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Eurasian 1C swine influenza A virus exhibits high pandemic risk traits

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ABSTRACT

Recent surveillance has identified an expansion of swine H1 1C influenza viruses in Eurasian swine. Since 2010, at least 21 spillover events of 1C virus into humans have been detected and three of these occurred from July to December of 2023. Pandemic risk assessment of H1 1C influenza virus revealed that individuals born after 1950 had limited cross-reactive antibodies, confirming that they are antigenically novel viruses. The 1C virus exhibited phenotypic signatures similar to the 2009 pandemic H1N1 virus, including human receptor preference, productive replication in human airway cells, and robust environmental stability. Efficient inter- and intraspecies airborne transmission using the swine and ferret models was observed, including efficient airborne transmission to ferrets with pre-existing human seasonal H1N1 immunity. Together our data suggest H1 1C influenza virus poses a relatively high pandemic risk.

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KEYWORDS Influenza virus; swine; pre-existing immunity; transmission; pandemic risk assessment

Introduction

Influenza virus in swine, much like humans, causes an acute infection of the respiratory tract with typical recovery in 7–10 days. Interspecies transmission from swine to humans can occur, however, onward transmission from person-to-person is very rare, with the notable exception of the H1N1 influenza pandemic in 2009. Three predominant influenza A virus (IAV) subtypes are endemic in swine, H1N1, H1N2, H3N2. Genetically and antigenically distinct IAV swine H1 viruses are classified into three major lineages named 1A, 1B or 1C [1]. The Eurasian avian 1C lineage is geographically restricted with detections only in Europe and Asia in contemporary surveillance [2,3]. An avian H1N1 virus crossed the species barrier from wild ducks to swine in Europe in 1979 and donated the HA gene to the 1C lineage [4]. Eurasian 1C swine viruses were detected in the human population 29 times since 2011, >48% of those across 9 countries since 2021 (https://github.com/flu-crew/datasets/blob/main/h1n2-pandemic_risk/h1v-lineList-2010toPresent.xlsx). The broad geographical distribution of H1 1C viruses in swine populations and zoonotic spillover frequency suggests that these viruses have pandemic risk.

The 1C lineage is the dominant lineage in China [5–7] and serological surveys of swine production workers from 15 Chinese farms indicates an increased infection rate as compared to serum samples from ordinary Chinese households [5], suggesting repeated spillover events in individuals in close contact with swine. Additionally, 1C viruses appear antigenically novel as little to no cross-protection is observed in human serum samples from Asia [5,7–9], in combination with an increased number of swine detections in Europe [10,11], making spillover events into humans of increased concern. In Fall of 2021, multiple human cases of an H1N2 variant from clade 1C.2.4 were observed, including one in France. This was the first human detection of 1C.2.4 in France and

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represents an evolutionary expansion of 1C in the swine population. In this study, we utilize a decision tree [12] (Figure S1) to determine the pandemic potential of A/Bretagne/24241/2021, a 1C.2.4 variant virus (referred to herein as 1C H1N2v) through examination of viral phenotypic traits as well as interspecies and intraspecies transmission.

Materials and methods

Cells

Madin-Darby canine kidney (MDCK) or MDCK-London and 293 T cells were obtained from American Type Culture Collection (ATCC) and maintained in Minimum Essential medium and Dulbecco's modified Eagle's medium (DMEM), respectively. Medium was supplemented with 10% fetal bovine serum, 2 mM L-glutamine and 2 mM penicillin/streptomycin. Primary human bronchiole epithelial cell cultures were differentiated from human lung tissue and cultured at an air–liquid interface using a protocol approved by the institutional review board at the University of Pittsburgh [13]. All cells were incubated at 37°C with 5% CO_2.

Rescue of virus from plasmids using co-cultured cells

The reverse genetic plasmids of A/California/07/2009 (H1N1pdm09) were a generous gift from Dr. Jesse Bloom (Fred Hutch Cancer Research Center, Seattle). Reverse genetics plasmids expressing A/Bretagne/24241/2021 (1C H1N2v) were synthesized based on sequences deposited in the GISAID (Accession numbers EPI1913510, EPI1913511, EPI1913512, EPI1913513, EPI1913514, EPI1913515, EPI1913516, EPI1913517 doi:10.6084/m9.figshare.27194397), with noncoding regions determined from consensus alignment of swine H1 1C viruses. The A/Bretagne/24241/2021 virus was selected as it represents a candidate vaccine virus (CVV) selected by the World Health Organization for pandemic preparedness [2]. The HA gene is classified within the Eurasian swine lineage 1C.2.4 clade [1] and this diverse group of viruses has become one of the most frequently detected in Europe [14]. The A/Bretagne24241/2021 retains 87% identity across the HA1 to the most representative 1C.2.4 HA gene selected using PARNAS v.0.1.6 [15]. To fully characterize the 1C.2.4 HA genes collected between 2021 and 2023, it would be necessary to select a currently unfeasible number of strains, i.e. to cover all HA genes within a 5% divergence threshold it would be necessary to characterize 18 strains [15,16].

The A/Bretagne/24241/2021 (H1N2v) co-culturing of 293 T cells and MDCK or MDCK-London cells was performed in a 12-well plate at a ratio of 4×10^5: 4×10^4 cells/well using Opti-MEM®I complete media (Life Technologies, Waltham, MA). The 12-well plate was incubated at 37°C with 5% CO_2 and used for transfection within 24 h. The confluence of the co-cultured monolayer cells used for transfection was about 60%. Each plasmid containing the 8 segments of A/Bretagne/24241/2021 was diluted to a concentration of 100 ng/mL and a total of 500 ng of each gene segment was combined with Opti-MEM®I up to 100 and 8 µL of TransIT-LT1 transfection reagent was added into the mixture (Mirus Bio LCC, Madison, WI). The transfection mixture was incubated at room temperature for 45 min and transferred to the co-cultured cells monolayer. After 24 h of incubation at 37°C with 5% CO_2, the transfection mixture was discarded and replaced with 1 mL of virus growth media Opti-MEM®I (Life Technologies, Waltham, MA) containing antibiotics/antimycotics and 1 µg of tosyl sulfonyl phenylalanyl chloromethyl ketone (TPCK) trypsin (Worthington Biochemical Corp., Lakewood, NJ) and incubated at 37°C with 5% CO_2. Following 48 h post-transfection, hemagglutination activity was checked by hemagglutination (HA) assays [17]. A blind-passage of the rescued virus was performed in MDCK-London cells in a T25 cm^2 flask. The flask was monitored for cytopathic effect (CPE) for 48 h post-inoculation. The supernatant was collected, HA activity was checked, and sequencing was submitted to the Iowa State University Veterinary Diagnostic Laboratory for sequencing to confirm the homology of the 8 gene segments of the 1C.2.4 H1N2 rg-A/Bretagne/24241/2021 strain.

Virus titration

Nasal wash samples from A/Bretagne/24241/2021-infected ferrets were titred in MDCK cell cultures. Ten-fold serial dilutions were made with 20 µL of sample being diluted into 180 µL of Minimum Essential medium supplemented with 2 mM L-glutamine and 1× Anti–Anti in 96-well plates of confluent MDCK cells and performed in quadruplicate. MDCK cells were observed 4 dpi for cytopathic effect (CPE). Virus titres were calculated using Reed and Muench method [18] and expressed as $\log_{10}$ $TCID_{50}$/mL. The limit of detection for this assay is $10^{1.2}$ $TCID_{50}$/mL.

Human subjects research ethics statement

The University of Pittsburgh Institutional Review Board approved protocol STUDY20030228 for the collection of human serum samples from healthy adult donors who provided written informed consent for their samples to be used in infectious disease research. All participants self-reported their age, sex, race, ethnicity, residential zip code, history of travel and immunization. Human bronchiole epithelial cell cultures were obtained from deidentified patients under the University of Pittsburgh Institutional

Review Board-approved protocol STUDY19100326. Plasma samples were collected from individuals vaccinated with FluLaval Quadrivalent in Philadelphia, PA under the University of Pennsylvania Institutional Review Board approved study 849398.

Microneutralization assay

The titre of neutralizing antibodies against A/California/07/2009 and A/Bretagne/24241/2021 was determined from human sera that had been heat inactivated at 56°C for 30 min. Briefly, two-fold serial dilutions of heat-inactivated human serum in a volume of 125 µL were incubated with 125 χ of $10^{3.3}$ $TCID_{50}$ of influenza virus for 1 h at room temperature with continuous rocking. 150 µL of Minimum Essential medium supplemented with 2 mM L-glutamine and 1× Anti–Anti with TPCK was added to 96-well plates with confluent MDCKs before 50 µL of virus:serum mixture was added in quadruplicate. After 4 days, CPE was determined, and the neutralizing antibody titre was expressed as the reciprocal of the highest dilution of serum required to completely neutralize the infectivity of each virus on MDCK cells. The concentration of antibody required to neutralize 100 $TCID_{50}$ of virus was calculated based on the neutralizing titre dilution divided by the initial dilution factor, multiplied by the antibody concentration. The limit of detection for this assay is 14.

