New reassortant and enzootic European swine influenza viruses transmit efficiently through direct contact in the ferret model

Kristina Fobian,1 Thomas P. Fabrizio,2 Sun-Woo Yoon,2‡ Mette Sif Hansen,3 Richard J. Webby2 and Lars E. Larsen1

Correspondence
Kristina Fobian
kfobian@gmail.com

1Section of Virology, National Veterinary Institute, Technical University of Denmark, Bülowsvej 27, 1870 Frederiksberg C, Denmark
2Division of Virology, Department of Infectious Diseases, St Jude Children’s Research Hospital, 262 Danny Thomas Place, Memphis, TN 38105-3678, USA
3Section of Bacteriology, Pathology and Parasitology, National Veterinary Institute, Technical University of Denmark, Bülowsvej 27, 1870 Frederiksberg C, Denmark

The reverse zoonotic events that introduced the 2009 pandemic influenza virus into pigs have drastically increased the diversity of swine influenza viruses in Europe. The pandemic potential of these novel reassortments is still unclear, necessitating enhanced surveillance of European pigs with additional focus on risk assessment of these new viruses. In this study, four European swine influenza viruses were assessed for their zoonotic potential. Two of the four viruses were enzootic viruses of subtype H1N2 (with avian-like H1) and H3N2, and two were new reassortants, one with avian-like H1 and human-like N2 and one with 2009 pandemic H1 and swine-like N2. All viruses replicated to high titres in nasal wash and nasal turbinate samples from inoculated ferrets and transmitted efficiently by direct contact. Only the H3N2 virus transmitted to naïve ferrets via the airborne route. Growth kinetics using a differentiated human bronchial epithelial cell line showed that all four viruses were able to replicate to high titres. Further, the viruses revealed preferential binding to the 2,6-α-sialylated glycans and investigation of the antiviral susceptibility of the viruses revealed that all were sensitive to neuraminidase inhibitors. These findings suggested that these viruses have the potential to infect humans and further underline the need for continued surveillance as well as biological characterization of new influenza A viruses.

INTRODUCTION

Influenza A viruses (IAVs) causes disease in humans, birds and some domestic animals, including swine. Swine influenza viruses are enzootic in pigs worldwide, and infection with the virus causes substantial economic loss for farmers due to secondary infections and reduced weight gain in affected pigs. Furthermore, the presence of IAV in swine also poses a potential health risk to humans due to the development of new reassortant viruses with zoonotic potential, as recently seen with the 2009 H1N1 pandemic virus (H1N1pdm09) (Smith et al., 2009).

Within the last few years, several new reassortant swine influenza viruses have been detected throughout Europe, Asia and the USA (Breum et al., 2013; Moreno et al., 2011; Pascua et al., 2013; Starick et al., 2011, 2012; Tremblay et al., 2011), with many carrying segments from enzootic swine influenza viruses and the H1N1pdm09 virus. In Denmark, at least four swine influenza virus subtypes are enzootic in pigs. These include the avian-like H1N1 (H1N1), being the result of a transmission event from birds to swine (Abusugra et al., 1987; Schulz et al., 1991), and the European reassortant human-like H3N2 (H3N2), with surface glycoproteins of human origin and a backbone derived from the avian-like H1N1 (Castrucci et al., 1993). The third subtype is an avian-like H1N2 (H1N2) containing seven segments originating from H1N1 and N2 from H3N2 (Trebbien et al., 2013). Finally, in 2010, a reverse zoonotic event led to the introduction of H1N1pdm09 into Danish pigs. The virus spread and rapidly achieved a prevalence of 15–20 % of swine influenza viruses isolated in

1Present address: Viral Infectious Disease Research Center, Korea Research Institute of Bioscience & Biotechnology (KRIBB), 125 Gwahak-ro, Youseong-gu, Daejeon 305-806, South Korea.

The GenBank/EMBL/DDBJ accession numbers for haemagglutinin for A/swine/Denmark/10845-1/2012(H1N2), A/swine/Denmark/101394-1/2011(H1N2) and A/swine/Denmark/101501-1/2010(H3N2) are KP202355, KP202356 and KP202357, respectively.

