

Cell culture adaptation of H3N2 influenza virus impacts acid stability and reduces ferret airborne transmission

Valerie Le Sage<sup>1</sup>, Karen A. Kormuth<sup>1^</sup>, Eric Nturibi<sup>1</sup>, Juhye M. Lee<sup>2,3</sup>, Sheila A. Frizzell<sup>5</sup>, Michael M. Myerburg<sup>5</sup>, Jesse D. Bloom<sup>2,3,4</sup> and Seema S. Lakdawala<sup>1,6\*</sup>

<sup>1</sup>Department of Microbiology and Molecular Genetics, University of Pittsburgh School of Medicine, Pennsylvania

<sup>2</sup>Division of Basic Sciences and Computational Biology Program, Fred Hutchinson Cancer Research Center, Washington

<sup>3</sup>Department of Genome Sciences, University of Washington, Washington

<sup>4</sup>Howard Hughes Medical Institute, Washington

<sup>5</sup>Department of Medicine, Division of Pulmonary, Allergy, and Critical Care Medicine, University of Pittsburgh School of Medicine, Pennsylvania

<sup>6</sup>Center for Vaccine Research, University of Pittsburgh School of Medicine, Pennsylvania

\*Direct correspondence to [Lakdawala@pitt.edu](mailto:Lakdawala@pitt.edu)

<sup>^</sup> KAK current address Bethany College

## Abstract

Airborne transmission of seasonal and pandemic influenza viruses is responsible for their epidemiological success and public health burden in humans. Efficient airborne transmission of H1N1 influenza virus relies on receptor specificity and pH of fusion of the surface glycoprotein hemagglutinin (HA). In this study, we examine the role of HA pH of fusion on transmissibility of a cell culture-adapted H3N2 virus. Mutations in the HA head at positions 78 and 212 of A/Perth/16/2009 (H3N2), which were selected after cell culture adaptation, decrease the acid stability of the virus from a pH of 5.5 (WT) to 5.8 (mutant). In addition, we observed that this mutant H3N2 virus replicated to higher titers in cell culture but had reduced

airborne transmission in the ferret model. These data demonstrate that, like H1N1 HA, the pH of fusion for H3N2 HA is a determinant of efficient airborne transmission. Surprisingly, we demonstrate that the NA segment noncoding regions can impact the pH of fusion of reassortant viruses. Taken together, our data confirm that HA acid stability is an important characteristic of epidemiologically successful human influenza viruses and is influenced by HA/NA balance.

**Keywords**

Influenza virus, transmission, pH, hemagglutinin

## Introduction

Influenza A viruses cause acute respiratory disease in mammals and birds whereas aquatic avian species and bats act as zoonotic reservoirs. Host tropism is determined by a combination of viral proteins and host factors, which allow for efficient replication and transmission within a given species. Influenza virus particles have two major surface antigens, hemagglutinin (HA) and neuraminidase (NA). The trimeric membrane-bound HA binds sialic acid on receptors and is a major host tropism determinant. Avian HA proteins preferentially bind to a sialic acid with an  $\alpha$ 2,3-linkage, whereas human viruses an  $\alpha$ 2,6-linkage [1-3]. HA is functionally balanced by the tetrameric receptor-destroying NA. At later stages of infection, NA plays a major function in removing sialic acids from host cell receptors as well as from newly synthesized HA and NA on nascent virions, which are sialylated during the cellular glycosylation process [4, 5]. Sialic acid removal by NA prevents virion aggregation and promotes spread to new target cells by preventing binding to the same dying host cell via HA [5]. The functional balance between HA and NA is important to maintain viral fitness as NA needs to be active enough to disaggregate virions upon release but not so much that HA receptor attachment is decreased [6].

