New reassortant and enzootic European swine influenza 1 viruses transmits efficiently through direct contact in the ferret model

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

Running head: Zoonotic potential of four European SIVs

Contents category: Animal viruses – Negative-strand RNA

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The GenBank accession number for the sequences of 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.
ABSTRACT

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 titers 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 titers. Further, the viruses revealed preferential binding to the α2,6-sialylated glycans and investigation of the antiviral susceptibility of the viruses revealed that all were sensitive to neuraminidase inhibitors. These findings suggest 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 virus (IAV) causes disease in humans, birds and some domestic animals, including swine. Swine influenza viruses (SIV) 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. Further, the presence of IAV in swine also pose a potential health risk to humans due to 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 SIV have been detected throughout Europe, Asia and the US (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 SIVs and the H1N1pdm09 virus.

In Denmark, at least four SIV subtypes are enzootic in pigs, the avian-like H1N1 (H1N1), being the result of a transmission event from birds to swine (Abusugra et al., 1987; Schultz et al., 1991), 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 the H1N1pdm09 into Danish pigs. The virus spread and rapidly achieved a prevalence of 15-20% of SIVs isolated in the country (unpublished data). During the national passive surveillance program 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 1990-ies (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 SIV’s, the H1avN2hu and H1pdmN2sw, were compared to H1N2 and H3N2 enzootic European SIV 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 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 DC ferrets (Fig. 1) as assessed by isolation of virus from nasal washes. Infectious virus was detected in nasal washes until approximately 7 dpi for donor ferrets and 9 dpi for ferrets inoculated with H3N2. The day of observed peak titers for individual animals infected via direct contact, was found to vary in the four different groups (Fig. 1). Airborne transmission (AT) was only observed in ferrets inoculated with H3N2 (Fig. 1). One of the AT ferrets died at 11 dpi although it was concluded that the cause of death was not related to the infection. Since both AT ferrets in the group inoculated with H3N2 shed virus and virus was detected in nasal wash from the deceased ferret at 11 dpi, the results of the airborne transmission were not compromised by its death.
In comparison to the observed DC transmission of the four European SIVs, 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 SIV’s were readily transmissible via direct contact (Barman et al., 2012).

Nasal wash samples from all days post infection were tested by real time RT-PCR to determine if virus could be replicating at levels too low to be detected by the TCID\textsubscript{50} assay. The real time RT-PCR results was found to support the results obtained from the TCID\textsubscript{50} assay. Furthermore, low viral load was detected in one of the AT ferrets in the H1pdmN2sw group at 3 and 5 dpi, suggesting a potential for this virus to also become transmissible among ferrets via the airborne route. Taken together, it could appear that the H1N2/H1avN2hu viruses carrying avian-origin HA, play a less significant role in transmission compared to 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). The ELISA assay was chosen for antibody detection, due to a too high background response in the HI assay. At 19 and 21 dpi, inoculated and DC ferrets had all seroconverted.

**Viral load in respiratory organs**

From each group, two inoculated ferrets were euthanized at 5 dpi for determination of viral titers in nasal turbinates, trachea, caudal and cranial lung lobe, lymph nodes, intestine, liver and spleen. No viral replication was detected in liver, spleen, intestines or lymph nodes as was expected, since 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 lobe 1 and 2 (Fig. 3). Highest viral titers 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 titers 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 NA of this virus is not optimal, which could affect viral transmission.

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

**Histopathology**

Histopathological examination of the ferrets euthanized at 5 dpi, revealed no significant lesions in trachea or lung tissue of the control group (Fig. 4a). All four viruses caused hyperemia, edema and hemorrhage 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 among the groups depending on the virus used for inoculation.

Findings in trachea were alike in 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 and c) consisting of suppurative bronchiolitis and bronchitis, as well as epithelial necrosis of serous glands and perivascular accumulation of mononuclear cells. The inoculated ferrets furthermore had necrosis of bronchiolar epithelium, and in one of the H3N2 inoculated ferrets mixed bronchopneumonia was seen. 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 and e). Both groups of inoculated ferrets (euthanized 5 dpi) 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 SIV strains was assessed and compared in a human cell line (NHBE), a swine cell line (pSRECs) and MDCK cells. Cells were inoculated with a low MOI (0.01).