Received 2 December 2014
Accepted 14 February 2015
Denmark (unpublished data). During the national passive surveillance programme in 2012, at least two new reassortants were detected in Danish pigs, one with seven segments derived from H1N1 and a N2 gene most closely related to that of a human seasonal H3N2 virus that was circulating in the mid-1990s (H1avN2hu) (Breum et al., 2013), and one containing seven segments from H1N1pdm09 and a N2 gene from H3N2 (H1pdmN2sw) (unpublished data), also detected in German pig populations (Starick et al., 2012).

The increased detection of new reassortants and the lack of knowledge regarding their zoonotic potential is a worrying aspect, and it is therefore of significant importance to genetically and phenotypically characterize these new reassortants.

In this study, the pathogenicity and transmissibility of two new reassortant swine influenza viruses, i.e. H1avN2hu and H1pdmN2sw, were compared with H1N2 and H3N2 enzootic European swine influenza virus strains in the ferret model. The ferret model has been widely used for experimental infection with IAVs, due to similarities in clinical signs and pathogenesis associated with human disease, as well as receptor distribution in the respiratory tract (Baum & Paulson, 1990; Maher & DeStefano, 2004; van Riel et al., 2007).

**RESULTS AND DISCUSSION**

**Clinical signs and virus shedding**

Ferrets were inoculated with $10^6$ TCID$_{50}$ ml$^{-1}$ of each virus, a dose previously shown to initiate an infection (Lednicky et al., 2010; Stark et al., 2013). None of the ferrets developed fever, had nasal discharges or showed signs of lethargy during the study. The ferrets showed variable weight losses from mild to moderate (3–10%).

All four viruses replicated in inoculated ferrets and were also found to be transmitted to naïve direct-contact (DC) ferrets (Fig. 1) as assessed by isolation of virus from nasal washes. Infectious virus was detected in nasal washes until ~7 days post-infection (p.i.) for donor ferrets and 9 days p.i. for ferrets inoculated with H3N2. The day of observed peak titres for individual animals infected via DC was found to vary in the four different groups (Fig. 1). Airborne transmission (AT) was only observed in ferrets inoculated with H3N2. The AT ferrets died at 11 days p.i., although it was concluded that the cause of death was not related to the infection. As both AT ferrets in the group inoculated with H3N2 shed virus and virus was detected in nasal wash from the deceased ferret at 11 days p.i., the results of the AT were not compromised by its death.

![Fig. 1. Replication kinetics in the upper respiratory tract. Groups of four ferrets were intranasally inoculated with 1 ml $10^6$ TCID$_{50}$ (0.5 ml per nostril) of the respective viruses. Each virus group contained eight ferrets: four donor ferrets (until 5 days p.i.), two DC ferrets and two AT ferrets. (a) H1avN2hu, (b) H1N2, (c) H3N2 and (d) H1pdmN2sw. Nasal washes were collected on days 1, 3, 5, 7, 9 and 11 p.i. Data are presented as virus titre for individual ferrets ($\log_{10}$TCID$_{50}$ ml$^{-1}$) on the indicated day. Limit of detection was $10^1$ TCID$_{50}$ ml$^{-1}$.

Downloaded from www.microbiologyresearch.org by IP: 54.70.40.11
On: Sun, 16 Dec 2018 05:27:10
In comparison with the observed DC transmission of the four European swine influenza viruses, it has previously been found that DC transmission in ferrets of a European avian-like H1N1 was poor and no AT was observed, whereas North American triple-reassortant swine influenza viruses were readily transmissible via DC (Barman et al., 2012).

Nasal wash samples from all days p.i. were tested by real-time reverse transcription (RT)-PCR to determine whether virus could be replicating at levels too low to be detected by the TCID$_{50}$ assay. The real-time RT-PCR results were found to support the results obtained from the TCID$_{50}$ assay. Furthermore, low viral load was detected in one of the AT ferrets in the H1pdmN2sw group at 3 and 5 days p.i., suggesting a potential for this virus also to become transmissible amongst ferrets via the airborne route. Taken together, it could appear that the H1N2/H1avN2hu viruses carrying avian-origin haemagglutinin (HA) play a less significant role in transmission compared with the H3N2 and H1pdmN2sw virus carrying human-origin HAs, consistent with previous results (Barman et al., 2012).

By the end of the experiment, IAV antibodies were detected in serum from all inoculated and DC ferrets, as well as the AT ferret belonging to the H3N2 group (Fig. 2). ELISA was chosen for antibody detection, due to an overly high background response in the haemagglutination inhibition (HI) assay. At 19 and 21 days p.i., inoculated and DC ferrets had all seroconverted.