Glycan array

Viruses, A/Bretagne/24241/2021 H1N2v and A/California/07/2009 H1N1pdm09 were propagated in T225 flask of MDCK cells and inactivated using beta-propiolactone (BPL). Inactivation was confirmed by serial passage in MDCK cells. Inactivated virus was purified and labelled as described in [19]. Purification was performed on 25% sucrose cushion in NTE (100 mM NaCl, 10 mM Tris, 1 mM EDTA) buffer spun for 2 h at 28,000 rpm. Resuspend in PBS and test HA titre (used to normalize virus on the glycan array after labeling). Labeling of virus with Alexafluor 488 using NHS ester labeling kit (A20000, Invitrogen/Thermo Fisher Scientific) utilized 200uL of purified virus and 20 µL 1M NaHCO3 (pH 9.0) and 25 µL of 1ug/uL Alexafluor 488, mix at room temperature for 1 h in the dark. The labelled virus was dialysed in 1 L of 1× PBS overnight at 4°. Emory Center for Glycan Genomics was given the labelled virus and HA titres. Varying dilutions of the virus were run on the legacy nCFG version 6 array. Data analysis was based on category of terminal sialic acid and relative fluorescence units detected on the array.

Glycan binding by ELISA

Streptavidin-coated high binding capacity 384-well plates were washed five times with PBS before incubating 50 µL of a 1.8 µM solution of a biotinylated glycan in PBS overnight at 4°C. The plates were washed five times with PBS and blocked with 100 µL of 1% bovine serum albumin (BSA) in PBS containing 0.6 µM desthiobiotin at room temperature for 2 h. Purified His-tagged-HAs (100 µg mL^{-1}) premixed with anti-His mouse IgG2a antibody (Biolegend, cat no. 362616) and HRP-conjugated goat anti-mouse IgG (H + L) secondary antibody (Invitrogen, cat no. G21040) in a 4:2:1 ratio (w/w/w) with 1% BSA in PBS and incubated on ice for 30 min. These complexes were then subject to 3-fold serial dilution. Plates were washed five times with PBS containing 0.05% Tween20 (PBS-T) before incubating with 50 µL of diluted complex and incubated at room temperature for 2 h. The wells were rinsed with PBS-T 5 times and replenished with 50 µL of the 3,3′,5,5′ tetramethylbenzidine (TMB, Sigma-Aldrich, cat no. T0440) peroxidase substrate. The plates were incubated at room temperature for 5 min and then quenched with 50 µL of 2 M sulphuric acid. The absorbance at 450 nm was detected using a BioTek Synergy H1 microplate reader (Agilent). The assay was performed in triplicate.

Replication kinetics in human bronchiole epithelial cell cultures

Three different HBE patient cell cultures were used (HBE0405, HBE0408, HBE0409) with three transwells of each per time point. The apical surface of the HBE cells was washed in 150 µL phosphate-buffered saline (PBS) to remove any airway surface liquid for 10 min and collected for subsequent use. An inoculum of 10^3 $TCID_{50}$ of A/California/07/2009 or A/Bretagne/24241/2021 was added per 100 µL of HBE growth medium. The titre of each inoculum was determined to ensure that both strains were within $10^{0.5}$ $TCID_{50}$/mL of each other. After 1 h incubation, the inoculum was removed and the apical surface was washed three times with 150 µL of PBS. At the indicated time points, 150 µL of HBE medium was added to the apical surface for 10 min to capture released virus particles. Infectious virus was quantified by $TCID_{50}$ using the endpoint method [18]. The limit of detection for this assay is $10^{1.2}$ $TCID_{50}$/mL.

Stability of stationary droplets

Airway surface liquid (ASL) was collected from each HBE transwell using 150 µL of PBS for 10 min at 37°C. ASL from at least 12 patient transwells was pooled and stability experiments were performed with four different patient ASL. Virus stock was mixed with ASL at a ratio of 1:10 and used to generate ten 1 µL droplets in triplicate on a 6-well plate with tissue culture-treated plastic (ThermoFisher, Waltham,

MA). Virus:ASL droplets were incubated in a sealed desiccator chamber containing saturated salt solutions of potassium acetate, potassium carbonate, magnesium nitrate and sodium chloride to maintain desired 23%, 43%, 55% and 75% relative humidity (RH), respectively. Chambers were maintained in a biosafety cabinet for the duration of the experiment and a HOBO UX100011 data logger (Onset, Cape Cod, MA) was used to collect RH and temperature data. After 2 h, the droplets were collected in 500 µL of L-15 Leibovitz's medium (Gibco, Grand Island, NY), which was titred on MDCK cells using the $TCID_{50}$ endpoint method [18]. The limit of detection for this assay is $10^{1.2}$ $TCID_{50}$/mL. Decay was determined by subtracting the titre of the virus aged for 2 h from the titre of the virus that had been deposited and then immediately recovered.

Enzyme-linked lectin assay (ELLA)

A 96-well ultra-high binding polystyrene plate (ThermoFisher, Waltham, MA) was coated with 25 mg/mL of fetuin (Sigma-Aldrich, St. Louis, MO) diluted in coating buffer (SeraCare, Milford, MA) overnight at 4°C. Plates were washed 3 times with 150 µL of wash buffer (0.01M PBS pH 7.4 and 0.05% Tween 20) to remove excess fetuin. Once washed, 50 µL of a two-fold serial dilution series of $10^{7.5}$ $TCID_{50}$/mL A/California/07/2009 or A/Bretagne/24241/2021 virus stocks was added to the fetuin plate and incubated overnight at 37°C. To standardize the viruses between different plates, a two-fold serial dilution series of 62.5 µU/mL *Clostridium perfringes* neuraminidase (Sigma Aldrich, St. Louis, MO) was also performed and added to the fetuin plate. Plates were thoroughly washed 6 times with 150 µL of wash buffer and incubated with 100 µL/well of peroxidase-labelled peanut agglutinin (Sigma Aldrich, Burlington, MA) solution in the dark for 2 h. Plates were washed 3 times with 150 µL of wash buffer to remove the Peroxidase-labelled Peanut Agglutinin. 100 µL of O-phenylenediamine dihydrochloride substrate (Sigma-Aldrich, St. Louis, MO) was added. Plates were incubated for 10 min in the dark at room temperature, then the reaction was stopped by the addition of 100 µL/well of 1N sulphuric acid (Sigma Aldrich, St. Louis, MO). Absorbance was read at 490 nm. NA activity was assayed in duplicate and performed in three independent replicates.

pH inactivation assay

The pH of PBS titrated from 7.5 to 3.0 at roughly 0.5 pH increments using concentrated HCl. Virus stocks (10 µL) were incubated in 990 µL of pH-adjusted PBS for 1 h at 37°C. The pH was neutralized by immediately titering the infectious virus on MDCK cells using the $TCID_{50}$ endpoint titration method [18]. The limit of detection for this assay is $10^{1.2}$ $TCID_{50}$/mL. The pH that reduced the titre by 50% (EC_{50}) was calculated by regression analysis of the dose–response curves. Each experiment was performed in triplicate in at least three independent biological replicates.

Animal ethics statement

Ferret experiments were conducted in a BSL2 facility at the University of Pittsburgh in compliance with the guidelines of the Institutional Animal Care and Use Committee (approved protocol 22061230). Animals were sedated with isoflurane following approved methods for all nasal washes and survival blood draws. Ketamine and xylazine were used for sedation for all terminal procedures, followed by cardiac administration of euthanasia solution. Approved University of Pittsburgh Division of Laboratory Animal Resources (DLAR) staff administered euthanasia at time of sacrifice. Pigs and ferrets in the swine and swine to ferret transmission studies were housed in BSL2 containment in compliance with approved USDA-ARS NADC animal care and use protocols.

Ferret screening

Sera from four- to six-month-old male ferrets were screened for antibodies against circulating influenza A and B viruses using hemagglutination inhibition (HAI) before purchase (Triple F Farms, Sayre, PA). Briefly, sera were treated with receptor destroying enzyme (RDE) II (Hardy Diagnostics, Santa Maria, CA) overnight at 37°C to remove non-specific inhibitors and heat inactivated at 56°C for 30 min. Two-fold serial dilutions of sera were performed and incubated with eight hemagglutinating units of the following antigens obtained through the International Reagent Resource, Influenza Division, WHO Collaborating Center for Surveillance, Epidemiology and Control of Influenza, Centers for Disease Control and Prevention, Atlanta, GA, USA: 2018–2019 WHO Antigen, Influenza A (H3) Control Antigen (A/Singapore/INFIMH-16-0019/2016), BPL-Inactivated, FR-1606; 2014–2015 WHO Antigen, Influenza A (H1N1)pdm09 Control Antigen (A/California/07/2009 NYMC X-179A), BPL-Inactivated, FR-1184; 2018–2019 WHO Antigen, Influenza B Control Antigen, Victoria Lineage (B/Colorado/06/2017), BPL-Inactivated, FR-1607; 2015–2016 WHO Antigen, Influenza B Control Antigen, Yamagata Lineage (B/Phuket/3073/2013), BPL-Inactivated, FR-1403. After incubation for 15 min, an equal volume of 0.5% turkey red blood cells (Lampire Biological Laboratories, Pipersville, PA) were added and incubated for 30 min. The reciprocal of the highest dilution of

serum that inhibited hemagglutination was determined to be the HAI titre.

