Pathogenesis and transmission of novel HPAI H5N2 and H5N8 avian influenza viruses in ferrets and mice

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ABSTRACT

A novel highly pathogenic avian influenza (HPAI) H5N8 virus, first detected in January 2014 in poultry and wild birds in South Korea, has spread throughout Asia and Europe, and caused outbreaks in Canada and the United States by the end of the year. The spread of H5N8 and the novel reassortant viruses, H5N2 and H5N1 (H5Nx), in domestic poultry across multiple states in the U.S. pose a potential public health risk. To evaluate the potential of cross-species infection, we determined the pathogenesis and transmissibility of two Asian-origin H5Nx viruses in mammalian animal models. The newly isolated H5N2 and H5N8 viruses were able to cause severe disease in mice only at high doses. Both viruses replicated efficiently in the upper and lower respiratory tracts of ferrets; however clinical symptoms were generally mild and there was no evidence of systemic dissemination of virus to multiple organs. Moreover, these influenza H5Nx viruses lacked the ability to transmit between ferrets in a direct contact setting. We further assessed viral replication kinetics of the novel H5Nx viruses in a human bronchial epithelium cell line, Calu-3. Both H5Nx viruses replicated to a level comparable to a human seasonal H1N1 virus, but significantly lower than a virulent Asian-lineage H5N1 HPAI virus. Although the recently isolated H5N2 and H5N8 viruses displayed moderate pathogenicity in mammalian models, their ability to rapidly spread among avian species, reassort, and generate novel strains underscores the need for continued risk assessment in mammals.

40 **IMPORTANCE**

41 In 2015, highly pathogenic avian influenza (HPAI) H5 viruses have caused outbreaks in domestic poultry
42 in multiple U.S. states. The economic losses incurred with H5N8 and H5N2 subtype virus infection have
43 raised serious concerns for the poultry industry and the general public due to the potential risk of human
44 infection. This recent outbreak underscores the need to better understand the pathogenesis and
45 transmission of these viruses in mammals, which is an essential component of pandemic risk
46 assessment. This study demonstrates that the newly isolated H5N2 and H5N8 viruses lacked the ability
47 to transmit between ferrets and exhibited low to moderate virulence in mammals. In human bronchial
48 epithelial (Calu-3) cells, both H5N8 and H5N2 viruses replicated to a level comparable to a human
49 seasonal virus, but significantly lower than a virulent Asian-lineage H5N1 (A/Thailand/16/2004) virus. The
50 results of this study are important for the evaluation of public health risk.
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INTRODUCTION

On December 16, 2014, the United States Department of Agriculture (USDA) confirmed the presence of a novel highly pathogenic avian influenza (HPAI) H5N8 virus in a captive gyrfalcon and H5N2 virus in a northern pintail duck from Whatcom County, Washington (1). This represented the first appearance of HPAI H5 virus in the U.S. since 2004 when H5N2 subtype virus was confirmed on a poultry farm in Texas (2). HPAI H5N8 viruses were first reported in duck farms in eastern China in 2010 and in early 2014 a novel reassortant H5N8 virus was detected in poultry in South Korea (3-5). The novel virus, belonging to Eurasian lineage clade 2.3.4.4 (formerly clade 2.3.4.6), subsequently spread to China, Japan and five countries in Europe (6, 7). In November 2014, the virus was detected on chicken and turkey farms in British Columbia, Canada, followed by Washington State, USA the following month (8). The novel H5N8 virus continue to spread to multiple regions and has been found in three North America's flyways (Pacific, Central and Mississippi) where wild bird migrations occur (USDA, Animal and Plant Health Inspection Service [http://www.aphis.usda.gov]). In addition, genetic reassortment with circulating North American avian influenza viruses has resulted in novel HPAI viruses, including H5N2 and H5N1 subtypes. These novel reassortant viruses carry a Eurasian-origin hemagglutinin (HA) gene genetically related to H5N8 viruses detected in South Korea in 2014, and the NA gene from N8, N2, and N1 subtypes (8), and are collectively referred to as H5Nx. Because these HPAI viruses can be spread by asymptomatic wild birds (9) and cause significant mortality in domestic poultry, they pose significant international trade issues and are a potential risk for public health.