All four SIVs were able to infect and replicate to high titers in (8.0 - 9.4 log<sub>10</sub> TCID<sub>50</sub>/ml) NHBE cells. The H1avN2hu and H1pdmN2sw viruses progressively increased in titers until 48 hours post-inoculation (hpi). The H1N2 and H3N2 titers increased until 60 hpi (Fig. 5a). The high level of replication in NHBE cells of all four SIVs, suggested that these viruses have the ability to infect cells in the human respiratory airway.

In pSREC an increase in titers was observed until 36 hpi for H1avN2hu and H1N2 and for H1pdmN2sw and H3N2, an increase in titers was observed until 48hpi and 60 hpi, respectively (Fig. 5b). Mean peak titers in pSRECs were in the range from 6.6 - 7.2 log<sub>10</sub> TCID<sub>50</sub>/ml, where H1N2 was reaching the highest mean peak titer.

In MDCK cells, the virus titers progressively increased during the first 36 hpi with highest mean peak titers in the range from 6.3 log<sub>10</sub> TCID<sub>50</sub>/ml to 7.9 log<sub>10</sub> TCID<sub>50</sub>/ml and the highest mean peak titer was observed for H1N2 (Fig. 5c).
Receptor binding

Binding of the 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, glycan binding properties of the four viruses were investigated by testing for their ability to bind biotinylated sialylglycopolymers in a dose-dependent fashion.

Previously it has been shown that human IAVs bind preferentially to α₂,₆-SL and α₂,₆-SLN and to a lesser extent α₂,₃-SL as representatives of α₂,₆- and α₂,₃-linked 5-N-Acetylneuraminic acid receptors (Stevens et al., 2006). In this study, none of the SIVs exhibited strong binding preference towards the “avian” α₂,₃-SL and binding to this glycan barely exceeded the threshold. The European SIVs preferentially bound to the “human/swine” α₂,₆ sialylglycopolymers, with H3N2 and H1pdmN2sw showing the highest affinity for the “human/swine” α₂,₆-SLN. H1avN2hu and H3N2 were found to also bind α₂,₆-SL, the short version of the α₂,₆ sialylglycopolymers. For H1avN2hu and H3N2, it appeared that as the concentration of sialyglycopolymers were decreased the receptor preference shifted from the long to the short version of the α₂,₆ sialylglycopolymer. Results are summarized in Fig. 6. These findings are consistent with the efficient infection and transmission of the European H3N2 virus, since it was found to bind both α₂,₆-SL and α₂,₆-SLN, but does not explain the less efficient infection and transmission of the H1avN2hu, that was also shown to bind these two receptor analogs.

Sequencing

For examination of molecular determinants involved in receptor binding of the European SIVs, full length HA sequences were obtained from all four viruses using nasal washes from the day of their highest mean peak viral titers 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 to 265 and is formed by three secondary structures, termed the 130-loop, the 190-helix and the 220-loop (Gamblin et al., 2004).
The amino acid at residue 190 has been shown to play an important role in binding of SIVs and human IAVs to the α-2,6 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 to S mutation potentially affects binding of the H1avN2hu to the α2,6 receptor.

Residue 225 was found to vary between all four European SIVs, where H1avN2hu was found to possess 225E and H1pdmN2sw showing the “avian” 225D. In H1N2 an E225K mutation was observed during the study, with the 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 cohoused and thus suggest 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, but also 225E and 225K (Dunham et al., 2009).

Investigation of the receptor binding properties of the H3N2 virus showed that the European H3N2 SIV possessed 155Y and 158G (Table 1), mutations found to be present in human H3’s and both mutations have previously been shown to play a critical role in recognition of two major molecular species of sialic acids, namely 5-N-acetylneuraminic acid and 5-N-glycolylneuraminic acid, where 5-N-glycolylneuraminic acid is an analog of sialic acid, expressed in many animal tissues, but absent from humans (Chou et al., 1998; Matrosovich et al., 2000; Takahashi et al., 2009).

Further, amino acids at residue 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 has been found to be present in human viruses (Glaser et al., 2005; Matrosovich et al., 2000) and these “avian” residues were also present in the European avian-like H1 viruses.
The European SIV H3N2 was found to possess 226L and 228S (Table 1). These amino acids have previously been shown, for the human H3 subtype, to reduce the affinity for α2,3 receptor binding, as well as increasing the affinity for α2,6 receptors (Matrosovich et al., 2000; Nobusawa et al., 2000; Rogers et al., 1983). Taken together, these findings are in accordance with the observed ability of H3N2 to infect both human and swine cell lines, as well as infecting and transmitting between ferrets.