Proteolytic cleavage of the HA0 precursor by host proteases produces HA1 and HA2 subunits and reveals the fusion peptide, which is required for membrane fusion [7, 8]. Once bound to sialic acids, influenza virus is internalized via receptor-mediated endocytosis and HA mediates escape from early endosomes in a pH-dependent manner. At a fixed pH, HA undergoes an irreversible conformational change, which facilitates fusion of the viral and cellular membranes and releases viral genomes into the host cytoplasm [9]. The stability of HA at low pH varies by host origin of the virus strain. Avian influenza viruses with less acid stable HA proteins undergo this conformational change at a higher pH between 5.5 and 6.2 [10-15]. The HA proteins from circulating human influenza viruses are more acid stable at pH 5.0-5.4 [16-18]; whereas swine isolates have a higher pH of HA

activation between 5.6-5.7 [18, 19]. Stability of the HA protein is an important factor in host adaptation infectivity, transmissibility and pandemic potential [18, 20-22].

The importance of HA pH stability on cell adaptation and transmission of H3N2 viruses is unclear. In this study, we utilized a seasonal H3N2 virus from 2009 (A/Perth/16/2009) and demonstrate that adaptation of H3 HA and N2 NA to replication in Madin-Darby Canine Kidney (MDCK) cells resulted in HA mutations (G87D and T212I) [23] that decreased HA stability. We confirm that the H3N2 virus with these mutations (herein referred to as 'rPerth mutant') exhibits enhanced replication in MDCK cells as compared to their WT counterpart (herein referred to as 'rPerth WT'). However, in more physiologically relevant adenocarcinoma human alveolar basal epithelial (A549) and human bronchial epithelial (HBE) cells there is no growth difference. In ferrets, we show that although rPerth mutant replicates similarly to rPerth WT, the virus has reduced transmission efficiency by respiratory droplets between ferrets. Our observations suggest that similar to H1N1 viruses, H3N2 viruses with a higher pH of fusion have decreased airborne transmission, confirming the importance of this phenotype on transmission fitness.

## Material and Methods

**Cells and Viruses.** Madin-Darby Canine Kidney (MDCK) epithelial cells were maintained in Eagle's minimal essential medium (MEM) supplemented with 10% fetal bovine serum (FBS), penicillin/streptomycin and L-glutamine. A549 and 293T cells (ATCC) were maintained in Dulbecco's MEM (DMEM) supplemented with 10% FBS, penicillin/streptomycin and L-glutamine. Primary HBE cells derived from human lung tissue were differentiated and cultured at an air-liquid interface using an institutional review board-approved protocol [24]. H3N2 viruses A/Panama/2007/2009, A/Perth/16/2009, A/Wyoming/3/2003 and A/Wisconsin/67/2005 were a generous gift from Dr. Zhiping Ye (Center for Biologics Evaluation and Research, FDA). A/Minnesota/11/2010 H3N2v and sw/OK/011506/2007 H3N2 viruses were a kind gift from Dr. Kanta Subbarao.

Influenza virus titers were determined by TCID<sub>50</sub> assay on MDCK cells according to the method of Reed and Muench [25].

**Plasmid-based reverse genetics.** The viruses used in this study were generated by reverse genetics using bi-directional reverse genetics plasmids based of pHW2000 [26]. The plasmids were cloned from cDNA reverse transcribed from the stock of A/Perth/16/2009 (H3N2) virus described in the subsection immediately above, and are named pHW-Perth09-PB2-SL, pHW-Perth09-PB1-SL, pHW-Perth09-PA-SL, pHW-Perth09-HA-SL, pHW-Perth09-NP, pHW-Perth09-NA, pHW-Perth09-M, and pHW-Perth09-NS1. The noncoding regions of the pHW-Perth09-HA-SL and pHW-Perth09-NA-SL exactly match the noncoding regions of the comparable vRNAs deposited in Genbank by the WHO Collaborating Centre for influenza research in Australia (Genbank GQ293081 and GQ293082) except for single A nucleotide inserted after 21<sup>st</sup> nucleotide in noncoding region at 5' end of the NA vRNA. We also used the variant HA and NA plasmids described in [23] as pHW-Perth09-HA-G78D-T212I and pHW-Perth09-NA. These variant plasmids differ in two ways from the initial set of eight: they have noncoding regions from the lab-adapted X-31 strain, and the HA has two amino-acid mutations that were selected after passaging in cell culture (see [23] for details). The virus termed "rPerth WT" was generated from the pHW-Perth09-\*-SL series of plasmids, whereas the virus termed "rPerth mutant" used the pHW-Perth09-HA-G78D-T212I and pHW-Perth09-NA plasmids and the other six genes from the pHW-Perth09-\*-SL series. The sequences of all plasmids are provided in Supplementary File 1.

