Pandemic swine H1N1 influenza viruses with almost undetectable neuraminidase activity do not transmit via aerosols in ferrets and are inhibited by human mucus, but not swine mucus.

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Abstract

A balance between the functions of the influenza virus surface proteins hemagglutinin (HA) and neuraminidase (NA) is thought to be important for the transmission of viruses between humans. Here we describe two pandemic H1N1 viruses, A/swine/Virginia/1814-1/2012 and A/swine/Virginia/1814-2/2012 (pH1N1low-1 and -2, respectively) that were isolated from swine symptomatic for influenza. The enzymatic activity of the NA of these viruses was almost undetectable whilst the HA binding affinity for $\alpha$2,6 sialic acids was greater than that of highly homologous pH1N1 viruses, A/swine/Pennsylvania/2436/2012 and A/swine/Minnesota/2499/2012 (pH1N1-1 and -2), which exhibited better balanced HA and NA activities. *In vitro* growth kinetics of pH1N1low and pH1N1 viruses were similar but aerosol transmission of pH1N1low-1 was abrogated and transmission via direct contact was significantly impaired in ferrets compared to pH1N1-1, which transmitted by direct and aerosol contact. In normal human bronchial epithelial cells, pH1N1low-1 was significantly inhibited by mucus but pH1N1-1 was not. In Madin-Darby canine kidney cell cultures overlaid with human or swine mucus, human mucus inhibited pH1N1low-1 but swine mucus did not. These data shows that the interaction between viruses and mucus may be an important factor in viral transmissibility and could be a barrier for interspecies transmission between humans and swine for influenza viruses.

Importance

A balance between the function of the influenza virus surface proteins hemagglutinin (HA) and neuraminidase (NA) is thought to be important for transmission of viruses from swine to humans. Here we show that a swine virus with extremely functionally mismatched HA and NAs (pH1N1low-1) cannot transmit via aerosol in ferrets, whilst another highly homologous virus with HA and NAs that are better matched functionally (pH1N1-1) can transmit via aerosol. These viruses show similar growth kinetics in...
Madin Darby canine kidney (MDCK) cells but pH1N1low-1 is significantly inhibited by mucus in normal human bronchial epithelial cells whilst pH1N1-1 is not. Further, human mucus could inhibit these viruses but swine mucus could not. These data shows that the interaction between viruses and mucus may be an important factor in viral transmissibility and could be a species barrier between humans and swine for influenza viruses.

Introduction

Hemagglutinin (HA) and neuraminidase (NA), the surface glycoproteins of influenza virus, play vital roles in the virus lifecycle. HA binds to sialic acids on the cell surface, initiating fusion of the cell and viral membranes. NA enzymatically cleaves sialic acids from glycans on the host cell surface, facilitating the release of budding progeny viruses (1, 2). Thus, HA and NA have opposing roles in the viral lifecycle and both are required for viral replication. A functional balance between HA and NA is important for both efficient replication and respiratory droplet transmission in humans. Four pandemic human viruses, A/California/04/2009 (H1N1) (CA/09), A/Hong Kong/68 (H3N2), A/Japan/305+/1957 (H2N2) and A/South Carolina/1/1918 (H1N1), all showed a functional balance between their HA and NA proteins (3). Further, the inefficient respiratory-droplet transmission of a triple-reassortant swine virus, A/swine/Hong Kong/915/2004 (H1N2), was enhanced upon replacing the NA with one of greater activity, making a better functional balance between HA and NA (4). This indicated that an HA/NA functional balance in the recombinant swine virus was one of the factors that contributed to enhanced transmission (4). A balance has also been shown to be important for efficient viral replication in mice and \textit{in vitro} (4-8). In swine, an HA/NA balance seems less important for replication and transmission (3, 4). Whilst viruses with mismatched HA and NA can replicate in swine, they do not seem to transmit between humans effectively (3, 4).
Here we describe two pandemic swine H1N1 (pH1N1) viruses that were isolated from swine symptomatic for influenza in the same commercial farm in the United States. An extreme mismatching of the activities of HA and NA was evident in these viruses, A/swine/Virginia/1814-1/2012 (pH1N1low-1) and A/swine/Virginia/1814-2/2012 (pH1N1low-2), as the enzymatic activity of NA was almost undetectable but the binding affinity of HA for α2,6 sialic acids was greater in comparison to the highly homologous pH1N1 viruses A/swine/Pennsylvania/2436/2012 (pH1N1-1) and A/swine/Minnesota/2499/2012 (pH1N1-2) with more balanced HA/NA activities. Both pH1N1low-1 and -2 were isolated from animals in the same herd and were highly homologous (98% for all gene segments). We used these viruses to further study the transmissibility of swine viruses with functionally mismatched HA and NAs in the ferret model and we also studied the interaction of these viruses with human and swine mucus to gain an insight into why such viruses can apparently transmit in swine but not in humans.

Materials and methods

Viruses and cells

The viruses studied here were isolated from nasal swabs obtained from pigs in commercial swine herds in the United States that showed symptoms of influenza-like illness, such as coughing, thumping, nasal discharge, diarrhea and fever. A/swine/Virginia/1814-1/2012 (H1N1) (pH1N1low-1) and A/swine/Virginia/1814-2/2012 (H1N1) (pH1N1low-2), were isolated from pigs in the same herd in Virginia. A/swine/Pennsylvania/2436/2012 (H1N1) (pH1N1-1) was isolated from a pig in Pennsylvania and A/swine/Minnesota/2499/2012 (H1N1) (pH1N1-2) was isolated from a pig in Minnesota. Viruses
were propagated in swine testes epithelial (STE) cells (ATCC, Manassas, VA) and viral titers were determined using Madin-Darby canine kidney (MDCK) cells (ATCC). MDCK and STE cells were maintained in minimal essential medium (MEM) supplemented with 10% (v/v) fetal bovine serum (Thermo Scientific, Waltham, MA). Viruses were diluted in infection medium (MEM supplemented with 5% (v/v) bovine serum albumin (BSA) (Sigma, St Louis, MO)). Normal Human Bronchial Epithelial (NHBE) cells (Lonza, Walkersville, MD) from a single, healthy, non-smoking adult donor (lot # 04212004) were expanded, cryopreserved and cultured in an air-liquid interface system as previously described (9). The apical surface of the cells was exposed to a humidified 95% air/5% CO2 environment, and the basolateral medium was changed every two days. NHBE cells were washed by adding 150μL of infection medium to the apical surface for 10mins followed by gentle pipetting and aspiration of the wash solution.