**NA kinetics and antiviral susceptibility**

Previously it has been suggested that NA activity may facilitate the transmissibility of IAVs (Campbell et al., 2014) and hence NA enzyme kinetics of the four European SIVs were determined, using the MUNANA fluorogenic substrate. The Michaelis-Menten constant ($K_M$) is an estimate of the dissociation equilibrium for substrate binding to enzyme and thereby reflects the enzyme affinity for the substrate, where $V_{max}$ reflects the enzyme’s catalytic activity. All four European SIVs showed high $K_m$ values, ranging from 281 µM for H3N2 to 492 µM for H1N2, compared to N1 viruses (Hooper & Bloom, 2013; Ilyushina et al., 2010; Yen et al., 2011). $V_{max}$ values were found to be in the range from 842 U/sec for H3N2 to 1845 U/sec for H1pdmN2sw (Table 2).

It has previously been found that PR8 modified by reverse genetics to contain a pandemic matrix (M) gene, enhanced NA activity (Campbell et al., 2014). Of the European SIVs, only H1pdmN2sw contained a pandemic M gene and interestingly, the H1pdmN2sw showed higher enzyme activity (a higher $V_{max}$) than the H3N2 virus, despite comparable $K_M$ values. These findings suggest that high NA activity alone cannot be responsible for the airborne transmission observed for the H3N2 virus. It would be interesting though, to test if NA activity and transmission efficiency of the European H3N2 virus would increase further if the original M gene was replaced with a pandemic M gene.

Previously it has been shown that H1N1pdm09 was transmissible among ferrets by the airbourne route, in spite of a much lower NA activity (Yen et al., 2011), compared to the one observed for H1pdmN2sw. In the same study, it was suggested that an optimal HA-NA balance is required for
airborne transmission, and it could therefore be speculated that this balance is not optimal in the H1pdmN2sw virus.

The antiviral susceptibility of the four European SIVs to three of the most commonly used neuraminidase inhibitors (Oseltamivir carboxylase, Zanamivir and Peramivir) was also tested and all of the European SIVs were found to be sensitive to all of the neuraminidase inhibitors tested (Table 2).

Here we have shown that four European SIVs, including two new reassortants and two enzootic viruses, transmitted efficiently in the ferret model via direct contact. Furthermore, we showed that one of these viruses, H3N2, was the only virus able to transmit via the airborne route. These results, combined with the efficient transmission of this virus to DC ferrets and the high viral titers in both nasal turbinates and trachea, showed altogether that this virus was the most effective, of the four European SIVs, for replication and transmission in the ferret model. Furthermore, the ability of this virus to replicate in human respiratory cells suggests that this virus could potentially transmit to humans. For the past 25 years, the European H3N2 virus has been adapting to swine and the degree of antigenic divergence between this virus and the human seasonal H3N2 virus is likely to have increased dramatically. A recent study did show that contemporary human seasonal H3N2 viruses had obtained substantial antigenic distance from swine H3N2 viruses, even though the lineages shared a common ancestor. Hence, the authors speculated that this increasing distance could pose a risk for the youngest of the human population, since they could become increasingly susceptible to infections with swine H3N2 due to the lack of cross-reacting immunity (Lewis et al., 2014). These findings stress the need for a continued and systematic surveillance of the European SIVs in order to detect new reassortants as well as monitoring the evolution and zoonotic potential of both reassortant and enzootic SIV strains.
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 Madin-Darby canine kidney (MDCK) cells. Before inoculation, influenza viruses were passaged in the allantoic cavity of 10-day-old embryonated chicken eggs (Marshall Durbin, Birmingham, AL) 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, Carlsbad, CA, USA) containing 5% fetal calf serum (FCS), 2 mM L-glutamine, Non-essential amino acids (NEAA) and penicillin-streptomycin. Normal human bronchial epithelial cells (NHBE) in individual inserts were obtained from MatTek Corporation (Ashland, MA, USA). The cells were grown in AIR-100-ASY (MatTek Corporation Ashland, MA, USA) serum free media containing growth factors. The apical surface was washed to remove mucus and media was changed every other day.