Recombinant viruses were generated using the eight reverse genetics plasmids transfected into 293T cells using TransIT-LT1 transfection reagent (Mirus Bio LLC) in accordance with the manufacturer's protocol. After 24 and 48 hours, 293T cell supernatants were used to infect MDCK cells to rescue a CP1 stock of virus.

**Replication kinetics.** A549 and MDCK cells were infected with the indicated virus at a multiplicity of infection (MOI) of 0.01 or 1.0 (calculated on the basis of the

TCID<sub>50</sub>) in MEM containing 2% L-glutamine and supplemented with 1 µg/mL L-(tosylamido-2-phenyl) ethyl chloromethyl ketone (TPCK)-treated trypsin (Worthington Biochemical). Virus was allowed to adsorb for 1 hour before the inoculum was removed and replaced with fresh media. Supernatants were collected at the indicated time points and titered by TCID<sub>50</sub>.

Three different HBE patient cell line were used being HBE0176, HBE0256 and HBE259. The apical surface of HBE cells was washed in phosphate-buffered saline (PBS), and 10<sup>3</sup> TCID<sub>50</sub> of virus was added per 100 µL of HBE growth medium. After a 1 hour incubation at room temperature, the inoculum was removed and the apical surface was washed three times with PBS. At the indicated time points 150 µL of HBE medium was added to the apical surface for 10 minutes to capture released virus particles. The experiment was performed in triplicate in three different patient cell lines.

**Animal ethics statement.** Ferret experiments were conducted in an BSL2 facility at the University of Pittsburgh in compliance with the guidelines of the Institutional Animal Care and Use Committee (approved protocol #16077170 and #19075697). Animals were sedated with isoflurane following approved methods for all nasal wash and survival blood draw. 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.

**Ferret screening.** Five to six month old male ferrets were purchased from Triple F Farms (Sayre, PA). All ferrets were screened for antibodies against circulating influenza A and B viruses by hemagglutinin inhibition assay, as described in [27], using 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.

**Transmission studies.** Our transmission caging setup is a modified Allentown ferret and rabbit bioisolator cage similar to those used in [27-29]. For each study, three ferrets were anesthetized by isoflurane and inoculated intranasally with  $10^6$  TCID<sub>50</sub>/500uL of A/Perth/16/2009 WT or mutant virus to act as donor animals. Twenty-four hours later, a recipient ferret was placed into the cage but separated from the donor animal by two staggered perforated metal plates welded together one inch apart. Recipients were exposed for 14 days. Nasal washes were collected from each donor and recipient every other day for 14 days. To prevent accidental contact or fomite transmission by investigators, the recipient ferret was handled first and extensive cleaning of all gloves, chambers, biosafety cabinet, and temperature monitoring wand was performed between each recipient and donor animal and between each pair of animals. Sera was collected from donor and recipient ferrets upon completion of experiments to confirm seroconversion. To ensure no accidental contact or fomite transmission during husbandry procedures, recipient animal sections of the cage were cleaned prior to the donor side, with three lab personnel participated in each husbandry event to ensure that a clean pair of hands handled bedding and food changes. One cage was done at a time with a 10 minute wait time between cages to remove contaminated air prior to moving on to the next cage. Fresh scrappers, gloves, and sleeve covers were used on subsequent cage cleaning. Clinical symptoms such as weight loss and temperature were recorded during each nasal wash procedure and other symptoms such as sneezing, coughing, lethargy or nasal discharge were noted during any handling events. Once animals reached 10% weight loss, their feed

was supplemented with A/D diet twice a day to entice eating. A summary of clinical symptoms are provided in Table 1.