MU-NANA neuraminidase activity assay

NA activity was measured via a fluorimetric assay using 2′-(4-methylumbelliferyl)-α-d-N-acetyleneuraminic acid (MUNANA; Sigma) as a substrate (10, 11). The assay involved incubating a 2-fold dilution series of virus with MUNANA at a final concentration of 100 μM for 30mins at 37°C. Input viruses were normalized by HA titer. The reaction was terminated by adding a stop solution of 25% ethanol and 12.5% glycine (Fisher Scientific, Rochester, NY) in distilled water. The fluorescence of the released 4-methylumbelliferone was measured with a Synergy 2 (BioTek Instruments, Winooski, VT) fluorimeter, using an excitation wavelength of 360 nm and an emission wavelength of 460 nm. The results are the means for 2 or 3 independent determinations.

Oseltamivir susceptibility assay

Viruses were preincubated for 30 min at 37°C in the presence of various concentrations of the NA inhibitor oseltamivir carboxylate (10^-4 to 10^4 μM) prior to addition to MDCK cells. The viability of MDCK cells was then tested after 2 and 24 h incubation using the CellTiterGlo cell viability assay (Promega,
Madison, WI) according to the manufacturer’s instructions. Luminescence and absorbance were measured by using a Synergy 2 multimode microplate reader (BioTek Instruments). The mean value of the negative control in each plate was set at 100% luminescence, and the percentage of luminescence of each compound-containing well was determined in relation to this internal control. The 50% effective concentration (EC50) was determined by using the 4-parameter logistic nonlinear regression model equation in GraphPad Prism 5 software (GraphPad Software, La Jolla, CA).

**Protein gel electrophoresis**

STE cells were infected or mock infected with virus at 80% confluency and were harvested at 72 hours post infection. The resulting suspension was clarified by centrifugation at 900g for 5 mins followed by centrifugation at 100,000g for 1.5 hrs at 4°C to pellet the virus, which was resuspended in phosphate buffered saline, pH7.2 (PBS) (Corning, Danville, VA). Proteins were denatured by heating at 100°C for 5 mins in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (Sigma) and 10μg of each sample was separated on a 4-12% Bis/Tris gradient gel (Invitrogen, Carlsbad, CA), which were subsequently stained with 0.1%(w/v) Coomassie blue. Relative amounts of protein in each band were determined via densitometry using Image J (NIH, Bethesda, MA).

**HA binding affinity**

Viruses were grown in eggs, purified, and concentrated over a cushion of 25% sucrose in 1 x STE (0.1 M NaCl, 10 mM Tris-HCl, 1 mM EDTA pH8.0) buffer, and ultracentrifuged at 25,000 rpm for 1 h at 4°C. The general procedure of this solid-phase receptor-binding assay was described previously (12). For receptor binding assay, we used biotinylated glycans of α2,3’S (Neu5Aco2-3Galβ1-4Glcβ-PAA-Biotin), α2,6’S (Neu5Aco2-6Galβ1-4Glcβ-PAA-Biotin), and α2,6’S (Neu5Aco2-6Galβ1-4GlcNAcβ-PAA-Biotin) (GlycoTech, Gaithersburg, MD). Polyvinyl chloride EIA microplates (Corning) were coated with 10 μg of bovine fetuin per ml in PBS (200 μl/well) overnight and were washed with distilled water. Fetuin-coated
plates were blocked with 0.2 ml of PBS containing 5% BSA at room temperature for 1 h. The viruses, diluted in PBS to an HA titer of 1/32, were incubated in the wells of fetuin-coated plates (40 μl/well) at 4°C overnight; then the wells were washed with an ice-cold PBS washing buffer. Serial twofold dilutions of sialylglycopolymer in the reaction buffer (0.02% bovine serum albumin, 0.01% Tween 80, 1 μM zanamivir in PBS) were added to the wells (100 μl/well), followed by 2 h of incubation at 4°C. After five washings with PBS containing 0.05% Tween-20, 100 μl of streptavidin-peroxidase conjugates (Invitrogen), diluted 1/2,000 in reaction buffer, was added to each well, followed by 1 h of incubation at 4°C. After washing, the plates were incubated with 0.05 ml of and 3,3’,5,5’-Tetramethylbenzidine (TMB) (Sigma, MO) for 10 min at room temperature and the reaction was stopped with 0.05 ml of 50 mM HCl, and then optical density at 450 nm was measured in a Synergy 2 multi-mode microplate reader (BioTek Instruments).

Ferret experiments

We used three-month-old ferrets that were seronegative for influenza virus (Triple F Farms, Sayre, PA). Four ferrets per virus were lightly anesthetized with isoflurane and inoculated with 10^6 plaque forming units (pfu) of virus in 1 ml PBS (0.5 ml per nostril). These inoculated (I) ferrets were then moved into separate cages, each containing one naïve direct contact (DC) ferret. In an adjacent cage to each I/DC pair was a naïve aerosol contact (AC) ferret, separated by double-layered grills to allow unobstructed airflow but prevent direct contact. Weight, temperature, and clinical signs (sneezing, lethargy, and ruffled fur) were recorded every other day for 14 days. Nasal washes were collected on days 3, 5, 7, 9 and 11 post infection by flushing nostrils with 1 ml of PBS. Tissue culture infectious dose 50% (TCID_{50}) of the nasal wash samples were determined in MDCK cells according to the Reed and Muench method (13).

Infection and staining of normal human bronchial epithelial (NHBE) cells
Viruses were diluted in BEBM (Lonza) to equal titers as determined by plaque assay using MDCK cells. NHBE cells were washed with PBS to remove excess mucus secretion on the apical surface prior to infection. Viruses were allowed to adsorb for 1 h at 37°C and the inocula were then removed by aspiration. When used, exogenous bacterial NA (Roche, Basel, Switzerland) was added at a concentration of 1μU/ml to the virus inoculum. NHBE cells were incubated for the indicated times post infection at 37°C. Viruses released apically were harvested by the apical addition and collection of 300μL of 0.05% BSA-BEBM, which was allowed to equilibrate at 37°C for 30 min. Samples were stored at -80°C until assayed by TCID₅₀ assay. Cells were fixed using 3.7% (v/v) paraformaldehyde (Electron Microscopy Services, Hatfield, PA) in PBS for 30 mins at room temperature, after which cells were washed three times with PBS and stored at 4°C in PBS until staining was performed.

Fixed NHBE cells were permeabilized apically and basolaterally with 0.3% (v/v) Triton X-100 (Sigma) for 15 mins and then washed twice with 0.01% (v/v) Tween 20 (Sigma) in PBS for 5 mins, which was followed by blocking with 3% normal goat serum (Sigma) in PBS for 1hr at room temperature and cells were then washed twice. Primary antibodies (mouse anti-influenza virus nucleoprotein conjugated to fluorescein isothiocyanate (Abcam, Cambridge, MA) and mouse anti-β-tubulin conjugated to cy3 (Abcam)) were then added and incubated at 4°C overnight. Cells were then washed three times and filters were excised from the tissue culture inserts and mounted onto coverslips using Prolong Gold antifade reagent (Invitrogen) and viewed on a Nikon C2 confocal microscope.