**Viral load in respiratory organs**

From each group, two inoculated ferrets were euthanized at 5 days p.i. for determination of viral titres in nasal turbinates, trachea, caudal and cranial lung lobes, lymph nodes, intestine, liver and spleen. No viral replication was detected in liver, spleen, intestines or lymph nodes as was expected, as swine and human IAV replication is limited to the respiratory tract (Munster et al., 2009; Pascua et al., 2012; Pearce et al., 2012). For all viruses, infectious virus particles were recovered from nasal turbinates, trachea and lung lobes 1 and 2 (Fig. 3). Highest viral titres were observed in nasal turbinates, with the highest level of virus particles observed in ferrets inoculated with H1avN2hu and H1N2. In contrast to this result, H1avN2hu and H1N2 showed lower viral titres in the nasal washes. This could indicate that H1avN2hu replicates more efficiently in tissues but that release of new viral particles is not as efficient, and therefore suggests that the balance between the HA and neuraminidase (NA) of this virus is not optimal, which could affect viral transmission.

From the trachea, highest titres were observed in ferrets inoculated with H1avN2hu and H3N2. Viral replication in lung lobes 1 and 2 showed the lowest titres, with the highest level of virus particles observed in ferrets inoculated with H1pdmN2sw and H3N2.

**Histopathology**

Histopathological examination of the ferrets euthanized at 5 days p.i. revealed no significant lesions in trachea or lung tissue of the control group (Fig. 4a). All four viruses caused hyperaemia, oedema and haemorrhage into alveoli as well as varying degrees of non-suppurative interstitial pneumonia, dysplasia of the bronchiolar epithelium and hyperplasia of the bronchus-associated lymphoid tissue (BALT). Apart from these common characteristics, the severity of the lesions differed amongst the groups depending on the virus used for inoculation.

![Fig. 2](http://vir.sgmjournals.org)  
**Fig. 2.** Seroconversion of ferrets. Ferrets were tested for seroconversion at 19 and 21 days p.i. using a blocking ELISA detecting antibodies against the NP gene. In the H3N2 AT group, one of the animals died before seroconversion and hence only one animal is above the baseline. Serum samples were tested in duplicate and values are presented as mean ± SEM per cent seropositive.

![Fig. 3](http://vir.sgmjournals.org)  
**Fig. 3.** Comparison of European swine influenza virus titres recovered from ferret tissues. Ferrets were inoculated intranasally with 1 ml $10^6$ TCID$_{50}$ (0.5 ml per nostril) with H1N2, H1avN2hu, H3N2 or H1pdmN2sw and tissues were collected at day 5 p.i. Titres are expressed as log$_{10}$TCID$_{50}$ (g tissue)$^{-1}$. Each bar represents one ferret. Data are presented as virus titre (log$_{10}$TCID$_{50}$ g$^{-1}$) from the indicated tissue. Limit of detection was $10^1$ TCID$_{50}$ g$^{-1}$. 

http://vir.sgmjournals.org
Findings in the trachea were similar among the four virus groups, ranging from no lesions to mild dysplasia of the epithelium, moderate suppurative tracheitis and single-cell necrosis.

The viruses H3N2 and H1pdmN2sw caused the most severe lung lesions (Fig. 4b, c), consisting of suppurative bronchiolitis and bronchitis, as well as epithelial necrosis of serous glands and perivascular accumulation of mononuclear cells. Furthermore, the inoculated ferrets had necrosis of bronchiolar epithelium and mixed bronchopneumonia was seen in one of the H3N2-inoculated ferrets. The ability of H3N2 and H1pdmN2sw to induce the most severe lung lesions was consistent with the observed viral load in the lung lobes for these two viruses.

The H1N2 and H1avN2hu viruses caused infiltration of a few neutrophilic granulocytes and mononuclear cells in the alveoli (Fig. 4d, e). Both groups of inoculated ferrets (euthanized 5 days p.i.) had necrosis of the bronchiolar epithelium. Inoculation with H1avN2hu also induced perivascular accumulation of mononuclear cells, desquamation of bronchiolar epithelium, suppurative bronchiolitis and focal epithelial necrosis of serous glands.

**In vitro growth kinetics**

The in vitro replication capacity of the four European swine influenza virus strains was assessed and compared in human bronchial epithelial (NHBE) cells, primary swine
respiratory epithelial cells (pSRECs) and Madin-Darby canine kidney (MDCK) cells. Cells were inoculated with a low m.o.i. (0.01).

