation identified no anomalies in the transmission of patient results from the Sofia Analyzer to the authorized recipients. In addition to test results, patient age and patient status (ie, in- or outpatient) were optional fields for entry by the operator and, when entered, were transmitted with 100% accuracy for all patients. The test cassette lot number, instrument serial number (traceable to location), internal quality control results, and calibration results were transmitted automatically without operator action. Influenza A or B was detected by both Sofia and PCR from 25 specimens, including 31 by PCR and 30 by Sofia. Thus, compared to PCR, Sofia yielded a sensitivity of 81% (25/31), a specificity of 93% (64/69), and an overall accuracy of 89%. Conclusions: The Sophia Analyzer with cellular-wireless-based reporting capability was demonstrated to require minimal input by users in the point-of-care outpatient clinics and to reliably transmit data to public health authorities. The wireless capacity provided more immediate, extensive data to public health authorities and has the potential to report to additional organizations for whom rapid surveillance information is valued.

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Unpredictability of circulating influenza strains: pandemic and seasonal influenza surveillance during and post-pandemic periods in India (2009-2012)

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Background: Influenza surveillance in the community is an important tool for carrying out strain surveillance to identify emerging/reemerging strains and unusual epidemiological trends and to define seasonality. We describe the distinct patterns of circulating strains of the virus in different geographical areas in India, including the patterns of pandemic A/H1N1pdm09 from 2009 to 2012. Methods: Patients with influenza-like illness (ILI) presenting in outpatient departments of dispensaries/hospitals and those with severe acute respiratory infections (SARI) who were hospitalized were studied from January 2009 to December 2012. Nasopharyngeal swabs were tested for influenza viruses with real-time RT- PCR (since June 2009). Virus isolation was carried out in Madin-Darby canine kidney cells for representative specimens. Genotyping of influenza A/H3N2 (n = 197), A/H1N1pdm09 (n = 13) and influenza type B (n = 247) isolates were carried out by HA1 gene sequencing. Results: Of the 28,701 ILI/SARI cases from 2009 to 2012; 3982 (13.8%) were positive for influenza virus. Among these, 1260 (31.42 %) were due to A/H1N1pdm09, 937 (23.5%) were H3N2 and 1742 (43.7%) were due to influenza B. Peaks of influenza were observed during July-September in Delhi and Lucknow (north), Pune (west), Allaphuza (Kerala; southwest), Nagpur (central), Kolkata (east) and Dibrugarh (northeast), whereas Chennai (southeast) revealed peaks in October-November for each of the years, which coincided with the rainfall during the monsoon months. In contrast, the peak of influenza in Srinagar (northernmost state with temperate climate) occurred in December-February, similar to what has been observed for northern hemisphere countries. The patterns of circulating strains varied over the years. Phylogenetic analysis of the H1N1pdm09 (n = 13) 2009 isolates were genetically close to the 2009-2013 vaccine component A/California/07/2009, and all were sensitive for oseltamivir. Analysis of H3N2 isolates revealed similarities with A/Perth/16/2009 (the vaccine component of 2010-2012) in 2009 (n = 114) and 2010 (n = 19), whereas isolates from 2011 (n = 37) and 2012 (n = 8) were similar to A/Victoria/361/2011 (2012-2013 vaccine component). In addition, A/Brisbane/10/2007 (vaccine component for 2008-2010) also co-circulated throughout the study period. All H3N2 viruses were sensitive to oseltamivir and resistant to amantadine with S31N mutation. Likewise, among influenza B viruses we observed co-circulation of both Victoria and Yamagata lineages, with Victoria lineage (with similarity to vaccine strain B/Brisbane/60) being the most predominant strain (> 85%) in 2009 (12/14), 2010 (144/165), 2011 (34/34) and 2012 (29/34). Conclusions: Influenza A/(H1N1pdm09) and A/(H3N2) co-circulated in 2009, whereas in 2010, A/(H1N1pdm09) and type B were predominant. In 2011, predominance of A/(H3N2) was observed along with type B viruses. In 2012, increased activity of pandemic H1N1 was observed. Genetic characterization of influenza A /H3N2), A (H1N1pdm09) and type B isolates showed that Indian isolates were genetically close to the recommended vaccine component. Continued surveillance will help define regional differences in influenza seasonality, as well as determine optimal periods to implement influenza vaccination programs among priority populations. This study was supported by the Indian Council of Medical Research and the Centers for Disease Control and Prevention, Atlanta, USA (cooperative agreement IU51/IP000333).

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Molecular surveillance of Influenza viruses during pandemic (2009–2010) and post-pandemic (2010-2013) periods in western India

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Background: Pandemic influenza A (H1N1) 2009 virus first appeared in India in May 2009 and thereafter continued to circulate with considerable morbidity and mortality in the post-pandemic season in many parts of the country. This study aims to characterize influenza viruses circulating in India during the pandemic and post-pandemic periods and to determine the prevalence of antiviral-resistant viruses. Materials and Methods**:** Respiratory specimens were collected from patients with influenza-like illness on their first visit at outpatient clinics and from hospitalized patients with severe acute respiratory infections both during and in the post-pandemic period (2009 to August 2010 and September 2010 to 2013).Cycling probe real-time PCR assays were performed to detect influenza A subtypes and type B. Allelic discrimination-based real-time assay to detect H274Y mutation was used to detect antiviral resistance. Representative specimens were processed for virus isolation in Madin-Darby canine kidney cells. Sequencing and phylogenetic analysis of whole genomes, hemagglutinin (HA) and neuraminidase (NA) genes were done to characterize circulating strains. Results*:* In the pandemic and post-pandemic period, co-circulation of different types and subtypes of influenza viruses was observed. During the pandemic period, of the 36,010 samples analyzed, 8312 (23.08%) were A (H1N1) pdm09, 764 (2.12%) were A(H3N2), 193 (0.54%) were A(H1N1), 100 (0.28%) were type B and 1257 (3.5%) were influenza Type A viruses. All influenza A (H1N1) pdm 09, A (H3N2) and type B virus isolates were sensitive to oseltamivir. Out of 155 seasonal H1N1 specimens that were tested by allelic real-time RT-PCR, 87 specimens had H275Y substitution in the NA gene, leading to resistance to oseltamivir. In the post-pandemic period, of the 13,989 samples analyzed, 1724 (12.32%) were A (H1N1) pdm09, 495 (3.54%) were A (H3N2), 488 (3.5%) were type B and 67 (0.48 %) were type A viruses. Among A(H1N1)pdm09 isolates, a single isolate of 2013 showed reduced susceptibility to oseltamivir. All A (H3N2) viruses were amantadine resistant with S31N mutation. Phylogenetic analyses: Pandemic influenza A H1N1: HA gene-based phylogenetic analysis of A (H1N1) pdm09 isolates (n = 159) showed that they were genetically close to the 2009-2013 vaccine component A/California/07/2009 (group 0). HA sequences showed cluster of 7 subgroups based on reference viruses. On the basis of mutation S185T; groups 6 to 8 were differentiated from groups 0 to 5. The majority of 2013 HA sequences had double mutation D97N and K283E and clustered in subgroup of 6. The majority of 2012 viruses clustered with group 7, which had characteristic mutation A197T. Co-circulation of group 6 and 7 viruses was noted in 2010, 2011 and 2012; however, the majority of 2010 HA sequences clustered within group 6 possessing single mutation D97N. All the 2009 viruses were clustered in groups 0-5. Whole-genome analysis of 45 A (H1N1) pdm09 isolates showed the predominance of Clade 7 along with a few viruses from Clade 6 and 5. A(H3N2) viruses: HA gene (n = 35) analysis showed; only single isolate of 2009 clustered with contemporary vaccine component A/Brisbane/60/2007; however, the majority (n = 9) viruses clustered with A/Perth/16/2009, which was a 2010-2012 vaccine component. Twenty-five viruses of 2011-2012 were clustered with A/Victoria/361/2011, which was a vaccine component of 2012-2013. Influenza B viruses: HA gene (n = 30) analysis showed that Victoria (HA)-Yamagata (NA) reassortant B/Brisbane/60/2008-like viruses (n = 16) were circulating in 2010-2012; however, circulation of Yamagata lineage (n = 14) viruses was also noted during 2011-2013. Conclusion: India continued to experience significant levels of A (H1N1) pdm09 activity, which necessitated control measures. Genetic variants with mutations at antigenic sites were identified in A(H1N1)pdm09, and type-B viruses in the 2009-2012 season, but these did not show a change in antigenicity when compared with respective vaccine strains. Co-circulation of different types of influenza virus necessitates intense virological surveillance activity.

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Integrated disease surveillance and response system improves early detection of influenza season in Rwanda

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Background: In 2008, the Ministry of Health in collaboration with the US Centers for Disease Control and Prevention established an Influenza Sentinel Surveillance system (ISS) to describe the epidemiology of influenza and promptly detect influenza outbreaks in the country. ISS monitors patients with influenza-like illness (ILI) and severe acute respiratory illness (SARI) at six selected health facilities. For each case, demographic, clinical and epidemiologic data, and naso- and oropharyngeal specimens, are collected and analyzed. In 2011, a modified reporting system called the Rwanda Integrated Diseases Surveillance and Response system (IDSR) was instituted and influenza was added to this surveillance system. As the IDSR system grows, influenza is now monitored through two complementary systems in the country. The IDSR system allows for national baseline data for ILI and severe pneumonia in children < 5 years. We studied data collected through the IDSR system to determine whether the IDSR allows the early detection of the influenza season. Materials and Methods: The IDSR technical guidelines and training modules were amended to include standard case definitions (SCDs) of ILI and severe pneumonia in children < 5 years. Both, ILI and severe pneumonia < 5 years are part of weekly reportable diseases. SCDs were distributed to health facilities, and health care workers from all 45 district hospitals and 475 health centers were trained to identify cases using SCDs. Facilities are expected to submit weekly epidemiologic reports using the "electronic" IDSR system. If this is not done, the electronic system sends automatic reminders to the concerned data managers. We established thresholds of weekly IDSR data using the C2 CUSUM method designated to detect sudden changes in the mean value of a given count. When the number of cases exceeded the plotted C2 threshold value, an investigation was conducted to confirm the presence of influenza. We considered influenza season to be signaled by an increase in influenza laboratory positivity as determined in the sentinel system. The influenza IDSR data reported on a weekly basis was compared with the influenza sentinel surveillance system laboratory data to determine whether the IDSR allowed early detection of increased influenza activity. Results: From October 1, 2012, to May 13, 2013, 707,779 cases of ILI and 26,748 cases of severe pneumonia in children < 5 years were reported through the IDSR system. Average completeness of epidemiologic weekly reports was 98% and average timeliness was 90%. During the 32 weeks of surveillance, the number of ILI cases exceeded the threshold twice, during week 51 of 2012 and during weeks 4 to 11 of 2013. The number of cases of severe pneumonia < 5 exceeded the threshold during weeks 6 to 9 of 2013. During week 4, respiratory samples collected at Zaza and Nyange health centers of Kibungo District Hospital presenting with excess cases of ILI tested positive to influenza virus A(H1N1) pdm09 and A(H3N2) subtypes. From the sentinel surveillance sites, increased influenza positivity from 12.5% to 20% was seen from week 5 to week 19 of 2013 with a peak of influenza positivity rate of 37.5% during week 11. Subtyping data showed that influenza A(H1N1)pdm09 co-circulated with influenza A(H3N2) from week 9 to 19. Conclusions: Influenza is laboratory confirmed year round through the sentinel surveillance system, but the time outbreaks occur cannot be accurately predicted. Preliminary results of weekly reports of ILI and severe pneumonia < 5 into IDSR helped to detect seasonal influenza peaks 7 weeks before the peak of influenza positivity through the sentinel surveillance system. The peaks of influenza activity in both surveillance systems were characterized by co-circulation of influenza virus A(H1N1)pdm09 and A(H3N2) subtypes. The integration of ILI and severe pneumonia < 5 years into IDSR complements the sentinel surveillance system, as it provides baseline data to determine thresholds for influenza season activity per time, place and person.

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The global epidemiology of influenza B: disease burden and vaccine mismatches

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Background: Seasonal influenza is an important cause of morbidity and mortality, with the majority of influenza infections caused by influenza type A. Influenza type B also plays an important role in seasonal influenza, but most scientific literature has focused on influenza type A. The objective of this study is to present an overview of the global epidemiology of influenza B, including the disease burden by age group and the occurrence of vaccine mismatches (the 2 B lineages Victoria and Yamagata), based on surveillance data from around the world. Materials and methods: The study is based on epidemiological and virological surveillance data. The full data analysis will make use of 2 data sources: 1) detailed national surveillance databases and 2) the World Health Organization (WHO) FluNet database. It will include data from 25 countries covering all of the WHO regions. So far (June 2013), detailed national surveillance databases have been obtained from New Zealand (2000-2011), Turkey (2006-2009 and 2010-2012), and South Africa (2010-2012). We calculated the percent of influenza detections due to influenza B for each country and season. When this figure exceeded 50%, we defined the season as influenza B dominant, and when this figure was 20%-49%, we defined it as a season with influenza B co-circulation. When possible, we assessed influenza B detections for the following age groups (restricting the analysis to seasons where influenza B was ≥ 20% of total detections): 0-5 months, 6-35 months, 3-4 years, 5-17 years, 18-39 years, 40-64 years, and 65+ years. For each country and season (seasons where influenza B was ≥ 20% of total detections), we also determined whether there was a mismatch between the predominant influenza B lineage and the B lineage included in the vaccine. Results: Influenza B represented 20% or more of total detections during 6 of the 12 seasons in New Zealand, with 2 influenza B-dominant seasons (2008 and 2011). Lineage data from New Zealand indicated a B mismatch in 3 of the 6 seasons (2002, 2007, and 2008). In Turkey, influenza B represented 20% or more of total detections in all 5 seasons (it was dominant in 2010-2011). Lineage data for Turkey indicated a B mismatch in 4 of the 5 seasons (2007-2008, 2008-2009, 2010-2011, and 2011-2012). South Africa had fewer influenza B detections, with only 1 season where influenza B detections represented 20% or more of total detections (no season dominant). We did not have lineage data for South Africa. Detailed age-specific data were analyzed for the 12 seasons where influenza B represented ≥ 20% of total detections in the 3 countries. We found that more than 90% of influenza B infections were in persons aged 64 or younger (≤ 59 years for South Africa): 95% in New Zealand (n = 1742), 93% in Turkey (n = 458), and 96% in South Africa (n = 464). The full analysis, which will include an assessment of the characteristics of influenza B versus B epidemics (eg, timing and length of seasons) and data from 25 countries, is ongoing. Conclusions: We found that influenza B played a major role in the epidemiology of influenza in 3 different geographically dispersed countries. Our data suggest that influenza B predominantly affects persons aged less than 65 years (< 60 years in South Africa). We also found that influenza B vaccine mismatches were common in the 2 countries (New Zealand and Turkey) that could provide these data. Global data on the epidemiology of influenza B will help determine the public health value of introducing influenza vaccines containing 2 B lineages.

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Electronic school absenteeism system for influenza-like-illness surveillance in Hong Kong

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Background: Seasonal influenza epidemics cause great burden of illnesses, hospitalizations and deaths worldwide. Surveillance systems utilizing early indicator of disease activity would be useful for monitoring community disease pattern and facilitating timely decision making on public health interventions in an evidence-based manner. School absenteeism has been previously considered as a possible syndromic approach for monitoring influenza activity. We explored the feasibility and practicability of establishing an electronic school absenteeism surveillance system in Hong Kong for monitoring influenza-like illness (ILI) using automatically captured data employing smart card technology. Materials and Methods: The school absenteeism surveillance system was developed via the eAttendance module of the eClass system, a digital school administration platform operated by a local commercial vendor. In replacing the traditional paper-based roll call, students of the participating schools would swipe their smart card over a designated sensor at the school entrance each day on entering the school to register their attendance. Electronic summary reports can then be generated automatically for each school in the eClass system and transferred to our back-end server for analysis. The submitted records were anonymised to show only the total number of students and number of absentees in each grade and were further stratified into specific causes of absence, including ILI/upper respiratory tract illness, gastroenteritis, hand-foot-and-mouth disease, other illnesses with specification, and non–sickness-related absence in a subset of participating schools. All collected absenteeism data were aggregated weekly, matched and compared with viral isolation data from hospital laboratory surveillance system and a current outpatient-based influenza sentinel surveillance system. Feedback reports on absenteeism patterns together with interpretation of the overall influenza disease activity in the community were generated on a regular basis during the epidemic season and distributed electronically to all participating schools. Results: Our pilot surveillance initially began with 18 schools covering 17,255 students in 2008 and expanded in phases to 103, including 64 primary and 39 secondary schools covering 72,493 students in the current academic year of 2012-2013. The schools were distributed evenly across Hong Kong Island, Kowloon, and New Territory to cover different districts and represented approximately 10% of the registered number of schools and students in the territory. With the introduction of a more user-friendly dropdown list for entering the specific cause of absence in the user interface starting from this academic year, 35 (34%) of these schools were also submitting data for specific causes of absence in the system. When comparing the school absenteeism rates with the reference data from pre-existing surveillance systems during March 2008 to June 2011, covering a total of 7 influenza seasons, clear and sharp peaks were detected from both the overall and ILI-specific data during most of these seasons, which generally occurred 1-3 weeks ahead of the peak in the hospital laboratory surveillance data (range 1 to 5 and −1 to 4 weeks; median 3 and 0.5 weeks for overall and ILI-specific absenteeism data respectively). Generally the school absenteeism data also showed much sharper peaks than the outpatient sentinel data, possibly related to better coverage of influenza activities in the community, where sentinel data could only capture episodes that required visits to the doctor's clinic. Conclusions: The result demonstrated the feasibility and potential benefit of employing electronic school absenteeism data as captured automatically by a smart card system as an alternative data stream for monitoring influenza activities. The increasing popularity of usage of smart card technology in various community settings might also represent potentially timely and cost-effective opportunities for innovative surveillance systems.

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Influenza as a cause for hospital visit in children under 15 years old at 2 referral pediatric hospitals in Vietnam

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Background: Although sentinel surveillance for influenza-like illness (ILI) has been ongoing in Vietnam since 2006, the burden of influenza-associated ILI in the pediatric population is limited. The aim of this work is to describe epidemiological and laboratory characteristics of influenza in children with ILI at 2 referral pediatric hospitals in Vietnam. Materials and Methods: We selected 2 referral pediatric hospitals, 1 in Hanoi and the other in Ho Chi Minh City, for ILI surveillance. Each day during the period of 1 January 2006 through 30 December 2012, the first 2 pediatric patients who met the ILI case definition of fever (> 38˚C) and cough or sore throat were enrolled for further investigation. From each of the enrolled subjects, oropharyngeal specimens were collected for influenza testing by reverse transcriptase polymerase chain reaction assay. In addition, clinical and epidemiological data were collected. Results: During the study period, a total of 5656 ILI patients under 15 years old were enrolled and investigated. The median age was 2 years (IQR 1-4). There were more male children 3297/5656 (58.3%). Of the 5656 specimens tested, 1385 (24.5%) were positive for influenza. The highest prevalence of influenza was found in the age group of 10-15 years old 64/147 (43.5%), followed by the age group of 5-9 years old 364/892 (40.8%). Influenza positivity in the 1-4 years old and under 1 year old groups was 808/3579 (22.6%) and 149/1038 (14.4%), respectively. The influenza infection ratio was similar for both male and female children, with 804/3279 (24.4%) in males versus 581/2359 (24.6%) in females (*P* = .83). Among the influenza-positive cases, we identified 261 (18.8%) cases with seasonal influenza A/H1N1, 210 (15.2%) with pandemic A/H1N1 2009, 498 (35.9%) with influenza A/H3N2 and 413 (29.8%) with influenza B. Since July 2009, the seasonal A/H1N1 has been replaced by the pandemic A/H1N1 2009. Conclusions: High proportions of influenza positivity were found in pediatric ILI patients at 2 referral pediatric hospitals. Children between 6 and 10 years old had the greatest proportion of influenza positivity. Although studies have shown that vaccination can reduce the morbidity associated with seasonal influenza in children, there was no influenza vaccination strategy applied for children in Vietnam during the time of our study. Our data suggest that it may be important to better understand the influenza-associated disease and economic burden of influenza illness among children in Vietnam to estimate the value of influenza vaccination among young children. In addition, further study is recommended to understand the proportions of respiratory illness in children associated with other pathogens.

P2-663 G FELLOWSHIP GATES Fellowship

Influenza surveillance at ILI sentinel sites in Addis Ababa, Ethiopia: epidemiological and virological findings from 2012

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Background: Ethiopia launched influenza sentinel surveillance in 2008 during a period that “influenza” was virtually synonymous with “pandemic.” Until then, respiratory virus surveillance was nonexistent despite the known high burden of respiratory disease in a country with a population of over 80 million people. Because of the initial close association of influenza surveillance with pandemic preparedness, it was not until 2012 that influenza-like illness (ILI) surveillance assumed its proper role as a routinely

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functioning surveillance system that could also lend support to public health emergency preparedness efforts. Materials and Methods: Patients presenting daily to 3 designated ILI sentinel surveillance sites in Addis Ababa, namely Shiromeda, Kolfe and Akaki health centers, were screened. Those patients that met the ILI case definition were identified and throat swabs were collected from the first 5 randomly presenting cases each day, using commercial viral transport medium. Samples were maintained refrigerated (2°C-8°C) until transported to the national influenza laboratory, usually within a week of collection. RNA from specimens was extracted manually using a commercial extraction kit (QIAamp Viral RNA Mini-Kit, QIAGEN) and subjected to the CDC real-time RT-PCR protocol for influenza viruses using primers and probes provided by the CDC, Atlanta, Georgia, United States. Results: In 2012, a total of 788 ILI visits took place at outpatient facilities in 3 health centers across Addis Ababa, Ethiopia. Complete age, sex, date of specimen collection and influenza lab result information was available for 771 (98%) of the cases. Kolfe, Shiromeda and Akaki health centers each contributed 377 (49%), 311 (40%) and 83 (11%) of ILI cases, respectively. Of the total cases with full information, 340 (44%) were males and 431 (56%) were females. The age range was 0.6-87 years, with a median age of 18 years. A peak number of ILI cases (n = 222; 29%) was observed in the age category 14-25 years. The number of children less than 2 years accounted for 4% of the total ILI cases. Two hundred and eight specimens (27%) were positive for influenza virus across all age groups combined. By contrast, influenza positivity in children less than 2 years was 0.6%. Of influenza-positive cases, 170 (82%) and 38 (18%) were positive for influenza A and B viruses, respectively. Among influenza A positive ILI cases, 15 (9%) harbored seasonal H3 viruses while 155 (91%) harbored influenza A(H1N1)pdm09 viruses. All influenza A(H1N1)pdm09 viruses forwarded to WHO collaborating centers for influenza sequencing and characterization were sensitive to oseltamivir. Influenza viruses continued to be isolated from ILI cases throughout the year, but ILI cases peaked during October/November, with influenza positivity reaching 37%. Conclusions: Influenza viruses were found in the surveyed community year-round with 27% positivity for the entire period. Influenza A (H1N1)pdm 2009 was the most frequently detected virus, accounting for nearly 75% of the total influenza positive cases and 20% of all specimens collected during 2012. The bulk of ILI cases came from 2 health facilities (Kolfe and Shiromeda health centers). All ages were represented in the surveillance but the majority were young adults (14-25 years). Infants and young children were less likely to test influenza positive, suggesting other etiologies for ILI in this age group. Further investigations are warranted to identify other causes of mild respiratory illness and to achieve disease burden estimates due to influenza in specific age groups.

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Respiratory Multi Well System (MWS) r-gene approach to assess infectious agents involved in severe acute respiratory infections (SARI) in under-five years children in Côte d'Ivoire

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Background: Globally, one-third of all deaths in children under five years of age is due to SARI and pneumonia. This is especially true in sub-Saharan Africa, where 1.2 million children under the age of five years die each year from acute respiratory infection. In Côte d'Ivoire, SARI is the third leading cause of morbidity and mortality among children under five after malaria and diarrheal diseases. Among the SARI etiologies, viruses are recognized as the predominant causative agents within this age group. Over the past 10 years, nucleic acid amplification techniques have been developed for many respiratory viruses. Polymerase chain reaction (PCR) and reverse transcription-PCR (RT-PCR) assays provide faster results with equivalent or greater sensitivity than conventional methods. Furthermore, multiplex PCR assays have been used to detect the presence of two or more respiratory viruses in a single reaction tube. The MWS r-gene is one such platform utilizing real-time PCR technology for the simultaneous detection of infectious agents involved in respiratory diseases. In this study, we utilized the MWS platform to assess SARI etiologies in an influenza sentinel surveillance

study. Methods: This study was carried out in the context of sentinel surveillance for influenza viruses in Côte d'Ivoire from January 1, 2011, to December 31, 2013. Patients were recruited from sites performing pediatric surveillance for SARI located in the capital city (4 sites) and the interior of the country (5 sites). The WHO's 2006 SARI case definition was used until June 2012; SARI among children younger than 5 years was defined as a child 2 months to 4 years of age with cough or shortness of breath or difficulty breathing and any sign of severe illness. The WHO's 2011 SARI case definition was introduced in July 2012 and included all patients (> 31 days) presenting with current fever ≥ 38°C or history of fever and cough with onset in the past 7 days, and requires hospitalization. A nasopharyngeal sample was collected for each patient enrolled using a collection kit (Copan Universal Transport Medium kit for the preservation of virus, chlamydia, mycoplasma and ureaplasma). Epidemiologic and clinical information was collected through a standard survey form. RNA/DNA extractions were conducted using NucliSENS miniMAG kits from BioMerieux. Amplifications were performed using respiratory MWS r-gene reagent kits for the detection of influenza A and B viruses, human coronaviruses (229E, NL63, HKU1, OC43), respiratory syncytial virus (RSV), human metapneumovirus (HMPV), human parainfluenza virus (HPIV), rhinovirus, enterovirus, adenovirus, bocavirus, and *Chlamydophila pneumoniae/Mycoplasma pneumoniae*. The real-time PCR platform used was 7500 StepOne from AppliedBiosystems. Results: During the study period, 66 nasopharyngeal swabs were collected and tested, of which 16 (24.2%) were positive for at least one respiratory pathogen. Of the 16, four were positive for > 1 pathogen, two (12.5%) for influenza A(H1N1)pdm09, one (6.3%) for influenza A(H3N2), one (6.3%) for influenza A was unsubtypeable, one (6.3%) for influenza B, four (25%) for RSV, two (12.5%) for rhinovirus, two (12.5%) for coronavirus 229E, one (6.3%) for bocavirus, one (6.3%) for HPIV, one (6.3%) for HMPV, one (6.3%) for adenovirus and one (6.3%) for chlamydia. Coinfections were confirmed in four (25%) of the 16 positive cases, with two (12.5%) for influenza A(H1N1)pdm09 and RSV, one (6.3%) for influenza A(H3N2) and adenovirus, and one (6.3%) for rhinovirus and chlamydia. Of the 16 cases positive for at least one pathogen, 13 (81.3%) were in children aged 0-3 years. Influenza was detected in five (31.3%) of the 16 confirmed cases. Eight (50%) of the 16 SARI-positive cases were males while seven (43.7%) were females; information about gender was missing in one (6.3%) of the SARI-positive cases. Conclusion: Taken together, these data highlight the role that influenza plays in under-five SARI cases in Cote d'Ivoire, as well as the value of the MWS platform as a multiplex tool for detecting key respiratory pathogens involved in SARI pathogenesis.

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Performance of eHealth data sources in local influenza surveillance: a 5-year open cohort study using data from Google Flu Trends, telenursing call centres, health service provider web pages, and mass media coverage

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Background: Globally there has been much interest in using data from population-wide health information systems—often referred to as eHealth resources—to improve the effectiveness of infectious disease monitoring. The purpose of this study is to investigate the performance of a set of eHealth resources available for local public health and infectious disease control organizations as sources of syndromic data in influenza surveillance. The specific aim is to examine the timeliness and correlation patterns for data from Google Flu Trends (GFT), computer-supported telenursing call centres, and local health service Web page usage in relation to influenza case rates during seasonal and pandemic influenza outbreaks in a Swedish county. Materials and Methods: An open cohort

design was used for a 5-year study performed in Östergötland county (population 427,000), Sweden. Syndromic eHealth data were collected from GFT, computer-supported telenursing call centres, and local health service Web site visits at page level between October 2007 and May 2012 and stored in an administrative public health database. Data on media coverage of influenza outbreaks were collected from the online database of the largest newspaper in the county. The public health database also collected clinical data from care episodes in primary care and at hospitals in the county, and data from investigations at clinical laboratories. GFT data for the study period were collected using a Gmail account to download data on Google searches from the Östergötland region on influenza. Influenza-like-illness (ILI)-related telenursing call cases were identified by the chief complaint codes associated with influenza symptoms dyspnea, fever (child, adult), cough (child, adult), sore throat, lethargy, syncope, dizziness, and headache (child, adult)) from the fixed-field terminology register. Usage data from the County Council Web pages were collected beginning in May 2009. For technical reasons associated with a change of software provider, data could not be retrieved for the 2010-11 influenza season. Pearson's correlation coefficients (*r*) were examined to compare ILI case rates with the eHealth data sources, ie, GFT data and all possible combinations of telenursing chief complaints and Web site page visits with a two-week time lag to ILI case rates. The three groupings of chief complaints and combinations of Web site page types, respectively, with the strongest correlation to the influenza case rate for each time lag were listed. The chief complaint grouping and Web site page combination with the largest correlation strength were chosen to be used in the final analyses. Separate analyses were performed of correlations between media reports, ILI case rates, and the eHealth data sources, respectively. Results: Large strength correlations with influenza case rates were found for data from the three eHealth sources for the five influenza outbreaks covered by the study period, except for data from telenursing call centres for the first study year: GFT (r = 0.69; 95% confidence interval [CI] 0.22-0.90 to r = 0.96; 95% CI 0.88-0.99), telenursing call centres (r = 0.84; 95% CI 0.62-0.94 to r = 0.97; 95% CI 0.91-0.99), and health service provider Web pages (r = 0.75; 95% CI 0.45-0.90 to r = 0.94; 95% CI 0.84-0.98). eHealth data activity preceded case rates with two weeks during the first two seasonal outbreaks. This time lag was reduced to one week or none from the 2009 pandemic outbreak onward. Mass media coverage correlating with one week preceding time lag to influenza case rates was observed for the pandemic in 2009 (r = 0.74; 95% CI 0.42-0.90; P < .001) and the severe seasonal outbreak in 2011-12 (r = 0.79; 95% CI 0.42-0.93; P = .002). Conclusions: eHealth data showed strong correlations with influenza case rates in local surveillance, although the time lag varied between different influenza seasons. Mass media coverage reflecting a speculative warning viewpoint on potentially severe influenza outbreaks was associated with changes in the monitoring performance of the eHealth resources. Methods for analyses of eHealth data streams need to compensate for that media coverage and information-seeking behaviors in the population vary between influenza outbreaks.

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Respiratory viral pathogens among Singapore military servicemen: epidemiology and clinical characteristics

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Background: Seasonality and transmission of viral respiratory diseases in the tropics differ from temperate regions, but few studies have comprehensively described tropical respiratory disease surveillance. As influenza only accounts for 10%-53% of influenza-like illness (ILI), our study aims to determine the viral etiologies of ILI and compare the clinical characteristics of different viral etiologies, including influenza viruses, in a tropical setting. Materials and Methods: The study was conducted in the Singapore military where all males serve for two years after leaving high school. We tracked cases of febrile respiratory illness (FRI; temperature ≥ 37.5°C with cough or sore throat) in 5 sentinel primary care sites, from May 2009 through October 2012. Patients who met the criteria for FRI were

recruited. Health care workers obtained written informed consent, administered a questionnaire, obtained clinical specimens and performed a clinical examination on the participants. Repeat consults were excluded, and similar data and specimens were obtained from controls. Nasal washes were tested using a multiplex PCR strategy based on Resplex multiplex assays. The assays tested for the following viruses: adenovirus, bocavirus, coronavirus, coxsackie virus, enterovirus, human metapneumovirus, influenza A and B, parainfluenza, respiratory syncytial virus and rhinovirus. Additional in-house singleplex PCR assays were used to subtype influenza. Statistical significance was fixed at 0.05. The analysis was limited to viral monoinfections to allow the clinical presentations and symptom complexes associated with each pathogen to be discerned. Results: No distinct seasonality among the viral pathogens was observed. There was temporal association amongst influenza A(H1N1) (pH1N1), influenza B (FLU-B) and adenovirus E (ADV-E) with the fall in pH1N1 followed by a rise in FLU-B, and a subsequent surge in ADV-E as the number of FLU-B declined. Of the 7733 FRI cases, 3794 (49.1%) tested positive for at least one virus and 376 (9.9%) of these had multiple infections. A total of 4099 (53.0%) of the FRI cases fulfilled the definition of ILI, of which 2131 (52.0%) of these ILI cases were positive for at least one virus. The top etiologies for the monoinfections among FRI cases were pH1N1 (13.4%), FLU-B (12.9%) and coxsackie virus (9.4%). Influenza accounted for 40.1% of ILI monoinfections. The ILI case definition would have identified 69.0% of influenza infections among the FRI cases and 65.0% of ADV cases. The sensitivity, specificity, PPV and NPV of ILI for influenza among FRI cases was 72.2%, 48.1%, 40.1% and 69.3%, respectively. On multivariate regression analysis, influenza and ADV were more likely to be febrile. p(H1N1) and FLU-A(H3N2) less commonly resulted in sore throat, rhinovirus and enterovirus caused more rhinorrhea, and ADV less rhinorrhea. FLU-B was more likely to cause dry cough whereas coronavirus OC43 and human metapneumovirus were more likely to cause productive cough. Conclusions: There are multiple viral etiologies for FRI and ILI, with influenza accounting for less than half of the cases. In a tropical setting, influenza and adenoviruses tend to present more frequently with fever, a marker of severity, and vaccination should be considered. ILI as a surveillance tool for influenza has high sensitivity and NPV, and low specificity and PPV, unlike in temperate climates—hence the utility of ILI for tropical surveillance of influenza needs to be reviewed. The differences in presentation of the various etiologies support development of a clinical diagnostic model in settings where access to rapid laboratory testing is an issue.

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Molecular genetic characterization of influenza A and B virus strains circulated in Russia in 2010-2013

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Background: Genetic diversity of influenza A and B viruses that circulated in Russia in 2010-2013 was analyzed. Materials and Methods: Clinical specimens (nasopharyngeal swabs, bronchoalveolar lavages) from St. Petersburg hospitals and 49 regional base laboratories of the Federal Influenza Center were tested for influenza virus RNA using rRT-PCR according to CDC protocols. HA and NA genes were directly sequenced from virus isolates using a BigDye Terminator v3.1 Cycle Sequencing Kit on ABI GA 3130. All sequences were submitted to the GISAID database. Phylogenetic analysis was performed using MEGA5 software. Results: In the period from 2010 to 2013, influenza A viruses of subtypes H1N1pdm09 and H3N2, and influenza B viruses of Victoria and Yamagata lineages, circulated in Russia. In the epidemic season 2010/2011, influenza A/H1N1pdm09 strains belonged to 5 phylogenetic groups: A/St. Petersburg/27/2011-like (characterized by amino acid changes D97N, S185T in antigenic site Sb), A/St. Petersburg/100/2011-like (D97N, S143G in antigenic site Ca2 и S185T, A197T in antigenic site Sb), A/Christchurch/16/2010-like(N125D), A/Astrakhan/01/2011-like (D97N, R20K(Ca), I216V, V249L), и A/Toulon/1173/2011-like (A134T, S183P). In epidemic season 2011/2012, no influenza A/H1N1pdm09 was isolated. All A/H1N1pdm09 strains in epidemic season 2012/2013 belonged to clade 6 (A/St. Petersburg/27/2011-like). Influenza A/H3N2 virus isolates 2010/2011 belonged to A/Perth/10/2010-like strains (clade A/Victoria/208/2009) containing amino acid changes in antigenic site C (D53N, Y94H, I230V, E280A) or A/Victoria/210/2009-like strains (clade

A/Perth/16/2009) defined by amino acid changes E62K and N144K in antigenic site A and A212T in antigenic site D. All analyzed strains had K158N and N189K in antigenic sites B1 and B2, respectively. All influenza A/H3N2 strains 2011/2012 fell into the A/Victoria/208/2009 clade. The vast majority of these strains belonged to the A/Stockholm/18/2011 genetic group (subgroups 3A, 3B and 3C), defined by N145S in antigenic site A, V223I in site D, A198S in site B. A minor part fell into genetic group A/Perth/10/2010. Three strains constituted new outlying group characterized by amino acid changes I140K (site A); N158K, D188N, F193S, A196T (site B); V223I (site D). Influenza A/H3N2 strains isolated in 2013 are A/Victoria/361/2011-like (subgroup 3C of A/Victoria /208/2009 clade) defined by amino acid changes S45N, T48I, S145N, A198S, V223I и N312S. The majority of strains bore substitution T128A in HA1 presumably leading to the loss of the glycosylation site in position 126. Substitutions in antigenic site A (N121D, S124N, I140M) were common for these strains. All strains were predicted to be sensitive to neuraminidase inhibitors and adamantine antivirals. None of them carried H275Y substitution in NA, and all had S31N substitution in M2. Influenza B strains of the epidemic season 2010/2011 belonged to B/Victoria lineage (clade 1B, B/Brisbane/60/2008-like) and carried substitutions in loop 190 (T199I) and loop 120 (I117V) of HA1. In 2011-2013 there was co-circulation of strains of B/Victoria and B/Yamagata lineages. B/Victoria strains isolated in 2012 fell into clades 1A and 1B and had N197K and T199A substitutions in the receptor-binding site. Intraclade reassortant HA-1A/NA-4 was identified. B/Yamagata lineage strains belonged to clade 3 bearing N116K and N202S amino acid changes in loop 120 and 190, respectively, and T181K in antigenic site D. In 2012/2013 all strains of B/Victoria lineage fell into clade 1A and had common substitution of K209T in HA1. B/Yamagata lineage strains of clade 2 (B/Brisbane/03/2007-like) bore amino acid changes R48K (in antigenic site BC), I150S (in BA), Y165N (in BB2), T181A (in BD). Only two strains belonged to clade 3 (B/Wisconsin /01/2010-like). No changes conferring resistance to oseltamivir or zanamivir were found in influenza B strains isolated in 2010-2013. Conclusion: Marked heterogeneity of the population of circulating viruses, as well as their fast and unpredictable antigenic variability, dictates the need for permanent molecular epidemiologic surveillance of circulating influenza viruses for annual revision of the composition of influenza vaccines and recommendations for antiviral therapy.

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The etiology of influenza-like illness in Jiangsu province, China, 2012

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Background: The etiology of influenza-like illness (ILI) was not clear in China. Methods: Continuous surveillance of outpatients with ILI was conducted from January 2012 to December 2012 to detect 16 respiratory viruses in three cities (Nanjing, Suzhou, Taizhou) in Jiangsu Province, China. Throat swabs were collected for ILI from outpatients during medical visits in the four largest general hospitals and two children's hospitals. Respiratory etiology was detected using real-time PCR by four national networks of influenza laboratories. Results: Five hundred and twenty-three viruses were detected in samples from 600 (87.16%) patients with ILI. Among them, *Streptococcus pneumoniae* and *Haemophilus influenzae* type b accounted for 24.17% and 10.33%, respectively. The viruses detected most commonly were rhinoviruses (10.33%), influenza A (8.33%), herpes simplex virus (6.33%), adenovirus (4.67%), and HCoV-NL63 (4.16%). The rates of influenza A were much higher in April (25%; $P < .001$) and July (31.37%; $P < .001$) than the average of the whole year. Influenza B was also identified with a positive rate of 1.78% to 11.63% in January to May. Coinfections were detected in 29 (46.77%) influenza-positive patients and were found most commonly with *S pneumoniae* (29.03%), especially in children below 5 years of age. Conclusions: This study confirmed that multiple respiratory viruses may circulate concurrently in the population and account for a large proportion of ILI. Influenza virus was due to the summer peak of ILI in Jiang Province.