Although no human infections with the novel H5Nx viruses have yet been reported in the U.S., human infections with other subtypes of avian influenza viruses have occurred following direct or close contact with infected poultry. Such virus infections in humans have been associated with a number of symptoms ranging from mild influenza-like illness to sometimes fatal respiratory disease (10-12). Since information about the biologic and molecular properties is not sufficient to predict virulence and transmissibility of HPAI viruses in humans, it is imperative that their pathobiological properties be examined using mammalian models. Here we evaluated the virulence and transmission of two novel HPAI viruses: A/northern pintail/Washington/40964/2014 (Pin/WA/40964; H5N2) and

A/gyrfalcon/Washington/41088-6/2014 (Gyr/WA/41088-6; H5N8) isolated in Washington State in mid-December 2014. The H5N8 and H5N2 viruses were capable of causing severe disease in mice at high inoculation doses; however compared to highly virulent Asian H5N1 viruses (13), these novel H5Nx viruses showed less infectivity and lethality in mice and ferrets. However, unlike seasonal influenza H3N2 viruses, which mainly infect the upper respiratory tract of ferrets (14), both H5N8 and H5N2 viruses could be detected in lung tissues. The H5Nx viruses lacked the ability to transmit among co-housed ferrets, a characteristic feature of influenza A H5 viruses. Understanding the pathogenesis of H5Nx viruses and their capacity for human-to-human transmission is a critical requirement for guidance of public health responses.

MATERIALS AND METHODS

Viruses. Virus stocks of HPAI A/northern pintail/Washington/40964/2014 H5N2 (Pin/WA/40964), A/Gyrfalcon/Washington/41088-6/2014 H5N8 (Gyr/WA/41088-6), and A/Thailand/16/2004 H5N1 (Th/16) virus were propagated in the allantoic cavity of 10-day-old embryonated hens' eggs at 37°C for 24-26 h. Allantoic fluid was pooled from multiple eggs, clarified by centrifugation, and frozen in aliquots at -80°C. The seasonal H1N1 virus A/Brisbane/59/2007 (Bris/59) was propagated in Madin-Darby canine kidney (MDCK) (ATCC, Manassas, VA) cells at 37°C for 48 hrs. To determine the 50% egg infectious dose (EID_{50}) for each virus stock, eggs were inoculated with serially diluted virus and EID_{50} was calculated using the Reed and Muench method (15). Viruses were additionally tested by standard plaque assay in MDCK cells for determination of the titer in PFU/ml (16). The virus stocks were sequenced and real-time RT-PCR exclusivity tests were performed to rule out the presence of other subtypes of influenza virus. All research with HPAI viruses was conducted under biosafety level 3 containment, including enhancements required by the US Department of Agriculture and Select Agent Program outlined in Biosafety in Microbiological and Biomedical Laboratories (17).

Mouse Experiments. All animal experiments were performed under the guidance of the Centers for Disease Control and Prevention's Institutional Animal Care and Use Committee and were conducted in an

Association for Assessment and Accreditation of Laboratory Animal Care International-accredited animal facility. Female BALB/c mice (Charles River Laboratories, Wilmington, MA), 6 to 8 weeks old, were anesthetized intraperitoneally with 0.2 ml of 2,2,2-tribromoethanal in tert-amyl alcohol (Avertin; Aldrich Chemical Co., Milwaukee, WI) and inoculated intranasally (i.n.) with 50µl of virus diluted in phosphate-buffered saline (PBS). Fifty percent mouse infectious dose (MID_{50}) and fifty percent lethal dose (MLD_{50}) were determined by inoculating groups of eight mice with serial 10-fold dilutions of virus ranging from 10^0 to 10^7 EID_{50}. Three mice from each group were euthanized on day 3 post-inoculation (p.i.) and the lung and brain tissues were collected to determine viral titers. The remaining mice from each group were monitored daily for clinical signs and weight loss for 14 days p.i. Weight loss was determined from 5 mice per group on the dates indicated. Percent weight loss was determined from the mean original starting weight. The MID_{50}, as determined by the presence of virus in lung tissues, and MLD_{50} values were calculated using Reed and Muench method (15). Additional groups of four mice were inoculated i.n. with 10^3, 10^5 and 10^6 EID_{50} of each virus and euthanized 6 days p.i., with lung and brain tissues collected for virus titration in eggs. Any mouse which lost ≥25% of initial body weight was euthanized.