All four swine influenza viruses were able to infect and replicate to high titres (8.0–9.4 log10 TCID50 ml−1) in NHBE cells. The titres of H1avN2hu and H1pdmN2sw progressively increased until 48 h p.i. The H1N2 and H3N2 titres increased until 60 h p.i. (Fig. 5a). The high level of replication in NHBE cells of all four swine influenza viruses suggested that these viruses have the ability to infect cells in the human respiratory airway.

In pSREC, an increase in titres was observed until 36 h p.i. for H1avN2hu and H1N2; for H1pdmN2sw and H3N2, an increase in titres was observed until 48 and 60 h p.i., respectively (Fig. 5b). Mean peak titres in pSRECs were in the range from 6.6 to 7.2 log10 TCID50 ml−1, where H1N2 reached the highest mean peak titre.

In MDCK cells, the virus titres progressively increased during the first 36 h p.i. with highest mean peak titres in the range from 6.3 to 7.9 log10 TCID50 ml−1 and the highest mean peak titre was observed for H1N2 (Fig. 5c).

Receptor binding

Binding of viral HA to host receptors is known to be important for the determination of transmissibility efficiency and host range restriction (Matrosovich et al., 2004). Hence, the glycan-binding properties of the four viruses were investigated by testing for their ability to bind biotinylated sialylglycopolymers in a dose-dependent fashion.

It has been shown previously that human IAVs bind preferentially to 2,6-α-SLN (Neu5Ac2,6-α-Gal1,4-β-GlcNβ-PAA-biotin) and 2,6-α-SLN (Neu5Ac2,6-α-Gal1,4-β-Glcβ-PAA-biotin) and to a lesser extent 2,3-α-SL (Neu5Ac2,3-α-Gal1,4-β-Glcβ-PAA-biotin) as representatives of 2,6-α- and 2,3-α-linked 5-N-acetylenuraminic acid receptors (Stevens et al., 2006). In this study, none of the swine influenza viruses exhibited strong binding preference towards the ‘avian’ 2,3-α-SL and binding to this glycan barely exceeded the threshold. The European swine influenza viruses preferentially bind to the ‘human/swine’ 2,6-α sialylglycopolymers, with H3N2 and H1pdmN2sw showing the highest affinity for the ‘human/swine’ 2,6-α-SLN. H1avN2hu and H3N2 were found to also bind 2,6-α-SL, the short version of the 2,6-α sialylglycopolymers. For H1avN2hu and H3N2, it appeared that as the concentration of sialylglycopolymers was decreased, the receptor preference shifted from the long to the short version of the 2,6-α sialylglycopolymer. Results are summarized in Fig. 6. These findings are consistent with the efficient infection and transmission of the European H3N2 virus, as it was found to bind both 2,6-α-SL and 2,6-α-SLN, but does not explain the less efficient infection and transmission of H1avN2hu that was also shown to bind these two receptor analogues.

![Fig. 5. Replication kinetics of European swine influenza viruses in different cell lines. Growth curves were obtained by inoculating cells at m.o.i. 0.01 p.f.u. per cell with H1N2, H1avN2hu, H1pdmN2sw or H3N2. Supernatant was harvested and titrated in MDCK cells at 8, 10, 12, 18, 20, 24, 36, 48 and 60 h p.i. Data are expressed as mean ± SEM log10 TCID50 from two independent experiments titrated in quadruplicate.](http://vir.sgmjournals.org)

Sequencing

For examination of molecular determinants involved in receptor binding of the European swine influenza viruses, full-length HA sequences were obtained from all four viruses using nasal washes from the day of their highest mean peak viral titres as templates. Amino acids previously shown to be involved in receptor binding are located at the distal tip of the HA monomer in positions 111–265, which is formed by three secondary structures, termed the 130-loop, the 190-helix and the 220-loop (Gamblin et al., 2004).
Residue 190 has been shown to play an important role in binding of swine influenza viruses and human IAVs to the 2,6-α receptor in concert with the amino acid at position 225 (Matrosovich et al., 2000). For the H1 viruses, 190D was found for H1N2 and H1pdmN2sw, whereas H1avN2hu possessed 190S (Table 1). It could be speculated that the D→S mutation potentially affects binding of H1avN2hu to the 2,6-α receptor.