Primary swine respiratory epithelial cells (pSREC) were seeded into type VI collagen (Sigma-Aldrich, St. Louis, MO, USA)-coated tissue culture flasks and grown in Bronchial Epithelial Cell Growth medium (BEGM, Lonza, Walkersville, MD, USA) with SingleQuots™ Kit containing growth factors, and cytokines. Medium was further supplemented with 5% FCS and 1% penicillin/streptomycin/amphotericin (Sigma, St. Louis, MO, US) and passaged up to five times prior to infection. All cells were grown at 37°C in a 5% CO₂ atmosphere.
Infection and replication kinetics

For preparation of viral stocks, lung tissue was homogenized on TissueLyser (Qiagen, GmbH, Germany) in 1.5 mL MEM supplemented with penicillin-streptomycin and sterile filtered. MDCK cells were inoculated with 500 µL lung tissue homogenate for 30 min at 37°C, 5% CO₂. Following incubation, 10 mL MEM containing penicillin-streptomycin, NEAA, 2 mM L-glutamine and 2 µg/mL tosylsulfonfyl-phenylalanyl-chloromethyl-ketone (TPCK)-treated trypsin (Sigma-Aldrich, St. Louis, MO, USA) was added to the cells. The cells were incubated for 72 h and daily monitored for cytopathic effects (CPE). The supernatant was harvested and centrifuged at 2500 rpm for 30 minutes to clarify cell debris and then stored at -80°C until further use.

TCID₅₀ and pfu were determined by incubating serial dilutions of virus in MDCK cells at 37°C for 72 h. A hemagglutination assay was performed to determine the end point of infection and TCID₅₀ was calculated using the method of Reed and Muench (Reed & Muench, 1938).

Replication kinetics in MDCK cells were determined at a MOI of 0.01 pfu per cell. After 1 h of incubation the MDCK cells were washed and overlaid with infection medium (MEM containing 2% BSA, pen-strep, amino acids and TPCK-treated trypsin). Supernatants were collected and stored at -80°C for virus titration. Replication kinetics in differentiated NHBE cells were determined at a MOI of 0.01 pfu per cell. Cells were washed with PBS and were inoculated via the apical side with 200 µl diluted virus in the absence of trypsin. After 1 h of incubation at 37°C, viral inoculum was removed and cells were washed. Cells were then pre-incubated with 200 µl fresh medium at 37°C for 30 min prior to sample collection at specified time points. Samples were stored at -80°C. Replication kinetics in pSRECs was determined at an MOI of 0.01 pfu per cell. Cells were washed and infection medium (BEGM, Lonza, Walkersville, MD, USA) containing 0.5% BSA was added, supernatants were collected at specified time points and stored at -80°C for virus titration.
RNA purification and real time RT-PCR screening

Viral RNA was purified from cultured viruses by RNeasy Mini Kit (Qiagen, GmbH, Germany) according to manufacturer’s instructions. Cell culture supernatant was prepared by mixing 200 µl sample with 400 µl RLT-buffer containing β-mercaptoethanol. Total RNA was eluted in 60 µl RNase-free water and stored at -80°C.

The presence of influenza A virus was confirmed by real-time RT-PCR using an in-house modified assay for detection of the matrix 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., (Hoffmann et al., 2001) and the Superscript III One-Step RT-PCR kit with Platinum Taq High Fidelity (Invitrogen, Carlsbad, CA, USA). PCR cycling conditions for HA was as follows: 30 min at 55°C, 2 min at 94°C, four cycles of 94°C for 30 sec, 55°C for 30 sec, 68°C for 180 sec, followed by 40 cycles of 94°C for 30 sec and 68°C for 210 sec and then 68°C for 10 min. For NA the same conditions were used, except that reverse transcriptase temperature was 54°C and annealing temperature 58°C.

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

Full genome sequencing was performed on two cell culture propagated influenza positive samples by full length amplification of all 8 gene segments with in-house designed primers (primers available upon request) using SuperScript III OneStep RT-PCR System with Platinum Taq High Fidelity and PCR conditions similar to that described for HA full length amplification. Purified PCR products for all gene segments were pooled in equimolar quantity to a final amount of 1 µg and used for next generation
sequencing (NGS) on the Ion Torrent PGM™ sequencer (Life Technologies, Carlsbad, CA, USA).

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 A/S, Århus, Denmark). Sequences obtained by Sanger sequencing was analyzed using CLC Main Workbench Version 6.9 (CLC bio A/S).