Ferret Experiments. Male Fitch ferrets (Triple F Farms, Sayre, PA) six months of age were used for this study. Each animal was serologically negative for currently circulating influenza viruses as determined by standard hemagglutination inhibition assay. During experimentation ferrets were housed in Duo-Flo Bioclean mobile units (Lab Products Incorporated, Seaford, DE). Ferrets (3 ferrets per virus) were inoculated i.n. with 10^6 EID_{50} of virus diluted in PBS. The following day, a serologically naive ferret was placed in the same cage as each inoculated ferret for the assessment of virus transmission between ferrets in direct contact (18). The ferret pairs were observed daily for clinical signs of infection, and nasal washes and rectal swabs were collected every two days for two weeks. Three additional ferrets were inoculated and then euthanized on day 3 p.i. for the assessment of virus replication and systemic spread, as previously described (13).

Cell culture and viral replication. Human airway epithelial Calu-3 cells, obtained from American Type Culture Collection (ATCC; Manassas, VA), were cultured on 12-well plate transwells as described previously (19). Briefly, polarized Calu-3 cells grown on transwells were inoculated apically in triplicate with HPAI H5 or seasonal H1N1 viruses at a multiplicity of infection (MOI) of 0.01 for 1 h, washed, and then incubated at 33°C or 37°C in a 5% CO_2 atmosphere. Culture supernatants were collected at 2, 16, 24, 48, and 72 h p.i., and viral titers were determined in MDCK cells by standard plaque assay.

Nucleotide sequence accession numbers. The GenBank accession numbers are as follows: A/northern pintail/Washington/40964/2014 (H5N2), AJE30344; and A/Gyrfalcon/Washington/41088-6/2014 (H5N8), AJE30333.

RESULTS

Pathogenicity of H5N2 and H5N8 viruses in mice. Mice provide a reliable mammalian model for the study of HPAI H5 viral pathogenesis (20). Groups of BALB/c mice were i.n. inoculated with serial 10-fold dilutions of virus to determine viral MID_{50} and LD_{50} titers. Both H5N2 Pin/WA/40964 and H5N8 Gyr/WA/41088-6 viruses replicated in mouse lungs without prior adaptation but were lethal for mice only at high inoculation titers. Mice inoculated with $\geq 10^6$ EID_{50} of Pin/WA/40964 virus exhibited substantial weight loss and succumbed to infection by day 6 p.i. (Fig. 1A and C). Viral titers in the lungs of these mice were $10^{5.7-6.8}$ EID_{50}/ml on day 3 p.i. (Fig. 1E). Mice inoculated with 10^5 EID_{50} of Pin/WA/40964 virus exhibited 20% mortality with lung viral titers ranging from of $10^{4.3}$ to $10^{5.8}$ EID_{50}/ml. The MID_{50} and MLD_{50} values for Pin/WA/40964 virus were calculated to be $10^{2.0}$ EID_{50} and $10^{5.0}$ EID_{50}, respectively (Table 1). For the H5N8 Gyr/WA/41088-6 virus, severe morbidity and significant mortality were observed in mice inoculated with the highest dose (10^7 EID_{50}) of virus (Fig. 1B and D). Viral titers in the lungs of these mice were $10^{4.8}$ EID_{50}/ml and 3 of 4 mice succumbed to infection. With the exception of one animal which was euthanized for left side paralysis on day 13, mice challenged with 10^6 EID_{50} did not exhibit signs of severe disease. Nonetheless, infectious virus could be detected in the lungs of mice inoculated with 10^{4-7} EID_{50} of virus (Fig. 1F). The MID_{50} and MLD_{50} values for Gyr/WA/41088-6 virus were $10^{4.3}$ EID_{50} and $10^{6.4}$ EID_{50}, respectively (Table 1). In general, systemic spread of infectious virus to the brain was not detected

following either H5N8 or H5N2 virus infection. Only one mouse inoculated with 10^6 EID50 of Gyr/WA/41088-6 virus showed traces of infectious virus ($10^{2.25}$ EID50/ml) in brain tissue on day 6 p.i. Taken together, these results indicate that both H5Nx viruses have the ability to replicate in mice without prior adaptation, but generally demonstrated a moderate-pathogenicity phenotype compared with other avian HPAI H5 viruses (13).