Ferret-to-ferret transmission

Eighty-four days prior to the 1C H1N2v transmission experiment, four 5-month-old male ferrets were intranasally inoculated with 10^6 $TCID_{50}$ of A/California/07/2009 in 500 µL and allowed to recover from their illness, to later act as the H1N1pdm09-immune recipients. To act as donors for each transmission experiment, four 6-month-old male ferrets were inoculated intranasally with 10^6 $TCID_{50}$ of A/Bretagne/24241/2021 in 500 µL. Body weight and clinical signs (as previously described in [20]) were recorded upon collection of nasal wash samples on the indicated days. These 1C H1N2v-infected ferrets were placed on the donor side of the transmission cage and 24 h later a 6-month-old male naïve male ferret or a now 8-month-old H1N1pdm09-immune ferret was placed into the opposite side of the cage. The recipients were exposed to a donor for 48 h before being individually housed for the remainder of the experiment. Nasal washes, weight and disease signs were collected for each ferret on the indicated days post-infection or post-exposure.

Swine pathogenesis and swine-to-ferret transmission study design

Ten three-week-old pigs obtained from a herd free of IAV and porcine reproductive and respiratory syndrome virus. Upon arrival, pigs were treated prophylactically with Excede and Draxxin (Zoetis, Florham Park, NJ), according to the label directions, to reduce potential respiratory bacterial pathogens. Eight 4–6-month-old ferrets (males and females) were obtained from an influenza-free, high-health source for use as aerosol transmission contacts. All animals were screened for antibodies against influenza A nucleoprotein (NP) by a commercial enzyme-linked immunosorbent assay (ELISA) (MultiS ELISA; IDEXX, Westbrook, ME) to confirm the absence of pre-existing immunity from prior exposure or passively acquired maternal antibody. Pigs and ferrets received a subcutaneous radio frequency microchip (Deston Fearing, Dallas, TX) for identification and body temperature monitoring purposes. Five pigs were randomly assigned as the IAV-infected group and were experimentally inoculated intranasally with 2 mL of 10^6 $TCID_{50}$/mL of rg-A/Bretagne/24241/2021 strain diluted in PBS. The other five pigs were maintained as negative controls and placed in a separate containment room. At 2 dpi, four ferrets were placed individually in open-front cages as contacts at approximately 4 ft from the pig deck as previously described [21]. Ferrets were manipulated before pigs and decontamination of the outer gloves, gown, surfaces, and equipment using 70% ethanol was performed between individual ferrets. Body temperatures of pigs were monitored from −3 to 5 dpi and contact ferrets were monitored from −3 to 9 dpe. Febrile responses were considered when an animal had a temperature greater than two standard deviations above the mean of body temperatures before inoculation or exposure. Ferrets were weighed daily to evaluate weight loss throughout the experiment.

Swine and swine-to-ferret study sample collection

Nasal swab samples (FLOQSwabs; Copan Diagnostics, Murrieta, CA) were collected from pigs at 0, 1, 3, and 5 dpi. Pigs were humanely euthanized and necropsied at 5 dpi. Lung and trachea tissues were collected from pigs for histopathology analysis and bronco-alveolar fluid (BALF) to assess virus replication. Nasal wash samples were collected at 0, 1, 3, 5, 7, and 9 dpe to assess nasal shedding of recipient ferrets, as previously described [21]. On 21 dpe, contact ferrets were humanely euthanized and blood samples were collected to assess pig-to-ferret transmission by seroconversion using hemagglutination inhibition (HI) assays.

Virus detection and hemagglutination inhibition antibodies

We performed qPCR for IAV detection in all nasal swabs, nasal washes, and BALF samples using a commercial VetMAX™-Gold SIV Detection Kit (Thermo Fisher Scientific, MA). Virus isolation and virus titration were performed in MDCK-London cells on nasal swabs, nasal wash and BALF samples. Cytopathic effect on MDCK-L cells monolayers was monitored between 48-72 h post-inoculation and confirmed with an immunocytochemistry (ICC) protocol using an anti-influenza A NP monoclonal antibody [22]. A $TCID_{50}$ titre per mL was calculated for each positive sample.

Ferret antisera collected at 21 dpe were heat inactivated at 56 °C for 30 min then treated with a 20% Kaolin suspension (Sigma-Aldrich, St. Louis, MO), followed by adsorption with 0.75% guinea pig red blood cells to remove non-specific hemagglutination inhibitors. HI assays were performed in the presence of 20 nM of oseltamivir carboxylate and using 0.75% guinea pig red blood cells, as previously described [2].

Pathologic examination in pigs

On 5 dpi, the inoculated pigs were euthanized to evaluate respiratory lesions and collect BALF. The percentage of the lung affected by purple-red consolidation typical of IAV was visually scored [23]. Tissue

samples from the trachea and right middle or affected lung lobe were fixed in 10% buffered formalin for histopathologic examination. Tissues were processed by routine histopathologic procedures, and slides were stained with hematoxylin and eosin (H&E) or immunohistochemistry (IHC) [24]. Microscopic lesions in the lungs and trachea were scored as previously described [25,26].

Microbiological assays

Commercial qPCR assays for *Mycoplasma hyopneumoniae* and PRRSV were performed on BALF samples to exclude other causes of pneumonia in pigs, according to the manufacturer's recommendations (VetMax, Life Technologies, Carlsbad, CA, USA). In addition, a qPCR for porcine circovirus 2 and 3 PCR was conducted on BALF samples using an assay from the Iowa State University Veterinary Diagnostic Lab (ISU-VDL). Following the necropsy and collection of BALF samples, we cultured BALF for aerobic bacteria on blood agar and Casmin (NAD enriched) plates to indicate the presence of concurrent bacterial pneumonia.

Results and discussion

Mild disease of 1C H1N2v in the swine model

In swine, influenza virus causes an acute respiratory tract infection. Experimentally infected pigs with 1C H1N2v presented with mild febrile responses, spiking 1-day post-inoculation (dpi) with a group mean temperature of 41°C. Pigs necropsied at 5 dpi revealed that inoculation with 1C H1N2v induced microscopic and macroscopic lung lesions consistent with influenza infection (Figure 1(A,B)). In the trachea, infected pigs had a loss of cilia and a decrease in goblet cells, as well as mild to moderate tracheitis (Figure 1(C)). Furthermore, infectious virus was detected in the bronchoalveolar lavage fluid (BALF) (Figure 1(D)). Infected swine lungs had evident purulent bronchiolitis and an accumulation of a pluricellular inflammatory infiltrate (Figure 2(B–D)), as compared to uninfected lungs (Figure 2(A)). Tissue staining for viral antigen demonstrated virally infected cells in the lungs (Figure 2(E–G)) and trachea (Figure 2(H)). These observations are consistent with mild disease similar to other endemic H1 1C IAV in swine [27].

1C H1N2v is antigenically novel in a representative human population

Prior immunity against influenza viruses, specifically H1N1 and H3N2 viruses, can provide protection from emerging strains from the same subtype. To examine cross-protective immunity to 1C H1 in adults from the US, serum samples collected in Fall 2020 were tested for neutralizing antibodies against 1C H1N2v. Overall, 32% of individuals had levels of cross-neutralizing antibodies against 1C H1N2v that were above the limit of detection (Figure 3(A)) and these individuals were clustered by those born between 1940-1949. Of individuals born after 1950, only 27% had detectable cross-neutralizing antibodies. This protection is likely due to similarities in the avian origin HA from the 1C lineage to the 1918 H1N1 HA [4,28]. As expected, all individuals had higher levels of antibodies against the H1N1 pandemic virus strain, A/California/07/2009, from 2009 (H1N1pdm09) (Figure 3(A)), suggesting that the detectable 1C H1N2v cross-reactive antibodies are not equivalent to the dominant H1 response. However, if the minor broadly reactive H1 antibodies were generated from prior vaccination or H1 infection, it is feasible that the response could be boosted after vaccination. In a separate study, plasma collected from individuals prior to and 28 days post-vaccination with a commercial vaccine in 2021 were assessed for a rise in cross-reactive 1C H1N2v antibodies. As expected,