### Animals

All animal experiments were performed in Animal Biosafety Level 2 facilities at St. Jude Children 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 four to six month old ferrets (Triple F farms, Sayre, PA, US), weighing 0.8-1.6 kg, that had been tested negative to current circulating human influenza subtypes by hemagglutination inhibition (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 anesthetized with isoflurane and inoculated intranasally with $10^6$ TCID$_{50}$ influenza virus in 1 ml PBS with antibiotics and antimycotic (Sigma, St. Louis, MO; 100 U/ml penicillin, 100 mg streptomycin, and 0.25 mg amphotomycin per ml) with 500 µl in each nostril. Transmission experiments (1 donor+1 direct-contact (DC) recipient+1 airborne-transmission (AT) recipient) were conducted in duplicate for each virus. At day 1 post infection (dpi) each donor was co-housed with one naive DC ferret. One additional naive 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 dpi for donor ferrets and at 3, 5, 7, 9 and 11 dpi for all ferrets. Ferrets were anesthetized intramuscularly with 20-50 mg/kg of Ketamine, nostrils were flushed with 1 ml of PBS containing antibiotics and antimycotic (Sigma, St. Louis, MO, USA; 100 U/ml penicillin, 100 mg streptomycin, and 0.25 mg amphotericin per ml) with 500 µl in each nostril and collected into cups. Nasal washes were spun down and stored at -80°C until further analysis. TCID₅₀ were determined in MDCK cells and expressed as TCID₅₀/ml.

Ferret organ collection and virus titration
At 5 dpi two inoculated ferrets from each group were sacrificed for pathological examination. The remaining ferrets were sacrificed at 21 dpi 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 titers were determined in MDCK cells as described above and expressed as TCID₅₀/g tissue.

Serological tests
Serum samples collected at 0 and 1 dpi and at 19 and 21 dpi were tested for antibodies. The serum samples were tested in a blocking ELISA using the commercially available influenza A antibody test kit, detecting antibodies against the NP gene (IDEXX Laboratories A/S, Switzerland), according to manufacturer’s instructions. The ELISA antibody values were calculated as optical density (OD) from each sample and presented as percent transmittance.

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

**Receptor assay**

The four viruses were tested for their HA binding activity to the following glycans; α2,3-SL (Neu5Acα2-3Galβ1-4Glcβ-PAA-Biotin), α2,6-SL (Neu5Acα2-6Galβ1-4Glcβ-PAA-Biotin) and α2,6-SLN (Neu5Acα2-6Galβ1-4GlcNAcβ-PAA-Biotin) (Glycotech Corp., Gaithersburg, MD, US) as previously described (Matrosovich & Gambaryan, 2012).

**NA kinetics and antiviral susceptibility to neuraminidase inhibitors**

NA kinetics and antiviral susceptibility to NA inhibitors (NAI) were based on the method of Potier et al. (Potier *et al.*, 1979) using methylumbelliferone N-acetylneuraminic acid (MUNANA; Sigma-Aldrich, St Louis, MO) 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 Software, La Jolla, CA, USA) to determine the Michaelis Menten constant and maximum velocity ($V_{max}$) of substrate conversion.

Sensitivity of NA to oseltamivir carboxylate (oseltamivir) from Hoffmann-La Roche (Basel, Switzerland), zanamivir from Glaxo-SmithKline (Research Triangle Park, NC, USA), and peramivir from BioCryst Pharmaceuticals (Birmingham, AL, USA) was tested by using dilutions of inhibitors ranging from 5×10^{-7} µM to 50 µM. The drug concentration that inhibited 50% of the NA enzymatic activity (IC$_{50}$) was determined from the dose-response curve with GraphPad Software version 5. Results are expressed as the average of two independent tests.

**Statistical analysis**

Two-way ANOVA with Bonferroni’s posttest was performed using GraphPad Prism version 5.
Nucleotide sequence accession numbers

The GenBank accession numbers of HA 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.

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REFERENCES


Fig. 1. Replication kinetics in the upper respiratory tract. Groups of four ferrets were intranasally inoculated with 1 ml of $10^6$ TCID$_{50}$ (0.5 ml per nostril) of the respective viruses. Each virus group contained eight ferrets, four donor ferrets (until 5 dpi), two direct contacts and two airborne transmission contacts. (a) H1avN2hu (b) H1N2 (c) H3N2 (d) H1pdmN2sw. Nasal washes were collected on days 1, 3, 5, 7, 9 and 11 after infection. Data are presented as mean virus titer ± SEM ($\log_{10}$ TCID$_{50}$/ml) on the indicated day. Limit of detection was $10^1$ TCID$_{50}$/ml.