Pathogenicity and transmission of H5N2 and H5N8 viruses in ferrets. Evaluating the capacity for transmission of emerging influenza viruses is a key component of public health risk assessment. Ferrets are an excellent model for studying influenza transmission and also exhibit clinical signs of disease that are similar to those seen during human influenza infection (21). Six ferrets per group were i.n. inoculated with 10^6 EID_{50} of Pin/WA/40964 or Gyr/WA/41088-6 virus and three ferrets were observed for signs of infection. Nasal washes and rectal swabs were collected every two days to measure virus replication. The remaining three ferrets in each virus group were humanely euthanized on day 3 p.i. to assess viral spread in organs. Ferrets inoculated with Pin/WA/40964 (H5N2) virus exhibited mild lethargy during the peak of infection, minimal weight loss (1.2%), and no overt respiratory symptoms (Table 2). Mean maximum increase in body temperature was 1.4°C above baseline. On average, nasal wash titers of Pin/WA/40964 virus were in the range of $10^{2.0}$ to$10^{4.8}$ EID_{50}/mL on days 1, 3, 5 p.i.; two out of three ferrets had detectable virus on day 7 p.i. and virus was cleared from all animals by day 9 p.i. (Table 2, Fig 2A). Pin/WA/40964 virus was detected in nasal turbinates, trachea, and lungs of all inoculated ferrets at titers ranging from $10^{3.5}$ to $10^{5.5}$ EID_{50}/g of tissue or ml (Fig. 3A). Virus was not detected in rectal swabs collected up to 9 days p.i. (data not shown) or in intestinal tissue (day 3 p.i.), indicating that the virus did not spread to or replicate in the gastrointestinal tract. Low viral titers (<10^3 EID_{50}/g) were detected in olfactory bulbs of all three ferrets; however, the virus was not otherwise detected in the brain, blood, or other tissues outside of the respiratory tract.

The Gyr/WA/41088-6 (H5N8) virus caused mild lethargy, a moderate increase in body temperature (1.3°C above baseline), minimal weight loss (1.2%), and no overt respiratory symptoms

(Table 2). The amount of Gyr/WA/41088-6 virus detected in the nasal washes was generally lower compared to that of Pin/WA/40964-infected ferrets, as titers on days 1, 3, 5 p.i. were in the range of $10^{2.0}$ to $10^{4.3}$ EID_{50}/ml. One out of the three ferrets had detectable virus in nasal wash at day 7 p.i. but was cleared by day 9 p.i. (Fig. 2B). Infectious virus was detected in nasal turbinates of all three ferrets infected with Gyr/WA/41088-6 virus with a mean titer of$10^{3.0}$ EID_{50}/ml. Two of three ferrets in this group had virus present in trachea and lung tissues at titers ranging from $10^{2.2}$ to $10^{4.7}$ and in the olfactory bulb at low titers (≤10^{3} EID_{50}/g). Although virus was not detected in rectal swabs from any of the ferrets (data not shown), low titers of infectious virus were detected in intestinal tissues of two animals (Fig. 3B).

To evaluate the ability of Pin/WA/40964 and Gyr/WA/41088-6 viruses to transmit in the ferret model, a serologically naïve ferret was co-housed with each of three inoculated ferrets. Contact animals were observed for signs of infection while nasal wash and rectal swab samples were collected every two days after exposure to infected animals to test for virus shedding. None of the contact ferrets exhibited signs of infection and no virus was detected in nasal wash or rectal swab samples among contact animals. Furthermore, convalescent sera collected from contact ferrets and tested by hemagglutination inhibition assay confirmed that Pin/WA/40964 (Fig 2A) and Gyr/WA/41088-6 viruses (Fig. 2B) were not transmitted between the ferret pairs (data not shown). Taken together, these two novel HPAI viruses showed relatively mild virulence characteristics in ferrets and no transmission in the direct contact model.