**Serology assays.** Analysis of neutralizing antibodies from ferret sera was performed as previously described [27]. Briefly, the microneutralization assay was performed using  $10^{3.3}$  TCID<sub>50</sub> of either H3N2 virus incubated with 2-fold serial dilutions of heat-inactivated ferret sera. The neutralizing titer was defined as the reciprocal of the highest dilution of serum required to completely neutralize infectivity of  $10^{3.3}$  TCID<sub>50</sub> of virus on MDCK cells. The concentration of antibody required to neutralize 100 TCID<sub>50</sub> of virus was calculated based on the neutralizing titer dilution divided by the initial dilution factor, multiplied by the antibody concentration.

For hemagglutinin inhibition (HAI) assay serum samples were pretreated with receptor-destroying enzyme (Seiken) followed by hemadsorption. Briefly, sera were serially diluted 2-fold in a V-bottom plate and mixed with four agglutinating units of virus for 15 minutes. An equal volume of 0.5% turkey erythrocytes were gently added to each well and incubated for 30 minutes at room temperature. Agglutination was read and HAI titers were expressed as the inverse of the highest dilution that inhibited four agglutinating units of virus.

**Tissue sample collection.** Euthanized ferrets were dissected aseptically. Collection of respiratory tissue were performed in the following order: entire right middle lung, left cranial lung (a portion equivalent to the right middle lung lobe), one inch of trachea cut lengthwise, entire soft palate, and nasal turbinates. Tissues were harvested as described in [28] and frozen at -80 °C. Tissue samples were weighed and Leibovitz's L-15 medium (Invitrogen) was added to make a 10% w/v homogenate. Tissues were dissociated using an OMNI GLH (OMNI International Inc) homogenizer and cell debris was removed by centrifugation at 1500 RPMs for 10 minutes. Infectious virus was quantified by TICD<sub>50</sub> by the endpoint method [25].