Fig. 2. Seroconversion of ferrets. Ferrets were tested for seroconversion at 19 and 21 dpi, 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 mean values are presented as percent seropositive ± SEM.

Fig. 3. Comparison of European swine influenza virus titers recovered from ferret tissues. Ferrets were inoculated intranasally with 1 ml of $10^6$ TCID$_{50}$ (0.5 ml per nostril) with H1N2, H1avN2hu, H3N2 or H1pdmN2sw, and tissues were collected at day 5 p.i. Titers are expressed as $\log_{10}$ TCID$_{50}$/g per gram of tissue. Each bar represents two ferrets. Data are presented as mean virus titer ± SEM ($\log_{10}$ TCID$_{50}$/g) from the indicated tissue. Limit of detection was $10^1$ TCID$_{50}$/g.

Fig. 4. Lung pathology in ferrets inoculated with one of the four European swine influenza viruses and euthanized 5 days post infection a-e) Lung tissue from ferrets, hematoxylin and eosin, scale bar 50 µm. a) Normal lung tissue from control ferret; b) Ferret inoculated with H3N2, 5 days post infection (dpi). Suppurative bronchiolitis, dysplasia of bronchiolar epithelium and hyperplasia of the bronchus associated lymphoid tissue (BALT) (asterisks); c) Ferret inoculated with H1pdmN2sw, 5 dpi.
Suppurative bronchiolitis, dysplasia and desquamation (arrowhead) of bronchiolar epithelium and BALT hyperplasia (asterisks); d) Ferret inoculated with H1N2, 5 dpi. Suppurative bronchiolitis and peribronchiolar infiltration of mononuclear cells. Dysplasia, necrosis and desquamation (arrowhead) of bronchiolar epithelium; e) Ferret inoculated with H1avN2hu, 5 dpi. Suppurative bronchiolitis, and dysplasia, necrosis and desquamation (arrowhead) of bronchiolar epithelium.

**Fig. 5. Replication kinetics of European swine influenza viruses in different cell lines.** Growth curves were obtained by inoculating cells with an MOI of 0.01 PFU/cell with H1N2, H1avN2hu, H1pdmN2sw or H3N2, respectively. Supernatant was harvested and titrated in MDCK cells at 8, 10, 12, 18, 20, 24, 36, 48 and 60 hours post infection. Data are expressed as mean log_{10} TCID_{50} ± SEM from two independent experiments titered in quadruplicate.

**Fig. 6. Receptor specificity of four European swine influenza viruses.** The receptor-binding specificity of the four European swine influenza viruses H1N2, H1avN2hu, H1pdmN2sw and H3N2 were tested in a dose dependent glycan array assay against the sialyl glycans α2,6-SL and α2,6-SLN and α2,3SL.
Table 1. Sequencing data from important 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>
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<tbody>
<tr>
<td>155</td>
<td>-</td>
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<td>-</td>
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<tr>
<td>226</td>
<td>Q</td>
<td>Q</td>
<td>Q</td>
<td>L</td>
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<tr>
<td>228</td>
<td>G</td>
<td>G</td>
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**TABLE 2.** NA enzyme kinetics and antiviral susceptibility of European swine influenza viruses using MUNANA substrate

<table>
<thead>
<tr>
<th>Virus</th>
<th>Vmax [mean (95% CI)]</th>
<th>Km (µM) [mean (95% CI)]</th>
<th>IC_{50} (nM) [mean (95% CI)]</th>
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</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>
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<tr>
<td>H1pdmN2sw</td>
<td>1845 (1655-2036)</td>
<td>487 (320.5-653.6)</td>
<td>0.29 (0.19-0.44)</td>
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<tr>
<td>H1N2</td>
<td>1495 (1219-1772)</td>
<td>492 (191.7-792.4)</td>
<td>0.47 (0.31-0.72)</td>
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<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>

The Michaelis-Menten constant (Km) and maximum velocity (Vmax) of substrate conversion were fitted to the Michaelis-Menten kinetics by non-linear regression. CI, confidence interval.
Figure
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