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Timely alert of influenza-like illness from schoolchildren—using a newly established web-accessed syndromic surveillance system in Taipei, Taiwan

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Background: Schoolchildren have longer duration and higher levels of viremia after the infection with influenza. They are one of the major sources in transmitting the virus, with cluster cases on campus and in families, particularly for a novel influenza virus with a low level of herd immunity. In response to the challenge of 2009 influenza A (H1N1) pandemic, Taipei City government officials developed a Web-accessed Infectious Disease Syndromic Surveillance for Schoolchildren (WIDSSS) to replace the conventional facsimile reporting system. Materials and Methods: School teachers and nurses from preschools, primary, and secondary schools to universities in all the 12 districts in Taipei City are required to submit daily the numbers of symptomatic cases of influenza-like illness (ILI) through the WIDSSS. We retrieved these data from January 2010 to August 2011 and compared the temporal trends with the ILI cases obtained from the Emergency Department–based Real-Time Outbreak and Disease Surveillance (ED-RODS). Results: The WIDSSS detected 3046 cases of ILI from all the registered schools in Taipei City during the study period. Children in primary schools had the highest incidence rate, followed by preschools and secondary schools. In school-reported ILI cases, 98.88% had consistent reporting with a doctor's diagnosis and 96.62% requested sick leave. In addition, 10.64% of the cases reported that their family members presented with similar ILI symptoms. In fact, the WIDSSS detected 3 ILI peaks in 2010, one in January and the others before and after the summer vacation, with similar patterns as ED-RODS, except summer and winter vacations. Another ILI peak in January 2011 was detected occurring 2 weeks earlier than in ED-RODS, with a high correlation between the 2 systems [Pearson correlation = 0.95]. Spatial analysis found that the transmission pattern of ILI cases rose its incidence rate from suburban areas to the central business district in Taipei. In the aspect of public health management, this newly established web-accessed system in Taipei City reduced the cost of data processing by 95.5% and saved 59.7% of the time spent on the conventional facsimile system. Conclusions: Our WIDSSS is capable of early identification of suspected cases of ILI from schools in metropolitan Taipei. It is cost-effective and has a user-friendly interface for public health administrators to monitor daily data of ILI for more effective risk communication and timely control. Our temporal analysis implies that we can pay more attention to surveillance and put in more public health effort from the end of December because incidence rates of ILI were higher in January 2010 and 2011. Our valuable experience showed that the WIDSSS can be adapted in cities of other countries with higher population densities of schoolchildren to achieve more timely and effective surveillance for minimizing the public health threat of influenza pandemic.

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Virological surveillance of influenza B in Mexico in the period 2011-2013

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Background: Mexico is part of the International Network of Influenza surveillance. Influenza Surveillance Network System is conformed by a National Network of Public Health Labs (NNPHL) composed of 31 State Public Health laboratories, 6 laboratories of the National Institutes of Health System, and 716 Influenza Monitoring Units across Mexico. The system provides information on influenza A and B activity. Material and Methods: 10% of ILI and 100% of SARI of each point of care unit are processed according to their material resources and a decision making algorithm. From January 2011 to May 2013, 953 samples positive for influenza B were received at InDRE by Reference, were subject to RT-qPCR to identify Victoria and Yamagata lineages according to Norway NIC protocols. Briefly, confirmed positive samples were sent to virus isolation (inoculated into cell culture (MDCK) or embryonated hen eggs), isolates with HA ≥ 1:32 were characterized by HI with the WHO kit. 10% of selected samples were subject to partial DNA sequencing of matrix, haemagglutinin, and neuraminidase gene segments. Results: In 2011, 285 were positive for influenza B, 131 (46%), Victoria lineage 154 (54% unidentified). 102 samples were isolated, strains were identified by HAI (B/Brisbane/60/2008 and B/Victoria/02/87), the distribution was mainly in the central and southern states of the country. In 2012, 538 were positive for influenza B, 279 (51.85%) Victoria lineage, 181 (33.65%) Yamagata, 78 (14.5% unidentified). 167 samples were isolated, strains were identified by HAI (B/Brisbane/60/2008 and B/Wisconsin/01/2010) the distribution was mainly in the central of Mexico. In 2013, 91 were positive for influenza B, 38 (41.7%) Victoria lineage, 50 (55%) Yamagata, 3 (3.3% unidentified). 53 samples were isolated; strains were the same strains of 2012. Conclusions: Our results show that Yamagata lineage appeared late in the 2011-2012 season and it frequency was 5 times high in the 2012-2013 period. This is important for monitoring influenza in Mexico and its relationship to the composition of the vaccine that is issued annually by the WHO.

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Virological surveillance for early detection of new antigenic variants of influenza virus in Mexico in the 2009-2013 period

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Background: Comprehensive continuous Influenza Virus Surveillance is critical for the identification of circulating seasonal strains, as well as for the identification of uncommon influenza activity that may signal the appearance potentially pandemic influenza strains. A major reorganization of Mexico´s Influenza Surveillance System resulted from the lessons learned from the 2009 pandemic. Material and Methods: Currently, Mexico´s Influenza Surveillance Network System is formed by a National Network of Public Health laborites (NNPHL) composed of 31 State Public Health laboratories, 6

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laboratories of the National Institutes of Health System, and 716 Influenza Monitoring Units across Mexico. All of them coordinated, regulated, and assisted by the National Influenza Center (NIC) at InDRE and the General Epidemiology Directorate. Virological surveillance of influenza viruses in nonpandemic years included 10% of ILI cases, 100% of SARI cases, and 100% of associated deaths. All samples are analyzed according to a decision making algorithm. Testing methodologies included reverse transcription quantitative PCR (RT-qPCR), IHA, microneutralization, antiviral resistance, DNA sequencing of M1, M2, HA, NA gene segments and differential diagnosis with the xTAG® respiratory viral panel analysis on the Bioplex system. In 2009, the pandemic year, all cases of ILI and SARI were analyzed. Samples from severe cases and deceased were analyzed for oseltamivir resistance. Results: An outbreak of severe pneumonia cases from specialized hospital in Central Mexico signaled an unusual ILI activity back on April 2009. For this year, a total of 153,202 samples were analyses: 81,849 (53.4%) positive; 69,914 (85.6%) were positive for H1N1pdm09. The seasonal H1N1 circulating previous to the pandemic strain disappeared. The pandemic strain was also prevalent in 2009, 2011, and 2012. In 2010 and 2013, the prevalent strain was H3N2. A total of 11 oseltamivir-resistant samples have been identified between 2009 and 2013. Conclusions: On collaboration with CDC, Atlanta Influenza Division and Canada PHL, a response to the ongoing ILI unusual activity was mounted. No doubt, Mexico has today a comprehensive influenza surveillance system resulting from the lessons learned in the 2009 pandemic.

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Usefulness of nose-blowing specimens for cluster influenza surveillance in Yokohama, Japan

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Background: In Japan, influenza surveillance is conducted by virus surveillance, by hospitalization surveillance, and by cluster surveillance, which picks up common-cold outbreaks in the community. Cluster surveillance has been conducted for many years since 1973, and in Yokohama city, we had been collecting gargle fluid specimens and paired sera in new-onset influenza populations. However, because the virus isolation rate and gene detection rate from gargle fluid are low and paired sera cannot be obtained, we found ourselves in a quandary as to how to report the results. In 2007, nose-blowing specimens were approved for use with rapid influenza diagnostic kits. We noted that they were more sensitive than throat swab fluid and that the collection method was less stressful for patients, and since the 2008/2009 season we assessed their usefulness by using a combination of gargle fluid and nose-blowing specimens. Materials and Methods: We collected gargle fluid and nose-blowing specimens from up to 5 patients in an initial-onset population suspected of influenza within the jurisdiction of 18 public health center branch offices in the city of Yokohama. We had the gargle fluid collected with physiological saline solution and the nose-blowing specimens collected with specimen collection paper specially designed for use with the rapid influenza diagnostic kits, and we mixed each of them with virus transport medium. MDCK cells were used for virus isolation. The National Institute of Infectious Diseases' primers and probes were used for hemagglutinin (HA) gene detection by TaqMan real-time PCR. Results: A total of 188 specimens were collected in 4 seasons at 55 facilities. According to the subtype, the causative agent was AH1pdm at 5 institutions, AH3 at 45 institutions, and B at 5 institutions, and there was an overlapping AH1pdm and AH3 population at 1 institution. The mean interval in days between the onset of illness and sample collection was 2.6 days, and 92.2% were being treated with an NA inhibitor. Comparison of the virus isolation rates and gene detection rates in 3 seasons as a whole showed that the rates for gargle fluid were 56.9% and 43.6%, respectively, as opposed to 42.0% and 36.2%, respectively, for the nose-blowing specimens, and the rates for the nose-blowing specimens tended to be lower. However, combining the results for the 2 specimens yielded an isolation rate of 70.7% and detection rate of 59.6%, and thus both the isolation rate and detection rate increased considerably when the 2 were used in combination. In the comparison according to subtypes, the isolation rates when the results obtained from the 2 specimens

were combined were: B, 82.4%; AH1pdm, 81.3%; and AH3, 68.8%, and the detection rates were B, 76.5%; AH1pdm, 43.8%; and AH3, 60.0%. Eight different products have been approved in Japan for testing nose-blowing specimens with rapid influenza diagnostic kits. Their sensitivity is generally 90%, and the results are better than the approximately 80% achieved with throat swab fluid. On the other hand, the isolation rate and detection rate from nose-blowing specimens for cluster surveillance were not as high as expected, and possible reasons for this may be the fact that the nasal mucus was diluted with the transport medium for the culture test, that it took time to conduct the tests after collecting the specimens, interference with isolation by components of the nasal mucus and the small volume of nasal fluid soon after the onset, or that collecting the specimens was difficult because of nasal obstruction. Conclusion: Both the isolation rate and detection rate from nose-blowing specimens tended to be lower than from gargle fluid, but using a combination of both specimens improved the test results. We think that nose-blowing specimens are useful specimens for prevalence surveys and for virological searches in sudden outbreaks.

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Contribution of influenza and other respiratory viruses to hospitalization in Valencia, Spain, during the 2012-2013 winter season: a prospective hospital-based active-surveillance study

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Background: Few data are available on the age-specific hospitalization rates and circulation patterns of most respiratory viruses. As other than influenza respiratory viruses circulate during the winter season, taking into account the season and age impact on severe disease of those other respiratory viruses can improve the estimates on the burden of influenza obtained by conventional surveillance systems or statistical modeling. Using a prospective hospital-based active surveillance system, covering 1,266,899 inhabitants (27% of the population in Valencia, Spain), we estimated the overall and age-specific hospitalization rates for 14 respiratory viruses during the 2012-2013 winter season. Methods: Fieldwork began on epidemiologic week (EW) 46 (12 November 2012) and ended on EW 16 (20 April 2013). Study staff screened, Monday to Saturday, all previous 24 to 48 hours emergency admissions. The medical conditions that qualified an emergency admission as eligible were nonspecific acute respiratory infection, cough, fever, pneumonia, chronic obstructive pulmonary disease exacerbation, dyspnea, acute cardiovascular disease (heart failure or coronary syndrome), sepsis, systemic inflammatory response syndrome, metabolic failure, confusion, convulsions or myalgia. Symptom onset within seven days of admission was required. Patients were excluded if they were institutionalized or had been hospitalized in the previous 30 days. We obtained nasopharyngeal and nasal (< 14 years old) or pharyngeal (≥ 14 years) swabs. Four multiplex rtRT-PCR qualitative amplifications were performed: multiplex #1 for influenza virus type A and influenza virus type B; multiplex #2 for coronavirus, metapneumovirus, and bocavirus; multiplex # 3 for respiratory syncytial virus (RSV), adenovirus and parainfluenza virus; and multiplex #4 for rhinovirus. We estimated age-specific hospitalization rates per 100,000 for the age groups: 0 to 4; 5 to 17; 18 to 49; 50 to 64; 65 to 74; and 75 years of age or more. For the 0 to 4 years of age group, we estimated rates by age group in months: 0 to < 6; 6 to < 12; 12 to < 23; 24 to < 36; 36 to 59. The denominator was obtained from Valencia's Health Care Population Information System. Results: Overall, 510 patients had a positive rtRT-PCR result. Respiratory viruses were associated to 3 hospitalizations for each 1000 subjects less than 5 years of age, and one for each 1000 subjects 65 years old or older. The most frequent virus identified was influenza (n = 242 positives), followed by RSV (n = 154 positives), coronavirus (n = 53), rhinovirus (n = 51), bocavirus (n = 17) and adenovirus (n = 16); mixed infections, rhinovirus, metapneumovirus and parainfluenza were identified in less than 10 occasions. RSV was the most

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frequent virus identified in those less than five years of age (n = 106 RSV of 204 positives in this age group), while influenza (n = 145 of 220 positives in this age group) was the most frequent in those 65 years of age or older. For admissions with a positive PCR result, 70%, in those less than 5 years old, and 87%, in those 65 and over, were associated with those two viruses. Coronavirus was the third most frequently identified virus in both age groups (≈ 10%). During the 2012-2013 season, and for infants less than 6 months of age, one in 100 was hospitalized with a confirmed RSV infection, one in 800 was hospitalized with a confirmed coronavirus infection, and one in 1000 with a confirmed influenza infection. Conclusions: In both extremes of life, the majority of respiratory virus burden of disease was associated with influenza and RSV. RSV followed by influenza predominated in the young, and influenza followed by RSV in those 65 years of age or older. Burden of disease due to respiratory viruses was higher in those less than 4 years of age, followed by those over 65 years of age. Hospitalization rates were extremely high in those less than 6 months of age. The study was funded by a grant from Sanofi Pasteur.

P2-674 GATES FELLOWSHIP Gates Fellowship

Influenza among people of 50 years and above: national influenza surveillance in Bangladesh, 2008-2012

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Background: In 2007, the Institute of Epidemiology, Disease Control and Research, Bangladesh, and the International Centre for Diarrhoeal Diseases Research, Bangladesh, with the technical help of the US Centers for Disease Control and Prevention, established national hospital-based influenza surveillance in Bangladesh. The objectives of the surveillance are to characterize the epidemiology and diversity of circulating influenza strains and to identify people with severe influenza infection. Age-specific epidemiologic data permit evidence-based targeted public health programs to prevent influenza. Here we report the demographic and clinical characteristics of persons aged 50 years and above who sought care for mild and severe respiratory disease during January 2008-December 2012. Materials and Methods: Surveillance physicians in each of the 12 participating hospitals identified outpatients with influenza-like illness (ILI; with history of fever and cough or sore throat) and hospitalized persons with severe acute respiratory illness (SARI; aged ≥ 5 years with history of fever within 21 days and cough or sore throat). Staff collected demographics, clinical illness information and outcome during discharge. In addition, surveillance physicians obtained nasopharyngeal and throat swabs for rRT-PCR to detect influenza A and B RNA, and if found positive for influenza A, then further subtyping for influenza A (seasonal H1, H3, H5 and H1N1pdm09). We describe the laboratory results of swabs collected from persons ≥ 50 years of age along with their clinical characteristics. Results: During 2008-2012, a total of 2276 case-patients ≥ 50 years of age were enrolled in the surveillance; 1797 were inpatients and 479 were outpatients. Of the inpatients, 1308 (73%) were male whereas among the outpatients, 256 (53%) were male. The median age of influenza positive inpatients was 60 years (interquartile range, IQR: 55-70) and for outpatients it was 55 years (IQR: 50-60) ($P < .001$). A total of 300 (13.2%) samples tested positive for influenza, of which 245 (82%) were from inpatients. Irrespective of influenza status, the median duration of hospitalization of all case-patients was 4 days (IQR: 2-6). Of all influenza-positive case-patients, 207 (69%) had influenza A, 92 (31%) influenza B and 1 (0.3%) had both influenza A and B. Among the influenza A positive case-patients, 119 (57%) had influenza A(H3), 84 (41%) had influenza A(H1N1)pdm09 and 5 (2%) had seasonal influenza A(H1). Of 175 influenza-positive inpatients, the dominant influenza A subtype was A(H3) in 106 (61%) and of 33 influenza-positive outpatients it was (H1N1)pdm09 in 15 (45%) followed by A(H3) in 13 (39%) case-patients. In all the years except 2008 and 2011, among people ≥ 50 years, we exclusively detected influenza A(H3). Since 2009, influenza A(H1N1)pdm09 also was the predominant influenza subtype among this age group except in 2011, during which we only detected influenza A(H3). Among the inpatients, almost all (99%) had cough, 80% had difficulty breathing, 50% headache, 44% runny nose, and 34% had sore throat. A total of 532 (30%) of all inpatients and 67 (14%) of all outpatients

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had comorbidities (*P* < .001). Sixty-seven (22%) of all influenza-positive case-patients and 532 (27%) of all 1976 influenza-negative case-patients had either history of heart disease or lung disease. Among 1797 inpatients we identified 48 deaths (2.7%) during hospitalization. Of 245 inpatients tested positive for influenza, six patients died during hospitalization (case fatality proportion 2.4%); of these, three (50%) had either heart disease or lung disease. The median age for influenza-positive case-patients who died was 63.5 years (IQR: 60-70). Conclusions: Although both influenza A and B viruses are circulating among persons ≥ 50 years in Bangladesh, this age group is particularly affected by influenza A(H3). In low-income countries like Bangladesh, targeting people ≥ 50 years with comorbidities for influenza vaccination may help prevent the spread of influenza viruses, influenza-associated complications and fatal outcome.

P2-675

Severe acute respiratory infections in the 2012/2013 season studied by the Portuguese Laboratory Network for Influenza Diagnosis

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Background: During the 2009/10 influenza pandemic, a network of 14 laboratories located in the main reference hospitals in Portugal mainland, Madeira and Azores was established for the diagnosis of the new influenza A(H1N1)2009 pandemic strain. Since then, the network performs laboratory diagnosis of influenza as well as other respiratory pathogens, thus contributing to the laboratory diagnosis of respiratory disease in Portugal. This network is a valuable complement of the National Influenza Surveillance Programme (mainly based on primary health care units), enabling a more accurate knowledge of the aetiology of severe respiratory infections, especially in hospitalized cases. The present study describes the severe acute respiratory infections, in the 2012/2013 season, diagnosed by the laboratory network. Materials and Methods: Of the 14 laboratories, 11 reported cases of respiratory disease during the 2012/2013 season. The laboratory network performs diagnosis of influenza A and B viruses and other respiratory agents by PCR-based methods, also enabling the detection of mixed infections. All 14 laboratories perform the detection of influenza A(H1)pdm09, 4 perform the influenza A(H1) seasonal and A(H3) subtyping, and 10 participants also detect influenza B. Eight laboratories implemented methodologies for the detection of other infectious agents associated with respiratory disease. The antigenic characterization of 8 isolated viruses [3 A(H1)pdm09 and 5 B/Yamagata] was performed at the National Influenza Reference Laboratory. The genetic analysis of the HA1 subunit of the haemagglutinin gene was performed in 17 viruses [7 A(H1)pdm09, 1 A(H3) and 9 B/Yamagata]. Twenty-nine A(H1)pdm09 and 5 B/Yamagata were tested for antiviral susceptibility [PCR(NA)-H275Y and/or MUNANA phenotypic assays for oseltamivir and zanamivir]. Results: The 11 laboratories reported a total of 1470 respiratory disease cases, from week 39/2012 to 21/2013 (peak of 205 [13.9%] cases during week 10/2013). Influenza was identified in 504 cases. Influenza A was detected in 352 (70.0%) cases: 297 (59.0%) cases were A(H1)pdm09, 48 (10.0%) cases were not subtyped, and 7 (1.0%) cases were A(H3). Influenza B was identified in 152 (30%) of the influenza cases. During the 2012/2013 season, 311 (21.2%) reported cases were

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hospitalized in intensive care units (ICUs), the majority of them were aged 50-54 years (34; 10.9%), followed by the age groups 45-49 and 55-59 years (28; 9.0% each). The causal agent was identified in 160 (51.4%) ICU cases. Influenza was identified in 120 (38.5%) patients, other respiratory agents were detected in 40 (12.8%), within these, multiple infections were present in 18 (5.7%). Bacteria were identified in 31 (10.0%) cases mainly associated with RSV and hRV. Among ICU influenza cases, the most detected virus was A(H1)pdm09 (82; 62.0%). However, cases of A(H3) (3; 2.0%), A unsubtyped (8; 7.0%) and B (27; 23.0%) were also detected. As expected, the highest number of ICU influenza-positive cases was detected in week 10/2013 (18; 15.0%), coincident with the highest number of influenza cases during the entire season. ICU flu cases were detected predominantly in individuals aged 50-54 years (18; 15.0%). From the ICU-reported cases, 6 (1.9%) died. The influenza A(H1)pdm09 virus was detected in 2 men 50-59 years old from these 6 fatal outcomes. The isolated influenza A viruses were similar to the 2012/2013 vaccine strains. The influenza B/Yamagata viruses showed a greater antigenic and genetic variability. Conclusions: The Portuguese Laboratory Network for Influenza Diagnosis plays a major role in the diagnosis of acute respiratory infections in Portugal, providing a more accurate knowledge of the respiratory agents involved. During the 2012/2013 season, the influenza A(H1)pdm09 virus predominated in co-circulation with influenza B virus. The A(H1)pdm09 virus was responsible for the majority of the flu cases admitted to the ICU and may have been the cause of death in two cases. Bacterial and other viral agents have been identified in some of the severe cases reported. The majority of the characterized influenza viruses were similar to the vaccine strains, and none of the viruses showed reduced susceptibility to oseltamivir or zanamivir.

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Respiratory viruses and influenza in three winter seasons, in Portugal

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Background: Respiratory viral disease is a serious public health problem globally, with considerable social and economic impact. Respiratory viruses cause respiratory disease that ranges from mild to severe pneumonia, contributing to morbidity and mortality throughout the world. The etiologic diagnosis based on clinical presentation is very difficult, due to the similarity of clinical presentation of respiratory viral infections. To have a more complete knowledge of the viral etiology of influenza-like illness (ILI) in Portugal, a search for viral agents (including influenza) was done in the framework of the National Influenza Surveillance System, from seasons 2010/2011 to 2012/2013. Materials and Methods: During the 3 winter seasons, cases of ILI (according to the EU case definition) were notified by the sentinel and nonsentinel networks. For each studied case, a nasopharyngeal swab collected in the first seven days after symptoms onset was received in the National Influenza Reference Laboratory. Multiplex real-time PCR for influenza detection was performed on all the samples. All the influenza A were subtyped using the Prodesse Proflu+ (GeneProbe, Inc), and influenza B lineage was determined using the WHO recommended protocol. The other respiratory viruses—human rhinovirus, respiratory syncytial virus A and B, parainfluenza virus 1, 2 and 3, adenovirus and metapneumovirus—were detected by multiplex real-time PCR, allowing detection of multiple infections. From the first winter, 2010/2011, a random selection of 378 ILI cases (taking into account the time and age distribution of cases representing 37.2% of the reported cases) was analyzed. In the following seasons 2011/2012 and 2012/2013, all samples were tested for influenza and other respiratory viruses, corresponding to 678 cases and 1256 cases, respectively. For the 2012-13 season, the odds ratio (OR) of being positive for a specific respiratory virus for each sign/symptom was calculated. Results: A total of 2312 samples were analyzed for the 3 seasons. In each season the causal agent was identified in 68%, 52% and 62% of the samples. The flu virus was the most frequently detected agent, accounting for 53.4%, 37.5% and 43.0% of the cases analyzed. In co-circulation with influenza, hRV and RSV were detected in the highest percentage of cases. The PIV, hMPV and AdV viruses were detected sporadically. There were cases of mixed infections of 2 or more viruses (representing 1%-2%), mostly associated with the influenza virus. In the three seasons, the highest percentage of positive cases was detected in children aged 0-4 years. hRV was detected throughout the winter, while RSV circulated simultaneously with the influenza virus. In the 2012-13

season and considering signs and symptoms more associated with influenza virus, significant association was found with fever (OR = 3.13; 95% CI: 1.89–5.19) and cough (OR = 3.94; 95% CI: 2.52–6.16). These results were similar if we consider the influenza subtypes A(H1)pdm09 (fever: OR = 2.82; 95% CI: 1.4–5.66; cough: OR = 5.43; 95% CI: 2.36–12.48) and B Yamagata lineage (fever: OR = 2.24; 95% CI: 1.23-4.07; cough: OR = 1.74; 95% CI: 1.05–2.88). For hRV only, fever (OR = 0.45; 95% CI: 0.26-0.79) and sore throat (OR = 2.85; 95% CI: 1.51–5.39) were significantly associated, while for RSV only sore throat was associated (OR = 2.23; 95% CI: 1.05-4.72). For the remaining respiratory viruses (PIV, hMPV and AdV viruses), chills were the only symptom associated (OR = 4.04; 95% CI: 1.22–13.35). Conclusions: The detection of other respiratory viruses enabled the laboratory diagnosis of more than 50% of ILI cases. The other respiratory viruses accounted for 10%-15% of the ILI positives, with greater prevalence for hRV and RSV. Influenza and RSV co-circulated in the 3 winters as described previously for other countries. For the majority of the investigated respiratory viruses, the presence of one or two signs or symptoms was associated with being positive for that virus. These included fever/ cough (influenza), sore throat (RSV) and chills (PIV, hMPV and AdV viruses). The absence of fever was associated with the detection of hRv.

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Influenza virus detection capacity in the African region—efforts from WHO and its partners

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Background: The recent emergence of avian influenza A(H7N9) in China has again highlighted the importance of global virologic surveillance to detect emerging influenza viruses. The Global Influenza Surveillance and Response System (GISRS) was established in 1952 to serve as a global alert mechanism for influenza viruses of pandemic potential as well as monitoring the evolution of seasonal influenza viruses. Since the reemergence of highly pathogenic influenza A(H5N1) in 2003, many partners have supported WHO to develop and strengthen laboratory-based influenza surveillance globally. In recent years, additional support has been directed to the African region, where HIV/AIDS, malaria and TB, the leading causes of morbidity and mortality, have made the implementation of influenza surveillance challenging. Methods: In a collaborative project with USAID, the CDC, and the WHO AFRO regional office, countries in which capacity development efforts would be targeted were defined using two criteria: 1) establish or strengthen PCR capacity, and 2) strengthen sentinel site surveillance activities. Through this approach, countries with limited funds to implement routine influenza surveillance would be given the basic capacity to detect influenza viruses and, secondly, countries with weak sentinel surveillance systems would be strengthened to provide specimens on an ongoing basis for virologic surveillance. Results: Based on an assessment of current regional capacities including FluNet reporting, participation in the WHO External Quality Assessment Programme (EQAP), and virus sharing, 29 countries were identified with PCR testing capacity with capabilities ranging from partially functional to fully functional. Laboratories in two countries met the criteria for designation as a WHO National Influenza Center (NIC), ie, Zambia and Tanzania. Five countries were identified for PCR strengthening activities: Burundi, Malawi, Mozambique, Republic of Congo, and Zimbabwe, and two countries were identified for sentinel site strengthening activities: Burkina Faso and Sierra Leone. For countries that met the criteria for NIC designation, additional virus isolation training would be provided to strengthen their current capacity.
Conclusions: Since the implementation of the project, four onsite visits have been performed, focusing mainly on training for RT-PCR to detect influenza viruses. Through ongoing coordination with these countries, three have participated in WHO EQAP for the first time and one country has shared viruses with WHO GISRS. In the next phase of the project, sentinel surveillance activities will be implemented to complement the existing PCR capacity. Through the continued support provided by our partners, it is hoped that this strategic approach will assist WHO GISRS in broadening its capacities for influenza surveillance and detection in resource scarce countries.

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Influenza virological surveillance in Italy during the 2012-2013 winter season

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Background: This study presents the virologic data collected in Italy by the National Influenza Centre (NIC), during the 2012-2013 season, in the context of influenza surveillance activities along with the WHO Global Influenza Surveillance Network. Virologic surveillance is routinely carried out by the NIC, from week 46 to week 17 of the following year, in collaboration with a network of 14 peripheral laboratories (Influnet network), scattered throughout the country. The main objectives of this study were to evaluate the match of the circulating viruses with the vaccine strains and to investigate their evolutionary relationships, with particular regard to the hemagglutinin (HA) and neuraminidase (NA) genes. In addition, antiviral susceptibility analyses were performed on a selected number of virus isolates. Materials and Methods: Throat swabs were collected from patients with influenza-like illness by a network of sentinel physicians, according to a specific case definition, and sent to the peripheral laboratories. A selection of the isolated strains were sent to the NIC for further antigenic and molecular characterization. Virus isolation and antigenic analyses were performed according to standardized procedures (WHO manual for the laboratory diagnosis and virological surveillance of influenza, 2011). PCR amplicons were sequenced using an AB 3500 Genetic Analyzer (Applied Biosystems). HA and NA sequences were assembled and aligned using the Lasergene package (DNASTAR 4.0) and BioEdit 7.0.3. The MEGA5 software package was used to estimate phylogenies. The fluorescence-based enzyme inhibition assay (MUNANA) was used to define viral resistance to the NA inhibitor (NAI) drugs (Lackenby A, et al. *Euro Surveill.* 2008;13:8026). Results: During the 2012-2013 season, a total of 5535 clinical samples were tested, of which 2125 (39%) resulted positive for influenza virus. Of those, 899(42%) were type A and 1226 (58%) type B. Within type A viruses with subtype information, H1pdm09 subtype (80%) predominated over H3 viruses (13%). B viruses predominated, with the great majority belonging to the Yamagata lineage. Among this lineage, many viruses showed a slightly reduced HI reactivity with antiserum raised against B/Wisconsin/1/2010, whereas a better reactivity was observed with the B/Massachusetts /02/2012 strain included in the influenza vaccine composition for the 2013-2014 season. Phylogenetic analyses of the HA1 domain of these viruses showed that they fell into genetic clades 2 and 3, in similar proportions. With regard to the B/Victoria-lineage viruses, they were closely related to the B/Brisbane /60/2008 vaccine strain. The Italian A(H1N1)pdm09 viruses showed good antigenic reactivity with the vaccine virus, A/California/7/2009, and the HA1 sequence analyses indicated that they mainly fell into genetic group 6 and 7. Phylogenetic analyses of the A(H3N2) viruses showed that they mainly fell into genetic group 3C. Over 30% of the influenza strains received by NIC during this winter season were also tested for susceptibility to NAIs, using both phenotypic and sequence-based assays. All viruses tested were sensitive to both NAIs. Differences in the mean IC_{50} values for oseltamivir and zanamivir, according to subtype and NAI, were noted. B viruses were considerably more sensitive to zanamivir (mean IC_{50}: 3.3 nM) than to oseltamivir (mean IC_{50}: 42 nM); A(H1N1)pdm09 viruses showed higher levels of sensitivity to zanamivir than to oseltamivir (mean IC_{50}: 0.36 nM and 1.05 nM, respectively), whereas A(H3N2) isolates showed similar IC_{50} values for both drugs (mean IC_{50}: 0.35 nM for oseltamivir vs 0.27 nM for zanamivir). Conclusions: Influenza type A and B viruses co-circulated throughout the 2012-2013 season. The majority (58%) of the characterized influenza strains were type B, mostly belonging to the Yamagata lineage. Among type A, H1pdm09 and H3N2 viruses always co-circulated, but a gradual increase in H1pdm09 detections was observed during the season. Antigenic and molecular analyses of type A viruses showed a good match with the 2012-2013 seasonal vaccine; B viruses showed a slight reduced reactivity with B/Wisconsin/1/2010. None of the NA sequences analyzed presented mutations typically associated with oseltamivir and zanamivir resistance.

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Enhancement of the influenza sentinel surveillance network in Senegal

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Background: Implementation of surveillance tools and quality of data collected are necessary to improve public health decision-making. Influenza sentinel surveillance has been conducted in Dakar (the capital city of Senegal) since 1996. But, it was limited until 2012 to virologic surveillance and was not coupled with epidemiologic surveillance. We describe the challenges and steps involved in improving the sentinel surveillance system in Senegal and the well-timed information it provides. Methods: The network encompasses primary health care centres (sentinel sites) situated across the country and is managed by a steering committee that includes epidemiologists and virologists from IPD and public health managers from the MoH. The network has been expanded from 3 ILI sentinel sites in 2011 located in Dakar to 12 sentinel sites in 2013 to improve the geographic coverage and representativeness. Since March 2012, a daily report by cell phone has been introduced. Indeed, SGP report some data (number of patients with fever, number of ILI cases, et.) at least every day through an encrypted short message service. The sentinel surveillance system makes use of syndromic indicators to monitor the occurrence of selected diseases of importance for the country. The main criterion for inclusion of case-patients into the system is fever. Among patients with fever, other screening criteria (based on syndromic case definitions) are used to identify specific syndromes, namely, malaria, ILI and dengue-like syndromes. Standard WHO case definitions are used for comparability purposes. Virologic surveillance has been enhanced with the introduction of real-time multiplex RT-PCR targeting 16 respiratory viruses. Results: From week 11 in 2012 to week 23 in 2013, 89,512 visits were declared by the SGP. If we consider the different age groups, the distribution was 19,171 (21.4%) for the less than 1-year-old group, 25,839 (28.9%) for the 1-4 years group, 17,725 (19.8%) for the 5-14 years group, 7465 (8.3%) for the 15-24 years group and 19,310 (21.6) for the age group older than 24 years. In total, 18,222 febrile patients were declared (20.4% of all visits) and 3331 ILIs (18,3%). The distribution of ILI by age groups was 580 (17.4%) for the less than 1-year-old group, 1363 (40.9%) for the 1-4 years group, 690 (20.7%) for the 5-14 years group, 168 (5.0%) for the 15-24 years group and 497 (14.9%) for the age group older than 24 years. A total of 1416 viruses were detected among 1329 samples: 297 adenoviruses (21.0%), 228 rhinoviruses (16.1%), 201 enteroviruses (14.2%), 197 respiratory syncytial viruses (13.9%) (177 type A and 20 type B), 145 influenza B viruses (10.2%), 132 influenza A(H3N2) viruses (9.3%), 82 bocaviruses (5.8%), 55 human metapneumoviruses (3.9%), 41 coronaviruses (2.9%) and 38 human parainfluenza viruses (2.7%). During the period, adenoviruses, rhinoviruses, enteroviruses and bocaviruses were regularly detected. RSV were also present during numerous weeks. Influenza B viruses have been detected during the first months of 2013. The influenza A virus circulation was detected in October 2012; this result was different in comparison with prior years where the high level of circulation was between July and August. Conclusions: Our syndromic sentinel surveillance network represents the first nationwide real-time–like surveillance system ever established in Senegal. It is clear that the greatest advantage of this system is its low cost and ease of implementation. It proved to be a cost-effective system that can be easily established in medium- or low-resource countries.

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First year follow-up of respiratory viral infection through the cohort study in Dielmo and Ndiop (Senegal)

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Background: Acute respiratory infection (ARI) is one of the leading causes of child morbidity worldwide. In Senegal, with the decrease of malaria incidence, ARIs tend to become the main cause of morbidity in children under five years old. Since March 2012, a respiratory virus surveillance has been implemented in the two research platforms of Dielmo and Ndiop, initiated in 1990 to document the natural history of malaria. The aim of this work is to study the viral circulation among febrile patients. Methods: Historical clinical surveillance from Dielmo and Ndiop village has been enhanced by a systematic virologic surveillance targeting all the fever syndrome cases. A standardized clinical form was used for each patient of the two rural community cohorts and was entered into a database since 1990. Each compound was visited daily to rapidly detect new cases of fever. The dispensary at the two research sites remained open day and night and the care was free of charge. Since 2012, both nasopharyngeal swabs and blood samples were collected from each febrile patient (temperature ≥ 38°C). Influenza-like illness (ILI) was defined as a febrile patient with cough or sore throat; opposite, other febrile patients were included in non-ILI group. Furthermore, annual systematic blood sampling was carried out in all the inhabitants of the two villages for serologic studies among the healthy population. The swab samples were tested for the detection of 16 respiratory viruses by real-time RT-PCR. Results: From 1st March 2012 to 31st March 2013, a total of 2440 visits were identified and 567 fever syndromes (23.2%) were declared: 302 (53.3%) in Dielmo, 265 (46.7%) in Ndiop. Fever syndromes represented 33.7% in Dielmo and 17.2% in Ndiop of the global care activity. A total of 486 swab samples (85.7%) were collected among all the febrile patients. Of these samples, 310 (63.8%) were positive: 178 mono-infections, 88 dual infections and 44 infections with more than two viruses. In total, 499 viruses were identified: 114 adenoviruses (22.8%), 91 enteroviruses (18.2%), 90 rhinoviruses (18.0%), 56 influenza A(H3N2) viruses (11.2%), 49 influenza B viruses (9.8%), 32 respiratory syncytial viruses (6.4%) (28 type A and 4 type B), 20 metapneumoviruses (4.0%), 20 coronaviruses (4.0%) (14 OC43, 3 NL63, 3 229E), 18 bocaviruses (3.6%), 9 human-influenza viruses (1.8%) (2 HPIV-1 and 7 HPIV-4). Among fever syndromes, 329 were ILI (58.0%): 183 in Dielmo (60.6%) and 146 in Ndiop (55.1%). Among these patients, 284 (86.3%) were sampled and 209 (73.6%) were positive for at least one respiratory virus. Among the 238 non-ILI patients, 202 (84.9%) were sampled and 101 (50%) were positive for at least one respiratory virus. The positive rate was statistically different between ILI and non-ILI patients (OR = 2.7; 95% CI: 1.8-4.2; $P < .001$). However, 32.5% of the positive samples were from febrile non-ILI patients. Among all the positive influenza A and B samples, the non-ILI positive samples represented 17.8% and 22.5%, respectively. The proportion of detected viruses was statistically different between ILI and non-ILI patients for influenza A (respectively, 16.2% and 4.9%; OR = 3.7; 95% CI: 1.8-8.5; $P < .001$) and influenza B (respectively, 13.4% and 5.4%; OR = 2.7; 95% CI: 1.3-5.9; $P < .01$) but not for others viruses. Conclusions: The circulation of respiratory viruses is well documented. However, the burden measure needs to extend the identification among healthy inhabitants (serologic study). In this regard, the cohort is an interesting tool to document the epidemiology of respiratory viral infections. Furthermore, a well-documented cohort will be interesting for future vaccine clinical trial implementation.

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Rapid Determining HA Subtypes of Influenza A Viruses Using Restriction Fragment Length Polymorphism

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Background: Influenza A virus presents a threat to both animal and public health. To date, 17 different hemagglutinin (HA) subtypes (H1-H17) have been reported. Conventional methods for HA subtypes are based on either serological assay or genomic sequencing, and these HA subtyping methods are relatively time consuming. Materials and Methods: Here, we developed a novel restriction fragment length polymorphism (RFLP) based HA subtype identification method. The hypothesis of this method is that the influenza viruses in the same HA subtype have similar RFLP patterns, while those from different HA subtypes have different RFLP patterns. In this method, a Bayesian method is developed to learn the RFLP patterns conserved in the same HA subtypes but different in different HA subtypes. The enzymes will be ranked based on the performance in HA subtyping. This method can integrate multiple enzymes to improve performance by combining the posterior probabilities of each enzyme by product rule. Results: This method is applied in influenza A virus dataset containing 14,011 full length of HA sequences, which cover 17 HA subtypes. From 615 restriction enzymes, our method reached a top recognition rate of 80.49% with 1 enzyme (MunI). Recognition rate increased to 99.14% when RFLP patterns of 5 enzymes (MunI, XhoII, PsuI, BbvI, and BstMWI) were combined. Further analyses showed that, with these enzymes, RFLP patterns of test sequences were consistent with most sequences from their true subtype, while greatly different from other subtypes. Conclusions: The results demonstrated that the method could efficiently determining the viral HA subtype with RFLP patterns of a few restriction enzymes, and thus can be used to facilitate influenza A virus HA subtype identification.

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Influenza- and respiratory syncytial virus–associated hospitalizations among patients attending private hospitals in South Africa, 2006-2012

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Background: Influenza and respiratory syncytial virus (RSV) infections are common causes of pneumonia and are responsible for substantial global morbidity and mortality every year. The burden of influenza- and RSV-associated hospitalizations has been well characterized in developed countries, but such information remains scanty in low- and middle-income countries. Age-specific hospitalizations can inform policy-makers in sub-Saharan Africa how to design specific control measures targeting those most affected. We estimated the number of influenza- and RSV-associated hospitalizations among individuals of any age presenting to a national private hospital group in South Africa from 2006 through 2012. Methods: We estimated the number of influenza- and RSV-associated hospitalizations in 7 age categories (< 1, 1-4, 5-24, 25-44, 45-64, 65-74, and ≥ 75 years of age) using hospitalization data coded according to the *International Classification of Diseases, 10th Edition,* from a national private hospital group and influenza and RSV surveillance data from the National Health Laboratory Service Corporate Data Warehouse, a database that includes all patients tested for

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influenza and RSV by the national laboratory network. The private hospital group is the third largest in South Africa, representing 28% of private health care service providers. We modeled excess hospitalizations attributable to influenza and RSV infection by applying Poisson regression models to weekly all-respiratory (*ICD-10*: J00-J99), all-circulatory (*ICD-10*: I00-I99) and pneumonia and influenza (P&I) (*ICD-10*: J10-J18) hospitalizations. The full model included covariates for time trends and seasonal variations, as well as influenza and RSV detection rates. Results: Annually, seasonal RSV circulation peaked between March and April, followed by influenza that peaked between May and August. Increases in weekly hospitalization numbers coincided with peaks in RSV and influenza viral circulation. Over the study period, a mean of 530,345 hospitalizations was reported annually from the private hospital group, of which 61,947 (11.7%) were attributable to respiratory illnesses, 44,085 (8.3%) to circulatory illnesses and 20,014 (3.8%) to P&I as the primary cause of hospitalization. Our model estimated that the mean annual number of influenza-associated hospitalizations was 2680, 198 and 1625 among all-respiratory, all-circulatory and P&I cause of hospitalization, respectively. This on average accounted for 4.3%, 0.5% and 8.1% of the total hospitalizations in the same categories. RSV was associated with a mean of 3779 and 1395 hospitalizations accounting for 6.1% and 7.0% among all-respiratory and P&I causes of hospitalization, respectively. No RSV-associated hospitalizations were found among all-circulatory causes of hospitalization. There were variations in the percentages of influenza- and RSV-associated hospitalizations among the different age groups. Of the 1625 patients hospitalized with a diagnosis of P&I, influenza-associated cases accounted for 1.5% (66/4476) of P&I hospitalizations among patients aged < 1 year, 5.2% (138/2638) among those 1-4 years, 16.0% (380/2377) among those 5-24 years, 9.0% (335/3731) among those 25-44 years, 10.9% (380/3494) among those 45-64 years, 9.1% (132/1453) among those 65-74 years and 10.5% (194/1845) among those aged > 75 years. All the 1395 P&I hospitalizations associated with RSV were observed in patients below 25 years of age, accounting for 16.5% (739/4476) among those aged < 1 year, 17.6% (464/2638) among those 1-4 years, and 7.8% (185/2377) among those 5-24 years. Conclusions: Our model estimated that influenza and RSV substantially contributed to private-sector hospitalizations in South Africa in the period we studied. Influenza-associated hospitalizations were identified across all age-groups and accounted for a larger proportion of hospitalizations in the < 5 and ≥ 65 year age groups. The burden of RSV-associated hospitalizations was greatest in individuals < 5 years of age, whereas no RSV-associated hospitalizations were detected in individuals ≥ 25 years of age.