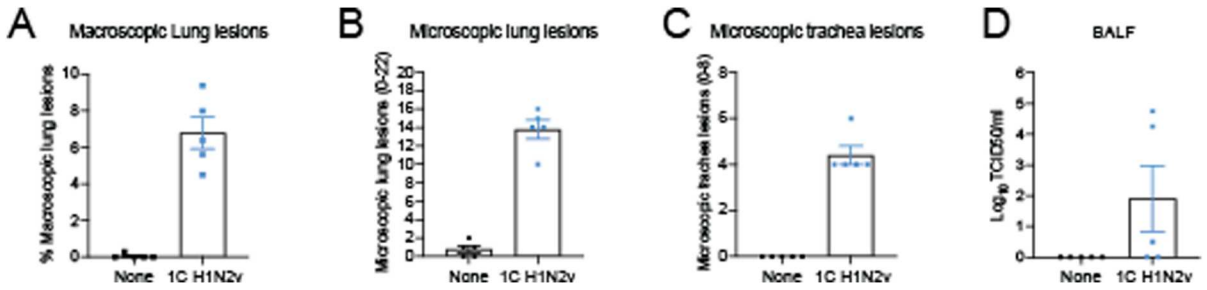

Figure 1. Pathological examination and virus shedding in pigs infected with 1C H1N2v. Five pigs were intranasally infected with 2 mL of 10^6 $TCID_{50}$/mL of 1C H1N2v and necropsied 5 days post-inoculation (dpi). A. Percentage of macroscopic lung lesions was significantly higher in 1C H1N2v-infected pigs at 6.8%. B. Composite of microscopic lung lesion scores on a scale of 0–22. 1C H1N2v-infected pigs had an average score of 13.8 and were characterized by purulent bronchiolitis with the accumulation of pyknotic and karyorrhectic debris admixed with neutrophils in the lumen of two bronchioles. C. Composite of microscopic trachea lesion scores on a scale of 0–8 with a group average composite score of 4.4 for 1C H1N2v-infecteds pigs. D. Virus titres in bronchoalveolar lavage fluid (BALF) at 5 dpi. Viral titres were measured by $TCID_{50}$ in MDCK cells and recorded as $\log_{10}$ $TCID_{50}$/mL.

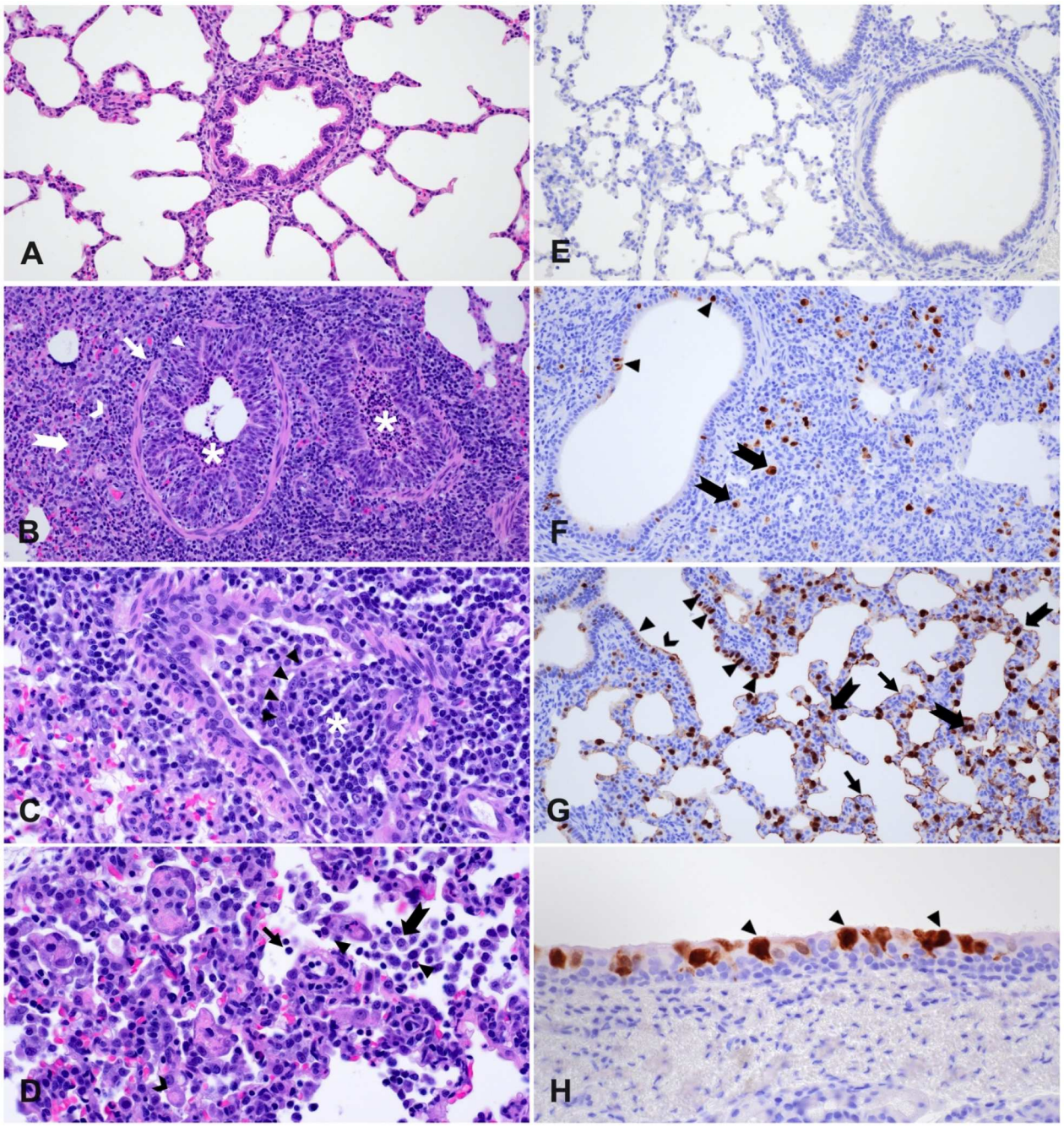

Figure 2. Representative microscopic lesions in the lung and trachea of pigs infected with 1C H1N2v at 5 dpi. A. Negative control swine lung (200× hematoxylin and eosin staining (H&E)). B. Infected swine lung with evident purulent bronchiolitis characterized by the accumulation of pyknotic and karyorrhectic debris admixed with neutrophils in the lumen of two bronchioles (white asterisks; 200× H&E). The bronchioles were surrounded and the propria submucosa was expanded by a pluricellular inflammatory infiltrate. In addition, bronchiolitis obliterans was observed in the lungs and characterized by partial obstruction of a bronchiole by intraluminal accumulation of inflammatory cells lined by attenuated epithelium. Accumulation of a pluricellular inflammatory infiltrate was observed in alveolar lumens consisting of lymphocytes, foamy alveolar macrophages and neutrophils. Bronchioles are surrounded and the propria submucosa is expanded by a pluricellular inflammatory infiltrate consisting of lymphocytes (white arrow), macrophages (white notched arrow), neutrophils (white arrowhead), and plasma cells (white chevron). C. Infected swine lung with bronchiolitis obliterans characterized by partial obstruction of a bronchiole by intraluminal accumulation of inflammatory cells (white asterisk) lined by attenuated epithelium (black arrowheads; 400× H&E). D. Infected swine lung with accumulation of a pluricellular inflammatory infiltrate in alveolar lumens consisting of lymphocytes (black arrow), foamy alveolar macrophages (black notched arrow), and neutrophils (black arrowhead). Alveolar lumens also contain alveolar macrophages phagocytizing proteinaceous fluid (black notched arrow). Alveolar septa are expanded by lymphocytes (black arrow) and neutrophils (black arrowhead; 400× H&E). E. Negative control swine lung (200×, immunohistochemistry (IHC)) F. Infected swine lung with IHC staining of IAV NP in the respiratory epithelium (black arrowhead) and alveolar macrophages (black notched arrow; 200× IHC). G. Infected swine lung with staining of IAV nucleoprotein (NP) by IHC, in the respiratory epithelium nuclei, indicating replication (black arrowhead) and in interstitial macrophages (black notched arrow). IAV NP staining lined the cilia of respiratory epithelium (black chevron) and pneumocytes (black arrow; 200× IHC). H. Trachea from infected pig with IHC staining of IAV NP in the tracheal respiratory epithelium with loss of cilia (arrowhead; 400× IHC).

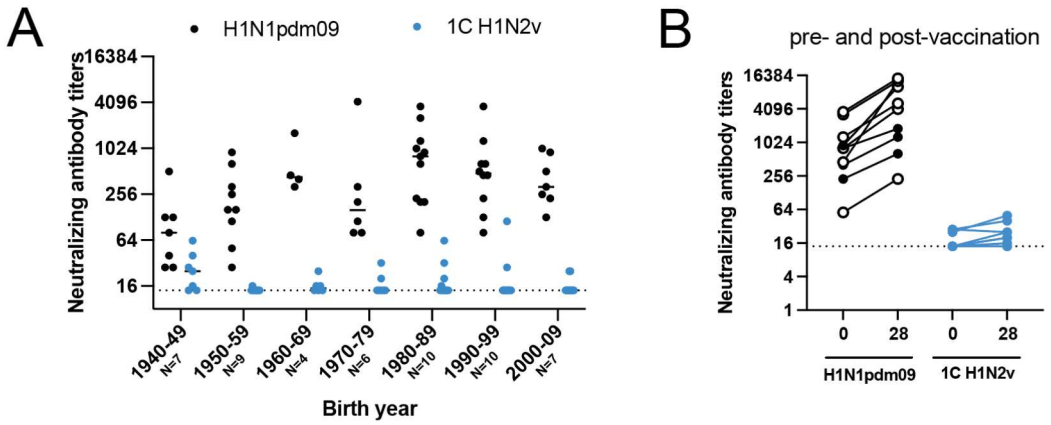

Figure 3. Low levels of cross-neutralizing antibodies against 1C H1N2v in the population. A. Sera from the indicated number of individuals collected in Fall 2020 from Pittsburgh, Pennsylvania for each decade of birth were tested for antibodies against H1N1pdm09 (black circles) and 1C H1N2v (blue circles). B. Plasma collected from ten individuals pre-vaccination and 28 days post-vaccination with GSK Fluvalal quadrivalent formulation in 2021/2022 were assessed for cross-neutralizing antibodies. Each dot represents an individual, and the open circles indicate individuals that had a greater than 4-fold rise in antibody titres. The dashed line indicates the limit of detection for each assay.