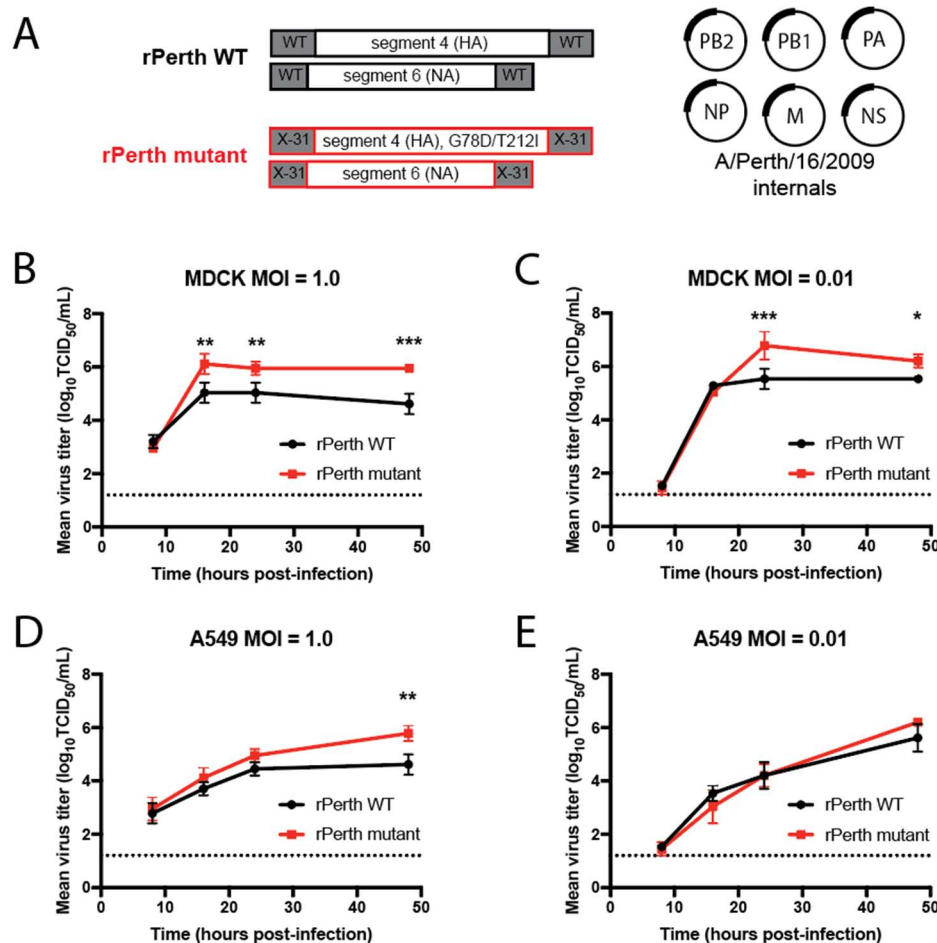


***In vitro* HA pH inactivation assay.** Virus stocks were incubated in PBS adjusted to the indicated pH values for 1 hour at 37 °C. The remaining virus was titered by TCID<sub>50</sub> by the endpoint method [25]. The pH that reduces the titer by 50% (EC<sub>50</sub>) was calculated by regression analysis of the dose-response curves. Each experiment was performed in triplicate at least twice.

## Results

**Viruses with cell culture-adaptive HA mutations replicate better in MDCK cells.** A previous study using a 6:2 reassortant virus of H3N2 virus with HA and NA Perth/16/2009 and internal WSN segments identified two mutations in the HA segment, G78D and T212I (H3 numbering), which arose after six serial passages in MDCK-SIAT1 cells expressing TMPRSS2 [23]. To compare the replication kinetics of viruses carrying these cell culture-adapted mutations, reverse genetics was used to generate both wild-type (WT) and mutant virus strains used in this study. The genes for all of these viruses were cloned from a stock of Perth/16/2009 (H3N2) virus; however, the HA and NA genes differed between the WT and mutant viruses. Recombinant A/Perth/16/2009 WT (rPerth WT) was generated from reverse genetics plasmids with native 5' and 3' untranslated regions (UTRs) for the HA and NA segments (Figure 1A). However, the mutant strain (rPerth mutant) was generated from plasmids in which the HA and NA were cloned into reverse genetic plasmids with the noncoding regions from the lab-adapted H3N2 reassortant strain X-31, with the HA also containing the two lab-adaptation mutations G78D and T212I (Figure 1A).

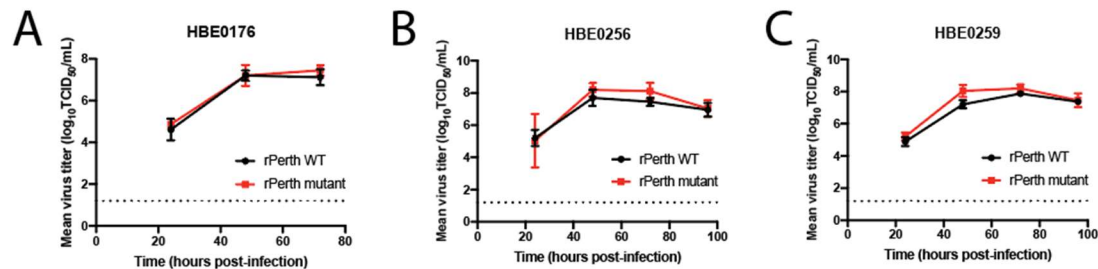
To address the replication kinetics of rPerth mutant in cell culture, we compared its growth kinetics in MDCK and A549 cells to rPerth WT. Each cell line was infected with rPerth WT or rPerth mutant virus at a multiplicity of infection (MOI) of 1.0 or 0.01 and the viral titers in infected cell supernatants were collected at the indicated time points. For the single cycle growth curve in MDCK cells, rPerth mutant had titers that were 1 log<sub>10</sub> greater at 16, 24 and 48 hours post-infection (hpi) as compared to rPerth WT (Figure 1B). Similarly, rPerth mutant grew better than rPerth WT at 24 and 48 hpi under multicycle growth curve (Figure 1C). In A549 cells, rPerth mutant replicated better than rPerth WT at 48 hpi at an MOI of 1.0 and to similar titers under all other conditions (Figure 1D and 1E).