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Evolutionary and genetic characterization of Romanian H5N1 highly-pathogenic avian influenza A virus

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Background: H5N1 highly-pathogenic avian influenza (HPAI) was first detected in farmed geese in the Guangdong province of southern China in 1996. Since then, H5N1 HPAI has undergone significant evolution, diversifying into 20 distinct clades, numbered 1-20, each with multiple sublineages. During an outbreak in an animal market in Hong Kong in 1997, the virus was detected in humans. Since then, the virus has geographically spread into Southeast Asia, India, Russia, Africa, the Middle East, and Europe, sporadically infecting humans with up to a 60% mortality rate. While sustained human-to-human transmission has not been detected, H5N1 HPAI remains a cause for concern, and a risk of becoming a human pandemic. Materials and Methods: In response to the H5N1 HPAI incursion into Europe in 2005, European member states set up an avian influenza surveillance network. Under this surveillance network, 90 H5N1 samples from outbreaks in Romania between 2005 and 2010 were collected. In this study, we have generated 78 complete H5N1 genomes from these samples through next-generation sequencing (NGS). Here, we investigate the number and timings of introductions into Romania, as well as assessing the incidence and frequency of mutations associated with virulence

and host-adaptation. Results: By combining these data with other H5N1 sequences in GenBank, we show that the spread of H5N1 across Asia and Europe follows the flyways of migratory birds. We further show that there have been at least 3 introductions of clade 2.2 H5N1 HPAI into Romania between 2005 and 2007, and that the 2010 outbreak was a separate introduction of a 2.3.2 clade. With these data, we see that the Romanian outbreak directly seeded subsequent European and African outbreaks. We also analyze our sequences and phylogenies in the context of mammalian adaptation and aerosol-transmission mutations. Conclusions: These results give an important insight into the evolutionary processes of avian H5N1; by understanding the patterns of transmission and the molecular evolution of the virus, we are better able to assess and respond to the threat of H5N1 HPAI to human public health.

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Influenza virus surveillance conducted by the Institute Adolfo Lutz, São Paulo, SP, Brazil: 2013 influenza season

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Background: Taking into account the shift in the period of influenza virus circulation regarding the influenza virus season 2013 in the state of São Paulo, Brazil, when compared with previous years, a surveillance task has been carried out as a means to cooperate with timely influenza virus variants detection and influenza virus strains selection for vaccine production. In contrast to many other vaccines, influenza vaccines are frequently updated so as to be most effective against newly evolving human influenza viruses that are likely to circulate in the following influenza season. Nowadays, in addition to the preventable measure represented by the influenza virus vaccine campaign, antiviral drugs reinforce the control and treatment of influenza. Facing the benefits provided by both strategies, monitoring influenza strains is the key for their successful utilization. Materials and Methods: Surveillance was conducted in 17 sites distributed among the Southeast, Midwest, North, and Northeast regions of Brazil. Patients with influenza-like illness enrolled in the study, provided nasopharyngeal swabs, and basic clinical data were collected on a standard form provided by the Ministry of Health of Brazil. From January to mid-June 2013, during the influenza season, a total of 7713 combined swabs specimens (nasopharyngeal and throats) were sent to the Institute Adolfo Lutz, was cooperates with the National and Global Influenza Surveillance Network. From these, 7111 came from São Paulo state; and 386, 96, and 120 respiratory specimens came from the Midwest, Northeast and North regions, respectively. The specimens were first tested by real time–reverse transcription polymerase chain reaction (rRT-PCR) by using the standard CDC protocol for (rRT-PCR). All specimens from sentinel sites positive by rRT-PCR were selected for virus isolation in MDCK cells and antigenic and molecular characterization have also been performed. Results: Virological surveillance reported the following results: 1214 (20%); 58 (1%), and 242 (4%) were identified as influenza A (H1N1) pdm09, A (H3) and influenza B, respectively. A total of 75% of the specimens collected from patients presenting with influenza-like syndrome yielded negative results for influenza virus infection. Conclusion: Influenza virus surveillance shows the mismatch between the World Health Organization (WHO) influenza B vaccine composition, recommended for the South Hemisphere, and the circulating influenza B viruses identified during the influenza season 2013. Antigenic characterization of influenza B viruses isolated revealed the circulation of influenza B/Victoria lineage; on the other hand, the B vaccine component selected for the South Hemisphere corresponds to B/Yamagata lineage. Influenza virus surveillance data will certainly assist the national public health authorities in order to make decisions on a quadrivalent vaccine composition. Antigenic characterization and molecular analysis have demonstrated that the influenza A/California/7/2009 (H1N1) pdm09-like virus are classified into 6 and 7 genetic groups which were antigenically indistinguishable. Influenza A/Victoria/361/2011(H3N2)–like virus has been identified, during influenza virus season 2013. Both H1 and H3 virus strains identified, in this surveillance task, match the WHO

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vaccine composition recommended for the South Hemisphere. These data show how important the follow-up of influenza virus surveillance is to keep the influenza vaccine composition updated.

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Disproportionate impact of influenza B viruses in influenza-associated pediatric deaths

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Background: In the United States, influenza-associated deaths in children < 18 years of age became a nationally notifiable condition in 2004. During the nonpandemic influenza seasons from 2004-05 to 2011-12, the annual number of deaths reported has ranged from 34 to 123. Although influenza A viruses are perceived to result in more severe illness, a large number of pediatric deaths were associated with influenza B virus infection. To better understand the role of influenza B viruses in severe illness in children, we compared children who died with influenza A virus infection to those with influenza B infection for both preschool and school-age children and compared the relative proportions of influenza A and B viruses reported in fatal cases to those reported through virologic surveillance. Materials and Methods: Virologic surveillance data were obtained from the US WHO collaborating laboratories system for persons aged 0-4 years and 5-24 years. Data from the Influenza-Associated Pediatric Mortality Reporting System were examined for influenza virus type, underlying medical conditions, bacterial coinfections, location of death, and number of days hospitalized prior to death for children 0-4 (preschool) and 5-17 (school-age) years of age. Data from the 2009 pandemic period were excluded from both data sources. Results: For all seasons combined, 25% of viruses among children 0-4 years and 32% of viruses among persons 5-24 years reported to the CDC by US WHO collaborating laboratories were influenza B viruses. Among pediatric deaths, 27% among preschool-age and 43% among school-age children were associated with influenza B infection. For preschoolers, the percentage of deaths due to influenza B viruses was slightly higher than that reported through surveillance during 4 of the 6 influenza seasons examined, but the differences were not statistically significant for any season or for all seasons combined. Among school-age children, the proportion of influenza B virus infections among children who died was higher than that seen in surveillance in all 6 seasons, and was statistically significant for the 2007-08 season (P =.003) and for all seasons combined ($P \leq .001$). Preschool children who died with influenza B virus infection were more likely to have an underlying medical condition (60% vs 41%; P = .02), have asthma (25% vs 6%; P = .0004), and to have asthma as their only underlying condition (17% vs 3%; P = .004) compared with those infected with influenza A viruses. In contrast, school-age children who died with an influenza B infection were less likely to have asthma (10%) than children with influenza A infection (23%; P = .01) and equally likely to have any underlying condition or asthma as their only underlying condition than children with influenza A infection. No statistically significant differences by virus type were seen for the presence of bacterial coinfections, location of death, or the number of days hospitalized prior to death for either age group. Conclusions: The proportion of children age 0-4 years who died of influenza B infection was similar to that seen in virologic surveillance data. However, for school-age children, the proportion with influenza B was higher among children who died compared with surveillance data, and they were less likely to have asthma. From this, it appears that influenza A infections are more severe than influenza B for younger children, but for older children, influenza B infections are the more severe and more likely to lead to death in previously healthy children than are influenza A infections.

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Epidemiological and clinical description of human metapneumovirus infectious diseases in Mbagathi District Hospital, Kenya, in 2008

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Background: Human metapneumovirus (hMPV), a recently identified respiratory virus, is a leading cause of acute respiratory tract infection in children, the elderly, and immune-compromised persons. Studies have been done in a hospitalized pediatric population in coastal Kenya. However, there is limited information about the prevalence, epidemiology, and clinical presentation of hMPV infections in the general population. The aim of this study was to outline the epidemiologic and clinical description of human metapneumovirus infectious disease in patients attending the outpatient department of Mbagathi District Hospital, Kenya, in 2008. Methods: A retrospective study was conducted from 2008 in patients ≥ 2 months of age presenting at the outpatient department of Mbagathi District Hospital for acute respiratory infection. Nasopharyngeal swabs were systematically tested for several respiratory viruses. Epidemiologic and clinical characteristics of hMPV-infected children were compared with those of patients with respiratory syncytial virus (RSV) and other viral infections. Results: A total of 498 patients were enrolled in this study. Viral investigations detected a total of 271 viruses. Of these, 77 (15.5%) were hMPV infections, 78 (15.7%) seasonal flu A, 60 (12%) seasonal flu B, 13 (2.6%) panenterovirus, 36 (7.2%) parainfluenza viruses, and 6 (1.2%) RSV infections. Human metapneumovirus infections were higher in males (43, 55%) than in females (34, 45%), and predominantly in children ≤ 5 years (97%), only 2 (3%) were aged between 6 and 9 years. The hMPV infection had peaked in January-February, and was uncommon after March. Most of the patients infected with hMPV were < 1 year of age, and cough (100%) and difficulty in breathing (75%) were the predominant diagnosis in these patients with clinical symptoms of a lower respiratory tract infection. The severity of the disease was similar to those of RSV patients. Conclusions: These results highlight that hMPV plays an important role in seasonal acute respiratory tract infections, especially in children, with a severity similar to RSV infections. This work is ongoing to cover the wider Kenyan population.

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Evaluation of two influenza surveillance systems in South Africa

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Background: Influenza surveillance programmes should provide timely, good quality virologic and epidemiologic data on mild and severe respiratory disease to facilitate epidemic and pandemic detection and guide clinical and public health interventions. Surveillance for outpatient influenza-like illness (ILI) and inpatient severe acute respiratory illness (SARI) can contribute to an understanding of the spectrum of influenza illness in resource-limited settings. This study evaluated two influenza surveillance systems in South Africa: surveillance for outpatient ILI through the Viral Watch (VW), and surveillance for hospitalized pneumonia through the SARI system. Methods: ILI surveillance is performed voluntarily at 205 sentinel sites in private and public clinics in all 9 provinces; it was established in 1984 to collect virologic influenza surveillance data. SARI surveillance is performed at 6 sentinel sites in public hospitals in 4 provinces by teams of dedicated personnel; it was established in February 2009 to collect detailed epidemiologic and virologic influenza surveillance data. Both systems were compared for weekly laboratory-confirmed influenza detection rates, peak detection rates, and season start and end dates (defined as influenza detection rates > 10% and < 10% for two consecutive weeks, respectively) from 2009 to 2012. Using criteria established by the US Centers for Disease Control and Prevention, nine surveillance system attributes were also assessed; this included quantitative analyses of data quality, timeliness, sensitivity, and positive predictive value, and qualitative descriptive analyses of representativeness, simplicity, flexibility, acceptability, and stability. Results: Influenza seasonality was analysed using surveillance data collected between 01 January 2009 and 31 December 2012 from 18,296 SARI patients and 11,102 ILI patients (VW). The nine surveillance system attributes were analysed using surveillance data collected between 01 January 2009 and 30 June 2012 from 15,271 SARI patients and 8654 ILI patients. Compared with the SARI system, VW registered the influenza season mean start date 2.3 weeks earlier, the mean peak 1.0 week earlier, and the mean season end date 1.0 week later. VW had consistently higher rates of influenza detection (mean = 40.5% positive; range = 38.3-43.0; n = 9104) than SARI (mean = 7.9% positive; range = 5.2-10.7; n = 18,293) in all four years analysed. The proportion of circulating influenza strains was similar between VW and SARI systems, except in 2011 when VW identified more A(H1N1)pdm09 than SARI (74.6% vs 39.5%). The current World Health Organization recommended ILI case definition was introduced into VW in March 2012; using enrolled ILI patients who did not meet the case definition as a comparator, this slightly improved sensitivity (89.8%; 95% CI: 88.4-91.1) and specificity (30.9%; 95% CI: 29.2-32.7) of identifying laboratory-proven influenza compared with the ILI case definition previously used (sensitivity 87.0% [95% CI: 85.4-88.4], specificity 26.4% [95% CI: 24.7-28.1]). Relative strengths of VW included simplicity, minimal cost, representativeness, long-term stability, and timeliness (97.9% of ILI cases had respiratory specimens collected within 7 days of symptom onset; n = 5659). Relative weaknesses of VW included poor data quality measures such as form completion (61.7% overall; n = 8556) and poor adherence to ILI case definitions (77.5% overall; n = 5237). VW also collects minimal epidemiologic patient data. By

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comparison, SARI surveillance collects detailed epidemiologic patient data and has excellent data quality (40/43 key data elements on patient interview forms had completeness measures above 90; n = 15,189 forms) and timeliness (90.5% had respiratory specimens collected within 7 days of symptom onset; median = 4 days). Limitations of the SARI programme included representativeness and simplicity, as the programme demands substantial investment, with operating costs totalling approximately US $1 million per year. Conclusions: The VW programme meets a number of virologic influenza surveillance objectives and is more representative, financially sustainable, and expandable than the SARI surveillance system. However, it is of limited use in characterising the epidemiology, risk factors, or burden of influenza. The SARI system meets many virologic and epidemiologic influenza surveillance objectives, and is better designed to improve our understanding of the epidemiology of influenza. Surveillance for influenza through the SARI system may be better suited to address questions of public health priority in South Africa.

P2-689 GATES FELLOWSHIP Gates Fellowship

Noninfluenza viral etiology associated with acute respiratory infection in children under five years in Cote d'Ivoire

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Background: Acute respiratory infections (ARIs) are one of the major causes of morbidity and mortality in young children throughout the world, especially in developing countries, where up to 1.9 million children die each year due to ARIs, 70% of them in Africa and Southeast Asia. The majority of acute lower respiratory tract infections (ALRTIs) in developed countries have been reported to be often due to viral pathogens. However, information on these viruses in developing countries is limited, probably because of the paucity of modern diagnostic molecular techniques. These infections are therefore treated unsuccessfully with antibiotics based on suspicion of bacterial causes. In Cote d'Ivoire, ARIs are the third cause of death, after malaria and diarrhoeal diseases, in children under 5 years of age. Previous studies have described the circulation of influenza viruses in this country, but only limited data have been reported for children on the epidemiology of the other respiratory viruses involved in ARIs. To further understand the epidemiology of ARIs in children, we analysed data collected throughout the influenza sentinel surveillance network to assess the contribution of the other respiratory viruses to childhood ALRTIs in Cote d'Ivoire. Materials and Methods: This was a surveillance-based study of children under five years of age recruited between January and May 2013 in the nine sentinel sites that compose the influenza surveillance network in Cote d'Ivoire. Patients enrolled in the study were selected according to the case definition proposed by WHO for ILI and SARI monitoring. A nasopharyngeal sample was collected for each patient, using a collection kit (Copan Universal Transport Medium for virus preservation) in conjunction with epidemiologic information including age, gender, hospitalization status and symptoms. RNA extractions were conducted using QIAamp viral mini-kit from QIAGEN. Amplification were performed using the QIAGEN OneStep RT-PCR kit for the detection of respiratory syncytial virus (A, B), metapneumovirus, parainfluenza virus (1, 2, 3, 4), human coronaviruses (229E, OC43), rhinovirus and enterovirus. All products were subjected to electrophoresis on a 2% agarose gel. Results: Other viral agents were detected in 128 specimens (30.2%) of the 424 negative samples for influenza processed. Human coronavirus 229E was the dominant virus detected (50.8%). Detection rates of 32.8%, 8.6%, 3.1%, 0.8%, and 0.8% were observed for parainfluenza viruses 1-4, rhinovirus, RSV, metapneumovirus, and human coronavirus OC43, respectively. The co-infection rate for at least two viruses was 3.1% (4/128), with one (0.8%) positive for metapneumovirus/parainfluenza virus 3, one (0.8%) for metapneumovirus/parainfluenza virus 4, one (0.8%) for human coronavirus 229E/parainfluenza virus 3 and one (0.8%) for RSV/parainfluenza virus 3. The positive cases were significantly younger than the nonpositive cases (mean age 15.7 months vs 20.4 months; $P < .01$). There was no significant difference in positive detection rate for hospitalized children versus nonhospitalized children (32.1% vs 30.1%; $P = .054$). Conclusions: This study has demonstrated the important burden of other respiratory viruses as major causes of childhood ARIs in Cote d'Ivoire. These data address a need for more studies on viral-associated respiratory tract infection to improve patient management and implementation of infection control precautions.

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Etiology of community-acquired pneumonia among hospitalized children in the United States: preliminary data from the CDC Etiology of Pneumonia in the Community (EPIC) study

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Background: Pneumonia is a common cause of hospitalization. Over the last 2 decades, molecular methods have improved diagnostic capability, and community-acquired pneumonia (CAP) etiology has likely changed due to antimicrobial use and expanded vaccine use. Using traditional and innovative methods, the Centers for Disease Control and Prevention (CDC) EPIC study aims to determine CAP etiology among hospitalized children. Materials and Methods: Children < 18 years old were enrolled in 3 hospitals in Memphis and Nashville, Tennessee, and Salt Lake City, Utah, United States. CAP was defined as an acute respiratory infection and new radiographic pneumonia in a child from and hospitalized in the catchment area without recent hospitalization or severe immunosuppression. Blood for culture and streptococcal polymerase chain-reaction (PCR) targeting *lyt-A* and *spy* genes and naso/oropharyngeal swabs (NP/OP) for PCR of respiratory viruses and atypical bacteria were collected as soon as possible after admission. If therapeutic thoracentesis was required, pleural fluid samples were tested by culture and PCR methods. Results: From January 1, 2010, to June 30, 2012, we enrolled 2628 (69%) of 3808 eligible children with CAP aged 0-23 months (46%), 2-4 years (26%), and 5-17 years (28%). The majority were white (39%), black (34%), or Hispanic (19%). Twenty percent of children were admitted to intensive care, 7% required invasive mechanical ventilation, and < 1% died. Antibiotics were prescribed to 25% of the children before hospitalization. Among children with blood sample timing available, 77% of 2348 first cultures and 38% of 2313 PCR samples were collected prior to inpatient antibiotic administration. Among 2431 children with blood (culture or PCR) or pleural fluid (culture or PCR) and NP/OP results, a virus (73%) or bacteria (13%) was detected. Co-infections were detected in 21% of children (378 cases with multiple viral, 139 cases with bacterial-viral). No pathogen was detected in 19% of children. The following pathogens were more commonly detected: rhinovirus (29%), respiratory syncytial virus (27%), human metapneumovirus (11%), adenovirus (11%), *Mycoplasma pneumoniae* (8%), parainfluenza viruses 1, 2, 3 (5%), coronaviruses (5%), influenza (5%), and *Streptococcus pneumoniae* (3%). Conclusions: Preliminary results from this multisite prospective study conducted in the United States indicate that viruses were commonly detected in children with CAP requiring hospitalization. Bacteria may have been underdetected due to limited sensitivity of available tests and prior antibiotic use. Additional etiologic testing, including serology in cases and NP/OP PCR in healthy controls, is underway to help further elucidate etiology and clinical relevance. Clinicians should test for viruses and bacteria in patients with suspected pneumonia to optimize antimicrobial use. The CDC EPIC study is ongoing and will continue to provide insights into pediatric CAP etiology and incidence.

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The role of Influenza infection and other respiratory pathogens: a contribution for improving surveillance of maternal mortality

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Background: Acute respiratory infections (ARI) are caused by different respiratory pathogens, which circulate within the population at the same time. Since these infections are clinically indistinguishable, the etiological identification depends on laboratory testing. Due to the social and public health impact, investigation of maternal deaths is necessary. However, to date, our surveillance system has relied on clinical/epidemiological investigation in such a way that the etiology of deaths by respiratory causes frequently remains unclear. The aim of this study was to investigate the role of different respiratory pathogens on maternal death. Materials and Methods: The study population was composed of 470 women of reproductive age (15-44 years) from Rio de Janeiro (southeast) and Rio Grande do Sul (south of Brazil) recruited in the scope of the Influenza Surveillance Program between April 2009 and February 2013. Therefore, all subjects consisted of ILI or SARI cases, according to WHO case definition. Clinical specimens (oro-nasal swabs) and demographic/clinical/epidemiological information was collected by the Epidemiological Surveillance teams using a nationally standardized questionnaire. After nucleic acid extraction (QIAmp MinElute Virus Spin kit, QIAGEN), detection of respiratory pathogens was performed by a multiplex real-time RT-PCR (FTD Respiratory Pathogens 21plus, Fast-Track Diagnostics), allowing detection of influenza A(H1N1)pdm09 (H1N1p), influenza A, influenza B; parainfluenza 1-4, *respiratory syncytial virus* (RSV A/B), rhinovirus, coronaviruses OC43, NL63, 229E, HKU, muman metapneumovirus (HMPV), human bocavirus (HBoV), adenovirus (AV), Enterovirus (EV), parechovirus (PV), *Mycoplasma pneumoniae* (Mpneu), *Chlamydia pneumonia* (Cpneu), *Haemophilus influenzae* (HiB), *Staphylococcus aureus* (Saur) and *Streptococcus pneumoniae* (Spneu). Descriptive, bivariate analyses (chi-square/Fisher's exact test and *t* test for means) and multiple logistic regressions (considering variables of epidemiological relevance/plausibility) were carried out and considered significant when $P < .05$. Results: Among the 470 studied women, 253 were pregnant (G1) and 217 were non-pregnant (G2), including 69 fatal cases (46 vs. 23, in G1 and G2, respectively). Within the fatal cases, no pathogen was detected in 10 (14.6%), whereas 1 pathogen was detected in 18 women (26.1%). Figures were not significantly different between G1 and G2. However, the same was not true for multiple detection, whose presence was significantly associated with a fatal outcome among pregnant women (67.4% vs. 43.5%, OR = 2.7, 95% CI 1.3-5.9; $P = .008$). Among fatal cases, mono- and multiple detections were found in 8 and 41 cases, respectively, where H1N1p played a major role. In about 80.0% (n = 33) of multiple-detection cases, ≥ 1 virus and bacterium were found, followed by multiviral detection (n = 5). In addition to Influenza, other pathogens were also detected, as follows: Spneu (n = 17). HiB (n = 16), HBoV (n = 6), AV (n = 2), Rhinovirus (n = 2), Mpneu, OC43, NL63, RSV, EV (n = 1, each). Within the subsample of 253 pregnant women (49 deceases and 207 resolved infection), fatal outcome was significantly associated with multiple detection (any pathogen) (77.5% vs. 58.0%, $P = .016$), bacterial infection (71.7% vs. 56.0%; $P = .035$), H1N1p (79.1% vs. 48.0%), H1N1p+bacteria (42.5% vs. 24.4%, $P = .020$), HBoV (13.0 vs. 4.8%, $P = .049$) and Spneu (37.0% vs. 17.4%, $P = .004$). After multiple logistic regressions (final model $\chi 2 = 22.7$; $P < .001$), infections by H1N1p (AOR = 4.3; 95% CI 1.6-11.4; $P = .003$). HBoV (AOR = 3.2; 95% CI 1.0-10.3; $P = .050$) and Spneu (AOR = 3.7; 95% CI 1.6-8.5; $P = .002$) were independently associated with progression to death. Conclusions: Besides influenza, other pathogens seem to play an important role in maternal mortality and should be considered during prenatal follow-up/care. Under a complementary perspective, these findings also highlight the relevance of immunoprevention during pregnancy. The detection of other pathogens in the scope of surveillance agenda (morbidity and mortality) is pivotal to elucidate the etiology of deaths by respiratory causes and to tailor/design preventive policies, especially those driven toward pregnant

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women. Financial support: Carlos Chagas Filho Foundation for Research endowment for Rio de Janeiro (FAPERJ), process n. 111.802/2012; Science and Technology Department (DECIT), Ministry of Health and National Counsel of Technological and Scientific Development (CNPq), Brazil.

P2-692

Setting up of a surveillance platform for acute respiratory infections in rural North India

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Background: Acute respiratory infections (ARIs) are a major cause of morbidity and mortality among children globally, with the greatest number of deaths occurring in developing countries. While estimates of total episodes of ARI and incidence of hospitalization due to pneumonia for India exist, no community-based systematic study has been carried out to date. We report the experience of setting up a surveillance platform to study the epidemiology and etiology of ARIs among children less than 10 years of age in rural north India. Methods: The surveillance platform initiated in August 2012 includes four villages in the Ballabgarh block of Haryana state in north India comprising 2754 children below 10 years. All children are screened weekly for the presence of any respiratory symptoms (cough, sore throat, earache/discharge, nasal discharge/congestion, shortness of breath, respiratory difficulty) or hospital admissions. Those screened positive are assessed clinically by trained nurses. Records of those hospitalized are scrutinized by medical officers. WHO's definition of ARI (AURI/ALRI) and pneumonia is used. Throat and/or nasopharyngeal swabs for bacterial and viral pathogens are collected from those meeting the case definition of ARI or pneumonia. Urine samples are also collected from pneumonia cases for pneumococcal antigen testing. Age- and village-matched asymptomatic controls of pneumonia patients also have their nasopharyngeal swabs taken to test for background carriage rates. Results: After initial pilot runs, the platform became fully functional from 14th August 2012. As of 28th March 2013 (90,031 child-weeks of surveillance), 10,561 ARI cases have been reported, for an incidence rate of 6.1 per child-year. Four hundred thirty-four cases of ALRI were diagnosed, giving an ALRI incidence rate of 0.25 per child-year. A total of 13 cases were hospitalized due to ARI, for a hospitalization rate of 7.5 per 1000 child-years. Of the 1000 cases tested for influenza, 99 (8%) were positive (25 for H1N1pdm09, 67 for influenza B and 4 for H3). Influenza positivity was higher among patients with upper respiratory infection (72/550; 13%) than in those with acute lower respiratory symptoms (24/450; 5.33%), whereas none of the 180 control specimens were positive for influenza. Influenza B accounted for 79% of influenza cases with AURI, whereas A/H1N1pdm09 was present in 50% of influenza cases with ALRI. Of the 295 samples tested for bacteriology, 20 were positive for *Streptococcus pneumoniae, 15 for Staphylococcus aureus* and 3 for *Haemophilus influenzae*. Of the 201 control specimens, only one tested positive for *S pneumoniae*. Tests for other viruses and urinary antigens are still to be carried out. Conclusions: A fully functional population-based surveillance platform has been set up that is documenting the burden due to pneumonia in this setting. This platform can serve as a potential vaccine trial site in the future.These studies were supported in part by cooperative agreements U01 IP000492 from the Centers for Disease Control and Prevention, Atlanta, Georgia, United States.

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Epidemiologic, laboratory, and clinical characteristics of humans cases of influenza A/H5N1 in Vietnam, 2003-2013

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Background: Vietnam has the third highest number of human cases of influenza A/H5N1 in the world, and continues to have H5N1 outbreaks in poultry (WHO 2012, OIE 2012). The H5N1 virus continues to pose a serious threat to public health. We sought to identify the epidemiologic, laboratory, and clinical characteristics of influenza H5N1 human cases in Vietnam from 2003 to 2013. Materials and Methods: A retrospective review was conducted on available case documents from regional official reports, provincial preventive medicine centers, and hospital medical records of all 125 H5N1 human cases reported in Vietnam from 2003 to 2013. All cases were confirmed by reverse transcription polymerase chain reaction using H5-specific primers and probes at the National Influenza Center in Hanoi, Vietnam. The case review was done by using a standardized questionnaire. Statistical analyses were performed in Stata/IC 10.1, with univariate analyses for possible associations, Wilcoxon rank-sum tests to compare the median of continuous variables, and chi-square and Fischer exact tests used otherwise. Results: The study indicated that 41 (65%) of 63 provinces/cities reported H5N1 cases, with more than 64% of the cases from northern provinces. Of cases, 25- to 64-year-olds were more affected (48.4%). By genetic analyses we found that H5N1 virus circulation in both animals and humans was clade 2.3.4 during the study period. Severe viral pneumonia was identified in 38 (63%) cases among 60 (48%) cases of respiratory infection, and other diseases as 13 (10.5%) cases. Median time from the last hospital admission to specimen collection = 1 day (IQR 0-1), and median time from the last admission to H5N1 laboratory confirmation = 3 days (IQR 2-5). The case fatality proportion was 62/125 (49.6%), and cases ≤ 18 years old were more likely to die than cases > 18 years (OR = 2.1; 95% CI = 1.0-4.6; *P* = .036). Antiretroviral treatment > 48 hours after the date of onset was significantly associated with increased mortality (OR = 9.1; 95% CI = 1.1-80.1; *P* = .02). A delay in hospital admission > 48 hours after illness onset significantly increased mortality (OR = 2.9; 95% CI = 1.1-7.6; *P* = .03). Conclusions: Case exposure histories and demographics provided no significant information regarding exposure and transmission risk factors, suggesting that more thorough questionnaires with more specific information be developed and reviewed with cases and their families. The cumulative H5N1 case fatality proportion was 50% in confirmed human cases in Vietnam from 2003-2013. A delay in hospital admission was significantly associated with higher mortality. Starting antiretroviral treatment within two days after illness onset was significantly associated with survival compared with that after this time period. We recommend that public health and health care professionals continue to practice and educate regarding the benefits of early hospital admission and early antiviral treatment of influenza A/H5N1 human cases.

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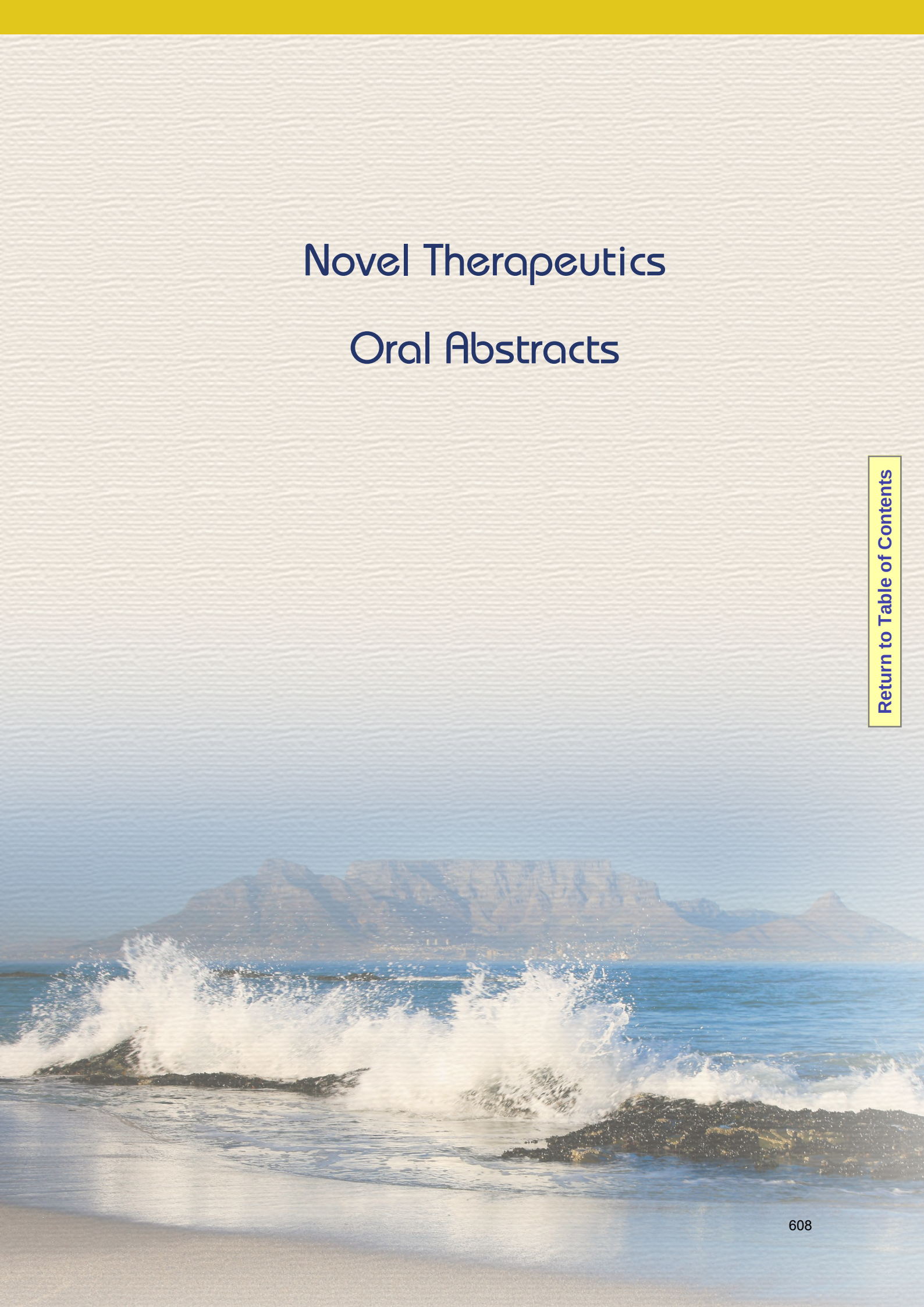

O-912

Host gene discovery and drug repurposing for treatment of emerging and re-emerging influenza virus

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Background: Classic anti-influenza drugs target viral proteins, providing specificity but enabling the development of drug resistance. The two classes of approved anti-influenza drugs target the virus neuraminidase and the M2 proton channel, typified by oseltamivir and amantadine, respectively. While most circulating human influenza viruses are susceptible to neuraminidase inhibitors, there are sporadic appearances of resistant viruses, and the emergence of resistant strains is an ongoing concern. An alternative approach for anti-viral drug development is to identify non-essential host factors required for virus replication and to block their expression or activity through drug repositioning or repurposing. Materials and Methods: An RNAi genome screen in human A549 respiratory epithelial cells was completed for influenza virus. Several genes were validated that, when silenced, reduced influenza virus replication without reducing cell viability. Pathway analysis of these genes revealed small-molecule inhibitors and existing licensed drugs that specifically target the genes or pathway. These drugs were analyzed and confirmed to silence the expression of the genes in both A549 cells and a mouse model and were then tested for prophylactic or therapeutic efficacy against influenza virus in vitro and in vivo. Results: Of the host genes validated as essential for influenza virus replication, several were tested, including organic anion transporter-3 gene (OAT3) and cell division cycle 25 B (CDC25B). Probenicid, an FDA-approved drug and inhibitor of organic ion transporters, including OAT3, was shown to reduce influenza virus replication in vitro and in a murine model of influenza virus infection. Similarly, NSC95397—a CDC25B inhibitor—was shown to interfere with influenza virus replication in vitro and in a mouse model of infection. Mechanisms of action for both drugs were identified. Antiviral drugs targeting host factors were shown to have broad efficacy against a range of seasonal zoonotic and potentially pandemic influenza viruses. Conclusions: Identifying existing drugs that block non-essential host genes critical for virus replication is a rapid and powerful approach to identifying anti-viral drugs that can be rapidly repositioned and implemented in our limited anti-viral arsenal.

O-913

TCN-032 (anti-M2e mAb) treatment leads to reductions in clinical symptoms and viral shedding in a human influenza challenge study

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Background: TCN-032 is a fully human mAb that targets a highly conserved conformational epitope on M2e, the sequence of which is present in > 99% of unique M2 sequences from human, swine, and avian influenza viruses, including H7N9. The mechanism of action of TCN-032 involves antibody-mediated effector functions, including ADCC and/or CDC, resulting in the lysis of infected cells and virus. TCN-032 protected mice from lethal challenge with H5N1 and H1N1 strains with reduction of viral spread to other organ systems; combination with oseltamivir provided additional efficacy benefit. Because of the broad binding ability and in vivo preclinical efficacy, TCN-032 may have potential as a universal therapeutic against influenza A infection. Materials and Methods: A randomized, double-blind, placebo-controlled study was conducted in serosusceptible healthy subjects (HAI titer ≤ 10) challenged with A/Wisconsin/67/2005 (H3N2) virus. Twenty-four hours following viral inoculation (Day 1), subjects were randomized (1:1) to receive study drug (TCN-032 40 mg/kg or placebo),

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administered intravenously. Subjects were monitored for clinical symptoms and viral shedding up to 10 days post-inoculation and for adverse events up to 28 days post-inoculation. Diary cards were collected 5x/day, which assessed 10 symptoms using a 4-point scale (0-3). Nasal swab specimens were collected 3x/day for viral quantitation by $TCID_{50}$ assay culture and qPCR. The study objectives included evaluation of TCN-032's effect on clinical symptoms, viral shedding, seroconversion, viral resistance, PK, immunogenicity, and safety. Clinical symptoms included the proportion of subjects with ≥ grade 2 symptoms, or pyrexia, from Days 1 to 7 (primary objective); total symptom scores; time to peak, time to resolution, and duration of symptoms; and nasal mucus weight. Viral shedding parameters included viral load by culture Days 1-7 and qPCR on Days 2-7 and time to peak, time to resolution, and duration of viral shedding. The efficacy analyses were conducted in subjects with laboratory-confirmed infection, defined as those with seroconversion (4-fold increase from baseline) or viral shedding by viral culture or qPCR (viral culture titer > 1.5 $\log_{10}$ $TCID_{50}$/mL on Days 1-7 or ≥ 2 positive detections of virus by qPCR [$TCID_{50}$/mL equivalent] on Days 2-7). One-sided *P* values are presented. Results: A total of 61 subjects were enrolled (TCN-032 n = 30 and placebo n = 31). All subjects received study drug with the exception of one subject in the TCN-032 arm, for a total of 60 subjects treated (TCN-032 n = 29 and placebo n = 31). A total of 48 subjects met the definition of laboratory-confirmed infection (TCN-032 n = 24 and placebo n = 24). A numerical reduction in the primary efficacy parameter of the proportion of subjects who developed any grade 2 or greater symptom or pyrexia was observed, but it was not statistically significant (*P* > .10). TCN-032–treated subjects had significant reductions in both total clinical symptoms and viral shedding via qPCR. Median clinical symptom AUC (Days 1-7) was reduced by 35% (*P* = .047, Wilcoxon rank-sum test) in TCN-032–treated subjects compared with placebo. Time to resolution, duration of symptoms, and nasal mucus weights were reduced in TCN-032–treated subjects compared with placebo. Median viral AUC (by qPCR, Days 2-7) was reduced by 2.2 $\log_{10}$ $TCID_{50}$/mL*Day (*P* = .095, Wilcoxon rank-sum test) in TCN-032–treated subjects compared with placebo. TCN-032 was safe and well tolerated, with no increase in adverse events compared with placebo, including lower respiratory tract symptoms. The TCN-032 pharmacokinetic profile was consistent with the Phase 1 study (half-life ~ 16 days), with no evidence of the development of anti-drug antibodies. Viral resistance analysis showed no change in the epitope recognized by TCN-032. Conclusions: The results of this study represent the first demonstration that a non-neutralizing antibody given parenterally may provide immediate immunity and therapeutic benefit in influenza A infection, with no apparent emergence of resistant virus. Clinical studies in natural infection are planned, including the target population of patients hospitalized with serious influenza A infection.