neutralizing antibody titres against H1N1pdm09 increased after vaccination, with seven of ten individuals having a greater than 4-fold rise in antibody titres, whereas little to no difference was observed in the neutralizing antibody titres against 1C H1N2v (Figure 3(B)). This indicates that a current influenza virus vaccine will not expand cross-protective antibodies against 1C H1N2v. Taken together, these data suggest that most North American individuals lack neutralizing antibodies against 1C H1N2v and is consistent with analysis of serum samples from Asia [5,7–9,29]. Emergence of an influenza virus that has a novel HA that is associated with lack of cross-protective immunity in the population should be considered to have pandemic potential and requires further in vitro characterization based on the decision tree (Figure S1).

1C H1N2v virus has a similar molecular phenotypic signature to H1N1pdm09 virus

Prior research on viral features necessary for the epidemiologic success of IAV in the human population has identified several viral phenotypic signatures that include receptor preference, pH of inactivation, neuraminidase (NA) activity, and environmental persistence [30–43]. To determine the sialic acid (SA) receptor preference of 1C H1N2v, a glycan array was performed using whole labelled influenza virus to measure HA binding to a panel of α2-3 and α2-6 linked SA (Table S1). Similar to H1N1pdm09, 1C H1N2v bound poorly to most α2-3 linked SA and had a α2-6 SA preference (Figure 4(A)). Further receptor preference analysis was performed using a biotinylated glycans containing terminal sequences with the avian (NeuAcα2-3Galβ1-4GlcNAc) or human (NeuAcα2-6Galβ1-4GlcNAc) type receptor, including three linear glycans with one, two or three N-acetyllactosamine (LN, Galβ1-4GlcNAc) repeats (3SLN1/2/3-L or 6SLN1/2/3-L) (Supplementary Figure 2A), and three biantennary N-linked glycans displaying the same sequences on both branches (3SLN1/2/3-N or 6SLN1/2/3-N) (Supplementary Figure 2B). On these refined glycan arrays, inactivated 1C H1N2v virus exhibited human-type specificity, binding two of three linear and all N-linked glycans containing α2-6 linked SA with no detectable binding to glycans containing α2-3 linked SA (Supplementary Figure 2C and 2D). Given the preference towards α2-6 sialic acids, replication efficiency of 1C H1N2v was determined in human bronchiole epithelial (HBE) cells. Three different patient cell cultures were infected with either H1N1pdm09 or 1C H1N2v and similar virus titres were observed for both viruses at 24- and 48-hours post-infection (Figure 4(B)). HA stability is an important determinant of influenza virus host range, infectivity, transmissibility, and therefore, pandemic potential [44]. H1N1pdm09 had a pH of inactivation of 4.9, whereas 1C H1N2v had a pH of 4.5, (Figure 4(C)) indicating that 1C H1N2v has a stable HA and is more human-adapted [37,45–48]. Higher NA activity of the H1N1pdm09 virus from the Eurasian NA segment has been associated with its efficient airborne transmission [34, 49]. 1C H1N2v displayed similar NA activity to H1N1pdm09 (Figure 4(D)). Finally, efficient airborne transmission requires viruses to remain viable under different environmental conditions, so the decay of 1C H1N2v was compared to that of H1N1pdm09 over a range of relative humidities in airway surface liquid (ASL) droplets. The viral decay of both 1C H1N2v and H1N1pdm09 was less than 1 $\log_{10}$ of decay, indicating that they are equally stable in ASL at all the relative humidity conditions tested

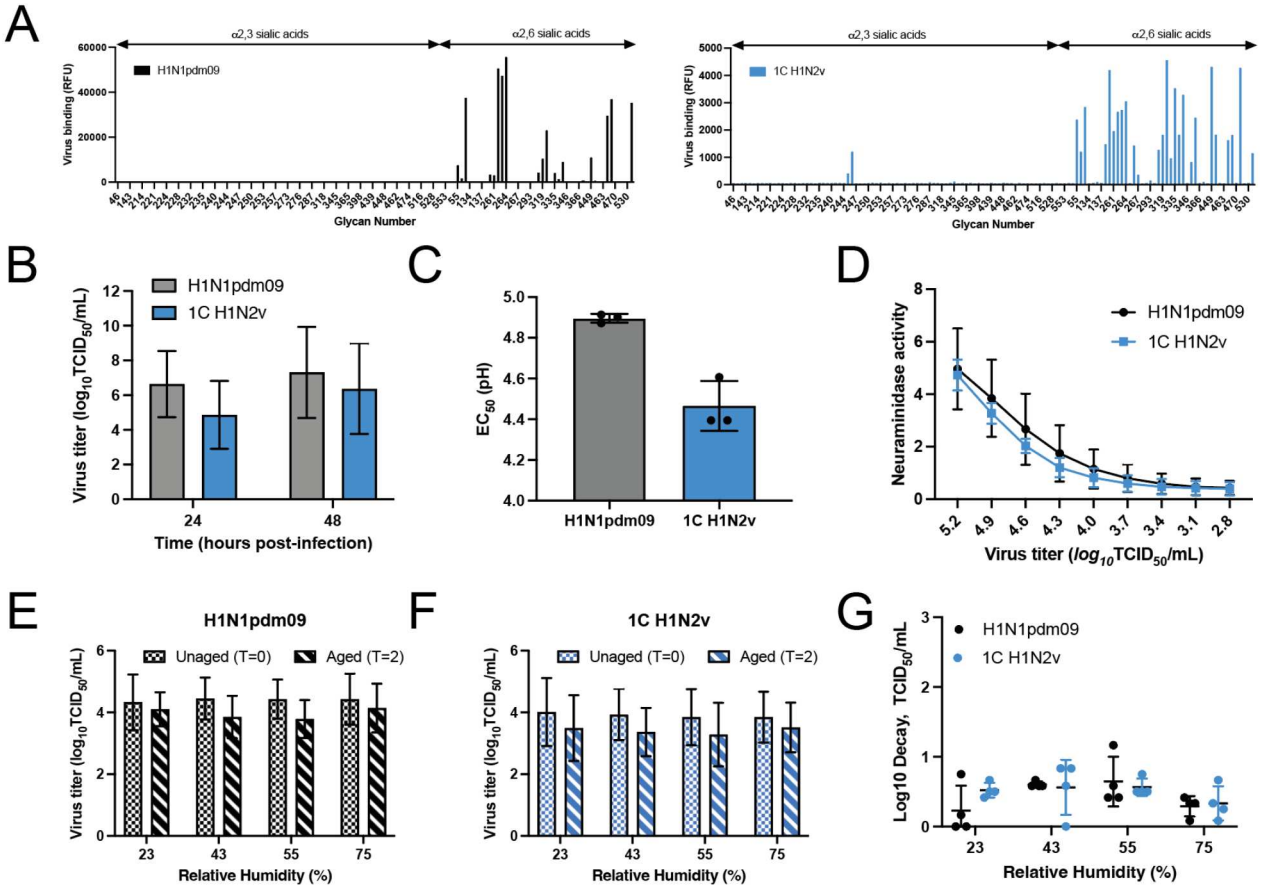

Figure 4. *In vitro* characterization of 1C H1N2v A. Binding of 1C H1N2v virus to a sialoside microarray. Printed glycans include those with α2-3 sialic acid linkages and α2-6 sialic acid linkages. Bars represent the mean fluorescent intensity for H1N1pdm09 (black bars) or 1C H1N2v (blue bars). Glycan numbers are shown on the x-axis and corresponding structures can be found in Supplemental Table 1. B. Replication of 1C H1N2v in primary HBE cells. Transwells of HBE cultures from three different patient donors were infected in triplicate with 10^3 $TCID_{50}$/mL of H1N1pdm09 or 1C H1N2v, and virus was collected at 24- and 48-hours post-infection for titration using MDCK cells. Data represent the mean values +/− standard deviation of three independent patient donors. C. H1N1pdm09 and 1C H1N2v were incubated in pH-adjusted PBS. Remaining virus titre was determined by $TCID_{50}$ assay. The data were fit with an asymmetric sigmoidal curve to determine the EC_{50}. pH of inactivation for H1N1pdm09 and 1C H1N2v as determined by regression analysis of the dose–response curve. The mean +/− standard deviation corresponds to three independent biological replicates, each performed in triplicate. D. Neuraminidase activity for H1N1pdm09 and 1C H1N2v was determined using an ELLA assay with fetuin as a substrate. The data are displayed as the mean +/− standard deviation of three independent assays performed in duplicate. H1N1pdm09 (E) and 1C H1N2v (F) virus stocks were diluted 1:10 in ASL collected from HBE cell cultures. Ten 1 µL droplets were generated and incubated for 2 h at the indicated relative humidity in a controlled chamber. Infectious titres were determined in unaged (time 0) and aged (time 2 h) G. Virus decay was calculated by comparing the virus titre at time 0 to that remaining after 2 h. Data represent the mean values +/− standard deviation from four independent replicates performed with ASL from four different HBE patient cell cultures.