Enzyme-linked lectin assay

Mucus secretion from NHBEs was measured by an enzyme-linked lectin assay (ELLA) essentially as described previously (14). Apical washes from NHBEs were coated onto high binding EIA/RIA flat bottom 96-well Costar plates (Corning, NY) overnight at 4°C. Porcine gastric mucin (Sigma) was used to generate a standard curve. Plates were washed three times with PBS containing Tween 0.05% (v/v) (Sigma) and
blocked by adding PBS containing 1% (v/v) bovine serum albumin (BSA) (Sigma) and incubating for 2 hours at room temperature. Plates were washed three times with PBS Tween and 50μL of a 0.3μg/mL solution of wheat germ agglutinin conjugated to horseradish peroxidase (WGA-HRP) (Sigma) diluted in PBS was added to each well and plates were incubated at 37°C for an hour. Plates were washed three times with PBS Tween and 3,3′,5,5′-Tetramethylbenzidine (TMB) (Sigma) was added to each well at room temperature. The reaction was stopped with H2SO4 (Sigma) and optical density was measured at 450 nm in a Synergy 2 multi-mode microplate reader (BioTek Instruments).

Viral infection of MDCK cells in the presence of human and swine mucus

MDCK cell cultures were prepared on 16-well chamber slides (Thermo Scientific). Human mucus was obtained from NHBE cell washes (Lonza) and swine mucus was obtained from freshly prepared swine tracheal explant washes. Explants were prepared from tracheal tissue samples obtained from full term, ready for market pigs as previously described (15, 16). Explants were maintained in 24-well plates on Transwell inserts (Corning) with the basal chamber containing bronchial epithelial cell basal medium (BEBM) supplemented with SingleQuot growth factors (Lonza). Medium was replaced after explant preparation every 3 hours, with a total three changes and then explants were incubated at 37°C (5% CO2) for 24 hours. Mucus was removed from the apical surface by washing using MEM. Equal amounts of human or swine mucus were added to MDCK cell cultures, as determined by ELLA, in 50μL total volume per well. Cells and mucus were incubated at 37°C (5% CO2) for 30mins prior to addition of a multiplicity of infection (MOI) of 1 in 50μL of infection medium per well. Cultures were then incubated for 1hr at 37°C and then washed and 200 μL of infection medium was then added to each well and cultures were incubated for 7hrs at 37°C (5% CO2). Cells were then fixed, permeabilized, stained for DAPI and influenza virus nucleoprotein and imaged as described above. Infected cells in five imaged fields
that best represented the entire well were counted using NIS-Elements software (Nikon, Melville, NY).

Images were taken at a magnification of 200x using a Nikon E800 microscope (Nikon).

Data analysis and statistics

Data collected were inputted and graphed using Graphpad Prism version 5.03 (Graphpad Software, San Diego, CA). Statistical analysis was performed using analysis of variance (ANOVA) with Bonferroni’s post-test, with \( p < 0.05 \) deemed as statistically significant.

Accession numbers

The sequences of the HA and NA genes of the viruses studied in this manuscript can be found on Genbank under the following accession numbers; A/swine/Virginia/1814-1/2012 (H1N1) (pH1N1low-1) KP938849 (HA), KP938850 (NA); A/swine/Virginia/1814-2/2012 (H1N1) (pH1N1low-2) KP938853 (HA), KP938854 (NA); A/swine/Pennsylvania/2436/2012 (H1N1) (pH1N1-1) KP938851 (HA), KP938852 (NA); A/swine/Minnesota/2499/2012 (H1N1) (pH1N1-2) KP938855 (HA), KP938856 (NA).

Results

pH1N1low viruses show decreased NA activity but increased HA binding affinity with no significant changes in protein content or replication \textit{in vitro}, compared to pH1N1 viruses.

NA assays, using MU-NANA or fetuin as substrates, revealed that pH1N1low-1 and -2 demonstrated almost undetectable NA activity, whilst the NA activity of pH1N1-1 and -2 were significantly greater (figure 1A, pH1N1low viruses = 140.6±55.2, pH1N1 viruses = 3990.6±53.6 mean units of NA, \( p < 0.01 \) at ½ dilution of virus, n=2, and, figure 1B, pH1N1low viruses = 0.13±0.04, pH1N1 viruses = 1.57±0.23 mean quantity of 4-MU, \( p < 0.001 \), n=4). The NA activity of pH1N1low viruses was also greatly reduced compared
to other pandemic H1N1 viruses (n=25, refer to table 1 for strain details) isolated from commercial pig farms in the United States that were obtained during surveillance undertaken by this group during the period of 2009 to 2011 (figure 1B, pH1N1low viruses = 0.13±0.04, pH1N1 viruses = 3.04±0.76 mean quantity of 4-MU p<0.001 at ½ dilution of virus). Enzyme kinetics data also showed that the NA of pH1N1low-1 was at the limit of detection, whilst pH1N1-1 showed greater kinetics using MU-NANA as a substrate ($V_{max}$ = 0.03 and 0.1, respectively, figure 1C). Both pH1N1low-1 and pH1N1-1 viruses showed NA enzyme kinetics at least four times less than another H1N1 virus, A/Brisbane/59/2007 (H1N1), used as a control ($V_{max}$=0.47, figure 1C). The Km values obtained using pH1N1-1 and A/Brisbane/59/2007 (H1N1) were similar (33.31 and 51.98, respectively) and were an order of magnitude lower than the Km value of pH1N1low-1 (413.3), indicating that pH1N1low-1 demonstrates lower affinity for the MUNANA substrate (figure 1C). Despite the difference in NA activity, pH1N1low-1 and -2 viruses show similar replication kinetics to pH1N1-1 and -2 viruses in vitro in MDCK cells (figure 1D). The undetectable/lack of NA activity was not due to a change in protein expression since there were no obvious differences observed between pH1N1low-1 and -2 viruses compared to pH1N1 viruses that would account for such a drastic reduction in NA activity, as determined by SDS PAGE and densitometry analysis (figures 1E and F).

Whilst we did not see any appreciable differences in growth kinetics between pH1N1low and pH1N1 viruses, plaques produced in MDCK cells by pH1N1low-1 were significantly smaller in diameter than those produced by pH1N1-1 (0.052±0.004cm versus 0.124±0.008cm, respectively, p<0.001, figure 2a, c and e). The addition of exogenous bacterial NA to the overlay significantly increased the diameter of plaques produced by both pH1N1low-1 and pH1N1-1 (0.106±0.008cm and 0.180±0.011cm, respectively, figure 2), however, there was a greater increase in the diameter of plaques produced by pH1N1low-1 compared to pH1N1-1 when exogenous NA was present (203.3% increase versus a 145% increase, respectively, figure
Exogenous NA was not effective in totally restoring the diameter of plaques produced by pH1N1low to those produced by pH1N1-1 but it did reduce the difference between them (0.106±0.007 cm versus 0.124±0.008 cm, respectively, \( p < 0.05 \), figure 2e).