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O-914

Carrageenan, a broad anti-viral active polymer, in combination with a specific influenza inhibitor—a novel concept for therapeutic interventions

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Background: The periodic appearance of new influenza variants, such as the recent H7N9 outbreak, poses a worldwide threat of a pandemic situation. Clinical data for the broadly active anti-viral polymer carrageenan support the concept of creating a protective physical barrier in the nasal cavity against respiratory infections such as influenza. Furthermore, we evaluated an intranasal treatment consisting of a combination of carrageenan and zanamivir for its potential synergistic anti-viral activity in the treatment of influenza. We present *in vivo* results for this combination against an infection with a low pathogenic H7N7 influenza virus [a virus carrying a closely related HA compared with the avian influenza A(H7N9) virus]. Materials and Methods: Clinical benefit and antiviral effectiveness are demonstrated in a pooled analysis of two randomized clinical trials in 254 subjects with early symptoms of the common cold treated with carrageenan. We also evaluated the anti-viral activity of the combination—carrageenan and zanamivir—in mice after a lethal infection with an H7N7 A/Turkey/Germany/R11/01 virus. Results: Intranasal administration of carrageenan in adults and children with early symptoms of common cold significantly (*P* = .002) reduced the duration of disease

by 2 days and promotes the significantly (*P* =.02) stronger elimination of viruses from the nasal cavity. Clinical and antiviral effectiveness was also confirmed in patients with influenza A virus infection. In comparison with patients treated with placebo, patients treated with carrageenan had a 3.3-d benefit in disease duration. A combination of carrageenan and the neuraminidase inhibitor zanamivir was tested for its potential to prevent death in a lethal H7N7 virus mouse model. Therapy with the combination resulted in a significant increase in survival in comparison with both monotherapies and placebo. A significant survival benefit was maintained even when the treatment started up to 48 hours post-infection (*P* = .001). Conclusions: The nasal spray containing carrageenan has shown antiviral effectiveness against a broad variety of respiratory viruses, including influenza, in several clinical trials and is already marketed in several countries under different brand names. The combination of carrageenan with zanamivir in an intranasal spray would provide a novel concept for therapeutic intervention against influenza. Patients would benefit from the fast and efficient treatment of influenza in the upper respiratory tract and reduced duration of illness. Moreover, the combination of both components in the nasal cavity would reduce the probability of emerging escape mutants. Additionally, due to the broad anti-viral effectiveness of carrageenan, concomitant viral infections in the upper respiratory tract that could lead to disease complications could be prevented. Finally, in the case of a new influenza virus outbreak, carrageenan in combination with zanamivir would provide an immediately available first-line intervention option while vaccines are being developed and manufactured.

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Resistance to the novel antiviral drug T-705 through increased fidelity of influenza A virus polymerase

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Background: With the potential problem of the emergence of influenza viruses that are resistant to existing antivirals, there is an intense need for new anti-influenza drugs that can be used as monotherapy and in combinations. T-705 is a novel broad-spectrum antiviral compound in a late stage of clinical development. It has demonstrated inhibitory activity against several RNA viruses, including seasonal influenza A and B viruses. We previously showed that T-705 can induce a high rate of viral genomic mutations that generate a nonviable influenza virus and that lethal mutagenesis is a key antiviral mechanism of T-705 *in vitro*. Importantly, we showed an absence of phenotypically resistant viruses after multiple rounds of replication in MDCK cells with T-705. Because this cell system may not be optimal, we investigated the development of T-705–resistant influenza viruses in a mammalian model under drug selection pressure. Materials and Methods: We performed 12 sequential lung-to-lung passages of A/Denmark/528/2009 (H1N1 pdm) influenza virus in DBA/2 mice treated with either T-705 (50 mg/kg/day) or vehicle (placebo). Virus-infected, untreated DBA/2 mice served as a control for the screening of host-selected mutations. The susceptibility of passaged variants to T-705 was assessed in a plaque reduction assay in MDCK cells. Whole genomes of wild-type and passaged variants were sequenced. Polymerase activity was determined in a mini-genome assay with a luciferase reporter plasmid and a mix of PB2, PB1, PA, and NP plasmids of A/Denmark/528/2009 virus. The mutation frequencies and mutation profiles of variants passaged in the presence of either T-705 or zanamivir (used as non-mutagenic antiviral agent) were measured by sequencing a subcloned 1523-kb region of the NP gene. Results: A dose of 50 mg/kg/day resulted in a reduction of ~ 2-log $TCID_{50}$/ml in virus titers in lungs compared with those of infected, non-treated mice, and this dose was selected for further studies. Virus isolated after 10 passages from T-705–treated mice was more pathogenic than that from placebo-treated animals, as confirmed by the higher virus titers in the lungs. Sequence analysis of T-705–treated variants revealed A36V (in 1 of 3 mice) and T97I (in 3 of 3 mice) substitutions in the PA gene after passage 10, whereas mixed population at those positions were identified after passage 5. A phenotypic assay of passaged variants in MDCK cells confirmed the difference in effective concentration (EC_{50}) for T-705–treated and placebo-treated viruses isolated after 10 passages. The mutation frequency of T-705–treated viruses did not change when increasing concentrations of T-705 were applied compared with a 10-fold increase in mutation frequency in placebo-treated viruses; this suggests an increased fidelity of RNA synthesis in the

presence of T-705. We then examined whether T-705– and placebo-treated viruses from passage 10 would bias the mutation profile toward specific nucleotide mutations. T-705 selection pressure did not change the mutation profile when increasing drug concentrations were applied to T-705–treated variants. Nonetheless, in placebo-treated variants, treatment with T-705 resulted in accumulation of G-to-T and C-to-T transition mutations, which increased linearly with the T-705 concentration, suggesting a dose-dependent effect. Treatment with zanamivir did not significantly increase the number of nucleotide changes and did not bias the natural mutation profile or shift in mutation profile in either T-705– or placebo-treated variants. Conclusions: We have identified an unusual mechanism of viral drug resistance in which drug-selected mutations increase resistance to a mutagen by enhancing polymerase fidelity. This mechanism has not been reported previously in influenza viruses.

O-916

A novel antiviral drug Ingavirin® restores the cellular antiviral response in influenza A virus–infected cells and enhances viral clearance in ferrets

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Background: In order to fight viral infection, mammalian cells are equipped with a rapid antiviral response reaction induced by extra- and intracellular danger signals. This includes activation of PKR, translocation of IRF3 and IRF7 into the nucleus, induction of type I interferon (IFN), and the induction of MxA. As viral RNA effectively induces those factors, most viruses express proteins, which suppress this cellular antiviral response. In this line, influenza A virus codes for non-structural protein NS1. Correspondingly, an influenza virus mutant lacking NS1 is replication defective. Ingavirin® (Imidazolyl Ethanamide Pentandioic Acid) is licensed in Russia for treatment of respiratory infections, because it was shown to target various types of viruses. We hypothesized that the mechanism of action of Ingavirin® is associated with the re-activation of antiviral cellular response pattern, which is usually suppressed by pathogenicity factors such as the influenza A virus NS1 protein. Materials and Methods: *In vitro*, A549 cells were infected with influenza A/PR/8/34 virus (wt) at an MOI of 3 in the presence or absence of 1 µg/ml Ingavirin®. We then determined mRNA levels by qPCR and/or protein levels by Western blot or immunocytochemistry, respectively, of the following factors of cellular antiviral response in a time-dependent manner: interferon (IFN) alpha receptor (IFNAR)1, IFNAR2, STAT-1, IRF3, IRF7, MxA, and phosphorylated PKR (pPKR). Expression of type I IFN, interleukin-6, and tumor necrosis factor was determined by ELISA. The replication defective influenza A/PR/8/34 delNS1 virus was used as a positive control. *In vivo*, 3 groups of ferrets (8 animals in a group) were intranasally infected with replicating wild-type influenza virus A/Vienna/1H/2009 (H1N1). Thirty-six hours after the infection, ferrets were orally administered Ingavirin® at a dose of 13 mg/kg or with oseltamivir (Tamiflu) at a dose of 5 mg/kg. Animals in group 3 received the vehicle buffer PBS (controls). Treatment of all animals was repeated in the same manner daily from day 3 (36 hr post-infection) until day 9. Nasal wash samples were collected 2, 4, 6, and 8 days post-infection and evaluated for virus replication by the rapid culture assay (RCA). Results: We found that Ingavirin® does not trigger production of IFNs or other pro-inflammatory cytokines in infected cells. However, treatment of wild-type virus–infected A549 cells with Ingavirin® restored and/or enhanced mRNA and protein levels of IFNAR1 and IFNAR2, which were otherwise downregulated by the viral infection. This upregulation of IFN receptors was associated with activation of interferon-dependent molecules such as Stat-1, pPKR, IRF3, and IRF7 and MxA protein, mimicking the molecular pattern of an infection with an NS1 deletion virus. Correspondingly, Ingavirin® significantly accelerated viral clearance from nasal washes of ferrets as compared with the control group on day 4. On day 6, ferrets receiving Ingavirin® had also cleared virus more significantly than the group receiving oseltamivir. No toxic side effects were observed by the addition of Ingavirin®. Conclusions: Our results indicate that Ingavirin® should be considered a potent antiviral compound. It appears to effectively restore the naturally evolved innate immune response selectively in virus infected cells. Moreover, this mechanism of action might be a valuable explanation for the proven clinical effectiveness of Ingavirin®.

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Influenza viruses and host cell signaling—identification and preclinical evaluation of novel targets for antiviral therapy

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Background: Influenza virus exploits a number of cellular signaling pathways during the course of its replication, rendering them potential targets for new therapeutic interventions. Several preclinical approaches are now focusing on cellular factors or pathways as a means of treating influenza. By targeting host factors, rather than viral mechanisms, these novel therapies may be effective against multiple virus strains and subtypes, and are less likely to elicit viral drug resistance. The most promising candidates are inhibitors of intracellular signaling cascades, which are essential for virus replication. This presentation reviews novel approaches and compounds that target the Raf/MEK/ERK signaling pathway and the NF-kappa B signaling cascade. In our previous work we were able to demonstrate that influenza virus replication is dependent on Raf/MEK/ERK signaling pathway activity and that inhibition of this pathway leads to a reduction in viral titers. Materials and Methods: Here, we questioned whether combination of MEK inhibitors with the neuraminidase inhibitor oseltamivir would have a synergistic effect against influenza virus *in vitro* and *in vivo*. Results: We found that MEK inhibitors CI-1040, PD0325901, RO5126766, PLX4032, and GSK-1120212 showed IC_{50} levels against influenza virus in the nanomolar range and that combination increased the antiviral capacity of oseltamivir in cell culture. No tendency of resistance was found for MEK-inhibitor treatment. Moreover, combination therapy was also successful in a mouse animal model. Concerning the use of NF-κB inhibitors to treat influenza, we were previously able to demonstrate *in vitro* and in mice that acetylsalicylate (ASA) and DL-lysine acetylsalicylate (LASAG) function as an antiviral against influenza virus. Both are efficient and quite selective inhibitors of the NF-κB–activating kinase IKK, acting directly on the IKK complex and consequently inhibiting phosphorylation and degradation of IκB. They efficiently blocked the replication of various influenza viruses, including H5N1 strains, in MDCK or A549 cells by several orders of magnitude in a concentration range that was not toxic for host cells. In animal studies it was demonstrated that aerosol, but not oral, administration of ASA reduced virus titers in the lung and significantly promoted the survival of lethally infected mice. Treatment was well tolerated and did not exhibit harmful side effects. Strikingly, the study also showed that ASA and LASAG, in contrast with the neuraminidase inhibitor oseltamivir or the M2 ion-channel blocker amantadine, did not lead to the generation of resistant virus variants in multipassaging experiments in cell culture. Conclusions: Although these new antiviral strategies are still in an early phase of preclinical development, results to date suggest that they offer a new approach to the treatment of influenza, supplementing direct-acting antiviral drugs.

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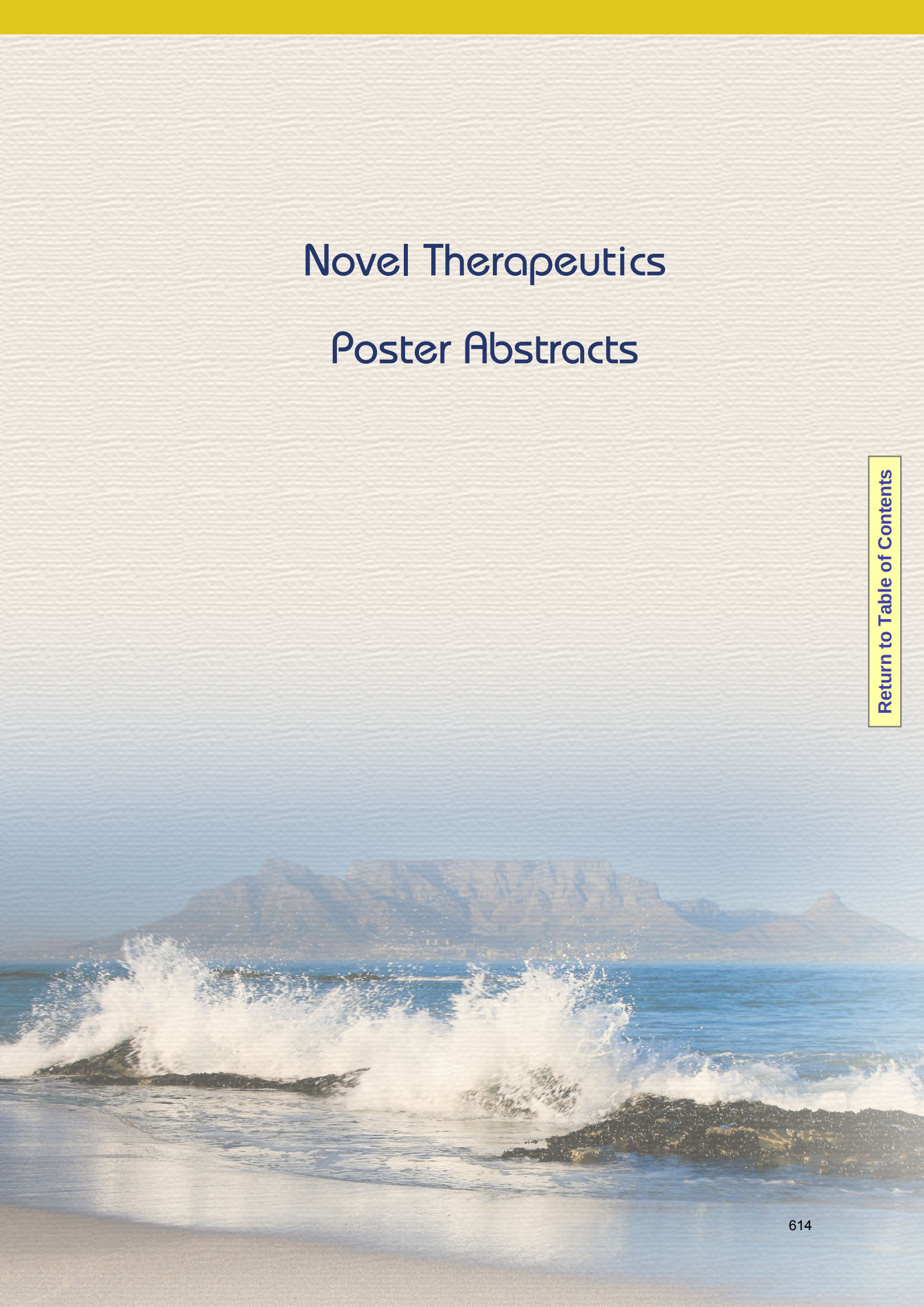

P2-695

Generation and characterization of recombinant scFv antibodies against haemagglutinin antigen of pandemic influenza 2009 (H1N1) virus

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Background: Phage display is a highly valuable technology for production of recombinant antibodies having high affinity towards the antigen. The coding sequences of the antibody variable (V) regions are fused to pIII, the minor coat protein of M13 phage using a phagemid vector. Materials and Methods: In this study, we generated recombinant single chain variable fragment (scFv) antibodies from spleen cells of mouse hyper-immunized with formalin-inactivated pandemic influenza 2009 (H1N1) virus. The variable light and variable heavy chain genes were amplified from mRNA isolated from mouse spleen cells and after cloning in phagemid vector, the recombinant antibody phage library underwent selection against HA antigen and inactivated virus. After 5 rounds of bio-panning, clones expressing HA specific recombinant scFv antibodies were selected and analyzed by ELISA for specific reactivity. Approximately 30% of clones showed binding activity to the inactivated virus and 7% to HA antigen of influenza A virus. Results: Our results confirmed that using phage display libraries is advantageous for the rapid development of molecules to detect target antigens. This suggests that it might be advisable to use a synthetic scFv phage display library by strategically considering the characteristics of target antigens and the potential situations. Conclusions: The analysis of the humoral immune response following influenza A virus infection using recombinant antibodies developed by us, may help in better understanding of the pathogenesis of the influenza virus infection. Further, the recombinant scFv antibodies can also be used for epitope mapping of HA antigen, which will be very useful for vaccine development.

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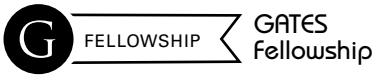

Sequence-specific cleavage of influenza A virus M2 gene transcript by circular DNAzymes: prolonged inhibition of viral RNA translation and replication

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Background: The influenza A virus M2 ion-channel protein is highly conserved among different viral strains and is essentially required during the trafficking, assembly, and budding processes of virus and is thus an attractive target for designing antiviral drugs. Although single-stranded DNAzymes (Dzs) may represent the most effective nucleic acid drug to date, they are nevertheless sensitive to nuclease degradation and require modification for *in vivo* application. The previously used PTO modifications have been shown to reduce the catalytic efficiency and are toxic when applied *in vivo*. Materials and Methods: We designed several 10-23 Dzs targeting different regions of the matrix gene (M2) of influenza A viruses and analyzed their ability to specifically cleave the target RNA in both cell-free systems as well as in cell culture using transient transfections. We further aimed to circularize the Dz to provide extended stability keeping the catalytic efficiency intact. Results: RT-PCR and real-time RT-PCR assays showed significant 75% inhibition of M2 gene of influenza A viruses upon specific Dz treatment. The transfection of MDCK cells with Dzs showed a reduced cytopathic effect caused by influenza A virus (A/PR/8/34-H1N1) and considerably reduced the M2 protein expression for a comparatively longer duration of time. The catalytic efficiency of both linear and circular Dzs was found to be comparable. As expected, the mutant Dz did not hinder in virus replication, showing a

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high level of specificity of designed Dz toward the target RNA. Conclusions: Our results demonstrate a substantial reduction in whole virus replication, thereby paving new dimensions in antiviral therapy. Our study, for the first time, has documented the antiviral potential of circular Dz against M2 transcript of influenza A virus. Thus, we propose that the 10-23 Dzs can be used as selective and effective inhibitors of viral RNA replication and that they be explored further for development of a potent therapeutic agent against influenza infection.

P2-697

Characterization of an H5N1 monoclonal antibody with therapeutic potential

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Background: Influenza H5N1 causes alarming mortality rates in immunologically naive populations and has the potential to cause the next influenza pandemic. The antigenic diversity of the virus hampers vaccination strategies, and emerging drug resistance among H5N1 isolates renders us defenceless against a potentially catastrophic H5N1 pandemic. Passive immunity has been suggested as a viable alternative solution, and, encouragingly, combination passive immunotherapy using non-competing neutralizing monoclonal antibodies (mAbs) can reduce the degree of escape mutations and decrease the total dosage of antibodies required. As such, the pre-pandemic generation, characterization and humanization of a library of neutralizing mAbs targeting H5N1 haemagglutinin (HA) could facilitate rapid accessibility to and selection of the appropriate combination of mAbs for passive immunotherapy in the event of a pandemic. Working toward this aim, we adopted a strategy that enables the generation and preliminary characterization of neutralizing mAbs within the confines of a BSL2 facility. This method resulted in the selection of mAb 9F4, which was found to bind to a novel and well-conserved epitope within HA that was distal to previously characterized antigenic sites on the H5 globular head, suggesting that it may be complementary to previously characterized anti-H5 mAbs. However, as murine antibodies will elicit a non-self immune response in humans, at least the chimerization of 9F4 is necessary for use in acute immunotherapy. Here we describe the generation and characterization of chimeric 9F4 IgG_1 in preparation for its potential use in combination passive immunotherapy of influenza H5N1. Materials and Methods: Mouse (o-) 9F4 was generated against recombinant baculovirus expressed HA protein of A/chicken/Hatay/2004 H5N1 virus by immunization of BALB/c mice and was selected based on its ability to neutralize pseudotyped lentiviral particles (HApp) carrying a firefly luciferase gene and harboring the H5N1 HA glycoprotein on the HApp envelope. The genes encoding for 9F4 variable regions of the heavy and light chains were obtained by reverse transcription PCR and ligated into an expression vector encoding for human kappa light and IgG_1 heavy constant chains. This vector was transiently transfected into 293FT cells to generate chimeric (xi-) 9F4. The ability of xi-9F4 to bind to H5 HA was tested by ELISA, HApp neutralization assay and fusion assay. The in vivo effects of both o-9F4 and xi-9F4 were also compared in a humanized mouse model created by adoptive transfer of human stem cells into NOD-scid Il2rg-/- (NSG) mice. Results: Both o- and xi-9F4 comparably bind to H5 HA and neutralize H5 HApp in a dose-dependent manner. xi-9F4 also retained its ability to reduce H5-mediated membrane fusion at a low pH compared with o-9F4. o-9F4, but not xi-9F4, led to an increase in IFN_Y, IL-8 and IL-6 expression in the humanized mouse model. Conclusions: Chimerization of 9F4 does not affect its binding or neutralizing ability and does not affect its ability to prevent H5 HA-mediated membrane fusion at low pH, suggesting that either the antibody variable regions were sufficient for 9F4 activity or that both mouse and human IgG Fc portions are equivalent in their contribution to binding to H5 HA. xi-9F4 induces a different cytokine profile compared with o-9F4 in humanized mouse model, suggesting that different effector functions are induced *in vivo*.

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Neuraminidase-inhibiting monoclonal antibodies: effective agents against seasonal and pandemic influenza viruses

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Background: The 2009 swine-origin H1N1 pandemic was not as severe as initially expected. Seroepidemiology and animal studies suggest that cross-reactive NA-specific antibodies elicited by seasonal H1N1 vaccination or infection contributed to protection against the pandemic virus. Since the molecular basis for this heterologous immunity was not known, we identified antigenic domains of the N1 subtype of NA that were conserved among influenza viruses using a panel of mouse monoclonal antibodies (mAbs). mAbs with the broadest reactivity bound the NA of seasonal H1N1 virus A/Brisbane/59/2007 (BR/07), 2009 pandemic virus A/California/7/2009 (CA/09), and 1918 pandemic H1N1 virus A/Brevig Mission/1918; in addition, they reacted with the NA of the H5N1 virus A/Vietnam/1203/2004 (VN/04). Our goal was to determine whether these N1-specific mouse mAbs as well as broadly reactive N1-specific human mAbs would protect mice against challenge with heterologous H1N1 and H5N1 viruses. Materials and Methods: mAbs were administered intraperitoneally at 10 or 15 mg/kg body weight to groups of 7-wk-old female DBA/2 mice. Twelve hours later the mice were anesthetized with isoflurane and challenged with 10 LD_{50} of BR/07 (seasonal H1N1), CA/09 (2009 H1N1pdm), or attenuated VN/04 (H5N1). Four mice from each group were euthanized on day 3 post-challenge, and the lung $TCID_{50}$ titers were determined in MDCK cells. Body weights and mortality of the remaining mice were monitored for up to 14 d. The prophylactic efficacy against challenge with wild-type, highly pathogenic A/Vietnam/1203/2004 (H5N1) was determined in immunocompetent BALB/c mice using the same dose, administration route, and procedures as in the experiments conducted in DBA/2 mice, with the exception that they were conducted in enhanced biosafety level 3 (BSL3+/ABSL3+) facilities. All mouse experiments were performed using protocols approved by institutional Animal Care and Use Committees following federal guidelines. Results: Both strain-specific and broadly reactive NA-inhibiting antibodies protected DBA/2 mice against lethal homologous virus challenge. mAbs that inhibited neuraminidase activity of heterologous H1N1 or H5N1 viruses also protected DBA/2 mice against challenge with these heterologous viruses. A broadly reactive non-inhibiting NA-specific human mAb did not protect DBA/2 mice against influenza challenge, suggesting that NA-specific antibody-mediated protection in this mouse model relies on blocking substrate cleavage. DBA/2 mice are deficient in complement; complement is likely to provide an additional mechanism of antibody-mediated protection since this latter antibody, which does not inhibit enzyme activity, was nevertheless effective at protecting immunocompetent BALB/c mice against challenge with 10 LD_{50} attenuated VN/04 (H5N1). Conclusions: We tested the therapeutic potential of broadly reactive mouse and human NA-specific mAbs in mice. Our results support the use of NA-specific mAbs for prophylaxis and therapy against influenza. NA-specific mAbs, particularly those that have broad reactivity, may be vital additions to the currently limited arsenal for prevention or treatment of severe H1N1 and H5N1 infections.

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Influenza viral hemagglutinin-targeted macrocycles as an antiviral agent

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Background: The constant threat imposed by influenza A viruses is one of the greatest public health issues. Although neuraminidase inhibitors are globally approved for influenza treatment, resistant mutants have already appeared; thus, alternative anti-influenza drugs are in high demand. Hemagglutinin (HA), one of the two glycoproteins that forms trimers on the surface of influenza virions, plays a critical role in the virus replication cycle; HA mediates cell attachment by binding to receptor molecules and low pH–dependent membrane fusion in endosomes. Although HA is classified into 16 subtypes based on its antigenicity, all HA molecules share the same fundamental biological function. Furthermore, HA is expressed on the virion surface; thus, penetrability through the cell or virion membrane is not required for HA-targeted antiviral agents. HA is considered to be alternative and ideal antiviral target. Since HA is the most immunogenic among influenza viral proteins, and HA-targeted antibodies have the potential to neutralize HA-mediated virion-cell binding, several HA-specific monoclonal antibodies have been established and demonstrated to be useful as anti-influenza drugs. The molecular size of antibodies, however, is comparable with that of the HA trimer; thus, the accessibility to HA functional domains should be limited. Materials and Methods: To devise smaller molecules capable of binding to the influenza viral HA and have the potential as an antiviral agent, we used an emerging technology, RaPID (Random non-standard Peptide Integrated Discovery) system, which enables the identification of high-affinity ligands against a protein of interest from easily prepared libraries consisting of more than a trillion macrocyclic peptides. A library of mRNA-linked nonstandard macrocyclic peptides was generated by this system using a reassigned initiator codon, and potent macrocyclic binders were selected against a recombinant HA derived from a highly pathogenic avian influenza virus—A/Vietnam/1203/04 (H5N1). The candidates for inhibitor HA (iHA) were screened for inhibition of plaque formation by various strains of influenza virus. Binding of the iHA candidates to influenza viruses was verified by virus pull-down assays. iHA-24, which exhibited the most potent inhibitory activity on viral replication among the screened iHAs, was selected for further characterization. To determine the mechanism of action underlying the viral replication inhibition of iHA-24, its effect on HA-mediated membrane fusion activity was tested by using red blood cell fusion assays and polykaryon formation assays. The potential binding site of iHA-24 to HA was mapped by isolating escape mutants from iHA-24 and by identifying the amino acid mutation responsible for the reduced sensitivity to iHA-24. The *in vivo* efficacy of iHA-24 against influenza virus infection was evaluated in a murine lethal infection model. Results: After five rounds of the RaPID system selection, we found an appreciable increase in the cDNA recovery rate. Sequencing of 69 molecular clones from the selected cDNAs revealed a total of 24 iHA candidates. Of the 24 candidates, seven iHA macrocycles inhibited plaque formation of A/duck/Hokkaido/Vac-3/07 (H5N1). The virus pull-down assays revealed that these iHA candidates indeed bound to influenza viruses. In particular, iHA-24 inhibited plaque formation of different clade H5N1 viruses, pandemic (H1N1) 2009 viruses, and a laboratory virus strain A/Puerto Rico/8/34 (H1N1). iHA24 impaired influenza virus–mediated red blood cell membrane fusions and polykaryon formations in a dose-dependent manner. The isolated escape mutants from iHA-24 possessed two mutations in their HAs; one of the mutations was located within the fusion peptide region of the HA2 subunit and was demonstrated to be responsible for the reduced sensitivity to iHA-24 by reverse genetics techniques. None of the mice treated with iHA-24 exhibited severe weight loss for 9 days post-infection, while all mock-treated mice experienced severe weight loss and died by 6 days. Virus titers in lungs from the

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iHA-24–treated mice were significantly lower than those of the mock-treated counterpart. Conclusions: These results indicate that this newly developed drug discovery technique allows for the identification of the HA fusion activity–targeted broad-spectrum antiviral candidates for influenza treatment.

P2-701

Testing the cross-reactivity of a neutralizing monoclonal antibody generated using baculovirus-insect cell expressed HA protein of H5N1

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Background: Since 2003, there have been reports of bird-to-human as well as human-to-human transmissions of the H5N1 avian influenza A virus, and this has raised the fear that the virus may be mutating to a form that can cause a flu pandemic with the magnitude of the Spanish flu pandemic of 1918. Recent studies have suggested that monoclonal antibody (mAb)–based passive immunotherapy is a promising new approach to combat the influenza A virus. In this study, we characterized murine mAbs generated using baculovirus-insect cell expressed HA protein of H5N1. Materials and Methods: In this study, several anti-H5 mAbs were generated from mice immunized with baculovirus-insect cell expressed HA protein of H5N1. Due to the lack of BSL3 facility, the abilities of these mAbs to inhibit viral entry were analyzed using a pseudotyped virus assay. This assay uses replication-incompetent retroviral particles comprising the firefly luciferase reporter gene and expressing HA proteins of H5N1 on the surface. Upon viral entry into permissive cell lines, luciferase expression provides a sensitive and quantitative measure of infection. In addition, enzyme-linked immunosorbent assay (ELISA) was used to determine whether these mAbs could cross-react with HA of other avian influenza viruses, namely H7N7, H7N9, and H9N2. Results: Our results showed that one of the mAbs generated, termed 4F3, prevented viral entry of the homologous virus, which is a clade 1 virus isolated in Vietnam in 2004, at concentrations of ~ 10 µg/ml. An ideal therapeutic mAb would be one that is able to tolerate the antigenic variability of circulating H5N1 variants and maintain its ability to neutralize the virus. Importantly, mAb 4F3 also prevented viral entry of both clade 2.1 and clade 2.2 viruses. Besides H5N1, other avian viruses—such as H9N2, H7N7, and H7N9—have also crossed the animal-human species barrier and caused diseases. Interestingly, ELISA showed that mAb 4F3 could also bind HA of the H7N7 and H7N9 subtypes but not the H9N2 subtype. Conclusions: A newly generated mAb 4F3, which was producing using the HA of H5N1, appears to cross-react with H7N7 and H7N9 but not with H9N2. This suggests that this mAb is binding to an epitope that is present in both H5 and H7 subtypes but is absent in H9. The current study focused on the evaluation of mAb 4F3's ability to prevent viral entry of H7N7 and H7N9.

P2-702

Antiviral activity of usnic acid derivatives against influenza virus in vivo

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Background: Despite success in chemotherapy and vaccine prophylaxis, influenza remains a poorly controlled infection that causes annual epidemics and pandemics. Usnic acid (UA), a dibenzofuran originally isolated from lichens, has been shown previously to act as a growth regulator in higher plants. In humans, it can possess anti-inflammatory, antimitotic, antineoplastic, antibacterial, and

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antimycotic activities. The aim of this study was to evaluate the anti-influenza properties of some derivatives of usnic acid. Materials and Methods: We had tested four compounds—(+) and (-) isomers of usnic acid and (+) and (-) isomers of its derivative (labeled as 575 and 612, respectively)—on a model of lethal influenza pneumonia in mice [influenza virus A/Aichi/2/68 (H3N2)]. Compounds 575 and 612 were chosen after previous testing of the antiviral activity of 30 usnic acid derivatives in vitro. All the substances were synthesized at the Novosibirsk Institute of Organic Chemistry. The drugs were administered at two doses: 0.5 and 0.25 LD50 (50% lethal doses). The virus A/Aichi/2/68 (H3N2) was administered intranasally at 1 and 10 LD50 under slight ether anesthesia. The following treatment scheme was used: 0.2 mL of a drug-water solution was administered 1 day before infection, 1-2 hours after infection, and daily afterward for 4 days. The animals were observed for 14 days, and deaths in the control and experimental groups were reported every day. Based on these data, the degree of animal protection was calculated. Results: Both forms of usnic acid have shown no protection. Compound 612 had 11.1% protection at both doses, but the best one was compound 575. The maximum level of protection of 33.3% was achieved when the animals were receiving 10 mg/kg of the substance daily. Conclusions: Compounds 612 and 575 can be considered as prospective for the prevention and/or treatment of influenza.

P2-704

Broad-spectrum and influenza-specific antiviral compounds identified via high-throughput screening

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Background: Resistance to both classes of antiviral drugs licensed for influenza has emphasized the need for the discovery and development of new anti-influenza compounds. Materials and Methods: Using a cell-based assay in 1536-well format and a novel influenza A virus that has been engineered to express *Renilla* luciferase, we screened a library of > 900,000 small molecules and identified those that inhibit influenza virus replication. The assay captured all stages of the virus life cycle, and the screen yielded a hit rate of 0.5%. Results: Following dose-response confirmation of antiviral activity and clustering of compounds based on structural similarity, 250 hits were subjected to validation studies. Here we report on five hits that all show potent anti-influenza virus activity with IC_{50} values in the low micromolar to nanomolar range in a human lung cell line. Two of these compounds have broad-spectrum antiviral activity and can efficiently inhibit the growth of both RNA and DNA viruses. We determined that one acts on the *de novo* pyrimidine synthesis pathway, while the other compound acts through a distinct mechanism. The other three compounds are specific for influenza A virus, and all show inhibition of both H1N1 and H5N1 subtypes; however, only one is active against the H3N2 subtype. Two compounds select for resistant viruses with mutations in the influenza virus NP protein, but in different areas of the protein, suggesting that they likely act through disparate mechanisms. The third compound profiles as an entry inhibitor and selects for resistance mutations in the stalk region of HA. This correlates with mechanism of action studies that show that the compound blocks HA fusion activity. Conclusions: In summary, this high-throughput screen has yielded anti-influenza compounds with targets distinct from existing influenza drugs as well as two broad-spectrum antiviral candidates that likely act on cellular targets.

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Angiopoietin-1 and keratinocyte growth factor restore the impaired alveolar fluid clearance induced by influenza H5N1 virus infection

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Background: Acute respiratory distress syndrome (ARDS) caused by high pathogenic avian influenza (HPAI) H5N1 virus infection has resulted in severe illness and high mortality rates among patients. Patients with ARDS are often characterized by impaired alveolar fluid clearance and alveolar edema. An understanding of the mechanism responsible for human alveolar edema will lead to the development of novel therapeutic treatments for ARDS patients. We hypothesized that the paracrine soluble factors angiopoietin-1 (Ang-1) and keratinocyte growth factor (KGF) can resolve alveolar fluid clearance by up-regulating the expression of major sodium and chloride transporters impaired by HPAI H5N1 virus infection. Materials and Methods: Human alveolar epithelial cells grown on transwell inserts were infected with HPAI H5N1 (A/HK/483/97) and low pathogenic avian influenza (LPAI) H1N1 (A/HK/54/98) viruses at MOI 0.1 or incubated with conditioned culture medium containing Ang-1 and/or KGF. At 24 and 48 h post-infection, the rate of alveolar fluid transport and protein permeability across the alveolar epithelium was measured. Protein expression of sodium and chloride transporters (Na-K-ATPase, CFTR, and epithelial sodium channel alpha subunit) was measured by qPCR, ELISA, and Western blot. Results: HPAI H5N1 (A/HK/483/97) virus infection significantly reduced net alveolar fluid transport and protein permeability when compared with H1N1 (A/HK/54/98) virus infection at 24 h post-infection and further reduced it at 48 h post-infection. This reduction in alveolar fluid clearance was associated with a substantial reduction in protein expression of Na-K-ATPase, CFTR, and epithelial sodium channel alpha subunit. The influenza virus–infected cells treated with Ang-1 and KGF restored the impaired alveolar edema fluid clearance and protein permeability after HPAI H5N1 virus infection. Furthermore, the paracrine soluble factors Ang-1 and KGF up-regulated the protein expression of the major sodium and chloride transporters resulting from the HPAI influenza virus infection. Conclusions: The paracrine soluble factors Ang-1 and KGF play an important role in maintaining human alveolar fluid clearance by up-regulating the sodium and chloride transporting systems in human alveolar epithelium. This study enriches the understanding of the development of ARDS in human H5N1 disease and may aid in the development of possible therapeutic applications.

P2-706

Immunomodulatory and anti-viral effects of statins in influenza H5N1 virus infection of human alveolar epithelial cells and peripheral blood–derived macrophages

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Background: Highly pathogenic avian influenza (HPAI) H5N1 virus panzootic in poultry continues to spread. It causes zoonotic human disease with a high (> 60%) fatality rate and continues to pose a pandemic threat. Based on clinical, animal, and in vitro cell studies, we and others have suggested that differences in viral replication competence, tissue tropism, and cytokine dysregulation between H5N1 and low pathogenic viruses may contribute to disease pathogenesis. Statins as HMG-CoA inhibitors act to reduce cholesterol and have been demonstrated to have anti-inflammatory and immune-modulatory activities. However, there is controversy about the benefits of statin use on influenza infection in mice and humans. In this study, we aimed to evaluate the effects of statin

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treatment in influenza infection using physiologically relevant in vitro models—human alveolar epithelial cells (AECs) and peripheral blood–derived macrophages (PBDMs). Materials and Methods: Primary human AECs and PBDMs were infected with HPAI H5N1 (A/HK/483/97) and seasonal H1N1 (A/HK/54/98) viruses in the presence or absence of statin (simvastatin and sevastatin) treatment. Virus replication was monitored by measuring infectious viral particles in cell culture supernatants using $TCID_{50}$. Immuno-modulatory effects of statins were examined by measuring the mRNA and protein expression of cytokines and chemokines using qPCR and ELISA. In order to understand the intervention of statins and influenza infection, the gene expression profile of selected members of the sterol-biosynthesis pathway in influenza virus–infected AECs and PBDMs were also monitored. The responses of a variety of cytokine treatments on the genes of the sterol-biosynthesis pathway were investigated in AECs. Furthermore, the intracellular free cholesterol level was also examined by enzymatic assay in AECs infected with influenza virus. Results: We demonstrated that both simvastatin and mevastatin exhibited a dose-dependent inhibition of influenza virus replication for both HPAI H5N1 and seasonal H1N1 viruses in human AECs and PBDMs. The observed inhibitory effect of simvastatin and mevastatin occurred below the non-specific toxic effects to cells, which were measured by MTT assay. Treatment of simvastatin and mevastatin significantly suppressed H5N1 virus–induced pro-inflammatory cytokines such as TNF-α in PBDMs and chemokines, including IP-10 and MCP-1 secretion in both AECs and PBDMs at 24 hours post-infection. We further showed that human AECs and PBDMs infected with both HPAI H5N1 and seasonal H1N1 viruses had significant down-regulation of sterol pathway gene expression at 24 hours post-infection. AECs and PBDMs treated with IFN-γ or IFN-β but not IL-1β, TNF, or IL-6, showed down-regulation of sterol pathway gene expression. In addition, we found that the free cholesterol level was significantly reduced at 24 and 48 h post-H5N1 virus infection in AECs and in IFN-β–treated AECs. These results further support a specific modulation of the sterol metabolic pathway upon influenza virus infection. Conclusions: Taken together, the controversy about the beneficial effects of statin use in influenza infection and our data suggest that statins possess both the antiviral and immune-regulatory effects in H5N1-infected in vitro cell models. We also demonstrated a highly specific response of AECs and PBDMs through a coordinated negative regulation of multiple sterol pathway members upon influenza virus infection or treatment of interferon. Identification of a reduction in sterol pathway gene expression and cholesterol levels with IFN treatment in human AECs offers new insights on the host-mediated antiviral responses through the sterol metabolism pathway and opens new therapeutic options for human influenza disease.

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P2-707

Immune modulation by a novel narrow spectrum kinase inhibitor RV1088 as a therapeutic strategy for influenza virus infections

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Severe outcome following influenza infection has been linked to the over induction of the host innate immune response resulting a “cytokine storm.” In certain patient populations, a therapeutic approach to help control over induction of the innate immune response may be of benefit. However, concerns have been expressed that suppression of aspects of the host immune response might also lead to an increase in virus replication and directly enhance viral induced pathology. Using airway-liquid interface cultures of primary well-differentiated human airway epithelium (HAEs), we have demonstrated that a narrow spectrum kinase inhibitor, RV1088 (RespiVert Ltd.) can inhibit the induction of an array of human cytokines including IL6, IL8, IP10, and RANTES while at the same time not adversely increasing viral replication. In contrast, treatment of infected airway cells with fluticasone propionate, a steroid, did not inhibit any of the cytokine/chemokine responses measured. RV1088 also inhibited the viral induction of transcription from the interferon promoter acting at or below the level of MAVS. Used alone, RV1088 inhibited cytokine production by virus strains representative of all currently circulating subtypes and lineages of influenza A and B virus. Used in combination with Zanamivir, the virus titre released from HAE cells was suppressed even further than

for either drug alone. This molecule may represent a new generation of compounds suitable for the treatment of respiratory virus infections.