(Figure 4(E–G)). Together, based on decision tree criteria (Figure S1), these results indicate that 1C H1N2v has *in vitro* characteristics that are consistent with viruses capable of human airborne transmission.

Efficient interspecies airborne transmission of 1C H1N2v from swine-to-ferret

Interspecies transmission events of IAV occur sporadically, making them difficult to study, but likely contribute to the pandemic potential of a given virus. To experimentally analyse whether 1C H1N2v can transmit from swine to human, we evaluated virus transmission from infected pigs to ferrets, as the ferret is a model for studying human influenza virus transmissibility. Ferrets were placed into open-air caging within the same room, approximately four feet away from the same five pigs infected above with 1C H1N2v (Figure 5(A)). Ferrets were exposed to the pigs at 2 dpi (Figure 5(A)) and, albeit variable, nasal swab collection indicated that the pigs were shedding virus within the three-day exposure window (Figure 5(B)). No virus was detectable in nasal washes of exposed ferrets, and none displayed any febrile responses or significant weight loss. However, all four ferrets seroconverted at 21 days post-exposure (dpe) (Figure 5(C)), suggesting efficient transmission of 1C H1N2v from swine-to-ferret. The lack of nasal wash detection in the ferrets may be due to the exposure time occurring after

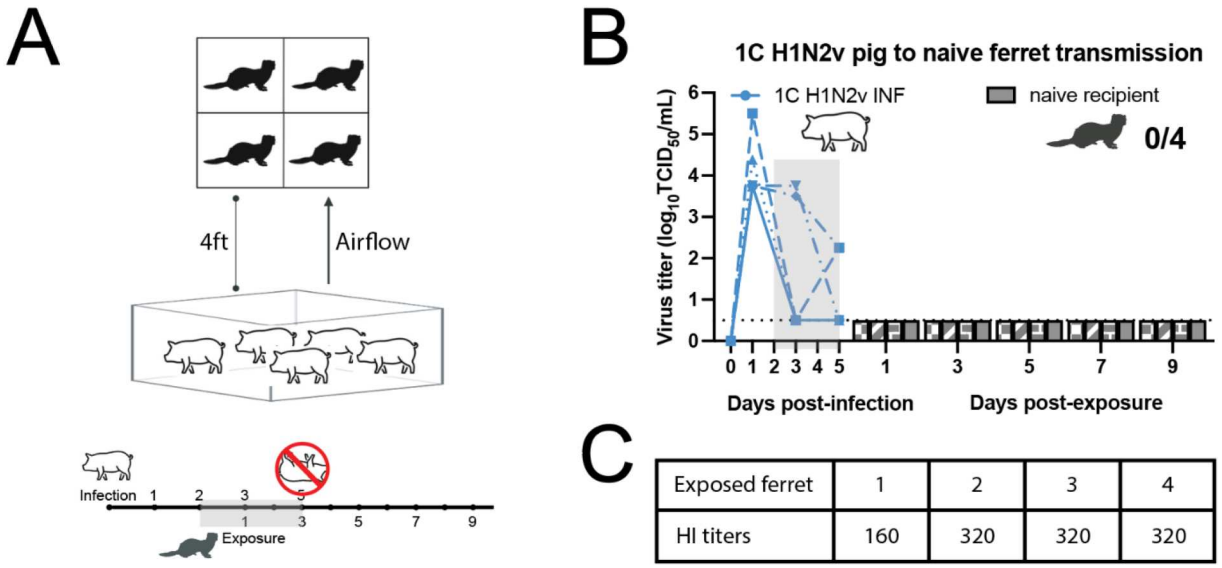

Figure 5. Airborne transmission of 1C H1N2v from pig-to-ferret. A. Schematic of experimental timeline whereby five pigs were intranasally infected with 2 mL of 10^6 $TCID_{50}$/mL of 1C H1N2v and nasal swabs collected on days 0, 1, 3 and 5 post-infection. Pigs were necropsied at 5 dpi. At 2 dpi, four feet away from the infected pig pen, four ferrets were placed into open cages with directional air flow from the pigs to ferrets. Nasal washes were collected from the ferrets on days 1, 3, 5, 7 and 9 post-exposure and sera were collected on day 21 post-exposure. B. Viral titres of nasal swabs from each infected pig, as indicated by blue lines and nasal washes from exposed naïve ferrets, grey bars. The number of recipient ferrets with detectable virus in nasal washes out of four is shown. The limit of detection is indicated by the dashed line. Grey box indicates the time during which the ferrets were exposed to the infected pigs. Viral titres were measured by $TCID_{50}$ in MDCK cells and recorded as $\log_{10}$ $TCID_{50}$/mL. C. HI titres of ferret sera collected at 21 days post-exposure. All schematics were created in BioRender.

peak viral shedding in swine or low levels of replication in the ferrets that were not captured by sampling. Although clinically mild, 1C H1N2v displayed the ability to cross the species barrier and transmit from swine-to-ferret, highlighting the zoonotic risk of this virus.

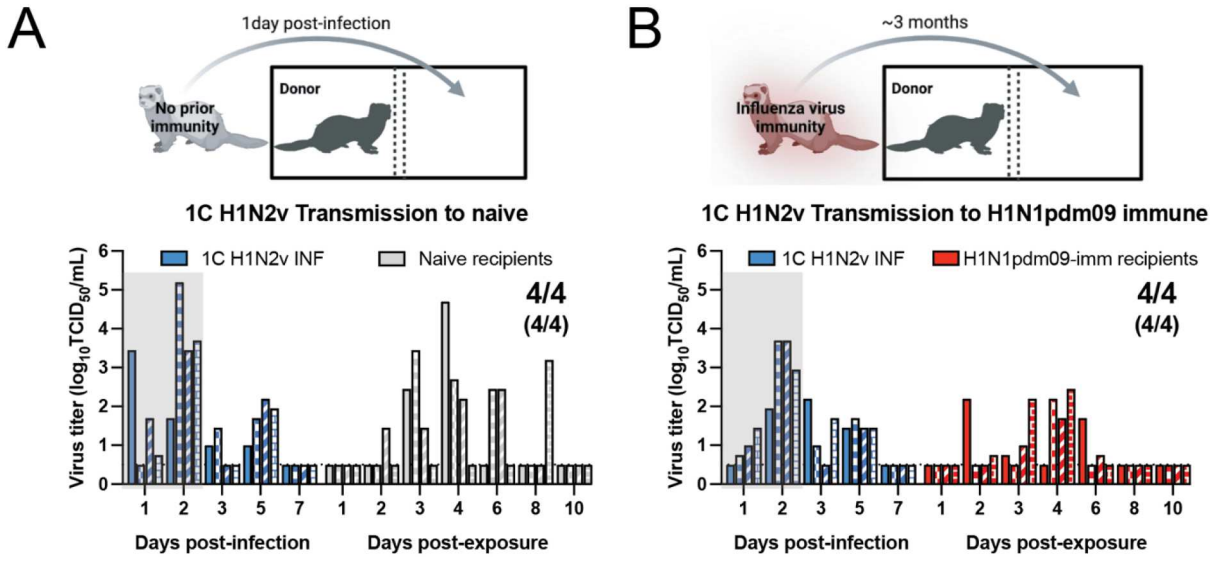

Figure 6. Swine 1C H1N2v transmits efficiently via the air after a short exposure. (A) Four donor ferrets were infected intranasally with 500 µl of 10^6 $TCID_{50}$/mL of 1C H1N2v (1C H1N2v INF), as in Methods. Schematic indicates that recipient ferrets with no prior immunity (naïve recipients) were placed in the adjacent cages at 24 h post-infection for two continuous days. Nasal washes were collected from all ferrets on the indicated days and titred for virus by $TCID_{50}$. Each bar indicates an individual ferret. (B) Schematic of procedure, whereby four ferrets were infected with H1N1pdm09 (H1N1pdm09-imm) between 84 and 110 days prior to acting as recipients to 1C H1N2v-infected donors. Four donor ferrets were infected with 1C H1N2v and H1N1pdm09-imm recipients were placed in the adjacent cage 24 h later. For all graphs, the number of recipient ferrets with detectable virus in nasal secretions out of four total is shown; the number of recipient animals that seroconverted at 14- or 21-days post-exposure out of four total is shown in parentheses. Grey shaded box indicates shedding of the donor during the exposure period. The limit of detection is indicated by the dashed line. All schematics were made in BioRender.