Replication kinetics of H5N2 and H5N8 viruses in human Calu-3 cells. We next investigated the replication efficiency of the H5Nx viruses in a relevant cell line, Calu-3, derived from human bronchial epithelium. Calu-3 cells were chosen because when grown on transwell inserts, they form tight, polar monolayers which share some similarities with human airway epithelium, the primary site of replication of influenza viruses in humans (16). Avian influenza viruses are adapted for growth at 40 - 41°C, the temperature of the avian enteric tract, and a proposed mammalian adaptation marker for avian influenza viruses is the ability to replicate at lower temperatures (33°C) found in the mammalian airway (22, 23). Replication kinetics of Pin/WA/40964 (H5N2) and Gyr/WA/41088-6 (H5N8) viruses were assessed in Calu-3 cells at 33°C and 37°C and compared to the replication kinetics of the seasonal H1N1 virus,

215 A/Brisbane/59/2007 (Bris/59) and HPAI H5N1 virus A/Thailand/16/2004 H5N1 (Th/16), which is highly
216 virulent for ferrets and mice (13). In general, all viruses tested resulted in a productive infection in Calu-3
217 cells at both 37°C and 33°C; all viruses reached high titers, approximately 10^{7-8} PFU/ml, by 72 h p.i. (Fig.
218 4). At 37°C, the Pin/WA/40964 and Gyr/WA/41088-6 viruses replicated with efficiency equal to that of the
219 seasonal Bris/59 virus, but significantly lower ($P < 0.05$) than titers recovered from H5N1 (Th/16) virus
220 infected cultures (Fig 4A). At 33°C, in general, the avian virus (H5N1, H5N2 and H5N8) replication was
221 delayed and less efficient at 33°C than at 37°C, especially during the early replication cycles. Only the
222 seasonal Bris/59 virus replicated equally well at both temperatures and reached similar titers at the
223 timepoints examined (Fig. 4A vs. B). Similar to the data at 37°C, at the lower 33°C temperature, the H5Nx
224 viruses replicated to significantly lower ($P < 0.001$) titers compared to H5N1 virus. In addition, the H5Nx
225 virus titers were significantly lower than that observed for Bris/59 virus at 16, 24, and 48 h p.i. ($P < 0.01$)
226 Taken together, these results demonstrate that in human airway cells H5Nx viruses do not replicate as
227 well as the virulent H5N1 virus at both 37°C and 33°C temperatures and the seasonal influenza virus at
228 the lower temperatures (33°C) found in the upper airway of mammals.
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231 **DISCUSSION**
232 Since their first appearance in Asia in 1997, HPAI H5N1 influenza viruses have spread to multiple
233 countries on several continents and caused considerable damage to the poultry industry and loss of
234 human life (24). Over 800 confirmed human cases of avian influenza (H5N1) have been reported to the
235 WHO since 2003, almost 60% of which were fatal (25). Since 1997, the HA genes of H5N1 have
236 diversified into multiple clades, including the novel clade 2.3.4.4 viruses (6, 7, 26, 27). There has been an
237 increased prevalence of clade 2.3.4.4 viruses in domestic and wild birds since 2010 and now the virus
238 reached North America (8). The H5N8 virus isolated in the U.S. showed 99% nucleotide identity with
239 viruses isolated in South Korea (8). Since its first appearance in North America, the H5N8 virus
240 reassorted in the Pacific flyway with North American avian influenza viruses resulting in novel HPAI
241 viruses, H5N2 and H5N1 (8). These highly pathogenic H5Nx viruses have caused several outbreaks in
242 domestic poultry in multiple states in the U.S., therefore raising significant concerns in the poultry industry

and general public due to the potential risk of human infection. In this study, we investigated the replication kinetics, pathogenesis, and transmission of Pin/WA/40964 (H5N2) and Gyr/WA/41088-6 (H5N8) viruses isolated from birds in Washington State in December 2014.