P2-708

Oral administration with KIOM-C attenuates lethal influenza A virus infection

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Background: At present, there are two major classes of anti-influenza drugs currently available: amantadine and rimantadine are adamantane derivatives that belong to the matrix protein 2 ion-channel class of inhibitors, whereas oseltamivir and zanamivir are two of the currently prescribed neuraminidase inhibitors. However, resistant viruses against these classes of prophylactic agents have arisen in recent years, requiring the need for additional anti-influenza therapy options. In recent years, the use of herbal extracts and compounds with anti-influenza virus activities and adjuvant-like or immunomodulatory effects have started to gain interest. Herbal extracts and compounds, naturally produced from oriental medicines, are believed to demonstrate multiple bioactivities and are thought to be safe for both oral and nasal applications. If proven effective, they would provide more affordable and broad-spectrum alternative means for mitigating influenza A virus infections. Therefore, this study investigated the antiviral effect of KIOM-C (herbal compound mixture) for the treatment of influenza A virus infection. Materials and Methods: More than 100 herbal medicine formulas are being tested by the Center for Herbal Medicine Improvement Research at the Korea Institute of Oriental Medicine, since 2010, to screen for novel antiviral compounds. These recent efforts have led to the discovery of KIOM-C—an herbal compound mixture. We evaluated the efficacy of KIOM-C against infection with a mouse-adapted A(H1N1)pdm09 A/Korea/CJ01/2009 (A/Korea/maCJ01/2009) virus. Following A/Korea/maCJ01/2009 infection, groups of BALB/c mice were orally administered KIOM-C, after which survival rates and immune responses in bronchoalveolar lavage fluid (BALF) were determined. Furthermore, we tested the potential anti-viral effects of KIOM-C in a ferret model by measuring the viral replication rate in their infected lungs. Results: Our results show that oral administration of KIOM-C conferred a survival benefit to mice infected with the 2009 pandemic H1N1 [A(H1N1)pdm09] virus and resulted in a 10- to 100-fold attenuation of viral replication in ferrets in a dose-dependent manner. Additionally, oral administration of KIOM-C increased the production of antiviral cytokines, including IFN-γ and TNF-α, and decreased levels of pro-inflammatory cytokines (IL-6) and chemokines (KC, MCP-1) in the BALF of A(H1N1)pdm-infected mice. These results indicate that KIOM-C can promote clearance of influenza virus in the respiratory tracts of mice and ferrets by modulating cytokine production in hosts. Conclusions: These findings suggest the therapeutic utility of the herbal compound mixture KIOM-C when used as an oral anti-influenza virus treatment. Data obtained since the herbal remedy was administered, on a daily basis for 4 to 5 days, 24 h after experimental inoculation, suggest the efficacy of KIOM-C in the early phase of virus exposure/infection. To elucidate whether the timing of treatment is significant for immune modulation along with the precise mechanism by which KIOM-C stimulates the host's antiviral functions, further studies will be needed. Regardless, our study shows that KIOM-C could be a novel, orally active antiviral agent with the potential to be an effective herbal remedy for prophylaxis and treatment of influenza virus infections.

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Antagonists of histamine receptors inhibit influenza virus replication through an off-target effect

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Background: Influenza virus is a highly contagious pathogen that causes annual epidemics and pandemics in the human population, resulting enormous suffering and economic losses. Drug repurposing can be an important part of new drug discovery program and has led to several blockbuster drugs. US Drug Collection from MicroSource Discovery Systems, Inc. consists of 1280 compounds that have reached clinical trial stages in the United States. All of the compounds in the library have known and well-characterized bioactivity, safety, and bioavailability—properties that could dramatically accelerate drug development and optimization. Using a cell-based assay, we identified a group of histamine antagonists that can inhibit influenza virus replication. Materials and Methods: Viruses and cells: The A/PuertoRico/8/34 ([PR/8] strain H1N1), Madin-Darby Canine Kidney Cells (MDCK) cells and a human pulmonary epithelial cell line (A549 cells) were used in the cell-based viral inhibition assay. Compounds: Cytotoxicity Assay: Cells were grown in 96-well plates for 16 hours. The medium was replaced with fresh medium containing serially diluted compounds, and the cells were further incubated for 48 hours. The cell viability is measured using MTT methods or commercial kit. Viral inhibition assay: Cells were infected with 100 TCID50:0.1 ml of the virus in the presence of different concentrations of the studied compounds at 37°C for 1 hour. After virus attachment, the cells were washed 3 times with D-Hank's solution and incubated with media containing the same concentration of compound at 37°C for 48 hours. The fluorescence intensity that resulted from the substrate cleavage by the viral neuraminidase activity was measured at 355 nm (excitation) and 485 nm (emission) using a multilabel plate reader. The effective concentrations that induced 50% inhibition of the viruses (EC50s) were calculated through a nonlinear regression analysis (sigmoidal dose-response variable slope) using the GraphPad Prism 5.0 software. Results: In order to find out if the infection of influenza virus lead to the release of histamine by the infected cells, the histamine in the supernatants of the virus-infected cells was detected by ELISA and HPLC and no change in the concentrations of histamine was observed. To understand whether histamine receptors are involved in the replication life cycle of influenza virus, the knock-down of histamine receptors by siRNAs and antibody blocking of histamine receptors were performed to analyze whether histamine receptors are required for viral replication. Our results suggested that the anti-viral activities were related with neither histamine nor histamine receptor. To identify the antiviral targets of these compounds, the time-of-addition assay results revealed that these compounds take effects at the very early steps of influenza virus life cycle. Based on the timing of inhibition and the properties of the compounds, we speculate that the histamine antagonists may lower the pH in endosome. And we found that these compounds increase the intralysosomal pH in mouse peritoneal macrophages. Further experiments in mice showed that some compounds increase the survival rate of mice infected by influenza virus. Conclusions: Cytokine storm during influenza virus infection is thought to be a prospective predictor of morbidity and mortality. Histamine antagonists have been used as immunomodulatory agents for many years, clinically used for the treatment of flu. Chlorphenamine is a component of Tynol. Here histamine antagonists were found to inhibit influenza virus replication. There is an expectation that combining immunomodulatory strategy with antiviral therapy is a rational approach to improve clinical outcome in influenza-infected patients. Once the key structures or scaffolds of each function identified, more potent antiviral agents with two functions can be developed. Our findings provide a new option to discover new agents against influenza virus infection.

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Exploring host targets for broad-spectrum antivirals against respiratory viruses

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Background: To identify host targets for a new generation of influenza antiviral therapy, several RNA interference (RNAi) screens have been performed to uncover required host factors. In total, these screens have implicated more than 1000 human proteins, but there is minimal overlap between the screens and greater evidence of the vital role of a particular host factor is required to support its selection as a drug target. Materials and Methods: To this end, we used an integrated "OMIC" approach to prioritize host factors with the most support for a role during influenza virus infection. Moreover, we have incorporated data on respiratory syncytial virus (RSV). Although these two viruses have distinct genetic structures, they encounter a similar host factor repertoire in the human respiratory tract; therefore, they likely share dependency on many cellular functions. We have conducted RNA-sequence experiments in parallel with influenza A virus and RSV to compare the profile of regulated host genes and then integrated these data with publicly available data sets for virus-host (and host-host) protein interactions, influenza virus, and RSV microarray data and from the influenza RNAi studies. Results: Using two complementary bioinformatic strategies, the data were filtered to reveal a subset of 53 host factors that are predicted to participate in the virus-host networks for both influenza virus and RSV. Inclusion of published data on quantitative protein expression in influenza virus– or RSV-infected cells reduces this further to 14 factors. Conclusions: We believe that this strategy greatly facilitates the identification of host factors to explore as targets for broad-spectrum antiviral drugs.

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RNA binding proteins regulate innate immune responses to avian influenza

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Background: Emerging avian influenza A viruses can cross the species barrier from birds into humans, exemplified by H5N1 (1997-present) and H7N9 (2013) outbreaks, causing severe pulmonary disease characterized by pneumonia, high levels of inflammatory cytokines and chemokines, and immunopathology. Avian viruses must typically acquire molecular adaptations in their heterotrimeric RNA-dependent RNA polymerase (PB1, PB2, and PA) and nucleoprotein (NP) to efficiently synthesize RNA in human cells. Materials and Methods: Using functional genomics (RNA interference), biological network analysis, and phylogenetics, we have explored interactions of H1N1, H5N1, and H7N9 viral polymerases with a network of human RNA binding proteins. Results: RNA binding proteins, including heteronuclear ribonucleoporoteins (hnRNP) and DExD/H-box RNA helicases, regulate virus RNA syntheses in human cells, suggesting a role for RNA binding proteins in the adaptation of avian strains to mammalian hosts. Intriguingly, RNA binding proteins, such as PKR, hnRNPA1, and NF90, that regulate avian polymerase function also affect induction of type I interferon and cellular RNA stress responses in influenza infection, possibly by binding immunostimulatory viral

RNA motifs. Conclusions: Targeting these virus-host interactions may offer a new line of therapeutic intervention against severe avian influenza in humans.

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The natural product aureonitol isolated from the fungus *Chaetomium coarctatum* impairs influenza A replication by inhibiting its hemagglutination

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Background: Antiviral drugs targeting influenza A and B viruses are a crucial component of pandemic preparedness. Recent data on oseltamivir (OST) use in patients with laboratory-diagnosed A (H1N1) pdm09 infection suggest that better clinical outcomes are achieved when treatment starts just after identification of symptoms. Two classes of anti-influenza drugs are licensed for clinical use: the M2 proton-channel blockers (adamantanes) and neuraminidase inhibitors (NAIs). The former class of compounds is no longer used due to massive circulation of resistant strains. Regarding NAIs, OST and zanamivir have been used more intensely since the 2009 pandemics. Consequently, an OST-resistant strain of A (H1N1) pdm09 has been found in various countries. This OST-resistant strain seems to occur at frequencies close to 2.0% and clusters of sustained transmission have been identified. Therefore, the search for novel anti-influenza drugs is pivotal. We have been studying secondary metabolites of endophytic and cosmopolitan fungi, *Chaetomium coarctatum*, found in Brazil, which constitute a fruitful source of bioactive compounds with a broad range of uses. This organism produces large amounts of a terpenoid known as aureonitol. Although there are some reports on the anti-bacterial activity of aureonitol, to the best of our knowledge, the antiviral activity of this compound is unknown. Materials and Methods: *Chaetomium* sp Kuntze (1817) was isolated and identified. A voucher specimen (CCFF/IOC 4613) was deposited at the Filamentous Fungal Culture Collection of Oswaldo Cruz Institute/FIOCRUZ. Aureonitol's chemical structure has been identified, and more than 99% purity was confirmed by RMN. Antiviral assays have been performed with laboratory-adapted influenza A and B viruses in MDCKs. Inhibition of hemagglutination and neuraminidase (NA) activity has been evaluated using standard procedures. Sequential passages of influenza virus in the presence of increasing concentrations of aureonitol have been carried out to monitor resistance mutations to aureonitol. In addition, *in silico* docking studies were performed with influenza hemagglutinin (HA). Results: The antiviral activity of aureonitol against influenza A (H3N2) virus resulted in the potent dose-dependent inhibition of virus replication, resulting in an EC_{50} equal to 100 ± 16 nM. For comparison, the reference NAI oseltavimir carboxylate (OST-c) EC_{50} value was 30 ± 2.3 nM, which was within the same sub-nanomolar range of aureonitol. Our compound presented low cytotoxicity, with a CC_{50} of 1426 ± 0.13 µM. This CC_{50} value is comparable with that found for OST-c (CC_{50} > 2000 µM). Based on the ratio between CC_{50} and EC_{50}, the selective indexes for aureonitol and OST-c were 14,260 and 66,666, respectively. NA inhibition assays, even those conducted at concentrations that exceed 10 times aureonitol's EC_{50}, showed no inhibitory effect. As a control, OST-c inhibited NA (IC_{50} of 0.1 ± 0.012 nM). Most importantly, aureonitol inhibited HA activity at 100 nM, comparable with the dilution of 1:1024 of WHO reference anti-sera. *In silico* docking studies with aureonitol and HA resulted in binding at the HA head. Hydrogen bridges between aureonitol with Glu190 and His183 were observed. Conclusions: Aureonitol presented more potent antiviral activity than other fungi natural compounds tested against influenza A (Nishihara, 2000; Jing, 2011). Our results suggest that the chemical structure of aureonitol is a promising one for the development of novel anti-influenza drugs.

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Is it possible to fight influenza by targeting intracellular redox state?

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Background: Influenza viruses induce a redox imbalance in the host cells, characterized by depletion of the main intracellular antioxidant glutathione (GSH) and an increase in reactive oxygen species (ROS). This oxidative stress may play a role in activating intracellular redox-sensitive pathways that are exploited by the virus for its own replication. The activation of the redox-regulated machinery may also play a role in the dysregulated proinflammatory responses and severe lung injury caused by highly pathogenic influenza viruses. We have recently demonstrated that it is possible to inhibit influenza virus replication by treating infected cells or mice with a derivative of GSH, GSH-C4. In particular, GSH-C4 is able to impair hemagglutinin (HA) maturation in the endoplasmic reticulum and, as a consequence, to block the progression of the virus replicative cycle. This effect is related to the correction of virus-induced GSH depletion that, in turn, affects the redox state of PDI, a cellular oxidoreductase involved in disulfide bond formation of glycoproteins. It has also been demonstrated that pro-GSH molecules, including GSH-C4, are able to increase the intramacrophage thiol content in vitro and in vivo as well as to shift the immune response toward Th1, which plays a pivotal role in antiviral immunity. On the basis of this background, our study was aimed at evaluating i) the ability of human and avian viral strains to induce an intracellular prooxidant state; and ii) the ability of redox modulating compounds other than GSH-derivative to inhibit influenza virus replication in lung epithelial cells and/or to prevent the excessive cytokine production induced in mice by lethal infection. Materials and Methods: For the study, human mucoepidermoid pulmonary carcinoma cells were infected with human and avian influenza A viruses (A/Puerto Rico/8/34 H1N1; A/ULSTER/H7N1) and treated after viral adsorption with redox modulating compounds such as GSH-C4, N-acetyl cysteine (NAC) and Trolox. After different hours of infection (3-6-8-24 hours), viral proteins, the intracellular redox state (ROS production) and mRNA of cytokines were evaluated through Western blot, FACS analysis and real-time PCR. Viral titer was measured through hemagglutination and plaque assays. Results: We found that human influenza virus induced a continuous and progressive increase of intracellular ROS levels from three to five hours after infection. Avian virus induced multiple waves of ROS hyperproduction, and the ROS levels were significantly higher than that observed in cells infected with human virus. Among different redox modulating compounds tested, differently from GSH-C4, NAC (a GSH precursor) and Trolox (a derivative of vitamin E with ROS-scavenging activity) exhibited short-term anti-influenza activity in infected lung epithelial cells, without interfering with viral protein expression or HA folding. In addition, the different compounds produced different effects on NF-κB activation and on the inflammatory cytokine production, suggesting that different redox-regulated pathways (GSH mediated or ROS mediated) may be specifically involved in the regulation of virus life cycle and/or inflammatory responses. Conclusions: Overall, these results indicate that GSH-C4 is effective in both inhibiting influenza virus replication and blocking the cytokine storm responsible for the lethality of some viral strains. Our data also demonstrate that not all the so-called "antioxidant drugs" are able to control viral infection and inflammatory responses. Thus, the efficacy of a specific compound depends on its real ability to affect redox-regulated pathways in different cellular contexts.

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Therapeutic or prophylactic treatment with an HA-stem antibody (VIS410) limits respiratory droplet transmission of influenza virus in the ferret model

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Background: Influenza virus infection is responsible for thousands of deaths each year, and novel treatment modalities are needed. Prophylaxis and therapy with monoclonal antibodies (mAbs) have been shown to limit the severity of disease in animal models of influenza. mAbs that target a conserved epitope in the hemagglutinin (HA) are likely to provide broad protection against viruses of different subtypes. Through protein structural analysis, we have identified a site on the influenza HA that is conserved across all influenza A virus subtypes and appears to be resistant to escape mutation. A human IgG 1 kappa mAb (VIS410) was engineered that targets this conserved region and demonstrates dose-dependent viral inhibition against influenza viruses with both Group 1 and Group 2 HAs. Mechanistic studies indicate that VIS410 inhibits HA fusion with the cell membrane. VIS410 has been previously shown to protect mice infected with a lethal dose of H1N1, H3N2, and H5N1 influenza viruses, both prophylactically and therapeutically. However, the effectiveness of this treatment option to limit the spread of disease remains unknown. In this study we addressed whether treatment of VIS410 therapeutically or prophylactically affects respiratory droplet transmission of the 2009 pandemic H1N1 (pH1N1) virus in ferrets. Materials and Methods: We evaluated therapy and prophylaxis with VIS410. The therapeutic arm included 6 donor ferrets inoculated intranasally with 10^6 $TCID_{50}$ of CA/07/09 pH1N1 virus. Eight hours post-infection (HPI), donor ferrets received 10 mg/kg intraperitoneal (IP) injections of either VIS410 (3 ferrets) or an irrelevant human IgG 1 kappa control antibody (3 ferrets). Untreated recipient ferrets were placed into the adjacent transmission cage 24 HPI and exposed to the donor animals for 13 days, after which all of the ferrets were euthanized and serum was collected. Nasal wash samples were collected from the donor animals on 1, 3, and 7 days post-infection (DPI) to assess viral titers. In the prophylactic arm, donor ferrets were inoculated as above; 8 hours later the uninfected recipient ferrets received 30 mg/kg IP injections of either VIS410 (3 ferrets) or control antibody (3 ferrets), and they were exposed to the infected ferrets for 48 hours starting 24 hours after the donor ferrets were infected. Three DPI the recipient ferrets were removed from the transmission cages and placed into individual housing units. Transmission efficiency of pH1N1 was determined in all groups by serology 14 or 22 DPI. In both studies, sera were collected on 1, 3, 7, 14, and 22 DPI from the animals receiving the antibody to determine the pharmacokinetics of VIS410 in ferrets. Results: Therapy with VIS410 resulted in a reduced pH1N1 transmission efficiency of 33% (1/3 recipient animals seroconverted) compared with a transmission efficiency of 100% (3/3 recipient animals seroconverted) in the control treatment group, although virus titers in the nasal washes from both groups of donor animals were equivalent. Prophylaxis with VIS410 also resulted in decreased pH1N1 transmission; only 1/3 recipient animals had a low antibody titer on 22 DPI compared with 2/3 recipient animals with high antibody titers in the control group. VIS410 and the control human mAb had similar kinetics in ferrets. Finally, we observed that VIS410 was cleared faster in ferrets as compared with mice. Conclusions: This is the first demonstration that treatment with a human mAb can reduce the spread of influenza via respiratory droplets in the ferret model. The decrease in respiratory droplet transmission was independent of the titer of virus present in nasal washes. Prophylaxis with VIS410 also reduced infection in spite of the rapid clearance of VIS410 in ferrets. Serum concentrations of VIS410 associated with reduced transmission are easily achievable in humans. These observations, in the context of data generated previously in the mouse model, suggest that therapeutic administration of VIS410 during an influenza outbreak can limit both the spread of the disease and the severity of illness in treated individuals.

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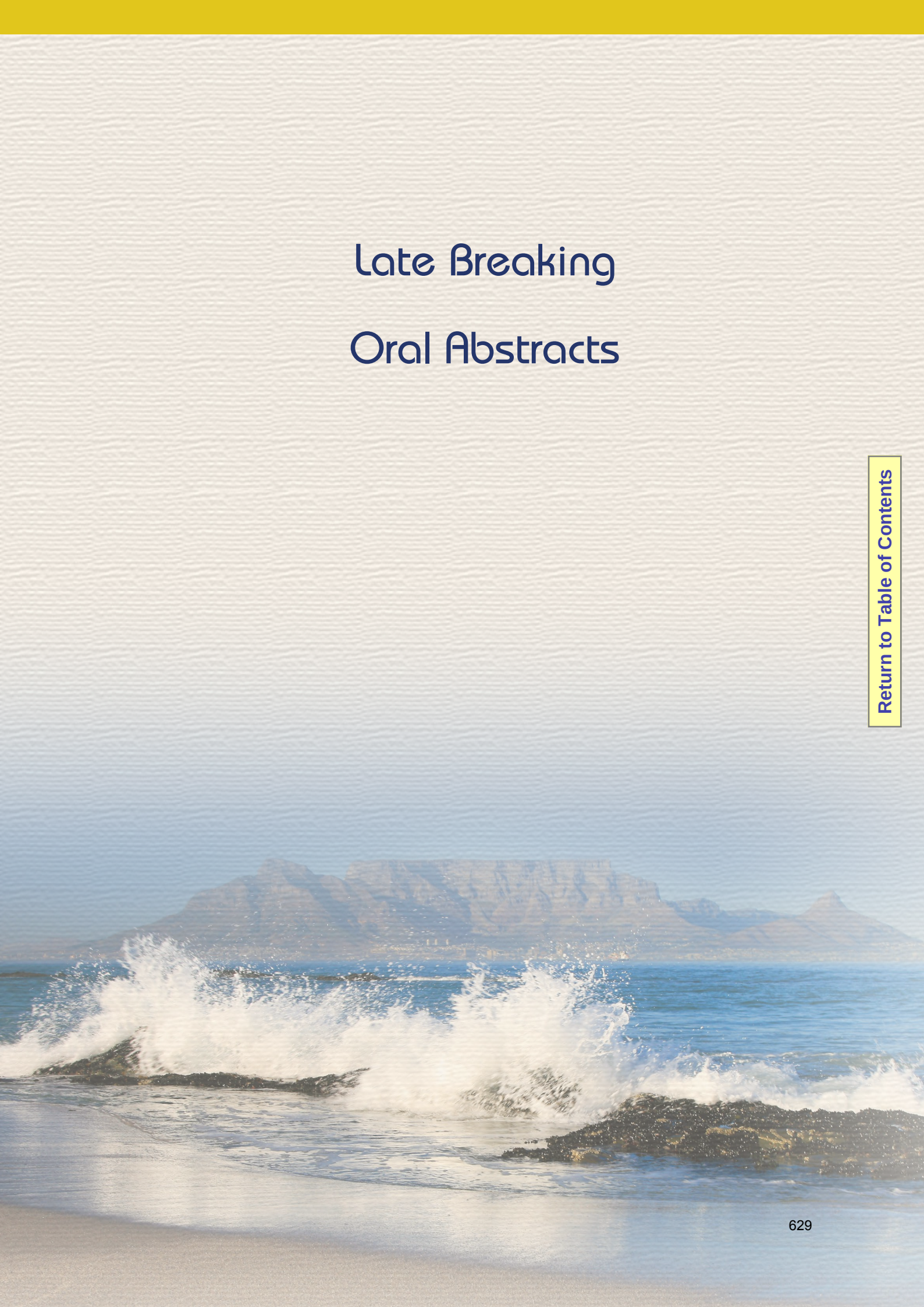
Late Breaking

Oral Abstracts



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LBA-O-001

Progress in building equitable influenza vaccine production capacity under the Global Action Plan for Influenza Vaccines (GAP) framework

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The world recognized in 2005 that there was insufficient vaccine production capacity to meet the world's needs in the event of an influenza pandemic, and that most of the available capacity was in high-income countries. This would make developing countries very dependent on the supply of pandemic vaccine from these countries, and vaccine supplies would be delayed and fall far short of the needs, as experienced during the 2009 H1N1 pandemic. The WHO Global Action Plan for Influenza Vaccines (GAP) aims to ensure rapid and equitable access to vaccines in an influenza pandemic, and one of its three objectives is the increase of global pandemic vaccine production capacity. Since 2006, WHO and partners have provided grants and technology transfer opportunities to manufacturers in Brazil, China, Egypt, India, Indonesia, Iran, Kazakhstan, Mexico, Romania, Serbia, South Africa, South Korea, Thailand and Vietnam. This has leveraged the increase in pandemic vaccine production capacity from these manufacturers to 330 million doses per annum, with six manufacturers having already achieved licensure of their locally produced influenza vaccines. A particular highlight has been the sublicensing by WHO of one of the two existing live attenuated influenza virus technologies to three emerging country vaccine manufacturers, one of which succeeded in obtaining WHO prequalification for its live attenuated H1N1(2009) pandemic vaccine. Building on these achievements, WHO is planning, with technical and financial support from the US government, to facilitate a further increase of the total production capacity of these developing-country manufacturers to exceed 500 million doses of pandemic vaccine per year, by 2016. The programme will strive to facilitate the registration of at least five new quality-assured influenza vaccines by 2016, and to support at least nine manufacturers to reach advanced development stage for their candidate vaccines by the same date. Challenges to be addressed include the optimisation of antigen-sparing technologies, obtaining national regulatory approvals and WHO prequalification, as well as building sustainable business models for the new producers. By the tenth anniversary of the GAP, this technology transfer programme will have increased the number of countries and regions able to respond to an influenza pandemic, and decreased the dependence of these countries on the vaccine produced in high-income countries.

LBA-O-002

Effectiveness of seasonal influenza vaccination of children in Senegal: year two of a cluster-randomized trial

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Background: Data are limited on the benefit of influenza vaccine to children in tropical countries. Additionally, because the pattern of influenza circulation and the role of children in transmission may differ markedly from those in temperate settings, population-level effects of wide-spread influenza vaccination of children, including total, indirect, and overall effectiveness of vaccination, are unknown. Therefore, we conducted 3 annual trials in rural Senegal to evaluate these effects after vaccination of children with trivalent inactivated influenza vaccine (TIV). This abstract reports results from the second annual evaluation. Materials and Methods: Twenty villages of the Niakhar Demographic Surveillance System were randomized 1:1 for vaccination of children 6 months through 10 years of

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age with TIV formulated for the 2010 Southern Hemisphere influenza season (Vaxigrip, Sanofi-Pasteur) or inactivated poliovirus vaccine (IPV) (Sanofi-Pasteur). Commercial presentations of vaccines were masked and coded before delivery to the site. From May-June 2010, children received dosing per the manufacturer's instructions (for vaccine-naive TIV recipients: two 0.25 mL doses for those 6-35 months, two 0.5 mL doses for those 3-8 years, and one 0.5 mL dose for those 9-10 years; children who received TIV in study year 1 received only 1 dose; and for IPV recipients: equivalent numbers of doses of the 0.5 mL presentation). After immunizations were completed, monitoring for laboratory-confirmed seasonal influenza among all persons in the community with protocol-defined febrile acute respiratory illness was conducted through active, community-based surveillance (weekly visits to households), and enhanced passive health post-based surveillance. Individuals reporting symptoms were assessed clinically, and nasal swab and throat swab specimens were collected for influenza testing using the US Center for Disease Control and Prevention (CDC) real-time reverse transcription polymerase chain reaction protocol. The primary study objective was an intention-to-treat analysis of total effectiveness of vaccination (VE_{Total}) (direct and indirect protection among vaccinees), with the cumulative incidence of laboratory-confirmed symptomatic influenza among consenting children in TIV villages compared with that in IPV villages and adjusted for village-randomization. Indirect effectiveness was estimated among age-ineligible unvaccinated children and adults, and overall effectiveness was measured among the entire community. Results: Of 12,115 children eligible for study vaccinations, 6076 were from TIV villages and 6039 were from IPV villages. Starting June 3, 2010, we enrolled 4703 children in TIV villages and 4646 children in IPV villages. Median village participation rates were 77% in both arms. During the second annual evaluation surveillance, beginning mid-July 2010 and ending April 2011, 575 laboratory-confirmed influenza cases were identified (134 A/H3, 98 $A/H1_{pdm}$ [2009], and 343 B). Nearly all A/H3 and B cases occurred during the rainy season from August through November 2010, and nearly all $A/H1_{pdm}$ occurred from January through March 2011. Overall, baseline attack rates were lower among consenting age-eligible children in the IPV group in this second year of the study as compared with the first year, ranging from 0.7% for $A/H1_{pdm}$ to 2.9% for influenza B. VE_{Total} against $A/H1_{pdm}$ was 57% (95% CI, 7% to 80%), 56% (95% CI, 18% to 76%) against A/H3, and 50% (95% CI, 31% to 64%) against influenza B. Indirect benefits to those not eligible for vaccination were variable: −11% (95% CI, −104% to 40%) for $A/H1_{pdm}$, 51% (95% CI, 37% to 63%) for A/H3, and 11% (95% CI, −34% to 40%) for influenza B. Finally, vaccination of children was overall 28% (95% CI, −37% to 62%) effective against for $A/H1_{pdm}$, overall 51% (95% CI, 14% to 72%) effective against A/H3, and overall 33% (95% CI, 8% to 51%) effective against influenza B in the entire population. Conclusions: This annual evaluation is a second in the largest influenza vaccine study conducted in sub-Saharan Africa. Our cluster randomized design allowed for the measurement of total, indirect, and overall vaccine effectiveness, and significant direct benefit (as VE_{Total}) to vaccinated children was measured. These data provide evidence that current inactivated influenza vaccines can provide benefit to developing country populations and may confer protection to unvaccinated contacts.

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LBA-O-003

Do changes in spirometry correlate with the severity and duration of infection in volunteers experimentally inoculated with influenza, RSV and HRV in the human viral challenge model

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Background: The human viral challenge model of infection provides a unique experimental opportunity in which healthy human volunteers are inoculated at a defined time point with a wild-type, attenuated virus under controlled conditions. This model has enhanced our understanding of how respiratory viruses like influenza behaves under such conditions. It has also increased our knowledge of the interplay between human immunity and infection with respiratory viruses. In addition, the model allows us to examine a number of other parameters related to experimental infection and disease severity using subjective (self-reported symptom diary card scores[SDC]) and objective (spirometry, vital signs) measures and their temporal relationship to viral shedding. Spirometry is routinely used in clinical practice for screening, diagnosis, and disease progression in chronic obstructive pulmonary

disease and has now superseded peak expiratory flow rate (PEFR) in monitoring disease progression in asthmatics. However there is little information on the use of spirometry in healthy volunteers in response to respiratory tract viral infection and its correlation with viral shedding and disease severity. Materials and Methods: Here we present data on healthy volunteers inoculated with wild-type strains of influenza (A/Wisconsin/67/2005, H3N2), respiratory syncytial virus (RSV-A, Memphis 37 strain), or human rhinovirus (HRV16) in a controlled quarantined facility. Healthy male and female volunteers aged between 18-45 years who met the protocol specific inclusion and exclusion criteria were considered. Only those volunteers who were inoculated and received placebo in each study were included in the analysis as a surrogate comparator to environmental infection. We examined the correlation among FEV1, FEV1/FVC ratio, and FEF25%-75%, viral shedding, disease severity (SDC score, mucus weight, temperature), and symptom duration in these volunteers and determined the use of serial spirometry to predict disease severity and duration .We also compared the changes seen in pulmonary function tests among the 3 viruses and assessed which is most likely to have a clinically significant impact on respiratory function. Results: Initial analysis suggested that inoculation with influenza and RSV caused a reduction in a number of clinically important spirometry parameters. These included a reduction in FEV1 and FEV1/FVC ratio, which correlates to the chronology of incubation and infection. In addition, there appeared to be a significant reduction in the value of FEF25%-75%. These reductions were temporary and returned to baseline values by safety day follow-up. There was no appreciable reduction in these values with HRV16. Further analysis of a larger data set is currently underway. Conclusions: It appears that healthy volunteers with no medical history of underlying airways disease challenged with attenuated wild-type virus shoed an appreciable reduction in a number of clinically important lung function values, including FEF25%-75%, during infection. This provides another useful objective measure to evaluate the efficacy of antiviral compounds in the human viral challenge model and may also be useful in clinical practice to predict patients who are more likely to be symptomatic during infection.

LBA-O-004

The association of age and clinical disease in ferrets infected with influenza A viruses

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Background: Ferrets (*Mustela putorius furo*) are considered ideal animal models for influenza research because they can be infected with a range of influenza viruses and exhibit clinical signs of influenza disease similar to those in humans. However, the model is not standardized and some aspects of the ferret model of influenza infection remain poorly understood. Materials and Methods: We sought to explore the effect of age (8-10 weeks vs 6-9 months) and inoculum volume (0.2, 0.5, or 1.0 mL) on clinical and pathological disease outcomes following intranasal administration of 10^6 $TCID_{50}$ of the influenza A/California/7/2009 (pH1N1) and A/Minnesota/11/2010 (H3N2 variant; H3N2v) viruses. Weight, temperature, and clinical scores were assessed for 6 days. Animals were sacrificed on days 1, 3, and 6 following virus administration, and the kinetics of virus replication and histopathology in the upper (URT) and lower (LRT) respiratory tracts were evaluated. We used a tissue dye to evaluate the distribution of the different volumes of inoculum throughout the URT and LRT. We also used a novel method of microcomputed tomography to investigate the anatomic features of the respiratory tract of the two age groups of ferrets. Results: Given similar doses and volume of inoculum, 8- to 10-week-old ferrets, infected with either pH1N1 or H3N2v, showed no significant clinical disease, whereas 6- to 9-month-old ferrets showed significant clinical disease when infected with either virus. Remarkably, high viral titers and severe lung pathology were present in both age groups, even in the younger animals that did not exhibit clinical disease. In the URT, we observed severe inflammation, necrosis, and abundant viral antigen deposition in columnar ciliated cells in both age groups. In contrast, the olfactory epithelium even directly adjacent to the inflamed respiratory epithelium was devoid of inflammation and viral antigen. Larger inoculum volumes (0.5 and 1 mL) resulted in delivery of inoculum to and more consistent replication in the LRT. Administration of 1 mL of dye into the respiratory tract of ferrets resulted in broad coverage of the URT and consistent delivery of inoculum to the LRT. Micro-CT analysis showed that the right main stem bronchus was

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consistently larger than the left and that the distal branches of the left bronchus were both longer and straighter than the branches supplying the right lung lobes. Conclusions: Replication of the pH1N1 and H3N2v influenza viruses was associated with clinical illness in older but not young ferrets despite similar anatomy, viral replication, and lung pathology. The optimal combination for experimental influenza virus infection and demonstration of clinical disease appears to be administration of 1 mL of virus to older ferrets.

LBA-O-005

Patterns of genetic reassortment between endemic swine influenza viruses and pandemic A(H1N1)2009 viruses in the Vietnamese pig population

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Background: Since its emergence in April 2009, human to pig transmissions of the pandemic 2009 (H1N1) (A[H1N1]pdm09) virus have been reported worldwide. Cocirculation of the A(H1N1)pdm09 viruses with endemic SIVs has resulted in reassortments among them in many countries/regions. Because reassortant SIVs with the internal genes from A(H1N1)pdm09 might have an ability to replicate better in humans than an epidemic SIV, it raises a concern that such reassortants could cause pig-to-human transmission as they have distinct antigenicity to human seasonal influenza viruses. We have conducted virological surveillance in Vietnam, the second largest pig producer in Asia, since 2010 to demonstrate incursions of the A(H1N1)pdm09 viruses into the pig populations as early as March 2010. Also, we isolated H3N2 and H1N2 SIVs possessing HA and NA genes originated from human seasonal viruses around 2005 and internal genes from North American SIVs, so-called “triple reassortant SIVs,” in Southern Vietnam at that time. To date, however, impact of the incursions of A(H1N1)pdm09 viruses to the pig population has not been evaluated in respect to genetic diversity of the Vietnamese SIVs. In this study, we report the results of the virological surveillance in pig farms and slaughterhouses in the Vietnamese pig population, and the isolates were subjected to genetic analyses to understand the impact of human to pig transmissions of A(H1N1)pdm09 viruses on the SIVs that have circulated in Vietnam. Materials and Methods: We conducted a total of 6 virological surveillances in 3 provinces in northern and 7 provinces in southern parts of Vietnam from August 2010 to December 2012. A total of 4675 nasal swabs were collected from clinically healthy pigs, consisting of suckling pigs, weaning pigs, fattening pigs, finishing pigs, sows, and boars, in 261 pig farms and 10 slaughterhouses. Nasal swab pools (2-5 nasal swabs per pool) were screened with a real-time PCR specific to the M genes of type A influenza virus. The nasal swabs that were positive by PCR were individually inoculated into MDCK cells and/or primary cultures of porcine alveolar epithelial cells for virus isolation. All of the 8 gene segments of the viruses isolated were sequenced. Phylogenetic analysis of each gene was carried out to determine the patterns of genetic reassortment of the isolates. Results: A total of 133 viruses consisting of 28 H1N1, 43 H1N2, and 62 H3N2 subtypes were isolated from weaning and fattening pigs aged less than 12 weeks in 14 farms. The virus isolation rate in pig farms was 3.1%, whereas no viruses were isolated from 375 finishing pigs in slaughterhouses. Sequences of HA genes of the H1N2 SIVs clustered in three distinct lineages, the triple reassortant H1 SIVs, human seasonal H1 viruses, and A(H1N1)pdm09 viruses. Five strains of the H1N2 SIVs were reassortants among triple reassortant SIVs, human seasonal viruses, and A(H1N1)pdm09 viruses. Twenty-nine H1N2 SIVs were reassortants between triple reassortant SIVs and A(H1N1)pdm09 viruses. The other 9 H1N2 SIVs were reassortants between triple reassortant SIVs and human seasonal H1 viruses. Sequences of HA genes of H3N2 SIVs clustered in two distinct lineages, human-like H3 and the triple reassortant H3N2 SIVs. Thirty H3N2 SIVs were reassortants among triple reassortant SIVs, human seasonal viruses, and A(H1N1)pdm09 viruses. The other 32 H3N2 SIVs were reassortants between triple reassortant SIVs and A(H1N1)pdm09 viruses. The entire genome of the H1N1 SIVs was derived from A(H1N1)pdm09 viruses. Conclusions: The SIVs isolated in this study were classified into 9 genotypes based on the pattern of their genome constellations. Although 4 genotypes out of them retained two or more genes

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originated from the triple reassortant SIVs that were endemic in 2010 in the Vietnamese pig population, 7 genotypes arose from several introductions of A(H1N1)pdm09 viruses from human to pig. Thus, these findings highlighted the importance of active surveillance for early detection of novel SIVs with undetermined risk for the swine industry as well as public health.

LBA-O-006

A mouse model to assess the utility of antiviral treatments for prevention of contact-dependent transmission of influenza A virus

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Background: Transmission of influenza A virus from an infected individual (index) to a naive susceptible individual (contact) occurs either via inhalation of viral particles shed from the respiratory tract as large droplets and fine aerosols or via direct contact with infectious fluids. Ferrets are routinely used to study transmission, as they develop respiratory signs of influenza illness similar to those observed in humans. However, a mouse transmission model would have greater capacity, scope of reagents, and genetically defined mouse strains at one's disposal than ferret models for which the immunologic reagents are limited, and ferrets can be expensive and difficult to house on a large scale. The use of a mouse model that can only examine contact-dependent transmission, would be of significant value in understanding transmission in close-contact exposure scenarios, for instance, those that might occur in households, schools, and workplaces. With this in mind, we have set out to investigate various factors that contribute to contact-dependent transmission between mice. In addition, we have utilised this model to evaluate the efficacy of a licensed antiviral (oseltamivir) as well as an experimental Toll-like receptor-2 (TLR2)-agonist (Pam2Cys) that stimulates innate immunity, in preventing contact dependent transmission. Materials and Methods: Index mice were infected intranasally with 104.5 plaque-forming units. After infection, index mice were housed separately for 6 hours to prevent contamination of the contacts with the initial viral inoculum. Thereafter, index and contact mice were cohoused; two index mice were introduced into a clean mouse box containing three naive contact mice. Transmission to contacts was determined by measuring the infectious viral titers in the nasal turbinates, trachea, and lung or the development of an antibody response to the viral hemagglutinin (HA) by testing their sera in hemagglutination inhibition (HI) assays. To test efficacy of antiviral therapies index mice were pretreated orally with oseltamivir or intranasally with Pam2Cys prior to cohousing, and viral loads in contact animals were determined four days later. Results: Strains within the H3N2 but not H1N1 subtype of influenza virus were found to be transmissible, and reverse-engineered viruses representing hybrids of these subtypes showed that the viral hemagglutinin was a determinant of the transmissible phenotype. Transmission to contact mice occurred within the first 6 to 54 hours after cohousing with directly infected index mice and was associated with a threshold level of virus present in the saliva of the index mice. In contrast, there was no correlation between greater transmission to contact animals and higher viral loads in the nose or lung of index animals. Treatment of index mice with oseltamivir or Pam2Cys completely prevented transmission to contacts, as no cohoused animals contained virus in any tissue of the respiratory tract. Conclusions: Our findings show that high viral loads in the saliva of index mice correlate with contact-dependent transmission of influenza A virus. Numerous strains within the H3N2 subtype were identified as transmissible, and these virus strains can be utilized in this transmission model for pilot studies to evaluate the efficacy of antiviral treatments and to optimize treatment regimes for novel interventions that can reduce contact-dependent transmission of H3N2 influenza A virus.