Considering the low NA activity of pH1N1low viruses, we were interested in their sensitivity to oseltamivir. pH1N1low-1 showed significantly less sensitivity to oseltamivir compared to pH1N1-1, which showed a sensitivity similar to A/Brisbane/59/2007 (H1N1) (mean EC_{50} (μM) = 33.94±19.59, 0.26±0.09 and 0.11±0.02, respectively, \( p < 0.01 \), figure 3). Considering that the mechanism of action of oseltamivir is competitive binding for the active site of NA, these results suggest that the low enzymatic activity of pH1N1low NA proteins may be due to an altered NA-sialic acid interaction.

We next measured HA binding affinity using a solid phase glycan binding array (figure 4). Surprisingly, considering the almost undetectable NA activity, pH1N1low-1 and -2 were able to bind to \( \alpha_{2,6} \) SLN sialic acids with significantly greater affinity than pH1N1-1 and -2 (figure 4c, pH1N1low viruses = 2.41±0.05, pH1N1 viruses = 0.338.6±0.14 mean OD_{450nm} at 5 μg/ml sialylglycopolymer, \( p < 0.05 \)). There were 17 amino acid differences in the HA common to pH1N1low viruses compared to pH1N1 viruses. Five of these changes, K118N (H3 numbering), introduced a putative N-glycosylation site (17). These mutations may have contributed to the different binding affinities observed. Taken together, these data show that the naturally occurring functional mismatching of HA and NA in pH1N1low viruses was not due to a change in NA protein expression compared to pH1N1 viruses and that the mismatching did not affect viral replication fitness in vitro.
Transmission of pH1N1$_{low}$-1 in ferrets is impaired compared to pH1N1-1

We next assessed the transmissibility of pH1N1$_{low}$-1 and pH1N1-1 in ferrets, which are a good correlate for aerosol transmission in humans (4). pH1N1-1 was shed from all four infected ferrets until seven days post infection (dpi), at which point one ferret died (figure 5a). pH1N1-1 was shed from all direct contact (DC) ferrets for 7dpi and was shed from aerosol contact (AC) ferrets from 7 to 11dpi (figure 5c). pH1N1$_{low}$-1 was also shed from all four infected ferrets, but only until 5dpi (figure 5b). pH1N1$_{low}$-1 was not detected in nasal washes obtained from AC ferrets and was only shed from two DC ferrets between 7 and 11dpi (figure 3d). At 5dpi, viral shedding was significantly greater from ferrets infected with pH1N1-1 than those infected with pH1N1$_{low}$-1 (10$^{5.625\pm 0.24}$ and 10$^{4.513\pm 0.29}$ mean TCID$_{50}$/mL, respectively. $p<0.05$, figure 5a and b). Further, viral shedding was significantly greater on days 3, 5 and 7 in ferrets in direct contact to pH1N1-1-infected ferrets than those in direct contact to pH1N1$_{low}$-1-infected ferrets on these days (pH1N1-1 3dpi; 10$^{4.211\pm 1.1}$, 5dpi; 10$^{5.66\pm 0.12}$, 7dpi; 10$^{5.16\pm 0.26}$. pH1N1$_{low}$-1 3dpi; 0, 5dpi; 0, 7dpi; 10$^{1.1}$ mean TCID$_{50}$/mL. 3dpi; $p<0.05$, 5dpi; $p<0.001$, 7dpi; $p<0.05$, respectively). Taken together, these results show that there were significant differences in the shedding of these viruses in ferrets and that transmission of pH1N1$_{low}$-1 to DC animals was significantly impaired compared to pH1N1-1 and aerosol transmission of pH1N1$_{low}$-1 was abrogated.

pH1N1$_{low}$-1 infection of NHBEs is inhibited by mucus

There were no significant differences between the growth kinetics of pH1N1$_{low}$ and pH1N1 viruses in MDCK cells but there was a significant difference in viral shedding from infected ferrets (figures 1d and 5). This suggests that either the replication of pH1N1$_{low}$-1 was less efficient in vivo or that shedding of
the virus in respiratory droplets was impaired. We hypothesized that mucus may have played a role in the differences observed between these viruses *in vitro* and *in vivo*. NA is important in the release of viruses from cells, but there is also evidence that it plays a role early in infection, by freeing viruses bound to sialic acids present on mucus in the airway (18-20). To determine if pH1N1<sub>low</sub>-1 was inhibited by mucus, we used washed and unwashed NHBE cells. However, before conducting this experiment, we first assessed how effective washing was in removing mucus from the cells. We washed three cultures of NHBE cells 20 times as described in the materials and methods and measured the mucus content of the washes by ELLA. We found that after 10 and 20 washes the mucus content of the washes decreased by 75.5% and 90%, respectively (data not shown). To reduce the risk of damaging the monolayers, we chose to perform 10 washes prior to infection.

We added pH1N1<sub>low</sub>-1, pH1N1-1 and A/Brisbane/59/2007 to washed and unwashed NHBE cells (total n=6 NHBE cultures per virus, 3 washed and 3 unwashed) at a MOI of 0.01 and measured viral titers by TCID<sub>50</sub> in apical washes collected at 24, 48, 72, 96 and 120hpi. At 24hpi, viral titers measured in washed wells were significantly lower in pH1N1<sub>low</sub>-1-infected wells compared to wells infected with pH1N1-1 and A/Brisbane/59/2007 (pH1N1<sub>low</sub>-1 = 10<sup>4.91±0.17</sup>, pH1N1-1 = 10<sup>7.67±0.42</sup>, p<0.01, A/Brisbane/59/2007 = 10<sup>7.41±0.17</sup> mean TCID<sub>50</sub>/mL, p<0.001, n=3, figure 6c and e, dashed lines). At subsequent time points, viral titers in washed wells were similar across all viruses (figure 6a, c and e, dashed lines). A different pattern was observed in unwashed wells. Viral titers measured in wells infected with pH1N1-1 and A/Brisbane/59/2007 were similar at each time point, however, we did not detect any virus in two of the three unwashed wells to which we added pH1N1<sub>low</sub>-1 at any time point (figure 6a, c and e, solid lines). In the remaining well, virus was also not detected at 24hpi. At 48hpi, the viral titer in this well was greatly reduced compared to washed pH1N1<sub>low</sub>-1-infected wells. Sequence analysis of the NA gene of viruses in
the unwashed well that supported replication did not reveal any new mutations (data not shown). The difference between viral titers measured in all washed compared to all unwashed pH1N1low-1-infected wells were statistically significant at 24 and 48hpi (24hpi; pH1N1low-1 unwashed = 0, pH1N1low-1 washed = 10^{4.91±0.17} mean TCID_{50}/mL, \ p<0.001, \ n=3. 48hpi; pH1N1low-1 unwashed = 10^{5.58±1.58}, \ pH1N1low-1 washed = 10^{6.25±0.14} mean TCID_{50}/mL, \ p<0.05, \ n=3. figure 6a). At later time points the viral titers in all pH1N1low-1-infected wells were similar to each other and to the other viruses (figure 6a, c and e, solid lines). Overall, these data shows that pH1N1low-1 infection and/or replication in NHBE cells was impeded by mucus.