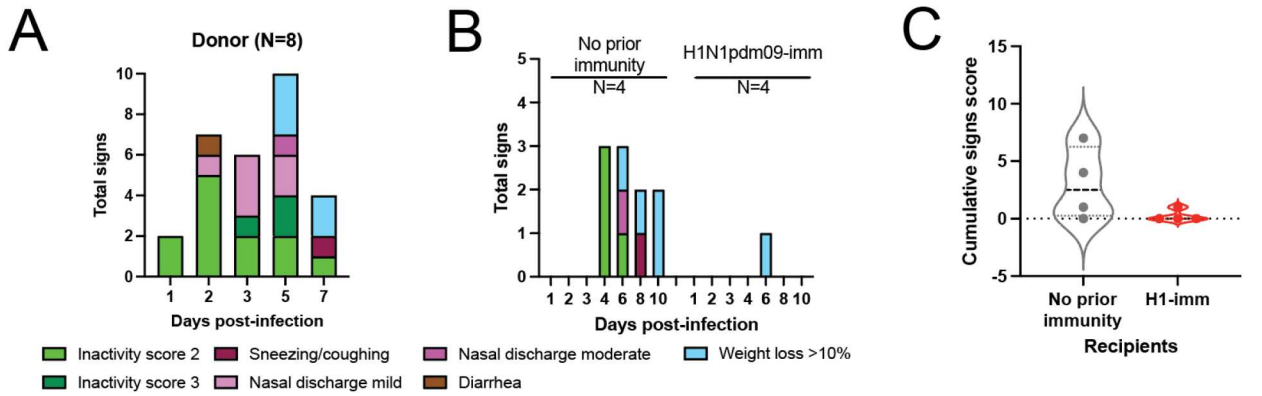

Figure 7. Pre-existing H1N1pdm09 immunity reduces 1C H1N2v influenza virus clinical signs in recipients. A. The total number of signs for each intranasally 1C H1N2-infected ferret from Figure 6 ($N = 8$) are indicated on the days post-infection. B. The total number of signs for each airborne 1C H1N2v-infected recipient having either no prior immunity ($N = 4$) or H1N1pdm09 pre-existing immunity ($N = 4$) from Figure 6 displayed on each day post-exposure. C. The symptoms for each airborne 1C H1N2v-infected recipient from panel B having either no prior immunity ($N = 4$) or H1N1pdm09-imm ($N = 4$) were added together to assign each animal a cumulative score. Each dot represents the cumulative sign score for a single ferret.

Pre-existing immunity to H1N1 viruses does not protect against natural infection of 1C H1N2v

Influenza viruses mainly spread human-to-human through infectious respiratory particles, and novel viruses emerge in the context of pre-existing immunity in the human population. Variable airborne and contact transmission efficiencies have been reported for 1C swine influenza virus isolates in ferrets [7]. To determine the transmissibility of 1C H1N2v, airborne transmission studies were conducted with ferrets that were either immunologically naïve to IAV or those imprinted with prior H1N1pdm09 infection. H1N1pdm09 was chosen as the imprint virus since a majority of the human population has been exposed to this strain. Donor ferrets were infected with 1C H1N2v and shed virus in nasal secretions for the first 5 days of infection (Figure 6(A,B), blue bars) and these animals presented with mild clinical signs (Figure 7(A)), consistent with those observed during infection with H1N1pdm09 [20]. Efficient airborne transmission to recipients without prior immunity was observed for all four animals (Figure 6(A)), indicating a robust airborne transmission potential. Figure 3(A) indicates that a vast number of individuals have cross-neutralizing antibodies against H1N1pdm09 and it has been previously shown that prior influenza virus immunity can serve as a barrier to influenza virus transmission [50,51]. Serum from ferrets infected with H1N1pdm09 did not have any cross-reactivity to the 1C H1N2v virus (Table S2); however, prior H1N1pdm09 infection has been shown to protect against airborne transmission in a neutralizing antibody-independent manner [50]. To assess whether prior H1N1pdm09 immunity could protect against airborne transmission of 1C H1N2v, recipients were infected with H1N1pdm09 (H1N1pdm09-imm) three months prior, then exposed to donor animals experimentally infected with 1C H1N2v virus. After a two-day exposure (Figure 6(B), grey box), all four H1N1pdm09-imm recipients shed virus in nasal secretions and seroconverted (Figure 6(B) and Table S2). Although no decrease in transmission efficiency was observed, H1N1pdm09-imm recipients displayed less clinical disease than naive recipients (Figure 7(B,C)). These results demonstrate that 1C H1N2v is highly transmissible between ferrets, even with pre-existing H1N1pdm09 immunity, and might therefore be highly transmissible between humans, posing a greater pandemic risk.

Conclusions

The endemic nature of swine H1 1C strains in the pig population in Europe and Asia and sporadic zoonotic events suggest a leaky barrier at the animal-human interface. Low levels of immunity against the virus in US and Asian populations and efficient inter- and intraspecies transmission suggest a pandemic threat of 1C H1N2 viruses. Although prior immunity with H1N1pdm09 decreased disease severity it did not disrupt the transmission of 1C H1N2v virus in ferrets, suggesting that H1 immunity in humans will not block airborne transmission. Taken together, risk assessment of 1C H1N2v virus would indicate that it is in the higher pandemic risk category and should be continued to be monitored for spillover into humans.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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Data availability

All raw data and sequence information uploaded to FigShare (10.6084/m9.figshare.27194397).

References

[1] Anderson TK, Macken CA, Lewis NS, et al. A phylogeny-based global nomenclature system and automated annotation tool for H1 hemagglutinin genes from swine influenza A viruses. mSphere. 2016;1(6):e00275–16. doi:10.1128/mSphere.00275-16

[2] Anderson TK, Chang J, Arendsee ZW, et al. Swine influenza A viruses and the tangled relationship with humans. Cold Spring Harb Perspect Med. 2021;11. doi:10.1101/cshperspect.a038737

[3] Zhao Y, Han L, Chen T, et al. Serological surveillance of the H1N1 and H3N2 swine influenza A virus in Chinese swine between 2016 and 2021. Biomed Res Int. 2022;2022:5833769. doi:10.1155/2022/5833769

[4] Pensaert M, Ottis K, Vandeputte J, et al. Evidence for the natural transmission of influenza A virus from wild ducts to swine and its potential importance for man. Bull World Health Organ. 1981;59:75–78.

[5] Sun H, Xiao Y, Liu J, et al. Prevalent Eurasian avian-like H1N1 swine influenza virus with 2009 pandemic viral genes facilitating human infection. Proc Natl Acad Sci U S A. 2020;117:17204–17210. doi:10.1073/pnas.1921186117

[6] Vijaykrishna D, Smith GJ, Pybus OG, et al. Long-term evolution and transmission dynamics of swine influenza A virus. Nature. 2011;473:519–522. doi:10.1038/nature10004

[7] Yang H, Chen Y, Qiao C, et al. Prevalence, genetics, and transmissibility in ferrets of Eurasian avian-like H1N1 swine influenza viruses. Proc Natl Acad Sci U S A. 2016;113:392–397. doi:10.1073/pnas.1522643113

[8] Cheung JTL, Tsang TK, Yen HL, et al. Determining existing human population immunity as part of assessing influenza pandemic risk. Emerg Infect Dis. 2022;28:977–985. doi:10.3201/eid2805.211965

[9] Wang DY, Qi SX, Li XY, et al. Human infection with Eurasian avian-like influenza A(H1N1) virus, China. Emerg Infect Dis. 2013;19:1709–1711. doi:10.3201/eid1910.130420

[10] Chastagner A, Herve S, Queguiner S, et al. Genetic and antigenic evolution of European swine influenza A viruses of HA-1C (avian-like) and HA-1B (human-like) lineages in France from 2000 to 2018. Viruses. 2020;12(11):1304. doi:10.3390/v12111304

[11] Chiapponi C, Prosperi A, Moreno A, et al. Genetic variability among swine influenza viruses in Italy: data analysis of the period 2017-2020. Viruses. 2021;14. doi:10.3390/v14010047

[12] Le Sage V, Rockey NC, French AJ, et al. Potential pandemic risk of circulating swine H1N2 influenza viruses. Nat Commun. 2024;15:5025. doi:10.1038/s41467-024-49117-z

[13] Myerburg MM, Harvey PR, Heidrich EM, et al. Acute regulation of the epithelial sodium channel in airway epithelia by proteases and trafficking. Am J Respir Cell Mol Biol. 2010;43:712–719. doi:10.1165/rcmb.2009-0348OC

[14] Richard G, Herve S, Chastagner A, et al. Major change in swine influenza virus diversity in France owing to emergence and widespread dissemination of a newly introduced H1N2 1C genotype in 2020. Virus Evol. 2025;11:veae112. doi:10.1093/ve/veae112

[15] Markin A, Wagle S, Grover S, et al. PARNAS: objectively selecting the most representative taxa on a phylogeny. Syst Biol. 2023;72:1052–1063. doi:10.1093/sysbio/syad028

[16] Offlu. 2023.