Residue 225 was found to vary between all four European swine influenza viruses, with H1avN2hu possessing 225E and H1pdmN2sw showing the ‘avian’ 225D. In H1N2, an E225K mutation was observed during the study, with 225E found in inoculum, an inoculated ferret and a DC ferret (Table 1). The 225K variation was found in an inoculated ferret and a DC ferret. The ferrets possessing the E225K mutation had not been co-housed and this suggests that this mutation may have been random. The receptor-binding domain at residue 225 has previously been found to be variable in European avian-like swine strains and has been shown to include the avian 225G, as well as 225E and 225K (Dunham et al., 2009).

Investigation of the receptor-binding properties of the H3N2 virus showed that the European H3N2 swine influenza virus possessed 155Y and 158G (Table 1), mutations found to be present in human H3s, and both mutations have previously been shown to play a critical role in recognition of two major molecular species of sialic acids, i.e. 5-N-acetylneuraminic acid and 5-N-glycolylneuraminic acid, where 5-N-glycolylneuraminic acid is an analogue of sialic acid, expressed in many animal tissues, but absent from humans (Chou et al., 1998; Matrosovich et al., 2000; Takahashi et al., 2009).

Furthermore, amino acids at residues 226 and 228 have also previously been shown to play a role in receptor binding (Matrosovich et al., 2000). In H1 HA, ‘avian’ residues 226Q and 228G have been found to be present in human viruses (Glaser et al., 2005; Matrosovich et al., 2000).

**Table 1. Sequencing data from important amino acid residues in the four swine influenza viruses**

<table>
<thead>
<tr>
<th>Residue</th>
<th>H1avN2hu</th>
<th>H1pdmN2sw</th>
<th>H1N2</th>
<th>H3N2</th>
</tr>
</thead>
<tbody>
<tr>
<td>155</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>Y</td>
</tr>
<tr>
<td>158</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>G</td>
</tr>
<tr>
<td>190</td>
<td>S</td>
<td>D</td>
<td>D</td>
<td>–</td>
</tr>
<tr>
<td>225</td>
<td>E</td>
<td>D</td>
<td>E/K</td>
<td>–</td>
</tr>
<tr>
<td>226</td>
<td>Q</td>
<td>Q</td>
<td>Q</td>
<td>L</td>
</tr>
<tr>
<td>228</td>
<td>G</td>
<td>G</td>
<td>G</td>
<td>S</td>
</tr>
</tbody>
</table>
The Michaelis–Menten constant \( K_m \) and maximum velocity \( V_{\text{max}} \) of substrate conversion were fitted to the Michaelis–Menten kinetics by non-linear regression. All values represent the mean (95% confidence interval).

<table>
<thead>
<tr>
<th>Virus</th>
<th>( V_{\text{max}} ) (µM min(^{-1}))</th>
<th>( K_m ) (µM)</th>
<th>IC(_{50}) (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Oseltamivir</td>
</tr>
<tr>
<td>H1avN2hu</td>
<td>1364 (1257–1472)</td>
<td>376.5 (272.6–480.4)</td>
<td>0.10 (0.07–0.14)</td>
</tr>
<tr>
<td>H1pdmN2sw</td>
<td>1845 (1655–2036)</td>
<td>487 (320.5–653.6)</td>
<td>0.29 (0.19–0.44)</td>
</tr>
<tr>
<td>H1N2</td>
<td>1495 (1219–1772)</td>
<td>492 (191.7–792.4)</td>
<td>0.47 (0.31–0.72)</td>
</tr>
<tr>
<td>H3N2</td>
<td>842.4 (743.9–940.9)</td>
<td>281.1 (158.7–403.5)</td>
<td>0.08 (0.05–0.12)</td>
</tr>
</tbody>
</table>
**METHODS**

**Viruses.** A/swine/Denmark/10302-2/2012(H1N2), A/swine/Denmark/10845-1/2012(H1N2), A/swine/Denmark/101394-1/2011(H1N2) and A/swine/Denmark/101501-1/2010(H3N2) (hereafter referred to as H1avN2hu, H1pdmN2sw, H1N2 and H3N2, respectively) were isolated from lung samples submitted for diagnostic purposes from swine with a history of respiratory disease. Viruses were grown and titrated in MDCK cells. Before inoculation, influenza viruses were passaged in the allantoic cavity of 10-day-old embryonated chicken eggs (Marshall Durbin) at 35 °C for 72 h. All isolates underwent a maximum of two passages in eggs and/or cells.