We also measured the mucus content of the apical washes obtained from NHBE cell cultures. The mucus content of apical washes from washed cultures was similar across all time points in cultures infected with pH1N1-1 and A/Brisbane/59/2007 (figure 6d and f, dashed lines). This pattern was not observed in washed cultures infected with pH1N1low-1. Pre-infection, mucus content in these wells was similar to washed wells infected with pH1N1-1 and A/Brisbane/59/2007 viruses (pH1N1low-1 = 0.35±0.01, pH1N1-1 = 0.38±0.02, A/Brisbane/59/2007 = 0.48±0.04mg/L mean mucus concentration, n=3). However, mucus content measured in washed pH1N1low-1-infected wells increased to significantly greater amounts at 48hpi compared to pre-infection amounts (0hpi = 0.36±0.01, 48hpi = 0.91±0.02mg/L mean mucus concentration, \ p<0.001, \ n=3, figure 5B, dashed lines). Mucus content then decreased such that, at 72hpi and later, amounts were similar to those measured in washed wells infected with other viruses (figure 6b, d and f, solid lines). These data indicate that viral infection interfered with mucus production.

In unwashed cultures infected with pH1N1-1 and A/Brisbane/59/2007, mucus content decreased to amounts similar to those measured in washed cultures post infection (figure 6d and f, solid lines). Again, this pattern was not observed in unwashed cultures infected with pH1N1low-1. In the two unwashed
cultures in which viral titers were absent, mucus levels increased significantly over time (pre-infection = 1.22±0.05, 120hpi = 1.81±0.01mg/L mean mucus concentration, p<0.05, n=2, Figure 6b). In the remaining culture, where viral titers were detected at 48hpi and later, mucus content began to decrease post 48hpi such that, by 120hpi, mucus content was similar to that in washed cultures (figure 6b).

Further, the mean mucus amount measured in all unwashed cultures infected with pH1N1low-1 was significantly greater than washed cultures at all time points but 120hpi (figure 6b). The changes observed are not likely to be due to the action of viral NA, as NA treatment of mucus does not impact the interaction of mucus with WGA (21, 22). We also investigated whether mucus production decreased due to declining cell health caused by viral infection. We measured cell health using the CellTiterGlo reagent but we did not find any correlation between cell health and mucus content (data not shown).

These data again show that viral replication interfered with mucus production.

We next performed a similar experiment to the one described previously using an MOI of 1 and fixing the NHBE cultures at 8hpi to gain a better insight into the earlier events occurring during these infections. Further, to determine if the apparent inhibition of pH1N1low-1 replication by mucus was due to low NA activity, we included exogenous bacterial NA during virus adsorption in some cultures. Overall, we found that numbers of infected cells correlated to the NA activity of the viruses. A/Brisbane/59/2007, which had much greater NA activity than the pH1N1 viruses (figure 1c), infected far more cells in both washed and unwashed wells compared to the pH1N1 viruses (figure 7a-h).

Further, pH1N1-1 infected more cells than pH1N1low-1 in washed cultures, although this was not statistically significant. (A/Brisbane/59/2007 – too numerous to count, pH1N1-1 = 363±64.5 infected cells, pH1N1low-1 = 120.3±36.1 mean number of infected cells, respectively, n=3 wells). The addition of exogenous NA did not have a significant impact on the numbers of infected cells counted in washed
cultures (figure 7i). In unwashed cultures, the numbers of infected cells were much lower. pH1N1-1 infected cultures had significantly greater numbers of infected cells compared to pH1N1low-1 (84.7±12.9 cells and 7±3 cells, respectively, p<0.05, n=3 wells, figure 6J). Again, exogenous NA increased the number of cells infected by pH1N1low-1, but the differences were not statistically significant (figure 7j). A comparison of the percentage reduction in numbers of infected cells between washed and unwashed cultures showed that pH1N1low-1 was more sensitive to the presence of mucus than pH1N1-1. A 76.7% decrease in numbers of infected cells was observed in pH1N1-1-infected unwashed cultures, whilst a 94.2% decrease was observed in pH1N1low-1-infected unwashed cultures. This difference was slightly lower in cultures where exogenous NA was added, at 92.8% (figure 7k). These data collectively indicate that NA activity is important early in infection to overcome the inhibitory actions of mucus.

Human mucus inhibits viral infectivity but swine mucus does not

Since it is likely that pH1N1low-1 could productively infect swine, we were interested in the inhibitory properties of swine mucus. We obtained swine mucus by washing cultures of freshly prepared swine tracheal explants and overlaid MDCK cultures with swine mucus or human mucus obtained from NHBE cell culture washes prior to addition of pH1N1low-1, pH1N1-1 or A/Brisbane/59/2007 (H1N1). We then fixed the cells at 7hpi and identified infected cells using an anti-influenza virus nucleoprotein antibody. The infectivity of pH1N1-1 and A/Brisbane/59/2007 in cultures overlaid with swine mucus was similar to cultures without a mucus overlay (figure 8). Interestingly, the infectivity of pH1N1low-1 was greater in cultures overlaid with swine mucus compared to cultures without a mucus overlay (percentage of cells infected with no overlay = 9.52±1.537, percentage of cells infected with swine mucus overlay = 15.35±0.1904, p<0.01, figure 9). In contrast, human mucus greatly decreased the infectivity of all viruses. The percentage decrease in infectivity observed between cultures overlaid with human mucus
compared to those without mucus was 90.8% (p<0.01), 89.8% (p<0.001) and 76.2% (p<0.05) for pH1N1low-1, pH1N1-1 and A/Brisbane/59/2007 viruses, respectively (figure 9). The inhibition observed inversely correlated to NA activity, although differences were not statistically significant. These data indicate that human mucus significantly interfered with the infectivity of these viruses whilst swine mucus did not.