[17] Kitikoon P, Gauger PC, Vincent AL. Hemagglutinin inhibition assay with swine sera. Methods Mol Biol. 2014;1161:295–301. doi:10.1007/978-1-4939-0758-8_24

[18] Reed LJ, Muench H. A simple method of estimating fifty per cent endpoints. Am. J. Epidemiol. 1938;27:493–497. doi:10.1093/oxfordjournals.aje.a118408

[19] Byrd-Leotis L, Liu R, Bradley KC, et al. Shotgun glycomics of pig lung identifies natural endogenous receptors for influenza viruses. Proc Natl Acad Sci U S A. 2014;111:E2241–E2250. doi:10.1073/pnas.1323162111

[20] Mueller Brown K, Le Sage V, French AJ, et al. Secondary infection with streptococcus pneumoniae decreases influenza virus replication and is linked to severe disease. FEMS Microbes. 2022;3:xtac007.

[21] Kaplan BS, Kimble JB, Chang J, et al. Aerosol transmission from infected swine to ferrets of an H3N2 virus collected from an agricultural fair and associated with human variant infections. J Virol. 2020;94. doi:10.1128/JVI.01009-20

[22] Kitikoon P, Nilubol D, Erickson BJ, et al. The immune response and maternal antibody interference to a heterologous H1N1 swine influenza virus infection following vaccination. Vet Immunol Immunopathol. 2006;112:117–128. doi:10.1016/j.vetimm.2006.02.008

[23] Gauger PC, Vincent AL, Loving CL, et al. Kinetics of lung lesion development and pro-inflammatory cytokine response in pigs with vaccine-associated enhanced respiratory disease induced by challenge with pandemic (2009) A/H1N1 influenza virus. Vet Pathol. 2012;49:900–912. doi:10.1177/0300985812439724

[24] Vincent LL, Janke BH, Paul PS, et al. A monoclonal-antibody-based immunohistochemical method for the detection of swine influenza virus in formalin-fixed, paraffin-embedded tissues. J Vet Diagn Invest. 1997;9:191–195. doi:10.1177/104063879700900214

[25] Gauger PC, Loving CL, Khurana S, et al. Live attenuated influenza A virus vaccine protects against A(H1N1)pdm09 heterologous challenge without vaccine associated enhanced respiratory disease. Virology. 2014;471-473:93–104. doi:10.1016/j.virol.2014.10.003
[26] Morgan SB, Hemmink JD, Porter E, et al. Aerosol delivery of a candidate universal influenza vaccine reduces viral load in pigs challenged with pandemic H1N1 virus. J Immunol. 2016;196:5014–5023. doi:10.4049/jimmunol.1502632
[27] Deblanc C, Queguiner S, Gorin S, et al. Evaluation of the pathogenicity and the escape from vaccine protection of a New antigenic variant derived from the European human-like reassortant swine H1N2 influenza virus. Viruses. 2020;12(10):1155. doi:10.3390/v12101155
[28] Worobey M, Han GZ, Rambaut A. Genesis and pathogenesis of the 1918 pandemic H1N1 influenza A virus. Proc Natl Acad Sci U S A. 2014;111:8107–8112. doi:10.1073/pnas.1324197111
[29] Belser JA, Katz JM, Tumpey TM. The ferret as a model organism to study influenza A virus infection. Dis Model Mech. 2011;4:575–579. doi:10.1242/dmm.007823
[30] Campbell PJ, Danzy S, Kyriakis CS, et al. The M segment of the 2009 pandemic influenza virus confers increased neuraminidase activity, filamentous morphology, and efficient contact transmissibility to A/Puerto Rico/8/1934-based reassortant viruses. J Virol. 2014;88:3802–3814. doi:10.1128/JVI.03607-13
[31] Chen LM, Blixt O, Stevens J, et al. In vitro evolution of H5N1 avian influenza virus toward human-type receptor specificity. Virology. 2012;422:105–113. doi:10.1016/j.virol.2011.10.006
[32] Herfst S, Schrauwen EJ, Linster M, et al. Airborne transmission of influenza A/H5N1 virus between ferrets. Science. 2012;336:1534–1541. doi:10.1126/science.1213362
[33] Imai M, Watanabe T, Hatta M, et al. Experimental adaptation of an influenza H5 HA confers respiratory droplet transmission to a reassortant H5 HA/H1N1 virus in ferrets. Nature. 2012;486:420–428. doi:10.1038/nature10831
[34] Lakdawala SS, Lamirande EW, Suguitan AL, et al. Eurasian-origin gene segments contribute to the transmissibility, aerosol release, and morphology of the 2009 pandemic H1N1 influenza virus. PLoS Pathog. 2011;7:e1002443. doi:10.1371/journal.ppat.1002443
[35] Pappas C, Viswanathan K, Chandrasekaran A, et al. Receptor specificity and transmission of H2N2 subtype viruses isolated from the pandemic of 1957. PLoS One. 2010;5:e11158. doi:10.1371/journal.pone.0011158
[36] Roberts KL, Shelton H, Scull M, et al. Lack of transmission of a human influenza virus with avian receptor specificity between ferrets is not due to decreased virus shedding but rather a lower infectivity in vivo. J Gen Virol. 2011;92:1822–1831. doi:10.1099/vir.0.031203-0
[37] Russier M, Yang G, Rehg JE, et al. Molecular requirements for a pandemic influenza virus: An acid-stable hemagglutinin protein. Proc Natl Acad Sci U S A. 2016;113:1636–1641. doi:10.1073/pnas.1524384113
[38] Steel J, Lowen AC, Mubareka S, et al. Transmission of influenza virus in a mammalian host is increased by PB2 amino acids 627 K or 627E/701N. PLoS Pathog. 2009;5:e1000252. doi:10.1371/journal.ppat.1000252
[39] Van Hoeven N, Pappas C, Belser JA, et al. Human HA and polymerase subunit PB2 proteins confer transmission of an avian influenza virus through the air. Proc Natl Acad Sci U S A. 2009;106:3366–3371. doi:10.1073/pnas.0813172106
[40] Yen HL, Liang CH, Wu CY, et al. Hemagglutinin-neuraminidase balance confers respiratory-droplet transmissibility of the pandemic H1N1 influenza virus in ferrets. Proc Natl Acad Sci U S A. 2011;108:14264–14269. doi:10.1073/pnas.1111000108
[41] Zanin M, Marathe B, Wong SS, et al. Pandemic swine H1N1 influenza viruses with almost undetectable neuraminidase activity Are Not transmitted via aerosols in ferrets and Are inhibited by human mucus but Not swine mucus. J Virol. 2015;89:5935–5948. doi:10.1128/JVI.02537-14
[42] Zaraket H, Bridges OA, Duan S, et al. Increased acid stability of the hemagglutinin protein enhances H5N1 influenza virus growth in the upper respiratory tract but is insufficient for transmission in ferrets. J Virol. 2013;87:9911–9922. doi:10.1128/JVI.01175-13
[43] Yang G, Ojha CR, Russell CJ. Relationship between hemagglutinin stability and influenza virus persistence after exposure to low pH or supraphysiological heating. PLoS Pathog. 2021;17:e1009910. doi:10.1371/journal.ppat.1009910
[44] Russell CJ, Hu M, Okda FA. Influenza hemagglutinin protein stability, activation, and pandemic risk. Trends Microbiol. 2018;26:841–853. doi:10.1016/j.tim.2018.03.005
[45] Shelton H, Roberts KL, Molesti E, et al. Mutations in haemagglutinin that affect receptor binding and pH stability increase replication of a PR8 influenza virus with H5 HA in the upper respiratory tract of ferrets and may contribute to transmissibility. J Gen Virol. 2013;94:1220–1229. doi:10.1099/vir.0.050526-0
[46] Singanayagam A, Zambon M, Barclay WS. Influenza virus with increased pH of hemagglutinin activation Has improved replication in cell culture but at the cost of infectivity in human airway epithelium. J Virol. 2019;93. doi:10.1128/JVI.00058-19
[47] Tosheva II, Saygan KS, Mijnhardt SM, et al. Hemagglutinin stability as a key determinant of influenza A virus transmission via air. Curr Opin Virol. 2023;61:101335. doi:10.1016/j.coviro.2023.101335
[48] Le Sage V, Kormuth KA, Nturibi E, et al. Cell-Culture adaptation of H3N2 influenza virus impacts acid stability and reduces airborne transmission in ferret model. Viruses. 2021;13(5):719. doi:10.3390/v13050719
[49] Gaymard A, Le Briand N, Frobert E, et al. Functional balance between neuraminidase and haemagglutinin in influenza viruses. Clin Microbisol Infect. 2016;22:975–983. doi:10.1016/j.cmi.2016.07.007
[50] Le Sage V, Jones JE, Kormuth KA, et al. Pre-existing heterosubtypic immunity provides a barrier to airborne transmission of influenza viruses. P*LoS Pathog*. 2021;17:e1009273. doi:10.1371/journal.ppat.1009273
[51] Tan J, O'Dell G, Hernandez MM, et al. Human anti-neuraminidase antibodies reduce airborne transmission of clinical influenza virus isolates in the Guinea Pig model. J Virol. 2022;96:e0142121.