**Cell cultures.** MDCK cells were grown in minimum essential medium Eagle (MEM; Gibco) containing 5 % FCS, 2 mM l-glutamine, non-essential amino acids (NEAA) and penicillin/streptomycin. NHBE cells in individual inserts were obtained from MatTek. The cells were grown in AIR-100-ASY (MatTek) serum-free media containing growth factors. The apical surface was washed to remove mucus and media was changed every other day.

pSRECs were seeded into type VI collagen (Sigma-Aldrich)-coated tissue culture flasks and grown in bronchial epithelial cell growth medium (BEGM; Lonza) with a SingleQuots kit containing growth factors and cytokines. Medium was further supplemented with 5 % FCS and 1 % penicillin/streptomycin/amphotericin (Sigma), and passed up to five times prior to infection. All cells were grown at 37 °C, 5 % CO2.

**Infection and replication kinetics.** For preparation of viral stock, lung tissue was homogenized using a TissueLyser (Qiagen) in 1.5 ml RLT-buffer with 2 ml TRIzol (Life Technologies). Viruses were extracted and purified using a RNeasy Mini kit (Qiagen) according to manufacturer’s instructions. Cell culture supernatant was prepared by mixing 200 ml sample with 400 ml RLT-buffer containing 10 mg/ml proteinase K and 10 mg/ml Triton X-100, then stored at −80 °C.

**RNA purification and real-time RT-PCR screening.** Viral RNA was purified from cultured viruses by a RNeasy Mini kit (Qiagen) according to manufacturer’s instructions. Cell culture supernatant was prepared by mixing 200 ml sample with 400 ml RLT-buffer containing 10 mg/ml proteinase K and 10 mg/ml Triton X-100, then stored at −80 °C.

The presence of IAV was confirmed by real-time RT-PCR using an in-house modified assay for detection of the M gene (De Vleeschauwer et al., 2009).

**Full-genome sequencing and RT-PCR.** Nucleic acid amplification was performed by one-step RT-PCR using primers modified from Hoffmann et al. (2001) and a SuperScript III One-Step RT-PCR kit with Platinum Taq High Fidelity (Invitrogen). PCR cycling conditions for HA were: 30 min at 55 °C, 2 min at 94 °C, four cycles of 94 °C for 30 s, 55 °C for 30 s, 68 °C for 180 s, followed by 40 cycles of 94 °C for 30 s and 68 °C for 210 s, and then 68 °C for 10 min. The same conditions were used for NA, except that the reverse transcriptase temperature was 54 °C and the annealing temperature was 58 °C.

The PCR products were visualized by gel electrophoresis using E-Gel 0.8 % agarose gels (Invitrogen) and purified with a High Pure PCR Product Purification kit (Roche Diagnostics). Purified PCR products were sent for single-sample sequencing at LGC Genomics.

Full-genome sequencing was performed on two cell culture-propagated influenza-positive samples by full-length amplification of all eight gene segments with in-house designed primers (primers available upon request) using a SuperScript III One-Step RT-PCR system with Platinum Taq High Fidelity and PCR conditions similar to those described for HA full-length amplification. Purified PCR products for all gene segments were pooled in equimolar quantities to a final amount of 1 µg and used for next-generation sequencing (NGS) on an Ion Torrent PGM sequencer (Life Technologies). NGS (including library preparation) was carried out at the Multi-Assay Core facility located at the Technical University of Denmark.

**Sequence analysis.** Sequences obtained by NGS were assembled using the **de novo** and reference assembly tools of CLC Genomics Workbench 4.6.1 (CLC bio). Sequences obtained by Sanger sequencing were analysed using CLC Main Workbench Version 6.9 (CLC bio).

**Animals.** All animal experiments were performed in Animal Biosafety Level 2 facilities at St. Jude Children’s Research Hospital (Memphis, TN, USA), in compliance with the policies of the National Institutes of Health and the Animal Welfare Act, and with the approval of the St. Jude Children’s Research Hospital Animal Care and Use Committee. In total, 36 ferrets (4–6 months old; Triple F farms), weighing 0.8–1.6 kg, that had been tested negative to current circulating human influenza subtypes by HI assay were used.