Discussion

The pandemic influenza A 2009 virus (pH1N1) arose from a reassortment event between a North American triple reassortant swine lineage virus and a Eurasian avian lineage swine virus. The NA and matrix (M) genes of the pH1N1 virus originated from the Eurasian swine virus and the remaining six genes originated from the North American triple reassortant swine virus (23). Transmission from swine to humans has been documented for both of the pH1N1 precursor viruses but secondary human cases have not been observed and swine viruses isolated prior to 2009 do not appear to be transmissible via respiratory droplets in ferrets (4, 24, 25). There is growing evidence that a functional balance between HA and NA is important for transmission of swine viruses to humans. The 2009 pH1N1 viruses became transmissible by respiratory droplet spread after attaining the Eurasian-origin NA, which showed increased activity over the North American-origin NA. However, another swine H1N1 virus containing this NA and an Eurasian-origin HA did not transmit by respiratory droplets in ferrets (4). Further, an HA/NA functional balance appears to exist in other pandemic viruses. A study of the activities of the HAs and NAs of the pandemic viruses A/Japan/305/1957 (H2N2), A/Hong Kong/1/1968 (H3N2), A/South Carolina/1/1918 (H1N1) and A/California/04/2009 (H1N1), showed that the activity of the NA of A/Japan/305/1957 and A/Hong Kong/1/1968 were relatively high but they were matched by HAs that also exhibited higher binding (3). The NAs of A/South Carolina/1/1918 and A/California/04/2009 showed
relatively less activity but the activity of the HAs of these viruses was also less (3). Therefore, all these
pandemic viruses isolated from humans demonstrated a functional balance between HA and NA, which
indicates that the selective pressures that lead to the emergence of viruses with functionally balanced
HAs and NAs in humans are lacking in swine.

The pH1N1low viruses studied here showed extreme functional mismatching between HA and NA but this
did not impact significantly on replication fitness in vitro. However, in vivo, shedding and transmission of
pH1N1low-1 was significantly reduced compared to pH1N1-1, indicating that an interaction with the host
had a negative impact on pH1N1low-1. Respiratory droplet formation may have been an important factor
in the reduced transmissibility of pH1N1low-1 in ferrets. Other ferret studies showed that the Eurasian-origin
NA and M genes facilitated the release of respiratory particles from infected ferrets (26). The
Eurasian-origin swine NA showed greater activity than that of the North American swine NA and the
increased release correlated with the increased activity of the Eurasian-origin NA (26). Whilst it is
possible that the low NA activity of pH1N1low-1 was a limiting factor in aerosol transmission of this virus
between ferrets due to reduced respiratory particle release, we did not examine this here.

NA plays an important role in the release of viruses from cells by cleaving sialic acids on the cell surface,
but sialic acids are also present on mucins, a major component of mucus (27, 28). This is an important
host defense mechanism, as pathogens trapped in mucus are expelled via mucociliary clearance before
they are able to bind to cells and initiate an infection (27, 29). The interaction between NA and mucins
was hypothesized as early as 1943 and, more recently, NA activity has been shown to be important in
initiating infection and viral penetration of mucus in vitro, by studying viral binding to MDCK cells, A549
cells (a human lung cell line), erythrocytes and isolated respiratory mucus (18, 20, 30-32). Mucus
overlying frozen human trachea/bronchial tissue sections bound to viruses in a manner which appeared to be mediated by sialic acids (20). In vivo, overexpression of MUC5AC, one of the major mucins in respiratory mucus, in mice significantly impaired viral replication in the lung (18). Further, mice that do not express MUC5B, the other major respiratory mucin, showed severely impaired mucociliary clearance, perturbed immune responses and succumb to bacterial lung infections (33). Interestingly, a previous study showed that an overlay of human mucus on MDCK cell monolayers seemed to be more inhibitory to viruses than an overlay of porcine mucus, despite a similar sialic acid content, although this effect showed some strain-specificity (20). The porcine and human respiratory glycomes show similarities, including the expression of α2,3 and α2,6 sialylated glycoproteins and large complex N-glycans with sialylated poly LacNAc chains, but differences have been characterized (34, 35). One difference is that humans express N-acetylneuraminic acid (Neu5Ac) and lack N-glycolylneuraminic acid (Neu5Gc), which is expressed in pigs (36, 37). In porcine epithelial cells, Neu5Ac glycans are more abundant than Neu5Gc glycans and NeuAC-containing glycans have been shown to be important for infection of swine cells (38).

We hypothesized that mucus may have been interfering with pH1N1low-1 in ferrets, considering the low NA activity and increased binding affinity of the HA of this virus. Similar phenomena were observed in viruses expressing NA proteins of differing stalk length by Blumenkrantz et al (2013). Viruses expressing NA proteins with shorter stalk lengths appear to show a similar phenotype to pH1N1low viruses. Replacing the NA of an efficiently-transmitted H5N1 virus with an avian NA expressing a stalk 20 amino acids shorter than the NA originally in the virus abrogated AC transmission and reduced the efficiency of DC transmission in ferrets (39). The enzymatic activity of the short stalk NA was significantly reduced compared to the NA with a longer stalk and the inhibitory effects of mucus on the virus expressing NA
proteins with short stalks was significantly greater compared to the virus with a long stalk NA in vitro (39). This is in agreement with our study, in that NA activity appears to be important for transmission and that the virus-mucus interaction is important for efficient transmission, particularly via aerosol droplets.

To further study the role of mucus, we used cultures of mucus-producing NHBE cells. It was evident that mucus had a profound effect on pH1N1low-1 replication, but not on pH1N1-1 replication (figures 6 and 7). There was also some evidence of a correlation between NA activity and infection efficiency, which was particularly evident at 8hpi in unwashed wells, where A/Brisbane/59/2007 infected more cells than pH1N1-1, which infected more cells than pH1N1low-1 (figure 7). Further, pH1N1low-1 seemed to be more sensitive to inhibition by mucus than pH1N1-1, as there was a 94.2% decrease in the number of infected cells in washed wells compare to unwashed wells, compared to the 76.7% decrease observed in pH1N1-1-infected wells (figure 7k). Interestingly, the addition of exogenous NA did not have a significant effect on this infection. This may point to the importance of HA in the virus-mucus interaction. Extrapolating, since the replication of pH1N1low-1 was similar to pH1N1-1 in the absence of mucus, these data suggests that little NA activity is required to successfully extract viruses from a cell. However, the interaction between virus and mucus appears more sensitive to NA activity, or HA/NA functional balance, as pH1N1low-1 was significantly inhibited by mucus but pH1N1-1 was not.