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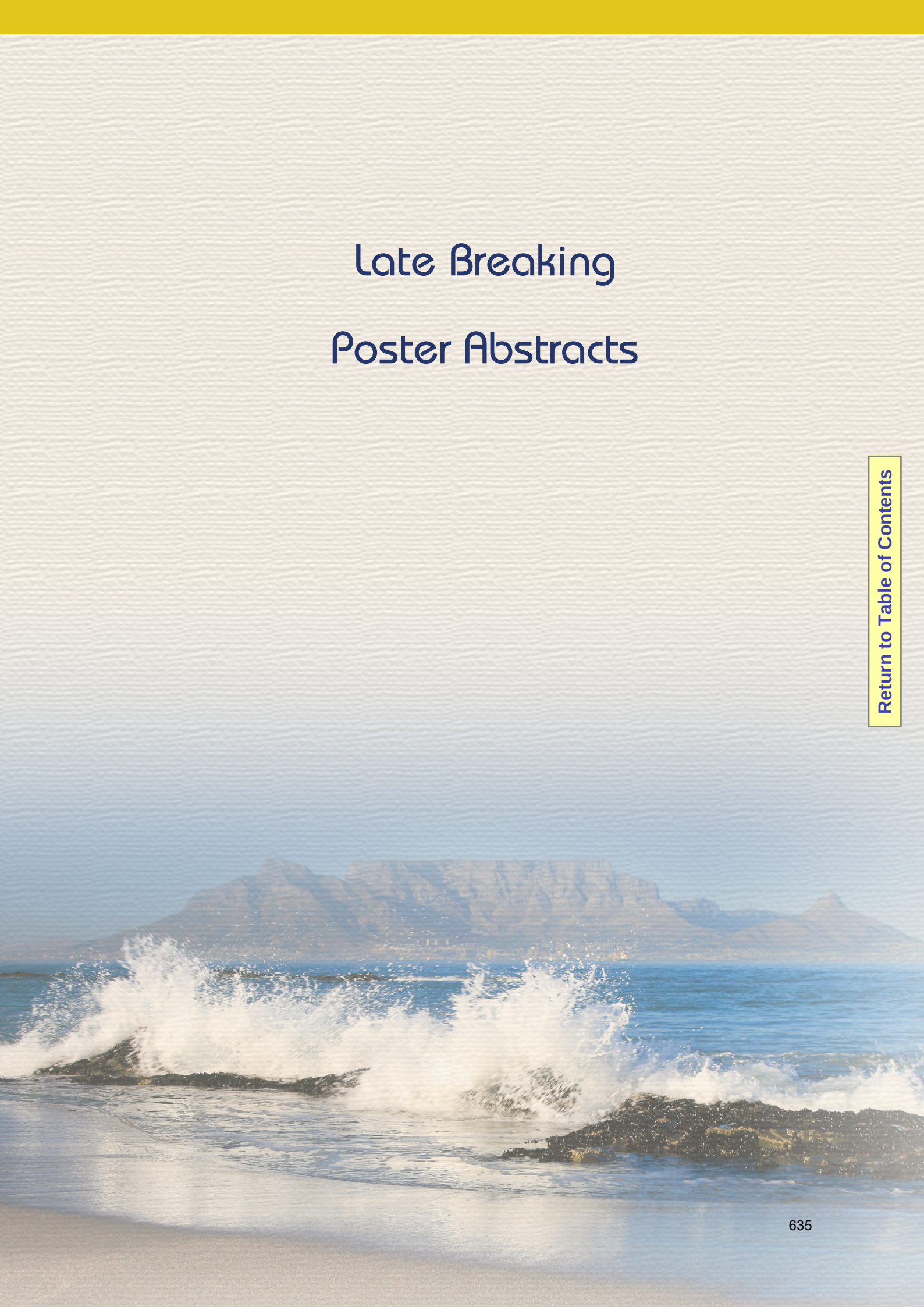
Late Breaking

Poster Abstracts



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LBA-P2-007

Improved immunity from a poly-ICLC adjuvanted Ad5-vectored avian influenza hemagglutinin vaccine against virus challenge in mice

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Background: The H5N1 avian influenza virus has been a continuing problem in the poultry industry for many years, and it is currently a threat to public health due to its high mortality rate when the virus crosses the species barrier into people. These studies evaluated the use of a synthetic dsRNA poly-ICLC as adjuvant to increase the immunogenicity of an Ad5-vectored influenza virus hemagglutinin (HA) vaccine against challenge infection with highly pathogenic A/Vietnam/1203/04 (H5N1) avian influenza virus in mice. Methods: Groups of six-week-old female BALB/c mice were vaccinated on study day 0 by the intranasal route. Placebo groups received physiological sterile saline by the same route. All mice received intranasal challenge with 1x LD_{90} of influenza A/Vietnam/1203/2004 (H5N1) on study day 28. Following challenge, all mice were observed for morbidity and weight loss through day 21. Kaplan-Meier survival curves were generated and compared by the log-rank test followed by pairwise analysis using the Gehan-Breslow-Wilcoxon test in Prism 5.0b (GraphPad Software Inc.). Mean body weights were analyzed by one-way analysis of variance (ANOVA) followed by the Tukey multiple comparison test. Evaluation of the immune response following vaccination included measurement of serum antibody levels by hemagglutination inhibition assay (HI) and IgA levels in lung lavage. Cellular immunity was evaluated by quantitation of cells, in lung lavage, releasing IFN-γ and IL-4 by ELISpot assay. The results from serologic assays and ELISpot assays were analyzed by ANOVA followed by the Tukey multiple comparison tests. Results: In a comparison of AdVN.H5 vaccines administered 30 minutes or 24 hours prior to administration of different doses of poly-ICLC, all treatment groups receiving the 10^8 dose of Ad-VN.H5 provided 100% protection from challenge infection, regardless of the concentration of poly-ICLC. In addition, four doses of AdVN.H5 (1.2×10^6, 1.2×10^7, 1.2×10^8, or 3.5×10^8 ifu/50 µL) vaccine administered 30 minutes prior to administration of 15-µg poly-ICLC provided 100% protection from challenge infection. Although, the 10^6 dose of AdVN.H5 combined with the 15-µg dose of poly-ICLC provided the greatest protection from weight loss of all vaccine doses evaluated. By day 28 post vaccination, all treatment groups receiving the 10^8 dose of AdVN.H5 showed significant HAI titers. In addition, all groups receiving the 10^8 doses of AdVN.H5 induced significant levels of IgA in lung lavage samples on day 14 and day 28 post vaccination. In addition, on day 14 post vaccination, only groups receiving the 10^8 dose of AdVN.H5 combined with the 15-µg dose of poly-ICLC induced a significant increase in the number of IFN-γ producing cells isolated and cultured from lung lavage. However, by day 28 all groups receiving the 10^8 dose of AdVN.H5 showed significant levels of IFN-γ producing cells. On day 14, only the group receiving the 10^8 dose of AdVN.H5 combined with 15 µg of poly-ICLC showed a significant increase in the number of IL-4 producing cells. However, by day 28 post vaccination, all treatment groups showed an increase in the number of IL-4 producing cells. Conclusions: The survival and weight loss data indicate that the 10^8 dose of AdVN.H5 is protective regardless of the concentration of poly-ICLC. However, even lower doses of vaccine can be protective if combined with adjuvant. In addition, groups of mice receiving 10^8 or $10^{8.5}$ dose of AdVN.H5 combined with the 15-µg dose of poly-ICLC showed significant increases in HAI titers in serum, and IgA titers in lung lavage on both days 14 and 28 following vaccination. Only the group receiving 10^8 AdVN.H5 combined with poly-ICLC showed a significant increase in IFN-γ producing cells on day 14. Although, all groups receiving 10^8 or $10^{8.5}$ AdVN.H5 showed significant increases in IFN-γ producing cells on day 28 following vaccination. Supported by Contract HHSN272201000039I from the Respiratory Diseases Branch, NIAID, NIH.

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LBA-P2-008

Anti-H5N1 specific polyclonal immunoglobulins harbor a high neutralization activity on heterologous strains of H5N1 virus

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Background: Since 2003, highly pathogenic avian influenza virus (H5N1) has been a major global health concern. The regular emergence of new outbreaks is observed in Southeast Asia and in particular Cambodia. Over 630 human cases (with almost 60% of deaths) were recorded since the emergence of the virus. Fab'entech has developed anti-H5N1 specific polyclonal immunoglobulins that have been tested in an H5N1-infected mouse model where a proof-of-concept of efficacy has been established. Polyclonal immunoglobulins are known for their ability to present cross-reactivity capacities. The objective of this study was to confirm the neutralization activity of the anti-H5N1 specific polyclonal immunoglobulins on heterologous H5N1 viral strains representative of various clades/subclades that emerged and circulated worldwide since 2004. Materials and Methods: Microneutralization in vitro assay was used to investigate the neutralization activity of anti-H5N1 immunoglobulin fragments derived from horse plasma immunized with inactivated H5N1 A/Vietnam/1194/04 strain. One hundred TCID50 of 21 H5N1 strains belonging to various clades were incubated with a range of dilutions of immunoglobulins and then transferred onto MDCK cells for neutralization analysis. Results obtained were confirmed by using a hemagglutination inhibition assay (HIA). Results: Incubation of specific anti-H5N1 immunoglobulins developed against A/Vietnam/1194/04 strain with various H5N1 strains isolated between 2004 and 2013 that belonged to 6 different clades or subclades provided in vitro neutralization with titers comprised between 1:1000 to 1:8000. Results were confirmed by HIA. Conclusions: These data demonstrate the excellent cross-reactivity of these specific polyclonal immunoglobulins on 21 strains, isolated in different part of the world, and representative of six different clades/subclades that emerged worldwide after 10 years of avian H5N1 virus natural evolution. Fabenflu, which has been tested for safety in a phase 1 clinical trial (Tambyah PA, et al), is available in Singapore under buffer stock procedure. Compassionate use protocol is in development in countries from epidemic or endemic regions to ensure the availability of this agent for patients acutely infected or suspected to be infected with H5N1 influenza.

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LBA-P2-009

Influenza B circulation during twelve influenza seasons (2001-2013) in a tertiary hospital in Sao Paulo, Brazil

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Background: Two antigenically distinct lineages of influenza B viruses, comprising the Victoria-like and Yamagata-like strains, currently circulate among humans. Information about the circulation of these lineages is important to support the need for a quadrivalent influenza vaccine. Surveillance from the USA and Europe over the past 10 years showed that the chance of correct matching between vaccine and circulating lineage had been 50%. Materials and Methods: This retrospective study evaluated 2944 respiratory samples collected in a tertiary hospital in Sao Paulo from 2001 to May 2013. Influenza B was investigated by direct fluorescence assay, culture, RT-PCR and/or real-time PCR, and lineages were identified by real-time PCR. Results: Overall, influenza B was detected in 3.6% (105/2944) of samples; influenza B positivity rate was 4.1% of adult and 2.7% of children's samples; teenagers (13-18 years) had the highest rate. The mean age of patients with influenza B was 23.9 years; 8.6% (9/105) were hospitalized, 19% (20/105) were immunocompromised and 10% (11/105) were vaccinated. Ninety-eight influenza B positive samples were analyzed by real-time PCR assay for virus lineage differentiation. Ninety-six samples could be characterized, 40 were Yamagata-like and 56 were Victoria-like strains. One lineage predominated during ten seasons but co-circulation

was found during 2002 and 2008. Mismatching between vaccine and circulating lineage was observed in 3 seasons (2002, 2008 and 2013). Conclusions: On the basis of data collected during a period of 12 years in a tertiary hospital of the largest city in the Southern Hemisphere, we found that influenza B was more frequent in teenagers and accounted for a variable proportion of influenza-positive samples. Co-circulation of both lineages was not common but occurred recently, and mismatch with vaccine strain should be evaluated to make correct decisions about vaccine composition.

LBA-P2-010

Influenza sentinel surveillance in Ukraine and characteristics of isolates during the season 2012-2013

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Background: Since 2008 the influenza sentinel surveillance system has existed in Ukraine. Clinical and epidemiologic information were collected from 18 adult and pediatric clinics in four cities located in different geographic regions of the country (Kyiv, Dnepropetrovsk, Odessa and Khmelnitsky). Epidemiologic and virologic information was collected during the entire year. Materials and Methods: Both epidemiologic and virologic data were collected on a weekly basis. The specimens (throat swabs) were tested for influenza by real-time polymerase chain reaction (RT-PCR), and viruses were isolated in MDCK and MDCK-SIAT cell culture from PCR-positive samples. Results: A total of 1419 samples from patients with influenza-like illness (ILI) and severe acute respiratory infection (SARI) were collected during the 2012-2013 influenza season. The numbers of positive samples in PCR were 245 (17.3%). But the proportion of laboratory-confirmed influenza among SARI and ILI cases reached 50% during the peak of the 2012-2013 season. The first influenza laboratory-confirmed cases (SARI) appeared in week 48 (November 2012). It was a long influenza season this year caused by all three viruses: A(H1N1)pdm, A(H3N2) and B/Yamagata linage. The last influenza laboratory-confirmed cases (SARI) were detected in week 22 (May 2013). Type A virus was responsible for 71% of the confirmed cases. The last high activity season caused by the B/Yamagata lineage was in 2006 in Ukraine. The National Influenza Center (NIC) and the sentinel laboratories in the 4 regions isolated 239 influenza viruses on cell culture; 116 of these isolates were identified by the NIC laboratory as A(H1N1)pdm subtype influenza virus, 54 as A(H3N2) virus and 69 as type B influenza virus. All A(H1N1)pdm isolates were related to A/California/07/2009-like strain, all A(H3N2) isolates were related to A/Victoria/361/2011-like strain, and all B isolates were related to B/Wisconsin/01/2010-like strain. For the most part, current A(H3N2) viruses did not agglutinate chicken red blood cells (RBCs). We used guinea pig RBCs. The high match between SARI hospitalizations and percent of influenza-positive samples demonstrates the adequacy of sentinel SARI surveillance in the country. Conclusions: The influenza sentinel surveillance in Ukraine reflected the influenza situation across the country. In the past influenza season, there was total or partial coincidence of influenza strains, which caused the influenza epidemic in Ukraine and in the countries of the North Hemisphere.

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LBA-P2-011 G FELLOWSHIP GATES Fellowship

Etiological structure and treatment of acute respiratory viral infections in among epidemic period

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Background: Influenza and other acute respiratory viral infections (ARVI) are the most common among human diseases, they account for almost 70% of infectious diseases. Treatment of patients with influenza and other ARVI is the great challenge. It is known, that drugs based on yeast RNA have antiviral activity of broad-spectrum antibiotics, anti-inflammatory and immunomodulating activity. Nuklex was recently registered in Ukraine and contains highly purified RNA, consisting mainly of 20-25 nucleotides. This remedy has already proven itself in many infectious diseases as antiviral agent. Materials and methods: 118 young and middle-aged patients who had influenza and other acute respiratory viral infection, were surveyed. Patients were divided into 2 groups: I (main) – included 59 patients who received nuklex, and II (control) – 59 patients who received standard treatment. Nuklex was administered as 500 mg 4 times a day, after meals, for 5-7 days. Efficiency of treatment was assessed by clinical data and PCR. Results: There were 12.7% patients with ARVIs who had complications of pneumonia, 13.6% of them were in group I, 11.9% in group II. In group I, 47.4% of patients had severe diseases, 52.6% had moderate disease; in the control group, it was 35.1% and 64.9%, respectively. The majority of patients were under 30 years of age; 53.5%, and 14.2%, respectively, were older than 60 years. Using a PCR method (during hospitalization and on the first 3 days of illness), a positive outcome was obtained in 83.9% of patients, including 3.0% (3 patients) who had different combinations with 2 exciters simultaneously. The spectrum of pathogens was as follows: in group I, influenza A virus was found in 28.8%, RS virus in 25.4%, parainfluenza virus in 15.2%, adenovirus in 8.4%, and a combination of viruses was diagnosed in 5.1% of patients (influenza A virus + parainfluenza [2 patients] and influenza A virus + RS virus [1 patient]); the virus was not determined in 27.1%; in group II, influenza A virus was found in 23.8%, RS virus in 23.8%, parainfluenza virus in 11.9%, adenovirus in 11.9%, a combination of viruses in 2.1% of patients (adenovirus + RS virus), and etiology were not determined in 30.5%. A probable contraction of temperature reaction was noted in 87.8% ± 3.9% of patients in group I whose fever lasted 1-2 days; in the II group, this occurred in only 42.6% ± 6.2%, $P < .001$. Fever that lasted 3 days or more was found less frequently in the study group than in the control group: 10.2% ± 5.1% to 16.8% ± 6.9%, $P > .05$. In 46.4% ± 11.3% patients in group I, cough ended in 2-3 days; in the control group, this occurred in only 11.4% ± 6.5% patients, $P < .01$. Cough lasting 6 days or more occurred less frequently in group I than in group II: 16.5% ± 7.2% versus 59.4% ± 9.4%, $P < .001$. Of 46 people in group I who had viruses detected during hospitalization according to the PCR, only 17.3% had pathogens detected again in early convalescence; of 41 similar patients in the control group, 48.8% had the virus found again in early convalescence, $P < .001$. Furthermore, the results were as follows: in group I, influenza A virus remained in 5.9% (1 patient); in group II, the virus remained in 21.4% (3 patients), $P < .01$. Eradication of RS virus occurred in 86.7% (13 of 15), adenovirus in 40.0% (2 of 5), parainfluenza in 66.7% (6 of 9) patients in the main group; in the control group, eradication of RS virus occurred in 57.1% (8 out of 14), adenovirus in 28.5% (2 of 7), and parainfluenza in 42.8% (3 of 7) patients. Thus, treatment with nuklex contributed a lot to eradication of the virus from the nasal mucosa and oropharynx, because the recovery of the patients' organisms in the main group occurred more often ($P < .01$), than in the control group. Conclusions: Complex treatment of patients with influenza A, parainfluenza, RS virus, adenovirus infection and other ARVI (unidentified), including the antiviral drug nuklex, can significantly improve clinical results, including mitigation of fever and cough. Clinical application of nuklex will promote eradication of influenza viruses A, parainfluenza, RS virus, and adenoviruses among patients with ARVI.

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LBA-P2-012

GATES Fellowship

Oseltamivir resistance among influenza viruses: surveillance in northern Viet Nam, 2009-2012

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Background: Antiviral resistance has been reported in seasonal influenza A viruses and avian influenza A(H5N1) viruses in Viet Nam, raising concerns about the efficacy of treatment. Methods: We analysed specimens from two sources during the period 2009-2012: influenza-positive samples from influenza-like illness patients at sentinel clinics in northern Viet Nam and isolates from patients with confirmed A(H5N1) infections. Pyrosequencing was used to detect mutations: H275Y [for A(H1N1) and A(H5N1)], E119V [for A(H3N2)] and I117V [for A(H5N1)]. A neuraminidase inhibition assay was used to determine the inhibitory concentration 50 (IC) values for all influenza A and B isolates. Results: There were 341 influenza A positive samples identified; influenza A(H1N1)pdm09 was identified most frequently (n = 215). In 2009, oseltamivir resistance was observed in 100% (19 of 19) of seasonal A(H1N1) isolates and 1.4% (3/215) of A(H1N1)pdm09 isolates. This H275Y mutation was not found in influenza subtypes A(H5N1) or A(H3N2) isolates. Conclusions: In Viet Nam, seasonal and A(H5N1) influenza vaccines are not currently available; thus, effective treatment is required. The presence of oseltamivir-resistant viruses is therefore a concern. Active surveillance for oseltamivir resistance among influenza viruses circulating in Viet Nam should be continued.

LBA-P2-013

Molecular characterisation of the Haemagglutinin gene of Confirmed cases of Pandemic A H1N1(2009) in Mauritius

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The first case of pandemic A H1N1 (2009) was diagnosed in Mauritius in June 2009, with approximately 150 laboratory-confirmed cases. Of these, 63 samples were selected and a temporal-spatial study was carried out to determine whether the virus has evolved. Molecular analysis of the hemagglutinin (HA) gene, crucial for the monitoring of modification in viral genome related to pathogenesis and transmissibility was carried out by PCR using newly designed primers to span the whole HA gene. The amplicons were then purified and sequenced. Sequences data obtained were analysed for homology and variations with the reference vaccine strain A/California 07/09 using BioEdit and MEGA 5.0 Bioinformatics software. A phylogenetic analysis was then carried out. The effect of positional amino acid substitutions on the mean hydrophobicity compared with the reference sequence was investigated using the Einsenberg mean hydrophobicity scale. Characterisation of the HA gene revealed conserved sequences at the antigenic sites and variations due to amino acid substitutions, potentially resulting in an N-linked glycosylation site. Novel mutations previously unreported were found, some of these variations were seen to affect the mean hydrophobicity at the concerned sites drastically. Continuous monitoring of the evolution of influenza is thus important and advocated.

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LBA-P2-014

Molecular evolution of the hemagglutinin (HA) gene of laboratory-confirmed cases of pandemic influenza A H1N1(2009) in Mauritius

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Background: Molecular analysis of the hemagglutinin (HA) gene of the AH1N1 influenza is crucial for the monitoring of modification in viral genome related to pathogenesis and transmissibility. The first case of pandemic A H1N1 (2009) was diagnosed in Mauritius in June 2009, with approximately 150 laboratory-confirmed cases. A temporal-spatial study was carried out to determine whether the virus has evolved Materials and Methods: Of the samples, 63 were selected and molecular characterization was carried out by conventional PCR using 6 sets of newly designed primers to span the whole HA gene. The amplicons obtained were then purified and sequenced. The sequences data obtained were analysed for homology and variations with the reference vaccine strain A/California 07/09 using BioEdit and MEGA 5.0 Bioinformatics software. A phylogenetic analysis was then carried out. The effect of positional amino acid substitutions on the mean hydrophobicity compared with the reference sequence was investigated using the Einsenberg mean hydrophobicity scale. Results: Characterisation of the HA gene revealed conserved sequences at the antigenic sites and variations due to amino acid substitutions, potentially resulting in the N-linked glycosylation site. Novel mutations previously unreported were found, some of these variations were seen to affect the mean hydrophobicity at the concerned sites drastically. Conclusions: The present study has confirmed conserved sequences and discovered various variations due to amino acid substitutions at the receptor-binding site and, hence, the antigenic sites including the potential N-linked glycosylation site of HA. It has been suggested that the presence or absence of N-linked glycosylation may cause increase or loss of function because it can initiate and maintain folding, stability, solubility, transportation, antigenicity and immunogenicity. Continuous monitoring of the evolution of influenza is thus important and advocated.

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LBA-P2-015

What more may multiple influenza surveillances tell you than one?

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Background: As the design of the surveillance determines what may be learned from that particular surveillance, influenza surveillance is a good example to discuss data collection, usage, ongoing analysis methods and information dissemination. Influenza surveillance in Japan is mainly focused on capturing the trend of the disease in the season. Influenza affects all age groups, genders, ethnicities, and geographic areas in different scales, throughout the year with or without seasonality. Not only global collaboration, but also regional collaboration and data incorporation from other existing surveillances will enhance the value of information obtained from the surveillances. Materials and Methods: The national surveillances and other open-access surveillances were summarized and compared to provide an overview and introduction to multiple surveillances in Japan. The usefulness of the each surveillance was qualitatively evaluated (ie, how it has been and could be used in the country). Results: The history of influenza-like illness (ILI) surveillance goes back a hundred years in Japan. Sound bases of domestic notification system exist for sentinel ILI case number reporting and viral strain surveillance. Recently, interest in influenza-related deaths such as severe pneumonia and encephalitis cases rose, as over 30% of the Japanese population is aged > 65 years. An additional surveillance was introduced to respond to these needs. Moreover, multiple case surveillances and syndromic surveillances by different definition were introduced during the decade for influenza pandemic preparation after the global epidemic of severe acute respiratory syndrome. The pathogen surveillance to observe changing trends of dominating or newly staged influenza virus strains has

been carried out steadily alongside the national notification system by the public health laboratory network. This surveillance focused on antiviral resistance in isolated influenza strains was initiated in 2009 by the National Institute of Infectious Diseases and Japan Association of Prefectural and Municipal Health Institutes. It started with oseltamivir and zanamivir resistance and then included peramivir and laninamivir from late 2010. However, the key information from clinical sites such as choice of flu medicine, dose, duration of treatment and how it was given (oral, nasal or injection) is still only available from hospital-based surveillances in Japan. The biggest challenge with all surveillance data is not being able to interlink. The surveillance populations and their characteristics are not the same. However, overlaying analysis data may shed some light on possible linked interpretation of existing multiple surveillances. Conclusions: Lack of disease surveillance is a large public health issue. However, running multiple surveillances can also be confusing in some cases. Surveillance evaluation is necessary and important to improve each surveillance and effectively use limited resources.

LBA-P2-016

Detection of influenza H7N9: all molecular tests are not equal

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Background: An avian influenza A virus (H7N9) was recently identified in China, and to date, there have been 132 human cases and 32 deaths. Since rapid and accurate detection of novel influenza viruses is the cornerstone of pandemic preparedness, many countries worldwide have been validating methods to rapidly identify this pathogen. Nucleic acid amplification tests (NAATs) such as RT-PCR have become the gold standard for the detection of influenza viruses and a number of commercial NAATs are currently available; however, the ability of these NAATs to detect novel influenza viruses must be experimentally evaluated. Materials and Methods: This study compared the performance of several commercially available NAATs to "in-house" real-time RT-PCR assays from public health or reference laboratories across Canada that use primer pairs designed by the Centers for Disease Control and Prevention (CDC) targeting the M, H7, or N9 genes of influenza A. The lower limit of detection (LoD) of each method was determined by testing 10-fold serial dilutions of RNA extracted from influenza A/Anhui/01/2013 (H7N9), which were tested on five independent runs. To reduce interlaboratory variability, specimen preparation, nucleic acid extraction and dilutions were carried out at the National Microbiology Laboratory in Winnipeg (Manitoba, Canada). All commercial assays were carried out as recommended by the manufacturer. The LoD was defined by Probit analysis using a probability of 95% and values were expressed as TCID50/mL or the number of target copies/mL. Results: The CDC-based in-house real-time RT-PCR assays targeting the M, H7, or N9 genes were consistently more sensitive then all commercial assays, despite some variability in protocols, reagents, and equipment. Unlike the LoD of the in-house methods, the analytic sensitivity of the different commercial assays was highly variable and some were unable to detect H7N9 at any RNA concentration. Conclusions: Given the poor sensitivity of some commercial assays, front-line laboratories, clinicians, other stakeholders like public health need to understand the limitations of these assays, and that a negative result cannot reliably rule out infection in circumstances in which there is a high index of suspicion for H7N9 infection. In these cases, further testing at a local public health or other reference laboratories that use the CDC-based M, H7, or N9 assays will be essential.

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LBA-P2-017 G FELLOWSHIP GATES Fellowship

Strengthening of the surveillance system for influenza, ARI and SARI in the Republic of Moldova

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Background: Currently the Republic of Moldova has a surveillance system for influenza-like illness (ILI), acute viral respiratory infection (ARI) and severe acute respiratory infection (SARI) adjusted to the international requirements, which is connected to the European (EuroFlu) and Global (FluNet) WHO networks. This system includes the National Influenza Center, which has been recognized on March 18, 2013, by the WHO and is certified as a member of the Global Influenza Surveillance and Response System. Materials and Methods: During the 2012-2013 season, 664 samples of nasopharyngeal exudates from patients were investigated by real-time RT-PCR, including 177 samples from patients with ILI, 384 with ARI and 103 with SARI. Results*:* In 301 (45.3%) samples, influenza and other respiratory pathogens were detected. Influenza viruses were detected in 86 (22.4%) of 384 samples from patients with ARI. Also, influenza viruses were detected in 117 (66.1%) of 177 patients with ILI, and in those with SARI in 34 (34.7%) cases. This flu season has been etiologically caused by 3 influenza viruses: A(H1N1)pdm, A(H3N2) and type B with dominance of the A(H1N1)pdm virus. The share of A(H1N1)pdm virus in the etiologic structure of influenza was 48.0%, of the influenza virus B – 40.8%, A(H3N2) – 9.3%, and of the viruses A(H1N1)pdm + B – 1.7%. MDCK cell cultures were isolated and 8 strains of influenza viruses were identified. Two strains were identified as influenza virus A/California/7/2009 (H1N1)-like, 3 strains - as virus A/Victoria/361/2011 (H3N2)-like, and 3 strains as influenza virus B: a strain - B/Wisconsin/1/2010 – B/Yamagata/16/88 lineage, the second – B/Brisbane/60/2008 – B/Victoria/2/87 lineage, and the third – B/Florida/4/2006 – B/Yamagata/16/88 lineage. Isolated strains of influenza viruses were sensitive to oseltamivir and zanamivir. The epidemic process of influenza morbidity in the 2012-2013 season was manifested by widespread, medium intensity – $70.1^{0}/_{0000}$, the trend of upward and downward, $2.8^{0}/_{0000}$ and $2.2^{0}/_{0000}$, respectively, with moderate impact on the health system. Influenza morbidity from week 04/2013 was increased, peaking in week 08/2013 ($12.8^{0}/_{0000}$) and from week 09/2013 decreased successively, reducing in week 17/2013 to $0.1^{0}/_{0000}$ cases. Of note, influenza cases were not registered in 11 administrative territories of the Republic of Moldova. Influenza in this season affected mainly persons aged 15-64 years (62.6%); children (0-14 years) accounted for only 32.9%, and those aged ≥ 65 years – 4.5%. The share of pregnant women and some groups of patients with immunocompromised status (cardiovascular diseases, diabetes, kidney and liver failure) with laboratory-confirmed influenza was 22.1% and 22.5%, respectively. ARI morbidity from week 40/2012 increased, peaking in week 08/2013 ($468.6^{0}/_{0000}$); after that a successive reduction was registered to $68.4^{0}/_{0000}$ cases in week 20/2013. ARI morbidity exceeded epidemic threshold ($189.17^{0}/_{0000}$) in the weeks 5-14/2013; the other weeks of the season (weeks 40/2012 – 20/2013) were below the epidemic threshold. The incidence of SARI from week 40/2012 increased, peaking in week 08/2013 ($76.6^{0}/_{0000}$), then morbidity was subsequently decreased, reducing in week 20/2913 up to $24.9^{0}/_{0000}$ cases. Conclusions: Strengthening of the influenza, ARI and SARI surveillance system connected to the WHO and CDC has allowed us to monitor the epidemiologic situation in these infections, the epidemic process trend and spread forecast, and to develop control and response measures according to the situation and international requirements. SARI morbidity served as an argument for extending the range of population on increased risk quotas for influenza immunization for the 2012-2013 season with the vaccine recommended by the WHO. Acknowledgement: CDC Cooperative Agreement Number: 1U51IP000329, Funding Opportunity Nr: CDC-RFA-IP09-902.

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LBA-P2-021

Fabenflu, a novel anti-H5N1 specific polyclonal immunoglobulin (F(ab')2 fragments) is safe and can potentially be used in postexposure treatment of H5N1 avian influenza infection in humans

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Background: Highly pathogenic H5N1 influenza A viruses have spread relentlessly across the globe since 1997, infecting both birds and humans. In the past ten years, more than 600 human infections have been reported to the WHO with a high mortality rate (60%), mainly in the Asia-Pacific region. Antiviral therapy has had a very limited effect, leading to the search for novel therapeutic approaches. Fabenflu (FBF001) is composed of anti-H5N1 F(ab')2 fragments of equine immunoglobulin manufactured by the same process that is used for a product that has been effective in postexposure treatment of rabies. The efficacy of Fabenflu was demonstrated in vitro and in vivo on a mortality model in mice. A phase 1 clinical trial was designed to evaluate the safety and determine the pharmacokinetic profile of Fabenflu in humans. Methods: Sixteen healthy males aged 21 to 40 years were enrolled into a randomized, double-blind, placebo-controlled study at the Investigational Medicine Unit, National University Hospital, Singapore. Subjects were enrolled either in part A (single administration [FBF001 0.85 IU/kg or placebo]) or in part B (multiple administrations over 5 days (FBF001 0.85 IU/kg or placebo]) of the study. Subjects in both groups were followed for safety and pharmacokinetic parameters using a specific ELISA method through days 22-33. Results: All subjects completed the study through day 22 (part A) or day 33 (part B). No serious adverse events were reported during this study. Three adverse events were reported during the study, all were reported in the active group and were of mild intensity: one subject reported blepharospasm, one pyrexia and one sinusitis. These may not have been related to study drug administration. No other clinically relevant findings were observed in clinical examination, biochemical parameters, or ECG parameters .The pharmacokinetic parameters from Part A and Part B on day 1 were similar. After five consecutive administrations, both AUCt and AUCinf values were greater than those following single administration, evidence for a slight accumulation following repeated administrations at this dose regimen. Conclusions: This trial demonstrated the safety of Fabenflu (FBF001) in humans. Pharmacokinetic analysis also demonstrated the availability of Fabenflu in plasma compartment during the infusion and persisting after the last injection. This has the potential to markedly reduce the morbidity and mortality associated with serious infections due to H5N1 avian influenza in humans. A compassionate use protocol will be implemented to ensure the availability of this agent for patients acutely infected or suspected to be infected with H5N1 influenza.

LBA-P2-022

Development of a device that surveys the acquisition of human-type receptor binding specificity of avian H5N1 influenza viruses

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Background: Human and avian influenza A viruses preferentially recognize the human-type receptor, Neu5Acα2-6Galandavian-type receptor, Neu5Acα2-3Gal, respectively. Materials and Methods: A novel sialoglycan immunochromatography device that can rapidly assess virus receptor specificity

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and the potential emergence of human-type receptor-adapted H5N1 viruses has been developed. Results: The device enables detection within 15 minutes of sialoglycan binding of avian H5N1 mutants that have acquired the ability to bind to human-type receptors, in agreement with the ELISA method. Conclusion: This technology allows the first surveillance of mutated avian influenza viruses that have acquired adaptation to human-type receptors.

LBA-P2-023

Genetic evolution of H5N1 viruses in Egypt

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Highly pathogenic avian influenza H5N1 viruses in Egypt have been rapidly evolving genetically and antigenically. The first outbreak in early 2006 was caused by clade 2.2 viruses. By 2008, viruses were reclassified as clade 2.2.1 and in 2010 clade 2.2.1.1 viruses emerged. In this study, different isolates of H5N1 from 2006-2013 were sequenced to explore their genetic diversity. Three clusters were defined based on hemagglutinin sequences, the majority of viruses isolated in 2010-2012 fell into 2 of these clusters. A potential glycosylation site was introduced at position 72-74 due to a P74S substitution. Viruses lost a potential glycosylation site at position 154-6 due to T156A substitution. Some important mutations were observed at critical positions of antigenic sites (K140G, S141P, and K140R). Other substitutions were observed at the receptor binding pocket of HA-1. Human-like host specific markers were also observed. Amino acid changes associated with sensitivities to various antiviral drugs were found in Egyptian H5N1 isolates. Given the continuous evolution of H5 viruses in Egypt, surveillance, and whole genome sequencing should be routinely performed.

LBA-P2-024

The emergence of H9N2 virus in Egypt

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Avian influenza virus (AIV) subtype H9N2 has been widespread in the Middle East since the 1990s. For ambiguous reasons, H9N2 was not detected in Egypt until the summer of 2011. Circulation of H9N2 viruses in Egyptian poultry in the presence of the endemic highly pathogenic AI H5N1 subtype adds a huge risk factor to the Egyptian poultry industry. This work describes the prevalence of H9N2 viruses in poultry through active surveillance in various Egyptian governorates during the last 2 years. The genomic signatures and protein sequences of several H9N2 isolates were analyzed. Phylogenetic data showed that Egyptian isolated H9N2 viruses were closely related to viruses of the G1-like lineage isolated from neighboring countries. These viruses showed impressive replication rate in embryonated chicken eggs and mammalian cells. To investigate the causative segment for increasing replication rate of H9N2 viruses, we constructed a recombinant influenza virus strain of A/PR8/34 (PR8) expressing individual genes from an avian H9N2 influenza strain A/chicken/Egypt/S4456B/2011(H9N2). Using several in vitro models of virus replication, we observed an increasing in replication rate for a reassortant virus expressing the neuraminidase (NA) gene of A/chicken/Egypt/S4456B/2011(H9N2) relative to that of either parental virus or reassortant PR8 expressing other genes. This finding suggests that replication and antigen content of PR8-derived H5N1 influenza vaccine viruses can be improved by incorporation of NA segment of H9N2 to improve in ovo growth of vaccine strains and offers a promising strategy to differentiate infected from vaccinated animals (DIVA).

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LBA-P2-025

IgM as protective factor against uncontrolled inflammatory response and early mortality in critical post-pandemic influenza

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Background: Lower levels of the IgG2 subclass associated with cytokine dysregulation have been associated with severe pandemic influenza. Nonetheless, there is no information on the role of immunoglobulin isotypes other than IgG in the critical illness caused by influenza infection. This work aimed to describe host immunoglobulin subclasses and isotype profiles in critical influenza infections during the 2010-2011 postpandemic season. Materials and Methods: Forty patients with severe respiratory failure and community-acquired pneumonia with confirmed laboratory influenza infection (polymerase chain reaction and/or cell culture) were recruited. Levels of IgG1, IgG2, IgG3, IgG4, IgM, IgA, and 17 cytokines were measured by using multiplex assays on plasma samples collected in the first 24 hours following admission to the ICU. Association studies among immunoglobulin/cytokine levels, disease severity, and outcome were performed by using the Spearman test, Cox regression analysis and Kaplan-Meier curves. Multivariate analysis was adjusted by age, sex, APACHE score, immunosuppression, and bacterial infection. Results: Fifteen patients died. Concomitant immunosuppression was more frequent in nonsurvivors than in survivors (N = 9 and 3, respectively). Patients showing immunosuppression had lower levels of IgG2 and IgM and higher levels of IL-6, IL-8, and G-CSF than nonimmunosuppressed patients. Correlation studies revealed that the IgG2 and IgM levels were inversely associated with the APACHE score. IgM correlated inversely with 7 proinflammatory/immunomodulatory cytokines. On the contrary, cytokines levels were directly associated with severity. Results of Cox regression analysis for mortality censored at day 28 following ICU admission showed that IgM had a protective effect against mortality at 28 days, whereas IL-8, G-CSF, MCP-1, MIP-1β behaved as risk factors. Conclusions: This study showed that IgM in critically ill patients is a protective factor in postpandemic influenza, preventing uncontrolled hypercytokinemia, severe disease, and fatal outcome. Acknowledgments: The authors acknowledge the support of “Instituto de Salud Carlos III—Fondo de Investigaciones Sanitarias and Health Council, Junta de Castilla y León (JCYL-IECSCYL-SACYL): Programa de Investigación Comisionada en Gripe, GR09/0021, GR09/0022.

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LBA-P2-026

Intranasal H5N1 vaccines, adjuvanted with chitosan derivatives, protect ferrets against highly pathogenic avian influenza intranasal and intratracheal challenge

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Background: The sporadic transmission of H5N1 viruses from birds to humans with more than 60% fatalities has raised concern as to whether H5N1 viruses will be the next influenza pandemic. An important part of H5N1 pandemic preparedness is the development of effective vaccines against these viruses. Pandemic influenza vaccines should not only be immunogenic for the antigen contained within the vaccine but also for drifted variants that may arise. As such, adjuvants have been included in H5N1 vaccines to increase the magnitude and breadth of the immune response. In this study, two intranasal H5N1 subunit vaccines using a novel chitosan-based adjuvant delivery system were investigated in the H5N1 ferret challenge model. Materials and Methods: Six groups of 6 ferrets were intranasally vaccinated twice, 21 days apart, with placebo, antigen alone, CSN adjuvanted antigen, or TM-CSN adjuvanted antigen. Homologous and intrasubtypic antibody cross-reacting responses were assessed. Ferrets were inoculated intratracheally (all treatments) or intranasally (CSN-adjuvanted and placebo treatments only) with highly pathogenic avian Influenza (HPAI) A/Vietnam/1194/2004 (H5N1) virus 28 days after the second vaccination and subsequently monitored for morbidity and mortality outcomes. Clinical signs were assessed, and nasal as well as throat swabs were taken daily for virology. Samples of lung tissue, nasal turbinates, brain, and the olfactory bulb were analysed for the presence of virus and examined for histopathologic findings. Results: In contrast to animals vaccinated with antigen alone, the CSN and TM-CSN adjuvanted vaccines induced high levels of antibodies, protected ferrets from death, reduced viral replication, and abrogated disease after intratracheal challenge and in the case of CSN after intranasal challenge. In particular, the TM-CSN adjuvanted vaccine was highly effective at eliciting protective immunity from intratracheal challenge; serologically, protective titres were demonstrable after one vaccination. The 2-dose schedule with TM-CSN vaccine also induced cross-reactive antibodies to clade 2.1 and 2.2 viruses. Furthermore, ferrets immunised with TM-CSN had no detectable virus in both respiratory tract and brain, whereas there were signs of virus in the throat and lungs, albeit at significantly reduced levels, in CSN vaccinated animals. Conclusions: This study has demonstrated that CSN and in particular TM-CSN adjuvanted intranasal vaccines have the potential to protect against significant mortality and morbidity arising from infection with HPAI H5N1 virus.