Using this mouse model, we have demonstrated that viruses with a $MLD_{50} > 10^{6.5}$ are considered of low virulence, while viruses with $MLD_{50} < 10^{3}$ are considered to be highly virulent (28). Based on these criteria the novel H5N2 virus (MLD50 = $10^{5.0}$ EID_{50}) and H5N8 virus (MLD50 = $10^{6.4}$ EID_{50}) exhibited moderate pathogenicity in mice (13). Similarly, a low to moderate-pathogenicity phenotype was observed in the ferret model. Both viruses were shed in respiratory tract secretions for up to 7 days p.i.; but all animals recovered fully from the infection. Of note, infection with both H5Nx viruses led to detectable virus in the ferret lungs. Influenza virus replication in lung tissue is thought to be a pathogenic trait that may contribute to the severity of H5Nx infection, if humans became infected by close contact with H5Nx-infected poultry. Overall, our results are consistent with previous pathogenicity studies with clade 2.3.4.4 H5N8 viruses isolated from South Korea, and Netherlands (29) which also exhibited low to moderate virulence in mammalian models (4, 30). Furthermore, the virus titers we observed in ferret respiratory tract tissues were comparable to those observed with other avian influenza virus strains that exhibited moderate pathogenicity in ferrets (13).

In our hands, neither of the H5Nx viruses had the ability to efficiently spread systemically in ferrets, although virus was detected in the gastrointestinal tract of some ferrets. Virus detected in the ferret GI tract likely originates from virus swallowed during inoculation and not systemic spread of H5N1 virus (31). Conversely, a previous study reported low levels of H5N8 virus in spleen, liver, and brains of ferrets infected with A/mallard duck/Korea/W452/2014 virus, (4) indicating that some of the currently circulating H5N8 strains have the ability to disseminate to multiple extrapulmonary tissues in mammals. Virus detection in the olfactory bulb, but not the anterior or posterior brain, of H5N2 and H5N8 viruses in ferrets day 3 p.i. is typical among both human and avian influenza viruses which replicate to high titers in the nasal turbinates and likely does not indicate productive H5Nx virus replication in this tissue (32). Virulence and the ability to replicate outside the respiratory tract have been, in part, linked with the

presence of a multi-basic amino acid cleavage site in the HA (33). The HA of the novel H5Nx viruses possess multibasic residues at the HA cleavage site, albeit fewer basic residues (PLRERRRKR/GLF) compared to the highly virulent H5N1 Thai/16 virus, which has one additional arginine basic residue (PQRERRRKKR/GLF) upstream of the furin recognition motif (RXXR). It is currently not clear whether reduced number of basic residues at the HA cleavage site contributes to the reduced virulence of H5Nx virus in the mammalian models.

One of the factors that may limit avian influenza virus infection in humans is the inability to efficiently replicate at temperatures found in the upper human respiratory tract (23). Unlike human influenza viruses which replicate efficiently in respiratory tracts where the temperatures range from 32°C in the upper respiratory tract to 37°C in lower respiratory tract (34, 35), avian influenza viruses have adapted to replicate at the higher temperatures, 40-41°C, of the avian enteric tract (36). The upper respiratory tract provides a large surface area of susceptible cells and is usually the initial site of infection and likely the predominant site of influenza virus replication in mammalian species (37). Therefore, in order to replicate efficiently and spread among humans, it is believed that avian influenza viruses need to acquire the ability to replicate efficiently at lower temperatures. Replication kinetics of the H5N2 and H5N8 viruses in polarized Calu-3 cells at 37°C were similar to that of the human seasonal virus Bris/59, however, these viruses exhibited significantly delayed replication kinetics at 33°C, suggesting that that H5Nx viruses have not yet acquired all the features required for mammalian host adaptation. Efficiency of replication of avian influenza viruses at lower temperatures was previously linked to an amino acid substitution Glu627Lys in PB2 (38). These results were subsequently supported by a study showing increased replication of H5N1 viruses possessing Lys at position 627 in PB2 in the upper respiratory tract of mice (39). Both tested H5Nx viruses possess Glu at the position 627 in PB2 which could contribute to inefficient replication at lower temperatures. HPAI H5N1 Thai/16 virus, which has Lys at the 627 position of PB2, replicated to higher titers at 33°C than Pin/WA/40964 and Gyr/WA/41088-6, but all three avian viruses exhibited delayed replication kinetics at 33°C as compared to 37°C. Schull et al. demonstrated that amino acid substitution Glu627Lys in PB2 does not entirely account for conferring temperature-dependency in mammalian cells. It was suggested that viruses bearing avian or avian-like surface

glycoproteins have a reduced capacity to establish productive infection at the temperature of the human proximal airways (36).