There was an interesting pattern in mucus production in NHBE cell cultures. Where a productive infection was absent, amounts of mucus measured in unwashed cultures remained constant whilst, in washed cultures, mucus content returned to unwashed amounts within 24 hours and then remained constant (figure 6g). However, where a productive infection was present, mucus amounts decreased...
sharply and did not recover to uninfected amounts (figure 6b, d and f). pH1N1low-1 proved to be an exception to the pattern, as this virus replicated to significantly lower titers at 24hpi in washed cultures. In these wells, mucus production increased significantly to 48hpi, before dropping as viral titers increased (figure 6b). It should be noted that we collected samples from the apical surface of these cultures every 24 hours to measure mucus content and viral load, a process that washed some mucus off the cultures. Therefore, influenza virus infection of these cultures either stopped mucus production or severely curtailed it. Since we saw no correlation between decreasing mucus production and cell health at 24 or 48hpi, it is possible that a virus-specific mechanism was behind this phenomenon, which is an interesting hypothesis that may be worthy of further investigation.

We observed a clear difference between human and swine mucus. Human mucus significantly inhibited the infectivity of pH1N1low-1, pH1N1-1 and A/Brisbane/59/2007, whilst swine mucus did not (figures 8 and 9). These data point to an attribute of human mucus that was inhibitory to these viruses that was not present in swine mucus. Therefore, it is possible that mucus may be an important species barrier between humans and swine, although more work is needed to confirm this. It was interesting that pH1N1low-1 was the only virus of the three that showed increased infectivity in the presence of swine mucus. This was unexpected and more work is required to ascertain the significance of this observation.

In summary, we have characterized pH1N1 viruses isolated from swine that exhibit a high degree of functional mismatching between HA and NA. In ferrets, viral shedding was reduced and transmission efficiency was significantly reduced compared to a highly homologous virus with better balanced HA and NA. Therefore, whilst viruses with functionally mismatched HA and NAs can exist in swine, human to human spread is highly unlikely without further adaptation, possibly by reducing HA activity or
increasing NA activity to approach a functional balance. We have also shown that the interaction between influenza virus and mucus could inhibit the replication and/or transmission of viruses with functionally unbalanced HA and NAs in humans. This could potentially be an important species barrier between swine and humans.

Acknowledgements

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References


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Figure Legends

Figure 1. pH1N1low viruses show almost undetectable neuraminidase activity but no change in viral neuraminidase protein content was detected. The mean neuraminidase (NA) activity of pH1N1low viruses was significantly less than the mean NA activity of pH1N1 viruses, and almost undetectable, as measured MU-NANA (A, \( p < 0.01 \) at ½ dilution, \( n=4 \)) and fetuin (B, \( p < 0.001 \) at ½ dilution, \( n=4 \)) as substrates. The mean NA activity of pH1N1low viruses was also significantly less than the mean NA activity of other pandemic H1N1 viruses isolated in swine during the period 2009-2011 in the United States (pH1N1 viruses, \( p < 0.001 \) at ½ dilution, \( n=25 \). Refer to table 1 for strain information) (B). The enzyme kinetics of the NA in pH1N1low-1 and pH1N1-1 were also reduced compared to A/Brisbane/59/2007 (H1N1), as measured using MU-NANA as a substrate (\( V_{max} = 0.03, 0.10 \) and 0.47, respectively, \( K_m = 413.3, 33.31 \) and 51.98, respectively, \( n=2 \)). Viral growth kinetics in Madin Darby canine kidney (MDCK) cells showed that the replication kinetics of pH1N1low viruses (red and purple) were similar to pH1N1 viruses (blue and green) (D, \( n=3 \)). Separation of viral proteins by SDS-PAGE did not reveal any sizeable changes in protein content between these viruses, as measured using densitometry (E). M – Marker, 1; pH1N1low-1, 2; pH1N1low-2, 3; pH1N1-1, 4; pH1N1-2 (E). Comparing the intensity of the NA bands normalized to the nucleoprotein (NP) bands of each virus in (E) also did not reveal any appreciable differences between pH1N1low and pH1N1 viruses (F).

Figure 2. Exogenous neuraminidase increases the size of plaques formed by pH1N1-1 and pH1N1low-1 in Madin Darby Canine Kidney cells. Exogenous neuraminidase did not increase the number of plaques formed by pH1N1-1 (A and B) or pH1N1low-1 (C and D) but significantly increased the mean size of plaques formed by both pH1N1-1 and pH1N1low-1 (E, \( p < 0.001 \)). The mean size of plaques formed by pH1N1low-1 was significantly smaller than the mean size of plaques formed by pH1N1-1 (E, \( p < 0.001 \)).
difference between the mean size of plaques formed by pH1N1-1 and pH1N1low-1 in the presence of exogenous neuraminidase was also statistically significant (E, p<0.001). The mean size of plaques formed by pH1N1low-1 increased 203.3% in the presence of exogenous neuraminidase, compared to a 145% increase in mean plaque size formed by pH1N1-1, indicating exogenous neuraminidase had a greater effect on plaque formation by pH1N1low-1 compared to pH1N1-1 (E). Exogenous neuraminidase increased the mean size of plaques formed by pH1N1low-1 but mean plaque size was still significantly smaller than the mean size of plaques formed by pH1N1-1 (E, p<0.05). * - p<0.05, *** - p<0.001.

Figure 3. pH1N1low-1 is significantly less sensitive to oseltamivir compared to pH1N1-1 or A/Brisbane/59/2007 (H1N1).

Oseltamivir resistance assays using Madin Darby canine kidney (MDCK) cells showed that pH1N1low-1 was significantly less sensitive to oseltamivir compared to both pH1N1-1 (p<0.01) and A/Brisbane/59/2007 (p<0.01), which showed similar sensitivities (mean EC50 (μM) = 33.94±19.59, 0.26±0.09 and 0.11±0.02, respectively).

Figure 4. pH1N1low viruses show increased binding affinity of hemagglutinin to sialic acids. Binding affinity of all viruses to α2,3 sialic acids was almost undetectable for all viruses (A) whilst binding affinity of pH1N1low viruses to α2,6 sialic acids was greater than pH1N1 viruses (B and C). N.C. – negative control, allantoic fluid from uninfected eggs. ** - p<0.01, * - p<0.05, calculated by comparing the mean of pH1N1-1 and -2 to the mean of pH1N1low-1 and -2 at each dilution of sialylglycopolymer (n=2).

Figure 5. Transmissibility of pH1N1low-1 is significantly attenuated compared to pH1N1-1 in ferrets.