LBA-P2-027

Use of a RT-PCR for seasonally characterization of Victoria and Yamagata influenza B lineages

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Background: The Victoria and Yamagata lineages of influenza B cause seasonal epidemics without a homogeneous temporal epidemic pattern, but they can also cause sporadic cases during influenza A

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epidemics, and some of these cases are severe. These lineages have remained distinct since the 1970s, and because of their antigenic differences, infection with one of them does not protect against infection with the other. For this reason, characterization of the influenza B virus lineages is important for adequate virological surveillance during the flu season (in the Northern hemisphere, from week 40 [early October] to week 20 mid-May]), which will improve seasonal vaccine design. The objective of this study was to develop a multiplex RT-PCR technique that allows simultaneous identification of both influenza B lineages in respiratory samples and present data from 3 flu seasons and 1 nonsurveillance period. Materials and Methods: Samples were collected from patients attending Clinical University Hospital of Valladolid (Spain), Influenza Surveillance Sentinel Network (ISSN), and various hospitals in Castilla y Leon (Spain). Samples were analyzed by cellular culture, using monoclonal antibodies and immunofluorescence assays, and by RT-PCR molecular detection. Cellular culture was done using MDCK-Siat1 cells for viral growth and monoclonal antibodies against influenza A and B (Oxoid) for fluorescence stain and microscope examination. Molecular detection was done by multiplex RT-PCR using the Luminex 200 device and RVP Respiratory Viral Panel (Luminex). Genetic material from the samples was extracted using the automatic extractor device EasyMag (Biomerieux). After detection of influenza B, specific primers and probes to subtype the 2 Influenza B lineages were purchased (Tibmolbiol) to develop an RT-PCR using Abi 7500-Fast platform (Applied Biosystems) (Biere B, Bauer B, Schweiger B. Differentiation of influenza B virus lineages Yamagata and Victoria by real-time PCR. *J Clin Microbiol*. 2010;48:1425-1427). Samples positive for influenza B (cell culture or molecular diagnostic) were stored at -80ºC at the Valladolid National Influenza Center (Valladolid NIC) until their final characterization. Results: 147 respiratory samples having B virus were diagnosed from the 2010-2011, 2011-2012 and 2012-2012 influenza surveillance seasons and the 2012 nonsurveillance period. Most of the influenza viruses analyzed during the last 3 epidemic seasons were able to be characterized as 1 of 2 lineages. Among those, 12 Victoria lineages and 3 nonsubtypable samples were detected in the 2010-2011 epidemic season. Similarly, 7 Yamagata lineages and 4 Victoria lineages were detected in the 2011-2012 season. Finally, during the 2012 nonsurveillance period, a cluster of influenza B cases were detected that could be the prelude to the Spain 2012-2013 epidemic season. Seven Victoria lineages were detected from patients during the nonsurveillance period (from June to September 2012), and 75 B Yamagata lineage viruses, 35 Victoria and 5 nonsubtypable samples were detected during the 2012-2013 epidemic season. Conclusions: Characterization of B lineages by the method described is reliable and leads to a high percentage of B characterization. Although modest representation of influenza B during the 2010-2011 and 2011-2012 epidemic seasons was observed, the predominant activity in the last epidemic season was due to influenza B, mostly the Yamagata lineage and secondly the Victoria lineage. This sudden influenza B epidemic ascendancy, especially with a lineage with low occurrence in seasonal influenza vaccines in the last years, shows the importance of B-specific characterization for vaccination efficacy control. These findings support the use of a tetravalent vaccine against influenza viruses.

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LBA-P2-028 SCHOLARSHIP Promising Investigator Scholarship

Pathological and immunological comparison of H5N1 and H7N9 viruses in mice

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Background: Beginning in March 2013, a novel, avian-origin influenza A/H7N9 virus caused 133 infections with a mortality rate of approximately 28%, primarily in older adults. Due to its high mortality rate, disease severity, and avian origin, this H7 virus has been touted "H5N1 like" and is considered to be extremely virulent. To determine if the H7N9 induced an H5-like infection, a panel of highly pathogenic avian H5N1 and H7N9 viruses isolated from human infections were tested in mice for the "hallmarks" of H5 infection, including morbidity and mortality, systemic spread, immune response, cytokine storm, and in vitro replication in macrophages and normal human bronchial epithelial cells (NHBE). Because the majority of the reported H7N9 cases occurred in high-risk groups, comparative infections were also performed in aged mice and NHBE isolated from smokers, individuals with asthma, and persons with underlying lung disease (COPD). Materials and Methods: The MLD_{50} for

A/Vietnam/1203/04 (VN1203; H5N1), A/Hong Kong/213/03 (HK213; H5N1), A/Anhui/1/2013 (Anhui; H7N9), A/Shanghai/1/2013 (SH1; H7N9), and A/Shanghai/2/2013 (SH2; H7N9) was determined in C57BL/6 mice. Following MLD_{50} determination, young (6-8 weeks of age) or aged (> 1 year) mice were infected with 1 MLD_{50} of each virus, respectively, and tissues were collected at 3, 6, and 9 days postinfection (dpi) to determine viral load, pathology, immune response, and cytokine levels. Viruses were also used to infect MDCK, RAW, and NHBE (isolated from normal and high-risk populations) cells for viral replication. Results: Of the H5N1 viruses, VN1203 virus had the lowest MLD_{50}, with HK213 being significantly higher. The H7N9 Anhui and SH1 viruses had MLD_{50} more similar to that of VN1203, whereas SH2 virus was more similar to HK213 and had slower replication kinetics in both normal and high-risk NHBE. Indeed, even with genetic similarities, SH2 infection was significantly different in all respects vs the Anhui and SH1 strains. In spite of the differences in MLD_{50}, no significant differences were observed in nasal or lung virus titers; however, VN1203 was the only strain detected in the brain and kidneys in 100% of mice at 6 dpi. Furthermore, there were no significant differences in BALF or splenic immune cell populations at any time postinfection. In vitro, only the VN1203 virus replicated in macrophages and had significantly faster replication kinetics in all cell types tested. Intriguingly, the H7N9 viruses induced higher viral titers and were shed longer from the NHBE cells isolated from high-risk individuals as compared with the H5 viruses. Studies are underway to define any cytokine or pathological differences in young and aged mice. Conclusions: Although the H7N9 viruses do cause significant morbidity and mortality in mice, a higher dose is required to generate an “H5N1-like” infection. In addition, H7N9 infections lack the hallmark systemic spread and the ability to replicate in macrophages associated with highly pathogenic H5N1 viruses. Based on the epidemiologic and experimental data, H7N9 infection in high-risk groups appears to cause significant clinical disease, whereas H5N1 infection causes significant mortality even in normal individuals. Taken together, these data indicate that the H7N9 viruses are not necessarily similar to H5N1 viruses; however, further studies are required to understand the differences in pathogenicity among H7N9 strains. In addition, further surveillance is necessary, especially in high-risk groups, to prevent another outbreak from occurring in the upcoming winter months.

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LBA-P2-029

The use of statins to combat viral and bacterial pneumonia in obese mice

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Background: Aside from their lipid-lowering properties, the pleiotropic anti-inflammatory and immunomodulatory effects of 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors have been hypothesized to have beneficial effects against community-acquired pneumonia. Although several clinical trials have shown some benefits of statin use against pneumonia severity, overall benefits appear to be minimal except in severe cases. One potential reason for the minimal effect is confounding factors not generally analyzed in clinical trials, including differences in the immune response required to combat viral or bacterial infection alone or as a synergistic infection as well as altered response in high-risk states, such as obesity. Therefore, we attempted to tease apart the differential effects of pathogen type and obesity on the pleiotropic effects of statins during experimental models of pneumonia in mice. Materials and Methods: Wild-type (WT) and genetically obese (*ob/ob*) C57BL/6 mice were fed either a control diet or a diet containing simvastatin for 4 weeks. Following dietary treatment, mice were inoculated with 1 MLD_{50} of either influenza virus or *Streptococcus penumoniae* or administered a nonlethal dose of influenza followed by a nonlethal dose of *Streptococcus penumoniae* at day 7 post-influenza infection and monitored for morbidity and mortality. Influenza and bacterial titers were monitored following both primary and secondary infections. Results: As expected, obese mice fed a control diet had decreased susceptibility to bacterial challenge and increased susceptibility to influenza virus challenge compared with WT controls. Following consumption of the statin diet, no significant differences were observed in mortality from either virus or bacteria in WT mice. However, statin treatment significantly altered response in the obese mice by increasing mortality from bacterial challenge to almost 100% and decreasing influenza-associated mortality from 0 to approximately 50%. Increased mortality from bacterial infection and increased survival from influenza was associated with significantly increased titers in the

blood and decreased titers in the lungs of obese mice, respectively. Following secondary bacterial challenge, control fed *ob/ob* mice began to succumb to infection 4 days faster than WT controls; however, no beneficial effect of statins were observed in either the WT or obese groups, and no differences were observed in titer. Conclusions: Based on our results, it is clear that several factors need to be considered when using statins as a possible therapeutic agent for decreasing pneumonia morbidity and mortality. Diagnosis for the cause of pneumonia as well as BMI should be carefully considered when designing future studies for pleiotropic use. Future studies are focused on understanding the statin-induced changes in susceptibility to viral and bacterial challenge in the obese host and if these results are similar for all types of HMG Co-A reductase inhibitors or purely associated with simvastatin use.

LBA-P2-030

VIS410, a human antibody, provides additive efficacy when dosed with oseltamivir in a severe influenza model

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Background: VIS410 is a human monoclonal antibody directed against the stem of influenza hemagglutinin that has demonstrated the ability to neutralize group I (H1 and H5) and group II (H3) viruses in vitro and treat infected mice in vivo. One potential use for VIS410 is to treat hospitalized individuals suffering from severe influenza. In this setting, neuraminidase inhibitors, such as oseltamivir, are administered, although there is debate regarding effectiveness if administered after 48 hours of onset of symptoms. We therefore examined in a lethal mouse model of influenza infection the efficacy of VIS410 alone and in combination with oseltamivir on symptoms, weight loss, physical signs of illness, and death. Materials and Methods: At time zero, five groups of 10 BALB/c mice were administered mouse-adapted A/Victoria/3/75 (H3N2) intranasally. Animal cohorts were treated with either VIS410 (intraperitoneally), oseltamivir (orally), or both. VIS410 antibody was administered once 48 hours after exposure at 20, 10, 5, or 2.5 mg/kg, whereas oseltamivir was given at 60, 30, or 10 mg/kg twice daily for 7 days beginning 4 hours before exposure. Additional groups of ten mice received various combinations of VIS410 (2.5-20 mg/kg) and oseltamivir (10-60 mg/kg/d). Fifteen mice were treated with PBS as controls. Mice were weighed from virus exposure until day 15, and mice losing more than 30% body weight were euthanized. Symptoms such as ruffling of fur, lethargy, paralysis, incontinence, repetitive circular motion, and aggression were noted. Normality of the animal weight data was confirmed (D'Agostino & Pearson omnibus normality test), and statistical inferences were made by two-way analysis of variance followed by pairwise comparisons to compare each treatment group with the placebo group, considering the survival of both (Tukey multiple comparisons tests). Survival analysis was conducted using the Kaplan-Meier graphing method, and pairwise comparisons of survivor curves (placebo vs any treatment) were analyzed by the Mantel-Cox log-rank test. Results: When VIS410 was used as a single treatment, all concentrations of the drug protected mice against death (80%-100% survival; $P<0.05$-0.0001 compared with placebo-treated animals). Dose response and combination effects were readily distinguishable when weight loss was evaluated, which reflects the physiological effects of viral infection on animal behavior. Depending on dose, when mice were treated with VIS410 alone or in combination with oseltamivir, weight loss was much less severe than for mice receiving placebo. At the highest doses of VIS410 (20 mg/kg) and oseltamivir (60 mg/kg/d), minimal weight loss was observed, and the mice returned to their starting weight sooner compared with both placebo and either treatment alone. Significant reduction in weight loss was also observed for additional combinations of VIS410 and oseltamivir. For example, the combination of VIS410 at 10 mg/kg plus oseltamivir at 30 mg/kg/d mitigated weight loss compared with either treatment alone on days 5-8 post-virus exposure, the critical time frame in which weight loss is a good predictor of later mortality due to virus infection; the combination of VIS410 at 5 mg/kg plus oseltamivir at 30 mg/kg/d also significantly lessened weight loss from days 6-8 post-virus exposure. Conclusions: All doses of VIS410 protected mice against death (80%-100% survival) in a severe H3N2 influenza model. In addition, when amelioration of weight loss was evaluated, a significant additive effect was

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observed for oseltamivir combined with VIS410. These data suggest that VIS410 may be an effective treatment alone or when added to oseltamivir for patients suffering from severe influenza.

LBA-P2-031

A project to translate and exchange knowledge toward more effective, efficient, and equitable public health and primary care strategies for influenza and influenza-like illness (ILI) in Canada

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Background: Influenza and influenza-like illnesses (ILI) continue to be a challenging public health issue, including annual “seasonal” influenza and other respiratory pathogens or pandemics and regional outbreaks (eg, pandemic H1N1 and avian H7N9, respectively). In most public health jurisdictions, there are significant allocations of attention and resources to prevent and control influenza and ILI. These activities are based on many assumptions and/or observations regarding burden of illness, accuracy of diagnosis, and the effectiveness, efficiency, and equity of our strategies for prevention (eg, annual immunization) and treatment (including antiviral drugs). Despite the longstanding awareness and recurring events of influenza and other ILI, there remain many unanswered questions and unsolved problems with respect to the effectiveness, cost effectiveness, and equity of public health and primary care programs and services in Canada. To analyze data for synthesis and translation of knowledge for public health policy makers and practitioners, the National Collaborating Centre for Infectious Diseases (NCCID) is leading a collaborative project with the other five Canadian Public Health National Collaborating Centres. To obtain input on the scope of this project and the knowledge translation products that would be useful to Canadian public health practitioners, NCCID hosted a national consultation with public health practitioners and administrators, clinicians, epidemiologists, researchers, and others. This presentation will outline topic areas that the consultation participants prioritized for action and describe proposed activities to address these issues. Materials and Methods: Thirty-five individuals from diverse professional backgrounds were brought together for a one-day national consultation. Together, working from a list of issues related to the prevention and control of ILI from a workshop hosted by the Institute of Population and Public Health (IPPH) of the Canadian Institutes of Health Research (CIHR) (2011), consultation participants deliberated and narrowed the list down to five top priority issue groups. Over the course of the consultation, participants brainstormed activities and knowledge products that could help address these priority issues. Results: The five top priority issue groups identified by the consultation participants were: (1) vaccine efficacy, effectiveness, efficiency, and equity (subpoint example for consideration: knowledge basis for considering making vaccination mandatory for health care workers), (2) primary prevention of influenza and ILI other than vaccine (subpoint example: environmental barriers to disease transmission), (3) rapid diagnostics (subpoint example: more accurate diagnosis and surveillance; ability to evaluate vaccine and antiviral effectiveness), (4) surveillance and better estimates of burden of influenza and ILI (subpoint example: severity of illness, medical and nonmedical determinants of outcome inequalities), and (5) communication and messaging (subpoint example: how to increase public knowledge and understanding of burden, causes, risk factors, and prevention and treatment of ILI). Diverse activities and knowledge products were suggested for addressing the priority issues. These included synthesis of evidence from published literature, inventory of researchers and their research in the relevant areas, better recent and real-time estimates of burden of influenza and ILI, better (electronic) information-sharing mechanisms between primary health and public health, and evaluation of existing prevention and control strategies. Conclusions: The consultation reaffirmed that there are many important unanswered questions about influenza and other ILI with respect to the burden of illness, diagnosis, prevention, treatment, and professional and public engagement. The variety of participants focusing for one day on the consideration of practical knowledge needs resulted in a balanced and comprehensive set of priority topics to guide the two-year collaborative knowledge translation project.

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Our goal is to share these products widely, including at the next international influenza conference three years from now!

LBA-P2-032

Antigenic characterization of influenza viruses circulated in Burkina Faso in 2012 and their sensitivity assessment to two antiviral drugs, oseltamivir and zanamivir

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Background: Although Influenza is an important respiratory disease in Africa, virological surveillance for influenza in Burkina Faso is lacking. During the 2012-2013 influenza season, the virology laboratory at the Institut de Recherche en Sciences de la Santé collected and submitted influenza specimens to the WHO collaborating center for influenza at the US Centers for Diseases Control and Prevention (CDC) for further characterization. In this study, we report characteristics of influenza viruses circulating in Burkina Faso during the season and their susceptibility to two antiviral drugs, oseltamivir and zanamivir. Materials and Methods: We performed rRT-PCR assay using US CDC reagents, primers, and probes to detect influenza viruses circulating in Burkina Faso sentinel sites in 2012. Eight positive specimens for influenza viruses (four influenza B viruses, three influenza A[H1N1]pdm09 viruses and one A[H3N2] virus) were sent to the US CDC for characterization. Hemagglutination inhibition tests with a panel of postinfection ferret antisera for detailed antigenic characterization, a functional neuraminidase inhibition assay and/or genetic analysis to assess susceptibility of these viruses to the neuraminidase inhibitors oseltamivir and zanamivir, and sequence analysis (HA, NA, and M genes for type A viruses and HA, NA, and NS genes for type B viruses) were performed. Results: From the four influenza B viruses, two viruses belonged to the B/Victoria/2/87 lineage and were antigenically similar to the reference strain B/Brisbane/60/2008 virus, whereas the two other influenza B viruses were B/Yamagata lineage viruses and were antigenically similar to B/Wisconsin/01/2010, vaccine strain for the 2012-2013 season. The three influenza A(H1N1)pdm09 virus isolates were antigenically characterized as A/California/07/2009 like, and their HA sequences fell into genetic group 8 and were very similar to other H1N1pdm09 viruses isolated in 2011 from the African region. The A(H3N2)virus was antigenically similar to the A/Victoria/361/2011 virus, which was the H3 component of the 2012-2013 influenza vaccine for the northern hemisphere and 2013 influenza season for the southern hemisphere. All eight viruses were sensitive to oseltamivir and zanamivir. Conclusion: This is the first report describing characteristics of circulating influenza A(H1N1)pdm09 and A(H3N2) and influenza B viruses in Burkina Faso after the 2009 H1N1 pandemic, and it underscores the importance of enhancing influenza surveillance in the country.

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LBA-P2-033

Efficacy of inactivated trivalent influenza vaccine among children in rural India during 2009-2010

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Background: Neither the burden of influenza disease nor influenza vaccine efficacy has been well characterized in children in resource-constrained developing countries. In November 2009, a 3-year household-randomized, controlled, observer-blinded study was initiated in 3 rural villages in northern India to measure vaccine efficacy (VE) of seasonal trivalent inactivated influenza vaccine (IIV). We report here the assessment of total (direct + indirect) protection due to influenza vaccination among children in these communities during November 2009-October 2010. Methods: From November 2009 through January 2010, 3372 children aged 6 months to 10 years were administered intramuscularly either one (9-10 years) or two (6 months-8 years) age-appropriate doses (0.25 mL for 6-35 months and 0.5 mL for ≥ 36 months) of IIV (2009-2010 northern hemisphere formulation: A/Brisbane/59/2007 [H1N1], A/Brisbane/10/2007 (H3N2), and B/Brisbane/60/2008) or a control vaccine, inactivated poliovirus vaccine (IPV). In November 2009-October 2010, active surveillance of vaccine recipients was conducted via weekly home visits to identify all cases of febrile acute respiratory illness (FARI, defined as reported fever and any respiratory complaint [such as cough, sore throat, nasal congestion, runny nose, earache, or difficulty breathing] within the past 7 days). Nasal and/or throat swabs were collected from each FARI episode for influenza detection by real-time reverse transcription polymerase chain reaction (RT-PCR) assay. A proportional hazards model was constructed to calculate the VE of full vaccination of children with IIV, as compared with IPV, with a per-protocol analysis. We calculated VE separately by age group (6-35 months, 3-8 years, and 9-10 years). Results: Of the 3334 children included in the analysis, 1614 received IIV and 1720 received IPV. The IIV group included 1443 fully vaccinated (1 or 2 doses based on age) and 171 partially vaccinated (received 1 of 2 planned doses), whereas the IPV group included 1540 fully and 180 partially vaccinated. The groups were similar in regard to sex (55% and 56% male) and age (mean 5.8 vs 5.7 years) for IIV and IPV, respectively. Overall, we identified 508 influenza-positive FARI cases, of which 261 were A(H1N1)pdm09, 241 were influenza B, and 6 were A(H3N2). Two peaks of A(H1N1)pdm09 were identified: in November-December 2009 and July-October 2010. Influenza B was detected throughout the study period, and 98% of those characterized were identified as Victoria lineage, as contained in the vaccine formulation. Among those fully vaccinated, the IPV group experienced 132 influenza B FARI episodes compared with 80 in the IIV group (all ages VE 37.6%, CI 16.2, 53.6, p=0.002). Vaccine efficacy was 34.5% (CI −13.3, 62.1) in those 6-35 months, 39.5% (CI 9.2, 59.7) in those 3-8 years, and 37.0% (CI -30.8, 69.7) in ages 9-10 years. VE was 39.8% (CI −23.5, 70.7) in the period November 2009 to April 2010 and 37.3% (CI 13.7, 54.5, p=0.004) from May 2010 to October 2010. No statistically significant VE was seen against influenza A(H1N1)pdm09 in fully vaccinated children (VE 17.4%, CI −16.7, 41.6). Conclusions: In the first year of a multiyear VE study, A(H1N1)pdm09 emerged, and the only vaccine-matched influenza virus that circulated in the study community was influenza B. We found that full vaccination with IIV among children aged 6 months to 10 years resulted in a vaccine efficacy of 38% against antigenically matched influenza B strains when analysis included all age groups. Vaccine efficacy was similar in age groups analyzed separately, but only statistically significant among children aged 3-8 years. Further work in needed to understand the efficacy of influenza vaccines in this population.

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LBA-P2-034 G FELLOWSHIP GATES Fellowship

Evaluation of the influenza sentinel surveillance system, Kinshasa, DR Congo, 2009- 2011

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Background: Influenza is an important cause of severe acute respiratory infection in DR Congo. It is notified through the sentinel surveillance system in five sites among in- and outpatients. This study was conducted to evaluate the impact of the surveillance system in reducing the burden of the disease. Methods: A cross-sectional descriptive study was done, using data collected from sentinel sites and the national reference laboratory. At each of the 5 participating sites, 8 surveillance staff were interviewed via a questionnaire. Simplicity was determined based on the staff's mastery of the case definition. Acceptability was measured according to their participation rate; and the completeness according to proportion of files completed. Promptness was based on the proportion of reports received within 48 hours, and data quality refers to whether or not the data were verified. The sensitivity and positive predictive value were calculated based on the proportion of real patients detected and laboratory cases confirmed. Results: All 8 (100%) staff knew the influenza case definition; 75% participated in surveillance activities. Completeness of reporting was 99% in 2009, 98.5% in 2010 and 99.2% in 2011. 100% of the reports were verified. Influenza cases were reported within 48 hours. The sensitivity of the system is 74.1%, and the positive predictive value is 11.47%. The utility is demonstrated by the detection of H1N1 pandemic and seasonal viruses. Conclusion: The influenza sentinel surveillance system in Kinshasa city DR Congo is simple, acceptable, stable, useful, and contributes to the global effort for disease control.

LBA-P2-035

Avian influenza surveillance in Northern Eurasia in the last decade: importance for the Global Influenza Surveillance Network

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Background: The territory of Russia covering a large part of northern Eurasia is of special interest for influenza virus ecology and evolution. The importance of this region has been confirmed by the previous outbreaks of highly pathogenic avian influenza (HPAI) H5N1 among wild birds since 2005. The territory of Russia is situated in the center of the Eurasian continent. This area is crossed by three major migration routes—central Asia flyway, east Africa west Asia flyway, and Black Sea/Mediterranean flyway, combining bird populations from Asia, Africa, and Europe. Avian influenza virus (AIV) surveillance is an important task to complete before the fundamental study and introduction of these data into practice. The study was aimed at the collection of specific data on different AIV distribution and biodiversity, compared with the data from other parts of the world and filling the gap in the Global Influenza Surveillance Network in Russia. Materials and Methods: The specimens were tested for AIV by standard methods. AIVs were isolated and studied by standard virological and molecular biologic methods according to WHO and OIE manuals. Results: The report focuses on some epidemiological, ecological, and evolutionary aspects of AIVs including H5N1 isolated in Northern Eurasia in the last decade. During surveillance for AIV in northern Eurasia (Russia, Mongolia, Kazakhstan), more than 10,000 samples from wild birds were collected. More than 300 AIVs were isolated, and 25 different subtypes were detected. All of the isolated viruses belong to low pathogenic viruses except the H5N1 virus. Moreover, we isolated some rare subtypes including

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H1N2, H8N8, H13N8, H10N7, H15N4, H16N3, and LPAI H5N1. Some of them have not been detected in Russia earlier or elsewhere (according to databases). The biologic properties of these viruses were studied. Phylogenetic analysis of different genes showed a close relationship of Russian viruses with strains from different European, African, and Asian countries and from Australia. Phylogenetic analysis of the M gene of isolated viruses revealed different lineages of influenza viruses isolated. Evolutionary trends will be discussed. We hypothesized that some Russian lake systems may play a significant role in the circulation of avian influenza in Eurasia. We suggested enhancing surveillance programs in these areas (Sharshov et al, 2010). Our hypothesis was confirmed by isolation of HPAI H5N1 and interesting LPAI viruses. In that "hot spot," we isolated unique viruses, including an H13N8 subtype influenza virus from a herring gull. This virus was found to be a reassortant containing the HA, NA, MP, NP, PB1, and PB2 genes from gull-specific viruses and PA and NS genes from European classic Avian-like viruses of different subtypes. This was the first isolation of H13N8 AIV in Mongolia. Also, influenza (H15N4) virus isolation was the first case of H15 subtype isolation in the northern hemisphere. Overall, phylogenetic analysis results revealed that the H15N4 virus is a reassortant with novel HA and NA genes and endemically circulating Eurasian internal genes. The report contains more detailed comparative virological, molecular, and pathogenic characteristics of viruses. Some molecular epidemiological aspects will be discussed. Conclusions: Recent outbreaks of an influenza A (H7N9) virus of avian origin raise concern about the emergence of novel reassortant viruses in Eurasia and the potential threat to the human population. We showed circulation of different AIVs, including HPAI H5N1 among wild birds in the natural ecosystems of Northern Eurasia. Our study confirmed the significant role of AIV surveillance in Russia for the Global Influenza Surveillance Network. The results demonstrate the need for ongoing surveillance to detect new variants of influenza viruses and facilitate prevention of outbreaks. We suggest increasing efforts of AIV surveillance in "hot spots" in northern Eurasia. Acknowledgments: This study was supported by RFBR project 13-04-91179-GFEN-a, USDA grant 58-0210-2-040F.

LBA-P2-038

Full-length genome sequencing of influenza A on Ion Torrent PGM sequencer

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Background: The evolution of influenza A viruses represents a major challenge for both human and animal health. Influenza A viruses may cross species barriers and transmit to humans, with the potential to cause pandemics. Materials and Methods: We describe a rapid sequencing method and standard operating procedure using the Ion Torrent PGM sequencing platform for rapid and cost-effective influenza A virus typing. This method was technically validated within a network of researchers from public health institutes around the globe, and it includes the analysis, reporting, and sharing of data. Results: We amplified the 8 genome segments of influenza A in a single-tube RT-PCR (PathAmp Flu A reagents) and performed a standard sequencing reaction on the Ion Torrent PGM sequencer. Base calling, alignment, and coverage analysis were carried out with the Torrent suite software. Our results show the accuracy of NGS to define the full-length sequence of the influenza A genome. For example, comparison of NGS with Sanger sequencing on a single H1N1pdm isolate showed 100% nucleotide concordance. The high throughput obtained so far indicates that we can multiplex more than 10 samples for a single sequencing run on the Ion PGM 316 chip. Conclusions: The PathAmp FluA reagents provide a rapid, accurate, and sensitive solution for influenza A viral sequencing on the Ion PGM sequencer—and the resulting data can be used for outbreak monitoring as well as routine surveillance. The method described can generate the whole-genome assemblies for IAVs in a very short period of time and represents an indispensable tool both in human and animal surveillance activities.

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LBA-P2-039

Subtle changes in the multibasic cleavage site of the H5 hemagglutinin glycoprotein can affect viral pathogenicity in ferrets

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Background: Predicting the pathogenicity of whether emerging avian H5N1 isolates upon human infection can be largely determined by the presence of more than four basic amino acids in the cleavage site (CS) of the hemagglutinin (HA) glycoprotein. The number and type of basic amino acids within the CS vary across the numerous H5N1 clades; however, the relationship between CS sequence variation and viral pathogenesis for humans is unclear. It is not viable to address this relationship based on clinical data derived from human infections because mutations commonly occur in the polymerase genes of avian isolates during interspecies transmission, which are known to also affect virulence. We therefore investigated how subtle changes in the CS sequence of H5N1, independent of other mutations, affected its virulence in ferrets, an animal routinely used to model human influenza infection. In doing so, we sought to characterize factors associated with severe disease outcomes in ferrets that were infected with H5N1 viruses containing mutations in their CS sequence. Materials and Methods: A/Vietnam/1203/2004 contains a clade 1 $HA_{320\text{-}329}$ with a CS amino acid sequence of PQRERRRKKR. This sequence was altered via site directed mutagenesis to contain a deletion and a substitution to produce a clade 2.3.4-like CS sequence (PLRERRRK-R). Furthermore, we also produced a virus with a CS sequence containing only a single basic amino acid (PQRET----R). Five ferrets were anesthetised and challenged intranasally with 10^6 EID_{50} of each virus. Following infection, ferrets were monitored for signs of disease and euthanised if they progressed to a predetermined humane endpoint. Ferrets that did not progress to the humane endpoint were euthanised on day 7. Numerous systemic organs and tissues of the respiratory tract were collected and examined for infectious viral titres, viral associated lesions and the presence of viral antigen. Results: Ferrets infected with A/Vietnam/1203/2004 that contained a single basic amino acid in the cleavage site (PQRET----R) did not develop severe disease, and all animals recovered from infection and cleared virus by day 7. In contrast, A/Vietnam/1203/2004 that contained a wild-type CS sequence (PQRERRRKKR) caused severe disease in four of five infected ferrets. The disease was characterised by peak rectal temperatures at day 1 post infection of 41°C, greater than 10% loss in body weight and abdominal or neurological disease signs. These ferrets were also lymphopenic at days 1 and 3, and at the time of euthanasia pulmonary lesions were detected and virus was isolated from systemic organs beyond the respiratory tract. Interestingly, ferrets infected with A/Vietnam/1203/2004 containing a clade 2.3.4 CS sequence (PLRERRRK-R) displayed a peak in rectal temperature only on day 3, exhibited minimal evidence of pulmonary damage and virus was isolated at fewer sites outside the respiratory tract. The milder pathogenic profile of the A/Vietnam/1203/2004 (PLRERRRK-R) virus was not attributed to its inability to spread outside the respiratory tract because viral RNA was readily detected in plasma post infection and was also able to infect ferret venous endothelial cells in ex vivo cultures. Conclusions: In this study we have clearly shown that the presence of a multibasic CS in the HA of H5N1 is inextricably linked to its ability to disseminate beyond the respiratory tract. In examining the infectivity of A/Vietnam/1203/2004 without the influence of mutations in polymerase genes, subtle deletions and substitutions in the CS can reduce the severity of infection and therefore are additional factors affecting clade-specific pathogenesis.

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LBA-P2-040

A broadly protective monoclonal antibody targeting a universally conserved epitope in all influenza A neuraminidase subtypes

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Background: Hemagglutinin (HA) and neuraminidase (NA) are the two major surface glycoproteins of influenza A virus and the main targets of protective immune response. Current seasonal influenza vaccines can induce strain-specific neutralizing anti-HA antibodies (Abs). In contrast to HA, anti-NA Abs can reduce viral yield and spread by inhibiting NA enzymatic activity. While several broadly neutralizing Abs targeting conserved epitopes in diverse HA subtypes have been isolated, NA-specific antibodies could only cross-protect partially against homologous and heterologous strains from the same subtype. Thus, we tried here to identify highly conserved sequences in all influenza NA subtypes and determine their functional roles in viral replication and their potential as antiviral targets. Materials and Methods: Comprehensive bioinformatics analyses of all publicly available full-length NA sequences using multiple alignments and Shannon entropy analyses were conducted to identify conserved sequences in all NA subtypes. Reverse genetics (RG) recombinant viruses were generated containing the 7 genes of influenza A/Puerto Rico/08/34(H1N1) and either the wild-type (wt) NA gene or NA plasmids with single alanine substitutions within the identified conserved regions. Growth kinetics of wt virus and each RG variant was then analyzed in MDCK cells. Thermostability and enzymatic activity were determined using the 2-(4-methylumberlliferyl)-α-D- N-acetylneuraminic acid (MUNANA) assay. A rabbit monoclonal Ab, denoted as HCA-2 mAb, raised against one of the characterized sequences was then examined for its inhibitory effects against replication and enzymatic activity of wt viruses from all 9 NA subtypes as well as the RG variants using in vitro microneutralization and enzyme-linked lectin assay, respectively. Finally, in vivo prophylactic efficacy of HCA-2 mAb was tested in a murine model against lethal doses (5 x LD_{50}) of influenza A/FM/1/47/MA (H1N1) and influenza A/HK/1/68-MA20 (H3N2) viruses. Results: Multiple sequence alignments and Shannon entropy analyses of all NA sequences in the GenBank uncovered a universally conserved 9-mer peptide amongst all influenza A viral NA subtypes located between amino acids 222 and 230 (N2 numbering) and comprised of “ILRTQESEC.” Substitutions within this universal epitope significantly affected viral fitness by decreasing the catalytic activity, substrate binding and thermostability of the NA enzyme. Consistently, protein modeling suggested that these residues could either directly bind to the substrate or contribute to the formation of the active site in the enzyme. Importantly, the HCA-2 mAb, which targets this universal peptide, significantly decreased NA enzymatic activity and showed broad in vitro inhibitory effect against antigenically diverse influenza A strains from all NA subtypes (N1-N9). It also provided heterosubtypic protection in mice challenged with lethal doses of mouse-adapted H1N1 and H3N2 strains (representing group 1 and 2 viruses, respectively). Finally, we found that amino acid residues I222 and E227, located in close proximity to the active site of the enzyme, are indispensable for inhibition by this mAb. Conclusions: Collectively, these findings not only reveal the essential role of this unique highly conserved linear sequence in NA function and viral replication, but also provide the basis for evaluating the potential of this sequence in the development of targeted vaccines or in the design of novel antivirals. Moreover, the HCA-2 mAb described here could be developed/humanized for use as a broad-range agent for prophylactic and therapeutic interventions against diverse strains of influenza A viruses.

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LBA-P2-041

Optimal design of serological surveys for emerging respiratory pathogens

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Background: Influenza infection does not always cause illnesses, and uncertainty regarding the number of unobserved infections dominates many other epidemiologic uncertainties, especially during the early stages of a pandemic. One way to determine the true size of an influenza outbreak is to perform serologic surveillance. However, there are a number of alternative study designs, and it is not clear how to choose between them to design the best study for measuring the cumulative infection attack rate (CAR). Materials and Methods: We simulated the CAR with a known influenza epidemic up to different points in time with a mathematical model, and we estimated the distribution of baseline titres and postinfection boosting of titres from the literature. We calculated the true CAR and expected study-estimated CAR for cross-sectional and longitudinal study designs. We assumed that baseline sera were taken prior to the start of the epidemic, and we compared the accuracy of estimates of the CAR between day 10 and day 100 after the start of local transmission every 10 days. Results: Based on simulation studies, cross-sectional study design and longitudinal study design give comparable estimated CAR. When background level of immune response is moderate, however, cross-sectional study design generally provides more accurate estimates. Under these scenarios, neither study design provides reliable estimates: 1) when the background level is high relative to the postinfection boosting level of the circulating pathogen, or 2) when both the background and postinfection boosting level are low. These observations hold true regardless of the true CAR value. The differences can be explained by the different definitions being used to compute the seroconversion rate in the two survey designs, which was adopted from Wu JT, et al (*Clin Infect Dis*. 2010;51:1184-1191). Conclusion: Levels of boosting and background level of immune responses significantly affect the accuracy of IAR estimations, and depending on these levels of responses, different survey designs should be used to estimate IAR.

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LBA-P2-042

A morbillivirus vaccine vector expressing influenza A virus HA and NP proteins induces robust humoral and cellular immune responses

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Background: Influenza A viruses continue to cause annual epidemics that are associated with considerable morbidity and economic losses, as well as occasional pandemics with worldwide impact. Even though the available vaccines protect against antigenetically matched strains, the high genetic plasticity of these viruses makes it challenging to accurately predict emerging viruses, leaving the population unprotected. We have previously observed that a natural infection with influenza confers protection from reinfection with the same and in some cases heterologous strains. Here we investigated if this protection can be reproduced by presentation of influenza proteins in the context of a viral vaccine vector that also targets the respiratory tract. Materials and Methods: Toward this end, we produced a replication-competent canine distemper virus vaccine strain carrying the nucleo (NP)- and hemagglutinin (HA) proteins of a seasonal H1N1 influenza strain and assessed its ability to confer protection against homologous or heterologous H1N1 and H3N2 strains in ferrets. Results: After intranasal infection, the ferrets immunized with the influenza protein-carrying virus mounted CDV and influenza-specific humoral and cellular immune responses. However, the CDV-specific responses were weaker than those seen in animals immunized with the parental CDV strain. When challenged with the homologous and to a lesser extent heterologous influenza strains, the animals experienced a

shortened period of virus replication and fever. Conclusion:This morbidity reduction may be sufficient to alleviate the disease severity of a highly pathogenic influenza strain and thus provide a first line of defense against a future pandemic.

LBA-P2-043

Nasal washes and nasopharyngeal swabs to detect and quantify viral replication using the influenza Human Viral Challenge Model: how does the sampling methodology affect the result?

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Background: The Human Viral Challenge Model of infection provides a unique experimental opportunity whereby healthy human volunteers are inoculated at a defined time point with a wild-type, attenuated virus under controlled conditions. This has enhanced our understanding of how respiratory viruses like influenza behave under such conditions. The model allows us to examine a number of other parameters related to experimental infection and disease severity using subjective (self-reported symptom diary card scores), and objective (spirometry, vital signs) measures and their temporal relationship to viral shedding. Materials and Methods: As part of a program to develop the Human Viral Challenge Model, we explored the sampling methodology used to evaluate the level of virus replication in the volunteers. The Human Viral Challenge Model is unique in that the exact time of exposure to virus is known and sampling can be timed as required, allowing a detailed analysis of viral replication and its relationship to the signs and symptoms of illness. We wished to increase the frequency with which we could take nasal samples, but were limited to twice-daily sampling via nasal wash due to the possible effect of the procedure on viral replication and the tolerability for the volunteers; we therefore investigated the use of flocculated nasopharyngeal swabs three times daily. Here we present data on healthy volunteers inoculated with a wild-type strain of influenza (A/Wiscconsin/67/2005, H3N2) that was manufactured under GMP conditions and had previously been assessed extensively by ourselves for safety, pathogenicity and virulence. Results: We showed that flocculated nasopharyngeal swabs were more sensitive than nasal washes at detecting virus and provided a smoother viral shedding curve, which could improve statistical sensitivity when used in the model to evaluate the efficacy of novel antiviral compounds. We also observed that the sampling technique chosen and the frequency of the sampling could have an effect on subsequently observed results, in particular that the nasal wash appeared to have a substantial effect on subsequent samples for several hours following the procedure. Conclusions: Nasopharyngeal swabs are more sensitive and more consistent when sampling influenza virus replication in the Human Viral Challenge Model. Nasal washes have an effect on viral replication in nasal mucosa that affects subsequent sampling for several hours following the procedure. The use of nasopharyngeal swabs rather than washes is likely to improve the sensitivity of the Human Viral Challenge Model in evaluating new antiviral compounds.

LBA-P2-044

Virus shedding from the nasal mucosa is closely correlated with illness severity in the Human Viral Challenge Model

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Background: The Human Viral Challenge Model of infection provides a unique experimental opportunity whereby healthy human volunteers are inoculated at a defined time point with a wild-type, attenuated virus under controlled conditions. This has enhanced our understanding of how respiratory viruses like influenza behave under such conditions. The model allows us to examine a number of other parameters related to experimental infection and disease severity using subjective (self-reported

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symptom diary card scores), and objective (spirometry, vital signs) measures and their temporal relationship to viral shedding. Materials and Methods: As part of a program to develop the Human Viral Challenge Model, we analysed the placebo data from several previously conducted clinical trials that occurred over multiple quarantines in a purpose-built facility designed for the conduct of such trials. Importantly, the volunteers were isolated from each other in individual en-suite negative pressure rooms, rather than on open wards common to phase I units. Strict infection control procedures were followed during the trials to prevent cross-infection with the challenge virus between volunteers and the accidental introduction of another virus into the unit from a staff member or volunteer. The Human Viral Challenge Model is unique in that the exact time of exposure to virus is known and sampling can be timed as required along with recording of volunteer self-reported symptoms and physician-directed physical examination, thus allowing a detailed analysis of viral replication and its relationship to the signs and symptoms of illness over time. Here we present data from healthy volunteers inoculated with a wild-type strain of influenza (A/Wisconsin/67/2005, H3N2) that was manufactured under GMP conditions and had previously been assessed extensively by ourselves for safety, pathogenicity and virulence. Results: We found that there was a strong correlation between virus replication in the nasal mucosa and the severity of symptoms when plotted over time. Primarily we observed upper respiratory tract symptoms as expected with this model. Physician-directed examination were not particularly revealing. Importantly, we observed very similar results over multiple studies, multiple quarantines and for different influenza viruses. Conclusions: The severity of infection as determined by virus shedding is directly related to the severity of illness as determined by the self-reported symptoms of volunteers in the Human Viral Challenge Model. Upper respiratory tract symptoms are primarily observed in the Human Viral Challenge Model; however, mild lower respiratory tract symptoms are also observed. Consistent results are observed between multiple studies, multiple quarantine sessions and different influenza viruses.