Avian-adapted influenza viruses bind preferentially to the alpha 2,3-linked sialic acid receptors abundant in the gastrointestinal tract of birds, while human influenza viruses preferentially bind to alpha 2,6-linked sialic acid receptors on cells found in the upper respiratory tract (40). The distribution of influenza virus receptors in the ferret respiratory tract resembles the airways of humans which may contribute to the success of this animal model (41, 42). However, several differences between the ferret and human respiratory tract have been identified which could influence the outcome of infection. For example the presence of Sda epitopes that carry alpha 2,3-NeuAc in ferret respiratory tract is hypothesized to reduce potential binding sites for avian influenza viruses (43, 44). The HA of H5Nx viruses possess the key residues Gln226 and Gly228 (H3 numbering) required for 2,3-linked sialic acid binding indicating the virus has not adapted toward human-type receptor specificity. Interestingly, the novel H5N8 and H5N2 viruses circulating in U.S. have a Thr to Ala substitution in HA at position 160, which results in a loss of a glycosylation motif at asparagine residue 158. The removal of this glycosylation site has previously been shown to be critical for H5 subtype influenza viruses to gain enhanced receptor binding affinity to human-like receptors and transmission in mammalian hosts (45, 46). The weak binding of the H5Nx viruses to human-like receptors (30, 47) along with reduced replication efficiency at temperatures found in the upper respiratory tract of humans likely contribute to the poor transmission of the H5Nx viruses. Others have reported similar transmission results for clade 2.3.4.4 HPAI H5N8 viruses in ferrets (4) and guinea pigs (47). However, an H5N2 virus isolated in China (A/duck/Eastern China/1112/2011) that had high nucleotide identity with H5N8 viruses circulating in South Korea was able to efficiently transmit between cohoused guinea pigs (47). Overall, despite being highly pathogenic in chicken and turkeys, the novel H5Nx viruses exhibited low to moderate virulence in mammals. However, as these and other H5 viruses continue to circulate in wild birds and cause widespread outbreaks in commercial poultry operations and backyard flocks, the likelihood of human exposure increases. Each exposure event serves as an opportunity for these viruses to evolve and adapt to humans increasing the potential for an H5 pandemic. Moreover, the ability of these viruses to reassort

with other circulating influenza A viruses raises the concern that they may reassort with a human influenza lineage virus and increase their adaptation to mammalian hosts. Influenza virus surveillance and timely risk assessment of newly emerging strains is critical to pandemic preparedness and assessing the threat to public health.

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FIGURE LEGENDS

Figure 1. Pathogenicity of H5N2 and H5N8 viruses in mice. Groups of five mice were intranasally inoculated with serial 10-fold dilutions, ranging from 10^3 to 10^7 EID_{50}, of Pin/WA/40964 (H5N2) or Gyr/WA/41088-6 (H5N8) virus and observed for signs of morbidity and mortality for 14 days. Any mouse that lost ≥25% of initial body weight was euthanized. The percentage mean maximum weight loss (A, B) and percent survival (C, D) are shown. Additional groups of 3 mice infected with serial 10-fold dilutions ranging from 10^0 to 10^7 EID_{50} of (E) H5N2 (Pin/WA/40964) and (F) H5N8 (Gyr/WA/41088-6) were euthanized 3 days p.i and groups of 4 mice infected with 10^3,10^5,10^6 EID_{50} of H5NX viruses were euthanized 6 days p.i. and the lung tissues were collected for viral titer determination. Viral titers are presented as $\log_{10}$ EID_{50}/ml. The limit of detection is 1.5 $\log_{10}$ EID_{50}/ml. Mice infected with the highest dose of Pin/WA/40964 virus were euthanized before day 6 p.i. due to substantial weight loss, therefore lung titers are not represented for 10^6 EID_{50}.