Viral titers, expressed as log10 tissue culture infectious dose 50% (TCID50), were measured in ferret nasal...
washes on days 3, 5, 7, 9 and 11 post infection with pH1N1-1 (A and C) or pH1N1low-1 (B and D). All inoculated (I) ferrets shed virus, but duration of shedding was reduced in pH1N1low-1-infected ferrets (A and B). Transmission of pH1N1low-1 to direct contact (DC) ferrets was delayed compared to pH1N1-1 infected ferrets, and transmission of pH1N1low-1 to aerosol contact (AC) ferrets was abrogated (C and D). Each bar represents a viral titer obtained from a single ferret. + - ferret died 7dpi showing pathology typical of influenza virus infection. Asterisks denote significantly greater mean titers in samples taken from ferrets infected with pH1N1-1 compared to pH1N1low-1 at the indicated day post infection. * - \( p < 0.05; *** - p < 0.001. \)

**Figure 6.** pH1N1low-1 replication in normal human broncial epithelial cells is inhibited by mucus. Viral titers, expressed as log_{10} tissue culture infectious dose 50% (TCID_{50}), in washed and unwashed normal human bronical epithelial wells (NHBE) cells infected with pH1N1-1 and A/Brisbane/59/2007 were similar at each timepoint (C and E, dashed and solid lines). The mean viral titer in washed wells infected with pH1N1low-1 at 24 hours post infection (hpi) was significantly less than that in washed wells infected with pH1N1-1 and A/Brisbane/59/2007 at 24hpi (\( p < 0.01 \) and \( p < 0.001 \), respectively, n=3, A, C and E). Asterisks shown in panels C and E at 24hpi). Virus was not detected in unwashed wells two and three infected with pH1N1low-1 and well one was also negative at 24hpi (A, solid lines). Further, the viral titer at 48hpi in well one was approximately two fold less compared to washed wells infected with pH1N1low-1 (A). The differences between the mean viral titers in washed and unwashed wells infected with pH1N1low-1 was statistically significant at 24 and 48hpi (\( p < 0.001 \) and \( p < 0.05 \), respectively, A). Mucus content was similar in all washed wells at each timepoint (B, D and F, dashed lines). The differences between the mean mucus contents of washed and unwashed wells at time zero was statistically significant in all experiments (\( p < 0.001 \), n=3, B, D, F and G). In uninfected unwashed cells, mucus content...
remained stable throughout the experiment whilst in uninfected washed wells, mucus content recovered to unwashed amounts within 24hpi (G). In wells infected with pH1N1-1 and A/Brisbane/59/2007, mucus content in unwashed wells was similar to that in washed wells at 48hpi and later time points (D and F). However, in wells infected with pH1N1low-1, the mean mucus content in unwashed wells was significantly greater than washed wells at 48, 72 and 96hpi (B, \( p < 0.01 \) at 48hpi and \( p < 0.05 \) at subsequent timepoints, \( n=3 \)). Further, mucus content in pH1N1low-1-infected well three decreased coincidentally with increasing viral titer (A and B). * - \( p < 0.05 \), ** - \( p < 0.01 \), *** - \( p < 0.001 \).

Figure 7. Neuraminidase activity is important early in infection. Washed (A, C, E, G) and unwashed (B, D, F, H) normal human bronchial epithelial (NHBE) cells were infected at an MOI of 1 with respective viruses and incubated for 8 hours at 37°C then fixed and immunolabeled using monoclonal antibodies against β-tubulin (red), which stains bundles of cilia on the apical surface of the cells, and influenza nucleoprotein (green). Scale bar = 50μM. Counts of infected cells, as determined by positive influenza nucleoprotein staining, did not show significant differences between pH1N1-1 and pH1N1low-1 viruses in washed wells (I), although the numbers of cells infected by these viruses were much fewer compared to A/Brisbane/59/2007, where the number of infected cells were too numerous to count (G). In unwashed wells, the number of cells infected by pH1N1-1 was significantly greater than those infected with pH1N1low-1 (J, \( p < 0.05 \), \( n=3 \)). Again, the number of cells in unwashed wells infected with A/Brisbane/59/2007 were too numerous to count and much greater than those infected with the other viruses (H). Exogenous neuraminidase (NA) increased the number of cells infected by pH1N1low-1, but this was not statistically significant (I and J). The difference in numbers of infected cells between washed and unwashed cells was greater in pH1N1low-1-infected and pH1N1-1-infected wells (K). Numbers of cells...
infected by A/Brisbane/59/2007 were too numerous to count, therefore, they are not included in panels I, J and K. Bars show the mean ± SEM in I and J. * - p<0.05.

Figure 8. Infection is inhibited by human mucus but not swine mucus. Madin Darby canine kidney (MDCK) cell monolayers were overlayed with swine mucus (B, E and H), human mucus (C, F and I) or no mucus (A, D and G) prior to addition of pH1N1low-1, pH1N1-1 or A/Brisbane/59/2007 (H1N1) at MOI=1. Cultures were fixed 7 hours post infection and stained for influenza nucleoprotein (green) and cell nuclei with DAPI (blue). Human mucus greatly decreased the number of cells infected by all viruses, whilst cultures overlayed with swine mucus appeared similar to cultures to which mucus was not added. Scale bar = 10μM. Images are representative of 3 replicates of each experimental condition.

Figure 9. Human mucus significantly inhibits viral infectivity whilst swine mucus does not. Madin Darby canine kidney (MDCK) cell monolayers were overlayed with swine mucus, human mucus or no mucus prior to addition of pH1N1low-1 (A), pH1N1-1 (B) or A/Brisbane/59/2007 (H1N1) (C) at MOI=1. Cultures were fixed 7 hours post infection and both infected and uninfected cells were counted in five representative fields in each well. Data is presented as mean percentage of cells infected. * - p<0.05, ** - p<0.01, *** - p<0.001.
Figure 3

![Graph showing cell viability and oseltamivir concentration.](http://jvi.asm.org/)

### Susceptibility (Mean EC$_{50}$ ± SD, μM)

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Figure 5

A. pH1N1-1

B. pH1N1\textsubscript{low-1}

C. Viral titer (log\textsubscript{10} TCI\textsubscript{50}/mL) against Days post infection

D. Viral titer (log\textsubscript{10} TCI\textsubscript{50}/mL) against Days post infection
Figure 8

A: No mucus
B: Swine mucus
C: Human mucus

pH1N1 low-1

D: pH1N1 low-1

pH1N1-1

G: A/Brisbane/59/2007
Figure 9

A

\( \text{pH1N1}_{\text{low}}, 1 \)

\[ \begin{align*}
\text{Percentage of cells infected} \\
\text{Mucus overlay} \\
\text{None} & \quad \text{Swine} & \quad \text{Human} \\
\end{align*} \]

B

\( \text{pH1N1-1} \)

\[ \begin{align*}
\text{Percentage of cells infected} \\
\text{Mucus overlay} \\
\text{None} & \quad \text{Swine} & \quad \text{Human} \\
\end{align*} \]

C

\( \text{A/Brisbane/59/2007 (H1N1)} \)

\[ \begin{align*}
\text{Percentage of cells infected} \\
\text{Mucus overlay} \\
\text{None} & \quad \text{Swine} & \quad \text{Human} \\
\end{align*} \]
Table 1. Details of the virus strains used in the MU-NANA assay presented in figure 1B (pH1N1 viruses).

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