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LBA-P2-045

Using the Human Viral Challenge Model to design subsequent field-based studies

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Background: The Human Viral Challenge Model of infection provides a unique experimental opportunity whereby healthy human volunteers are inoculated at a defined time point with a wild-type, attenuated virus under controlled conditions. This has enhanced our understanding of how respiratory viruses like influenza behave under such conditions. The model allows us to examine a number of other parameters related to experimental infection and disease severity using subjective (self-reported symptom diary card scores), and objective (spirometry, vital signs) measures and their temporal relationship to viral shedding. Materials and Methods: As part of a program to develop the Human Viral Challenge Model, we have conducted a retrospective analysis of data from our own and other groups' studies to understand how results from such studies can be used to better design subsequent field-based studies. The Human Viral Challenge Model is unique in that the exact time of exposure to virus is known and sampling can be timed as required along with recording of volunteer self-reported symptoms and physician-directed physical examination. This allows for a detailed analysis of viral replication and its relationship to the signs and symptoms of illness over time and thus gives the opportunity to evaluate the efficacy of novel antivirals and vaccines. The FDA has described the model as useful to assist the design of subsequent field studies and specifically possible doses of the vaccine or antiviral and the dosing regimen. As a group we have inoculated more than 1200 volunteers with several different influenza viruses, respiratory syncytial virus and human rhinovirus; previously other groups have conducted similar research. Results: We found that the Human Viral Challenge Model has demonstrated utility in guiding the design of subsequent field trials conducted by other groups. In addition, the model has been useful in better understanding of the immune response to influenza at a humoral and cellular level, correlates of protection and the possible mechanisms of action of novel antiviral compounds or vaccines. Conclusions: The Human Viral Challenge Model can provide useful guidance for the subsequent design of larger field-based studies. By its nature, the model allows for the detailed examination of the mode of action of novel antivirals or vaccines with the

ability to take multiple samples from volunteers under controlled conditions. The Human Viral Challenge Model has been shown to be predictive of the results of subsequent field-based studies in several case studies.

LBA-P2-046

Development of a universal influenza vaccine based on a conserved epitope in HA2

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Background: Influenza is one of the most common and serious diseases caused by influenza viruses in the respiratory track. Although inactivated influenza viruses have been widely used as preventive influenza vaccines, they provide only limited protection to mostly homologous virus strains. Recently, it has been demonstrated that monoclonal antibodies to HA2 region exhibited neutralizing activity and conferred cross-protection. Materials and Methods: We investigated the efficacy of novel influenza vaccine based on a conserved epitope in HA2 region. In order to stabilize the epitope peptide and generate spatial multimeric structure, HA2 epitope was fused with host-originated surfactant protein A (SPA). After verifying the multimerization of SPA-HA2 epitope by native PAGE, the recombinant SPA-HA2 epitope fusion protein was injected into mice intramuscularly. Two weeks after the second immunization, lethal doses of influenza viruses (A/Puerto Rico/8/1934 and A/California/04/2009) were challenged. Results: While HA2 epitope alone induced little antibody response, the mice immunized with SPA-HA2 epitope fusion protein exhibited a substantial epitope-specific IgG response. More importantly, injection of recombinant SPA-HA2 conferred protective efficacy against the lethal dose ($10LD_{50}$) of A/Puerto Rico/8/1934 and A/California/04/2009 virus challenge. Conclusions: These data demonstrate that the conserved epitope in HA2 is a promising vaccine target for the induction of cross-reactive immune responses and SPA-fused multimeric HA2 epitope could be an effective influenza vaccine candidate.

LBA-P2-047

G FELLOWSHIP GATES Fellowship

Prevalence of avian influenza and associated risk factors in live birds retail stalls of Lahore District, Pakistan

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Background: Live-bird markets (LBMs) are an important component of traditional poultry-marketing systems in many developing countries. During the last decade, these markets, also-called 'wet' markets, have been recognized as important for the maintenance of and genetic exchange among avian influenza viruses (AIVs). The risks associated with these markets are exacerbated in places where large numbers of poultry are maintained with poor biosecurity (which is the case in many Asian countries) and provide good places where viral reassortment and interspecies transfer can take place because they bring humans and poultry of different types together, such as chicken, quails, pigeon, pheasant, guinea fowl, chukar, and partridge. These markets are therefore hypothesized to be a

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missing link in the epidemiology of avian influenza viruses. In Pakistan, live bird retail shops (LBRSs) are the preferred source of fresh poultry meat and are scattered throughout cities and rural areas, but their characteristics have not been studied. Usually the birds are slaughtered on-site. The markets sell mainly broiler and indigenous “desi” birds, but in some markets, quails, sparrows, and ducks are also sold. The risk of avian influenza is higher in these markets because of poor biosecurity, improper cleaning, and mixing of birds from multiple suppliers in the same shop. This study was planned to determine the prevalence of avian influenza subtypes H9N2, H5N1, and H7N3 in these shops and associated risk factors to that prevalence. Materials and Methods: A cross-sectional survey of LBRSs in Lahore district was conducted during December 2009-February 2010 using 2-stage cluster sampling with probability proportional to size sampling (PPS). Of 9 towns of Lahore, 8 clusters were selected randomly (8 clusters from 7 towns); that is, 1 town was selected twice as a primary sampling unit. From each cluster, 35 LBRSs were selected as enumerating units. For the systematic selection of each enumerating units, the first LBRS was selected from a starting point in each selected town ie, commercial area and then every fifth LBRS along the way on a random side until the desired number of the sample was obtained. At each LBRS, 5 tracheal swabs were obtained from birds of broiler or desi type, selected at random, and pooled. A total of 280 samples were collected and were diagnosed either positive for AIV (H9N2, H5N1, H7N7) or negative by real-time RT-PCR testing. A structured questionnaire was used to collect data about the prevalence and potential risk factors associated with that prevalence from owner/supervisors of selected LBRS. Data on 36 potential risk factors were obtained. Point estimates of virus prevalence in the LBRS with associated 95% CI, and prevalence odd ratios with 95% CI for each explanatory variable were calculated using survey and Zelig package in R software respectively. Results: There were no positive samples for H5 and H7 subtype while 28 samples were positive for H9. The overall prevalence of subtype H9N2 was 10.0% (95% CI: 6.4%-13.6%). The final weighted logistic regression model identified 3 variables as potential risk factors associated with AIV H9N2 in these LBRS. These included adding new birds to cages already having old birds (OR = 9.2, CI 95%: 2.4-35.1), mode of purchase of birds for stall, that is, either from wholesale dealer or mix sources of purchase (OR = 3.4, CI 95%: 1.3-8.8), and birds being housed both indoors and outdoors (partly inside the cages for some time and are allowed to come outside the cages during the day) (OR = 1.7, CI 95%: 1.0-3.0). Conclusions: This study confirmed the prevalence of AIV H9N2 in LBRs in Lahore and supported the hypothesis that H9N2 is circulating and possibly perpetuating in these LBRSs. These findings support the requirement for biosecurity and continuous monitoring of these LBRSs to control not only H5 and H7 HPAI infections but also H9N2 virus. The study results can be used to develop a risk-based approach for AIV risk reduction in LBRSs.

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LBA-P2-048

Differential cytokine responses in intranasally and intramuscularly VLP-vaccinated mice

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Background: Despite available conventional vaccines, outbreaks of influenza virus are a major public health burden worldwide. More recently, influenza virus-like particles (VLPs) have been suggested to be a promising vaccine approach. In this study, we investigated antibody responses and the levels of cytokines produced locally and systemically after influenza vaccination of mice intranasally and intramuscularly. Materials and Methods: We produced influenza virus-like particles (VLPs) containing matrix (M1) and hemagglutinin (HA) proteins derived from the A/ A/PR8 virus, and evaluated their efficacy as a vaccine in mice. Female inbred BALB/c mice aged 6 to 8 weeks were used. Mice (8 mice per group) were intranasally and intramuscularly immunized with 20 ug of VLPs 3 times (weeks 0, 2, and 4) in 50 ul of PBS at 2-week intervals. For virus challenge, anesthetized mice were intranasally infected with 10 LD50 (the 50% lethal dose) of A/PR8 virus in 50 ul of PBS per mouse 8 weeks after the final immunization. Influenza virus-specific antibodies (IgG and IgA) were determined in sera by ELISA and haemagglutination inhibition test. Gene expression of cytokines (IL-6, IL-10, IL-17, IL-28b, IFN-gamma and TNF-a) and CD73 (NT5E 5'-nucleotidase, ecto) in spleen and whole lung homogenates was measured by real-time PCR. Results: We found that influenza vaccinated mice elicited protective levels of serum haemagglutination inhibition antibodies (IgG) and a significant local

antibody response (IgA) in the lung. A significant increase in virus-specific IgG and IgA antibody levels was detected in all intranasally than intramuscularly vaccinated mice. Expression of IL-6, IL-10, IL-17, IL-28b, IFN-gamma, TNF-a, and CD73 mRNA was down-regulated 2- to 6-fold in both spleen and whole lung homogenates intranasally than intramuscularly vaccinated than mock control mice after virus challenge. A low but significantly level of IL-10 and CD73 was detected in whole lung homogenates of intranasally than intramuscularly mice. Conclusions: This study shows that VLP vaccination provides highly effective protection against the influenza virus. The high level of IL-10 and CD73 can improve lung dysfunction after influenza infection. Also, the cytokine profiles suggest that the route of immunization may influence vaccine outcomes.

LBA-P2-049

Community immunity: overall and indirect effectiveness of school-located influenza vaccination programs in Alachua County, Florida

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Background: In the United States (US), seasonal influenza kills about 36,000 people and costs ≈ $87 billion dollars each year. Despite high vaccination coverage in the elderly population, 90% of influenza-related deaths occur among those 65 and older. An alternative strategy motivated by mathematical models supports immunizing school-aged children to indirectly protect the elderly. This approach is supported by the facts: a) schools are an effective source of community transmission, b) children shed more virus and do so for a longer duration than adults, c) the live attenuated influenza vaccine (LAIV) is ≈ 80% effective in preventing illness and transmission in children. Within the past decade, communities across the United States have implemented school-located influenza vaccination (SLIV) programs, recognizing it as a method for increasing influenza vaccination among school-aged children. Since 2010/11, the Control Flu SLIV program has offered free LAIV to all healthy children attending public and private schools (Pre-K to 12th grade) in Alachua County, Florida. The overall immunization rate of school children through the Alachua SLIV program was ≈ 37% (2011/12) and ≈ 41% (2012/13). The program estimates an additional 10%-15% of children receive a vaccination from their provider. Here, we estimate the overall and indirect effectiveness of the SLIV program towards reducing burden of influenza-like illness (ILI) in Alachua relative to the other counties in Florida for the 2011/12 and 2012/13 influenza seasons. Materials and Methods: We collected surveillance data for the weekly number of visits and the proportion of visits associated with ILI (chief complaint with the word “influenza” or “flu” or that contains the word “fever” plus “cough” and/or “sore throat”). Data were obtained from the Florida Department of Health Electronic Surveillance System for the Early Notification of Community-based Epidemics (ESSENCE) system, which collects data from 180 hospital emergency departments and urgent care centers in the state. Data were available by county of residence and by age group (5-17, 18-44, 45-64, and 65+ years). County and age-specific population counts were obtained from the 2010 US Census. The Centers for Disease Control and Prevention guidelines were used to determine the 2 influenza seasons. Analysis: Crude attack rates (AR) for ILI-associated visits per 100,000 population were calculated by age group and for geographic regions: Alachua, Region-3 (12 counties surrounding Alachua), and Florida, excluding Alachua. The unadjusted effectiveness of the program was estimated by 1 minus the ratio

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of the AR's for an age group in Alachua versus Region-3 and Florida. The overall effectiveness in the program's target age group was estimated by comparing the AR among those aged 5-17 years and the indirect effectiveness was estimated for older age groups. All estimates are reported with 95% CI. Results: For all age groups and both epidemic seasons, Alachua had a substantially lower crude AR for ILI-associated visits than Region-3 or Florida. During the 2011/12 season, the AR among those aged 5-17 years was 103 per 100,000 (CI: 73, 145) in Alachua versus 597 (CI: 571, 625) in Region-3 and 523 (CI: 514, 531) in Florida. Large differences in AR were observed for the 18-44 and 45-64 age groups, and a smaller difference was observed among 65+ year-olds. The unadjusted overall effectiveness of the program was 80% (CI: 72%, 86%) among those aged 5-17 years in Alachua compared to Florida. The unadjusted indirect effectiveness among those aged 18-44 years was 59% (CI: 51%, 60%), 45-64 was 48% (CI: 26%, 64%) and 65+ was 22% (CI: -26%, 53%). The trend is similar for 2012/13, with significant effectiveness in all but the oldest age group; however, the unadjusted overall effectiveness and unadjusted indirect effectiveness was lower. Conclusions: Our results demonstrate that high immunization coverage among school-age children through the Alachua SLIV program has produced a substantial reduction in the risk of symptomatic influenza infection in the target age group and, more interestingly, among older members of the same community. These results support the conclusion that SLIV programs provide a viable and effective way to reduce the influenza burden in a community.

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LBA-P2-050

The importance of virus origin and red blood cells uniformity in the inhibition of haemagglutination assay for the titration of serum antibodies against influenza viruses

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Background: Influenza vaccines are marketed mainly based on their capacity to induce serological responses. The human immune system produces specific antibodies against the most antigenic viral proteins, mainly the haemagglutinin (HA) and neuraminidase surface proteins. The viral haemagglutinin has the ability to agglutinate red blood cells. Serum antibodies against the HA are often titrated using the inhibition of haemagglutination assay. This assay uses viruses at specific dilutions and red blood cells from a chosen animal species, mainly from turkey. The pattern of glycosylation of the viral HA protein and the sialic acids on the surface of red blood cells (alpha 2,3- and alpha 2,6-linked) are major determinants of assay variability. Interassay and interlaboratory assay reproducibility is a key aspect of this assay that should be closely monitored. This study characterizes the role of red blood cells source and the importance of the virus passage history in the titration of serum specific antibodies. Materials and Methods: 200 sera samples from healthy volunteers were first heat inactivated and then treated with recombinant neuraminidase to abolish nonspecific reaction. A/Perth/16/2009 Influenza A strain virus propagated on MDCK cells or allantoic fluid of embryonated chicken eggs was titrated for HA content using red blood cells from turkeys. Human sera were serially diluted in PBS and incubated with 4 to 8 haemagglutination units (HAU) for 30 minutes in 96-well V-bottom plates. 0.5% suspension of red blood cells from different turkeys was then added to the plates and after 30 minutes the haemagglutination was assessed. Commercial sheep anti serum was used as standard in all experiments; in addition human sera samples with known level of inhibition were included. Results: The method of virus propagation was shown to play an important role in determining the distribution of results and the lower limit of specific antibodies detection. There was a significant difference between results obtained with virus grown on embryonated eggs and MDCK cells, the former giving lower "not detectable antibodies" results when testing the same set of samples. Viruses propagated on MDCK cells tended to give higher HA inhibition units than those propagated on eggs and therefore increasing the sensitivity of the detection. When the same sera were tested against an identical virus using red blood cells from different turkeys, there was up to 10% difference in the number of "not detectable antibodies" samples. Conclusions: When assessing a serological status, it is crucial to design the assay accordingly. The virus used should preferably be propagated on cells, and animals from which red blood cells are obtained should be first extensively tested

against controlled reagents. Testing vaccine efficiency on sera samples pre- and postvaccination should be done using red blood cell from the same animal or animals with identical response patterns.

LBA-P2-052

Natural human interferon (α-n3) is active against both wild-type and Tamiflu-resistant avian H7N9 influenza virus

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Background: In February, 2013 H7N9, a new, deadly strain of avian influenza, was first identified in Shanghai but cases have since been confirmed in various eastern and inland Chinese provinces. There have been a total of 132 confirmed cases with 43 deaths to date with people over 60 appearing to be more susceptible to the disease. Patients have exhibited viral pneumonia, acute kidney failure, acute respiratory distress syndrome, diffuse intravascular coagulation and septic shock. Although H7N9 can be highly virulent in humans, in poultry H7N9 produces very few, if any, symptoms. This unusual property makes this outbreak harder to detect and monitor. Experts remain fearful of the possibility of the virus mutating into a form easily transmissible between humans, although to date, there has been no confirmation of human-to-human transmission. A recent study has shown that H7N9 strains with a dominant K292 were resistant to zanamivir, peramivir, and oseltamivir, although resistance can be masked by a mixed R/K viral population (Yen 2013). In studies in SARS, Alferon was tested against other interferons and was the most active interferon against SARS at clinically achievable serum levels that does not induce neutralizing antibodies (Tan 2004). Materials and Methods: Tamiflu (oseltamivir) and Alferon were tested in A549 cells for antiviral activity in vitro singly against the wild-type human H7N9 isolate called A/Anhui/1/2013 (wt Anhui 1), and a reverse genetically created neuraminidase mutant A/Shanghai/1/2013 (NA-292K; Shanghai 1-NA292K) with resistance to oseltamivir. Results: The wt Anhui 1 was sensitive to both Tamiflu and Alferon. The Shanghai 1-NA292K virus was resistant to Tamiflu treatment, but was sensitive to Alferon when tested in A549 cells. Conclusions: Initial results showed that Tamiflu and Alferon have significant inhibitory effect on the wild-type H7N9 virus. Only Alferon and not Tamiflu had an inhibitory effect on oseltamivir-resistant neuraminidase mutant Shanghai 1-NA292K. Our results suggest new therapeutic strategies to mitigate the health hazards associated with the infection and the potential pandemic spread of Tamiflu resistant human influenza viruses.

LBA-P2-053

Influenza and risk of mortality among people with chronic medical conditions

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Background: Chronic medical conditions, such as cardiovascular diseases, airway diseases, diabetes, are well recognised as risk factors for influenza complications, but there was weaker evidence of an association with mortality among populations with preexisting chronic medical conditions. The objective of our study was to estimate the effect of influenza circulation on mortality of people with chronic medical conditions, from a variety of causes in addition to respiratory death. Materials and Methods: A prospective cohort of Chinese people aged 65 years and over was followed up from July 1998 to May 2012 in Hong Kong, and linked with population vital records. The chronic medical conditions history was recorded at the beginning of the study. We fitted Cox proportional hazard

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models with time dependant variables of weekly influenza activity (by type and subtype) to estimate the hazard ratio [HR] of influenza on all-cause and cause-specific mortality. Influenza activity was measured by the weekly proportion of outpatient consultations with influenza-like illness multiplied by the proportion of laboratory specimens that tested positive for a specific type and subtype of influenza. Results were presented in terms of the difference in risk of mortality associated with influenza activities within the influenza season compared to outside the influenza season. Potential confounding factors such as temperature and humidity as well as other main respiratory virus circulation were included in the model. Results: The cohort included 61,560 subjects at Elderly Health Centres with a median follow-up period of 12 years. 54% of subjects have at least 1 of chronic diseases, including cardiovascular disease (48%), diabetes (12%), and chronic obstructive airways disease (COAD) (5%). For subjects with COAD, influenza was associated with a 47% increase in all-cause mortality rate (HR, 1.47; 95% CI, 1.41-1.53), a 2-fold increase in respiratory mortality rate (HR, 2.02; 95% CI] 1.86-2.21) compared with subjects without COAD. Influenza seasons were associated with an 11% (HR, 1.11; 95% CI, 1.07-1.14) increase in all-cause mortality among subjects with vs without cardiovascular diseases; were associated with a 28% (HR, 1.28; 95% CI, 1.24-1.33) increase in all-cause mortality and a 7% (HR, 1.07, 95% CI: 0.97-1.18) increase in respiratory mortality among subjects with vs without diabetes. Conclusions: There was substantially increased mortality risk among people with chronic medical conditions during influenza epidemics. Findings from our study highlight the need to encourage vaccine uptake wherever indicated, especially for those people with established chronic medical conditions in high influenza circulation seasons.

LBA-P2-054

Infection of the human-isolated H7N9 influenza virus in rats

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Background: The emergence of the H7N9 influenza virus in humans in Eastern China has raised concerns of a new influenza pandemic that might occur. This virus was generated through multiple reassortment events between H7, N9, and H9N2 avian influenza viruses, carrying a number of amino acids associated with mammalian adaptations. Whether a mammalian intermediate host was involved in the genesis and transmission of this virus is unknown. As the most prevalent small mammals in China, and probably the most frequently seen free mammals around the local wet markets, the susceptibility of domestic rats to this novel H7N9 was investigated. Materials and Methods: To test the infectivity and pathology of the H7N9 virus in rats, we intranasally inoculated 106 times the median tissue culture infectious dose (TCID50) of the A/Shanghai/02/2013 (SH2), an H7N9 human isolate from a fatal index case, to the Sprague Dawley® rats (n = 6). At 4 days postinoculation (dpi), 3 rats were sacrificed to examine the gross pathology and virus distributions in different tissue types. Seroconversions were evaluated using standard hemagglutination inhibition (HI) test. Results: Although the inoculated rats did not show overt clinical signs of infection within the 14 days postinoculation (dpi), virus replication was detected in the nasal turbinates, trachea, alveoli, and bronchioles at 4 dpi. Moderate inflammatory infiltrates with viral nucleoprotein-positive cells were evident in the lungs. All inoculated rats seroconverted by 21 dpi. These results suggest that the novel H7N9 influenza virus can readily infect domestic rats, causing mild respiratory infections and pneumonia. Conclusions: Domestic rats are not normally considered as the natural host for influenza A viruses. To achieve reproductive infections in the laboratory rat strains, such as SD rats, prior adaptation by serial passage in the host is needed. Here we demonstrated that the recent human-isolated H7N9 influenza virus in Eastern China can readily replicate in the respiratory tract of SD rats, and cause self-limited pneumonia. The possibility for the H7N9 virus to be spread by domestic rats cannot be excluded. Close monitoring of this virus in animals that may be exposed is warranted to ensure efficient disease control.

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LBA-P2-056

Cost effectiveness of a quadrivalent versus trivalent influenza vaccine in the United States

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Background: Currently used trivalent influenza vaccines (TIVs) contain two strains of influenza A and one strain of influenza B. However, cocirculation of two distinct B lineages and difficulties in predicting which lineage will predominate in the next season have led to frequent B-strain mismatches. Newly registered quadrivalent influenza vaccines (QIVs) include two B strains and might therefore provide better protection. The aim of this study was to assess the cost effectiveness of using QIVs vs TIVs for routine influenza vaccination of the US population. Methods: A decision-tree model was developed to determine the cost effectiveness of replacing TIVs with QIVs over a time horizon of 10 years. For this purpose, results from an age-structured dynamic transmission model were used, estimating the additional protection offered by QIV over TIV against symptomatic influenza B disease. Assumptions included a vaccine efficacy of 60% and a vaccination coverage varying from 30% to 68% among different age groups. Age-specific data on influenza-related outpatient visits, hospitalizations, quality-of-life losses, and deaths for the United States were obtained from published sources to estimate the medical and societal burden. Costs and health outcomes allowed calculating the incremental cost-effectiveness ratio (ICER) of QIVs vs TIVs. Results were presented from the societal perspective as well as from the third-party payer's (TPP) perspective. Costs and health effects were equally discounted at 3%. Results: Depending on the incidence of influenza B and other variables, the transmission model predicted that replacing TIVs with QIVs could prevent 11.7 to 19.5 million symptomatic influenza B cases. According to our model, this would result in a reduction of 10,700-16,400 deaths, 177,000-285,000 hospitalizations, and 4.3-7.2 million outpatient visits during a decade. Associated savings were estimated at $5.9-$9.3 billion for medical costs and $6.9-$10.6 billion for productivity losses. The total gain of quality-adjusted life years (QALYs) was estimated at 191,000-300,000. QIVs were dominant or cost effective at significant price premiums vs TIVs. Parameters significantly influencing cost effectiveness were the number of prevented influenza B cases as provided by the dynamic transmission model, incremental vaccine price, hospitalization probabilities, hospitalization costs (both perspectives), and productivity losses due to influenza-related death (societal perspective). Conclusion: Our model predicts that introducing QIVs into the immunization program of the United States prevents a significant number of deaths and reduces the disease burden and health care costs as well as societal costs. Moreover, dominance or favorable cost effectiveness was shown from both the societal and the TPP perspective for a broad range of vaccine prices.

LBA-P2-057

Molecular epidemiology of influenza A virus infections in swine at agricultural fairs in the United States of America

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Background: The swine-human interface is a critical element in the ecology and epidemiology of influenza A viruses infecting humans. Historical evidence demonstrates that bidirectional zoonotic

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virus transmission across this interface can result in influenza outbreaks, epidemics, epizootics, and/or pandemics. Agricultural fairs in the United States create a unique swine-human interface that is conducive to intra- and interspecies influenza A virus transmission. Following the unprecedented number of reported variant influenza A infections in humans in the United States during the previous three years, retrospective investigations have implicated the swine-human interface at fairs as the source of virus for the vast majority of the variant influenza A cases. Here we investigate the molecular epidemiology of influenza A virus infections occurring in exhibition swine at agricultural fairs. Materials and Methods: The focus of this study was 199 influenza A virus isolates recovered from swine at agricultural fairs in Ohio during 2009-2011. The isolates were recovered as part of an ongoing active influenza A virus surveillance project and represented 12 fair events (3 in 2009, 3 in 2010, and 6 in 2011). Comparative genomic analysis was conducted using the full-length nucleotide sequences of the 199 isolates and the full-genome sequences of all the swine-origin influenza A virus isolates dating back to 2000 that were publicly available in GenBank. Also included in the analysis were the sequences of variant influenza A virus isolates recovered from humans during 2009-2011. Multiple sequence alignments were performed for each viral genome segment, and maximum likelihood trees were inferred using GARLI with a general time-reversible nucleotide substitution model. Results: The analysis of each gene segment for our isolates recovered from swine at Ohio fairs show a clear segregation by year; demonstrating a distinctly different collection of viruses. Within each year there is minimal segregation by fair, indicating the same viral strains became disseminated across multiple fairs within each fair season. Nearly all the variant influenza A virus isolates recovered from humans in other states fall within, or very close to, the clades containing isolates from the pigs at Ohio fairs, suggesting exhibition swine across a larger geographic area were concurrently infected with the same viral strains. While there is a large amount of genetic diversity among influenza A viruses infecting North American swine, only a small proportion of that diversity was reflected in the isolates recovered from the exhibition swine sampled in this study. The general lack of diversity within each fair indicates the number of viral introductions at each fair is limited, but there is subsequent viral spread among the pigs during the exhibition. A total of 18 mixed infections were detected in our collection, and all mixed infections occurred in samples collected during 2011. The internal gene segments of the H1 and H3 subtypes from our collection are interleaved, demonstrating that reassortant influenza A viruses were recovered from swine at these agricultural fairs. Conclusions: The segregation of strains by year and the recovery of highly similar strains across multiple fairs within a year suggest that exhibition swine are subject to their own “seasonal influenza” epidemics. The viruses that become established in exhibition swine each year, and subsequently infect humans at agricultural fairs, appear to be spillover introductions from the larger commercial swine population. Pigs have been previously identified as “mixing vessels” for influenza A viruses, and the identification of mixed infections in our collection supports the hypothesis that fairs are settings that not only encourage intra- and interspecies influenza A virus transmission, but are also ideal locations for genomic reassortment and novel virus formation. The public health threat from emerging novel influenza A virus strains mandates steps be taken to mitigate the transmission of influenza A virus at agricultural fairs. Mitigation strategies aimed at controlling influenza A virus in exhibitions swine prior to the fairs should be used in conjunction with current strategies that are focused on preventing zoonotic transmission.

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LBA-P2-059

Assessing seasonal influenza vaccination coverage by global vaccine dose distribution (2004-2011)

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Background: Licensed influenza vaccines are safe and efficacious and can prevent significant annual morbidity and mortality. The World Health Organization (WHO) currently recommends seasonal influenza vaccination for pregnant women, children aged 6 to 59 months, the elderly, individuals with

specific chronic medical conditions, and health care workers. With an abundant industrial capacity to supply seasonal influenza vaccines, vaccination coverage rates could meet targets. Yet influenza vaccination coverage remains below recommended levels in many countries. In Europe, seasonal influenza vaccination coverage is around 44% of the target population, and in the USA it is approximately 42%. Consolidated worldwide data have not been available to assess seasonal influenza vaccine uptake, and to review progress toward vaccination coverage targets. In 2008, the International Federation of Pharmaceutical Manufacturers & Associations Influenza Vaccine Supply task force (IFPMA IVS) developed a methodology to serve as a proxy for assessing seasonal influenza vaccination coverage by accounting for numbers of vaccine doses distributed globally. Materials and Methods: Member companies of the IFPMA IVS, who collectively manufacture approximately three-quarters of the world's seasonal and pandemic influenza vaccines, provided information on the supply of seasonal trivalent influenza vaccine doses to all WHO member states in 2010 and 2011. To ensure compliance with antitrust regulations, the survey results were collected and aggregated by an independent third-party legal counsel and combined with the results of a 2004-2009 IFPMA IVS survey. Doses distributed by country and by year were aggregated and categorized by WHO region. To assess existing barriers and drivers of seasonal influenza vaccination, in 2010 and 2011, a sample of countries were surveyed according to 2008 levels of distribution: low distribution, defined as < 159 doses per 1000 population; and high distribution, ≥ 159 doses per 1000 population (the hurdle rate, defined as the number of doses required to immunize those aged ≥ 65 years in more developed nations. Results: The survey accounted for approximately 79% of doses distributed globally. Global distribution of seasonal influenza vaccines in 2011 represents an approximate 86.9% increase over 2004 distributed doses, but only an approximate 8.8% increase over doses distributed in 2009. Increases in distributed doses have been progressive in all WHO regions since 2004, except in EURO and EMRO, where distributed doses have been declining since 2008 (speculative explanatory factors are for a presentation/publication but not for an abstract). Gross national income did not correlate with dose distribution. Analyses of doses distribution rates showed considerable variability between countries within the same WHO region, and several countries in Europe even had declining dose distribution rates. Using dose distribution as a proxy for vaccination coverage, seasonal influenza vaccination coverage remains well below WHO targets. The WHO regions EMRO, SEARO and AFRO together constitute only 3.7% of all doses distributed, with very few countries accounting for the majority of the doses distributed. Conclusions: In the absence of consolidated global data on seasonal influenza vaccination rates, the IFPMA IVS survey method provides unique insights into the global vaccination coverage with seasonal influenza vaccines. Because the benefits or seasonal influenza immunization are widely recognized, it is surprising to note negative rates of change in dose distribution, particularly in countries of EURO, where the dose distribution per population is higher than in other WHO regions. Renewed and focused efforts to stimulate seasonal influenza vaccination are warranted to ensure that the benefits of vaccination are more widely and evenly distributed across and within WHO regions.

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LBA-P2-060

Influenza virus reassortment occurs with high efficiency in the absence of segment mismatch

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Background: Reassortment is a key mechanism by which antigenically novel influenza viruses arise. These novel viruses can have a significant impact on the population in the form of epidemics and pandemics and therefore a better understanding of the conditions that favor reassortment is needed. Previous studies have focused on co-infection between two divergent strains for risk assessment, to optimize vaccine production and to examine the concept of genetic mismatch. These studies have revealed that reassortment is significantly restricted by genetic incompatibility at either the RNA or protein level. In fact, this effect is so potent that it is often difficult to identify other factors that define reassortment efficiency. To understand reassortment at a more fundamental level, our goal was to determine its baseline frequency in the absence of segment mismatch and evaluate the effects of dosage and timing on that baseline frequency.Materials and Methods: To study reassortment in the

absence of segment mismatch, we developed a novel system in which parental and all reassortant progeny are of equal fitness, eliminating selection bias. A pair of phenotypically identical parent viruses was created through the introduction of silent mutations into each segment of A/Panama/2007/99 virus, resulting in a wild-type and a variant virus. In addition, HIS and HA protein tags, respectively, were incorporated into the HA of each virus. The proportion of co-infected cells was determined at 12h p.i. by flow cytometry. Progeny viruses collected at 12h p.i. were genotyped by RT-qPCR followed by high resolution melt analysis. To examine the mechanisms of super-infection exclusion, the quantity of α-2,6 sialic acids on the surface of MDCK cells was examined using conjugated SNA lectin and flow cytometry. The induction of IFN-β in MDCK cells was determined by qPCR, normalized to canine 18s mRNA. Results: Using this unbiased system, we found that reassortment occurred with high efficiency (86%) following co-infection at a high MOI in cell culture. By varying the multiplicity of infection, we found that the number of reassortant progeny increased exponentially with the number of co-infected cells. Similarly, the number of reassortant progeny obtained from co-infected guinea pigs was dependent on dose: on average, 30% and 59% of viruses isolated were reassortants after co-infections with 10^3 and 10^6 PFU, respectively. The introduction of a delay between primary and secondary infections significantly affected reassortment both in cell culture and in vivo. For robust reassortment, a second influenza virus infection must occur within 8h of the initial infection in cell culture and within 12h in vivo. Enumeration of co-infected cells indicated that super-infection interference was the primary mechanism leading to the reduction in reassortment at later time points. Preliminary experiments suggest that, under the high MOI conditions used, MDCK cells become refractory to super-infection over time due to reduction in cell-surface receptors and induction of IFN-β: surface presentation of α-2,6 sialic acids was reduced by approximately 70% and IFN-β was induced 100-fold relative to mock-infected control cells. Conclusion: Our results indicate that, in the absence of genetic mismatch, reassortment occurs efficiently in both a co-infected cell and a co-infected host but is strongly dependent on both the dosage and timing of infections.

LBA-P2-061

The 2009 pandemic H1N1 influenza virus M, NA, and HA segments are sufficient to promote efficient contact transmission of rPR8-based H1N1 viruses

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Background: Influenza pandemics pose a significant threat to global health, with the most recent 2009 H1N1 virus pandemic causing millions of cases worldwide within its first year of circulation. Although there have been sporadic human infections with swine lineage influenza viruses, the 2009 pandemic (pH1N1) virus was exceptional in its ability to transmit between people. The pH1N1 lineage arose from reassortment between the Eurasian avian-like swine and North American triple reassortant swine lineage viruses. Notably, both parental lineages lack sustained transmission in humans. The viral factors and underlying mechanisms conferring transmissibility to pH1N1 remain largely unknown. Materials and Methods: We utilized a plasmid-based reverse genetics system to rescue a panel of recombinant A/Puerto Rico/8/1934 (PR8) based viruses harboring segments (M, NA, M/ NA, NA/ HA, or M/ NA/ HA) from the pH1N1 strain A/Netherlands/602/2009 (NL602). *In vitro* replication kinetics in MDCK and HTBE cells at 37°C were assessed by plaque assay. Virion morphology was assessed by TEM and SEM of infected MDCK cells and by negatively stained TEM of purified virus. In addition, the fluorogenic sialoside, MUNANA, was used as a substrate for detection of viral neuraminidase activity levels, and resultant enzyme kinetic data was utilized in non-linear regression modeling using the Michaelis-Menten equation. Contact transmission between co-caged guinea pigs was assessed. Naive animals were exposed to intranasally inoculated guinea pigs (1000 PFU of virus) 24 hours post-infection. Virus contact transmission efficiencies and *in vivo* replication kinetics were determined by plaque assay titration of nasal washes from infected and exposed animals collected on days two, four, six, and eight post-infection. Results: The wild type rNL602 virus, but not the wild type rPR8 virus, transmitted efficiently (100%) between co-caged guinea pigs. Additionally, the rPR8 virus containing the M, NA, and HA segments from the pandemic NL602 strain recapitulated the high transmissibility

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(100%) of the wild type rNL602 virus. Removal of the NL HA segment resulted in a slight decrease in transmission efficiency (88%) for the M/ NA virus. As previously reported, inclusion of the pandemic M segment alone lead to an intermediate transmission phenotype (75%), while transmissibility was further reduced for viruses with either the NA (50%) or NA/ HA (25%) segments. A correlation emerged between efficient transmission and filamentous morphology: PR8 virions were largely spherical, whereas the PR8/NL602 M/ NA/ HA virus exhibited a similarly filamentous morphology to that of the rNL602 virus, and other viruses carrying the NL602 M segment had an intermediate morphological phenotype. The increase in total number of filamentous virions also correlated with increases in neuraminidase activity levels. Conclusions: The pH1N1 M segment, as well as the NA segment, was found to be important for the introduction of filamentous virions in the PR8 background. Data demonstrated a correlation between virion filament number and transmission efficiency. A correlation was also found between filament number and levels of viral neuraminidase activity. In addition, the pandemic HA, NA, and M segments resulted in the highest transmission efficiency of the rPR8 virus, recapitulating the phenotype of the wild type rNL602 strain and supporting a role for these segments in the highly transmissible phenotype of the pH1N1 virus. Characterization of the viral factors associated with human transmission will prove invaluable for the identification of novel influenza strains with pandemic potential.

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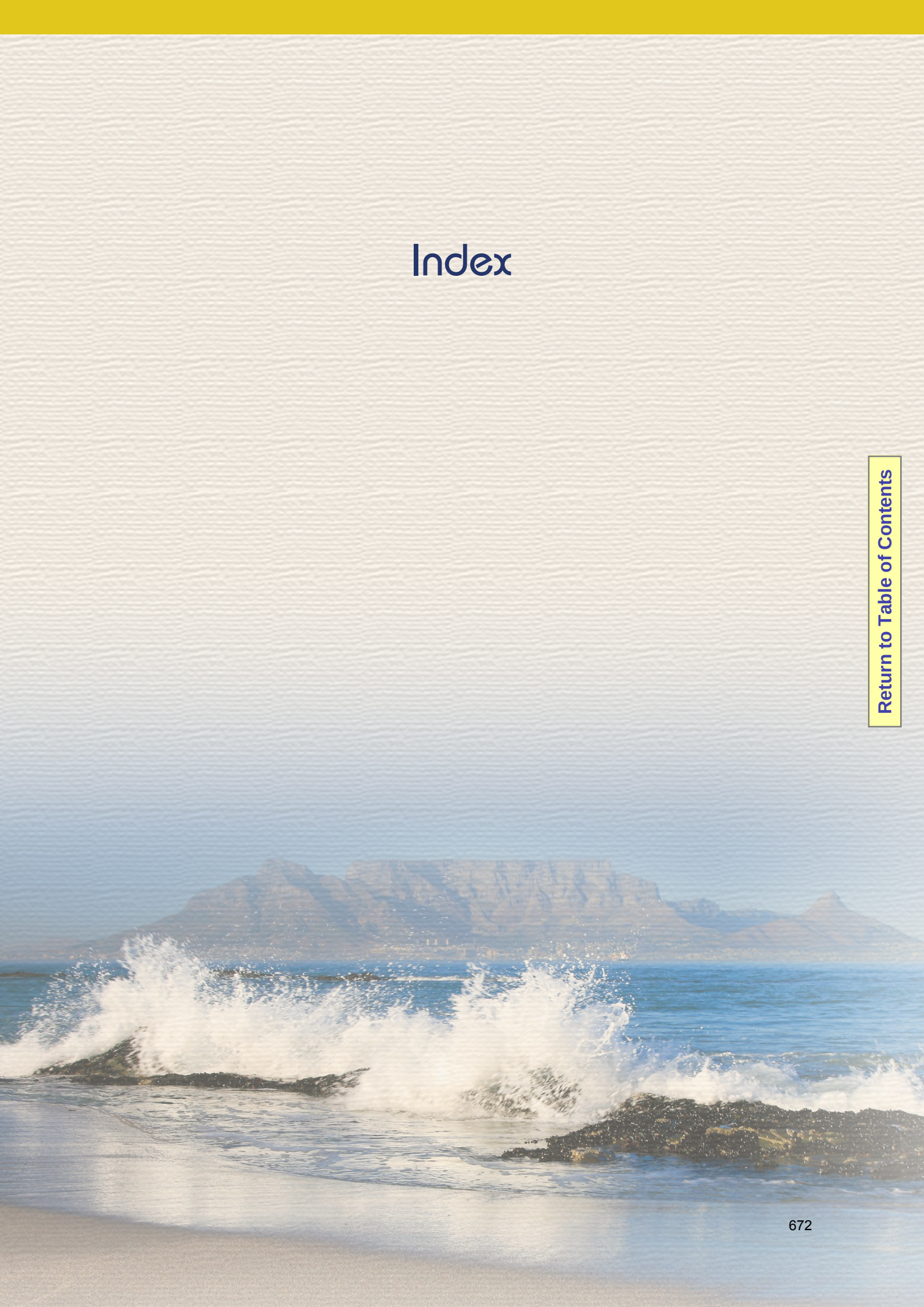

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