Figure 2. Transmissibility of H5N2 and H5N8 influenza viruses in ferrets. Groups of three ferrets were inoculated intranasally with 10^6 EID_{50} of (A) H5N2 (Pin/WA/40964) and (B) H5N8 (Gyr/WA/41088-6) virus. The following day, a serologically naive ferret was placed in the same cage with each inoculated ferret for the assessment of virus transmission between ferrets in direct contact. Nasal washes were collected from each ferret on the indicated days post inoculation or post contact. The results from individual ferrets are presented. Viral titers are presented as $\log_{10}$ EID_{50}/ml. The limit of detection is 1.5 $\log_{10}$ EID_{50}/ml.

Figure 3. Detection of H5N2 and H5N8 viruses in ferret tissues. Groups of three ferrets each were inoculated intranasally with 10^6 EID_{50} of (A) H5N2 (Pin/WA/40964) and (B) H5N8 (Gyr/WA/41088-6) virus. Tissues were collected on day 3 p.i. for assessment of viral titers. Blood and nasal turbinate viral titers are presented as $\log_{10}$ EID_{50}/ml and kidney, spleen, liver, intestines (pooled duodenum, jejuno-ileal loop, and descending colon), olfactory bulb (BnOB), brain (pooled anterior and posterior brain), lungs and trachea are presented as $\log_{10}$ EID_{50}/g of tissue. The limit of detection is 1.5 $\log_{10}$ EID_{50}/ml.

Figure 4. Replication kinetics of influenza viruses in polarized human airway epithelial cells. Calu-3 cells grown on transwells were infected apically in triplicate with 0.01MOI of Pin/WA/40964 (H5N2), Gyr/WA/41088-6 (H5N8), Bris/59 (H1N1), or Th/16 (H5N1). The cells were incubated at 37°C (A) or 33°C (B) and culture supernatants were collected at 16, 24, 48, and 72 hours p.i. for viral titer determination by standard plaques assay. Asterisks indicate statistical significance between Th/16 and other tested H5Nx viruses (* P < 0.05, ** P < 0.01, *** P < 0.001).

Table 1. Virulence of H5N2 and H5N8 viruses in mice.

			Day 3 lung titers[c]		Day 6 lung titers[c]			
Virus	Stock titer[a]	Wt loss (%)[b]	10^5	10^6	10^5	10^6	MID_{50}[d]	MLD_{50}[d]
Pin/WA/40964	8.5	25.0	5.8±0.1	5.7±0.1	4.3±0.4	ND[e]	2.0	5.0
Gyr/WA/41088-6	9.5	6.5	4.3±0.4	4.8±0.6	4.4±1.2	5.4±0.9	4.3	6.4

[a]Titer of stock viruses prepared in 10-day-embryonated eggs expressed as $\log_{10}$ EID_{50}/mL.
[b]The percentage mean maximum weight loss (five mice per group) following inoculation with 10^6 EID_{50} of virus.
[c]Mean viral titer following inoculation with 10^6 or 10^5 EID_{50} of virus on day 3 (three mice per group) or day 6 (four mice per group) p.i. expressed as $\log_{10}$ EID_{50}/ml ±SD
[d]MID_{50} and MLD_{50} are expressed as the $\log_{10}$ EID_{50} required to give one MLD_{50} or MLD_{50}, respectively.
[e]Mice were euthanized before day 6 p.i. due to severe morbidity. ND, not determined.

Table 2. Clinical signs and replication of H5N2 and H5N8 viruses in ferrets.

	Weight loss[a]	Temperature increase (C°)[b]	Sneezing[c]	Lethargy[d]	Nasal titer[e]
Pin/WA/40964	1.2 (2/3)	1.4	0/3	1.1 (3/3)	4.2±0.8 (d5)
Gyr/WA/41088-6	1.2 (1/3)	1.3	1/3	1 (1/3)	3.1±1 (d1)

[b]The percentage mean maximum weight loss observed during the first 10 dpi. Number of ferrets exhibiting weight loss is in parentheses.
[b]Mean maximum temperature increase over baseline (37.5-39.7 °C) during the first 10 dpi.
[c]Number of ferrets that exhibited sneezing during the first 10 dpi.
[d]Relative inactivity index of ferrets during the first 10 dpi. Number of ferrets showing lethargy is in parentheses.
[e]Mean maximum nasal wash titer expressed as $\log_{10}$ EID_{50} /ml ±SD, day in parenthesis. Limit of detection was 1 $\log_{10}$ EID_{50}/mL.