**Transmission and pathogenicity studies.** Before inoculation of the donor ferrets, baseline body temperatures and weights were documented for all ferrets. Donor and contact ferrets were housed separately. Four donor ferrets from each group were anaesthetized with isoflurane and inoculated intranasally with 106 TCID50 influenza virus in 1 ml PBS with antibiotics/antimycotic (Sigma; 100 U penicillin ml−1, 100 mg streptomycin ml−1 and 0.25 mg amphotericin ml−1) (0.3 ml per nostril). Transmission experiments (one donor plus one DC recipient plus one AT recipient) were conducted in duplicate for each virus. At day 1 p.i., each donor was co-housed with one naïve DC ferret. One additional naïve ferret was placed in an adjacent cage separated by double-layered perforated dividers that prevented physical contact, but allowed the passage of respiratory droplets, to assess for AT.

After inoculation, temperature, weight and clinical signs were recorded every other day for 11 days.

**Nasal washes.** Nasal washes were collected at 1 day p.i. for donor ferrets, and at 3, 5, 7, 9 and 11 days p.i. for all ferrets. Ferrets were anaesthetized intramuscularly with 20–50 mg ketamine kg−1, and nostrils were flushed with 1 ml PBS containing antibiotics/antimycotic (Sigma; 100 U ml−1 penicillin, 100 mg streptomycin ml−1 and 0.25 mg...
amphotericin B ml⁻¹ (0.5 ml per nostril) and collected into cups. Nasal washes were spun down and stored at −80 °C until further analysis. TCID₅₀ values were determined in MDCK cells and expressed as TCID₅₀ ml⁻¹.

**Ferret organ collection and virus titration.** At 5 days p.i., two inoculated ferrets from each group were sacrificed for pathological examination. The remaining ferrets were sacrificed at 21 days p.i. and the following tissues were collected from all animals: nasal turbinates, trachea, right/left caudal and cranial lung lobes, lymph nodes, intestine, liver, and spleen. Tissues were weighed and homogenized in MEM with antibiotics. Virus titres were determined in MDCK cells as described above and expressed as TCID₅₀ (g tissue)⁻¹.

**Serological tests.** Serum samples collected at 0 and 1 days p.i. and at 19 and 21 days p.i. were tested for antibodies. The serum samples were tested in a blocking ELISA using a commercially available influenza A antibody test kit, detecting antibodies against the NP gene (IDEXX Laboratories), according to the manufacturer’s instructions. The ELISA antibody values were calculated as optical density from each sample and presented as per cent seropositive.

**Histopathology.** Samples of trachea and the left/right cranial and caudal lung lobes were collected from two control ferrets and all inoculated ferrets at 5 days p.i., and the remaining ferrets at 21 days p.i. The tissues were fixed in 10 % neutral buffered formalin, embedded in paraffin, and slides were processed by routine methods for histochemistry, stained with haematoxylin/eosin and examined in a blinded fashion.

**Receptor assay.** The four viruses were tested for their HA binding activity to the following glycans: 2,3-α-SL, 2,6-α-SL and 2,6-α-SLN (see main text) (Glycotech), as described previously (Matrosovich & Gambaryan, 2012).

**NA kinetics and antiviral susceptibility to NA inhibitors.** NA kinetics and antiviral susceptibility to NA inhibitors were based on the method of Potier et al. (1979) using MUNANA (Sigma-Aldrich) substrate, as described by Jones et al. (2014).

Enzyme kinetics data were fitted by non-linear regression to the Michaelis–Menten equation using GraphPad Prism version 5 (GraphPad) to determine the Michaelis–Menten constant (Kₘ) and maximum velocity (Vₘₐₓ) of substrate conversion.

Sensitivity of NA to oseltamivir carboxylate (oseltamivir) from Hoffman-La Roche, zanamivir from Glaxo-SmithKline and peramivir from BioCryst Pharmaceuticals was tested by using dilutions of inhibitors ranging from 5 × 10⁻⁷ to 50 μM. The drug concentration that inhibited 50 % of the NA enzymatic activity (IC₅₀) was determined from the dose–response curve with GraphPad Prism version 5 and results expressed as the means of two independent tests.

**Statistical analysis.** Two-way ANOVA with Bonferroni’s post-test was performed using GraphPad Prism version 5.

**ACKNOWLEDGEMENTS**

We wish to thank Trushar Jeevan, Min-Suk Song, Jeri Carol Crompton and Jennifer Debeauchamp for technical assistance. This work was supported by National Institutes of Health.

**REFERENCES**