**Figure 1. *In vitro* replication of cell culture-adapted H3N2 virus. (A)** Schematic representation of recombinant (r) A/Perth/16/2009 wild-type (WT) and mutant virus constructs. MDCK (**B and C**) and A549 (**D and E**) cells were infected with rPerth WT (black circles) and HA mutant (red circles) viruses at a multiplicity of infection (MOI) of 1.0 or 0.01. Cells were infected in triplicate and supernatants collected at the indicated times and virus titers were determined on MDCK cells using TCID<sub>50</sub> assays. Graphs are representative of three independent experiments. Two-way ANOVA was used to determine statistical significance (\*  $p < 0.05$ , \*\*  $p < 0.005$ , \*\*\*  $p < 0.0005$ ). The dashed line denotes the limit of detection for the titration assay.

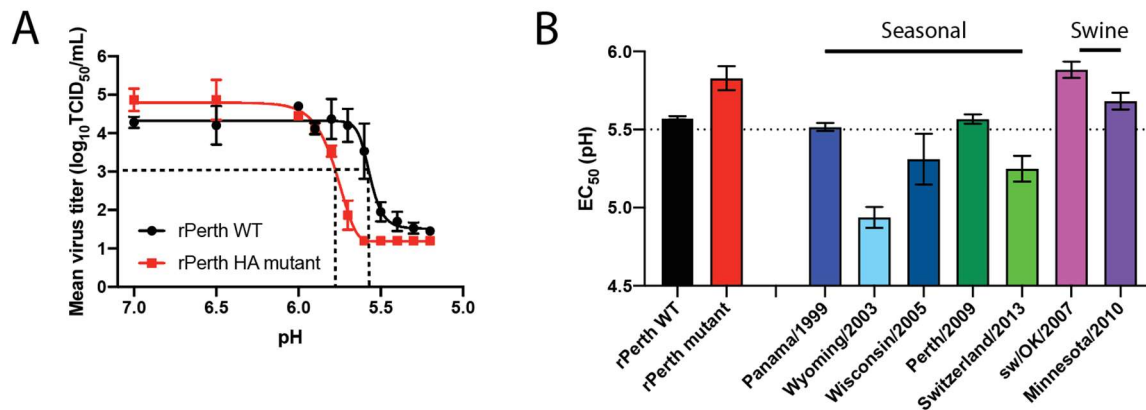
Primary HBE cell cultures are differentiated at an air-liquid interface and more closely mimic the lumen of the human airway because they produce mucus and are permissive to influenza viruses [24, 30]. We next characterized the replication capacity of rPerth mutant in 3 different HBE cell lines. HBE cells were infected with a low MOI of either rPerth WT or the mutant strain and the viruses were collected at the indicated times post-infection. Regardless of the patient cell line, we did not

observe a significant difference in replication between rPerth WT and mutant strains over time (Figure 2). Taken together, these data show that serial passage of the rPerth mutant on MDCK cells increases replication capacity in MDCK cells, but not in other cell lines or primary airway cultures.



**Figure 2. *In vitro* replication of rPerth WT and HA mutant in human bronchiole epithelial (HBE) cells.** Three different patient cell lines (A) HBE0176, (B) HBE0256 and (C) HBE0259 cells were infected at  $10^3$  TCID<sub>50</sub> per well with rPerth WT (black circles) or rPerth mutant (red circles). The apical supernatant was collected at the indicated time points and virus titers were determined on MDCK cells using TCID<sub>50</sub> assays. The experiment was performed in triplicate.

**The pH of inactivation rPerth mutant is higher than that of WT.** Cell culture adaptation has been associated with a broader pH range at which HA fuses with the host endosomal membrane early in the life cycle [31-33]. Treatment of virions with acidic pH causes HA to undergo a conformational change in HA, which results in premature activation of HA and an irreversible loss of viral infectivity [19]. To determine whether the HA changes associated with cell adaptation impacted HA acid stability, we tested the titer of virus incubated in pH-adjusted PBS after 1 hour (Figure 3A). The pH of inactivation was expressed as the EC<sub>50</sub>, which is the pH that reduces the titer by 50%. The EC<sub>50</sub> pH of inactivation for rPerth WT was calculated for each curve and resulted in a pH of inactivation of 5.57 while the rPerth mutant was 5.83 (Figure 3 A, black dashed lines). This experiment suggests that MDCK cell adaptive mutations results in a virus with decreased acid stability.

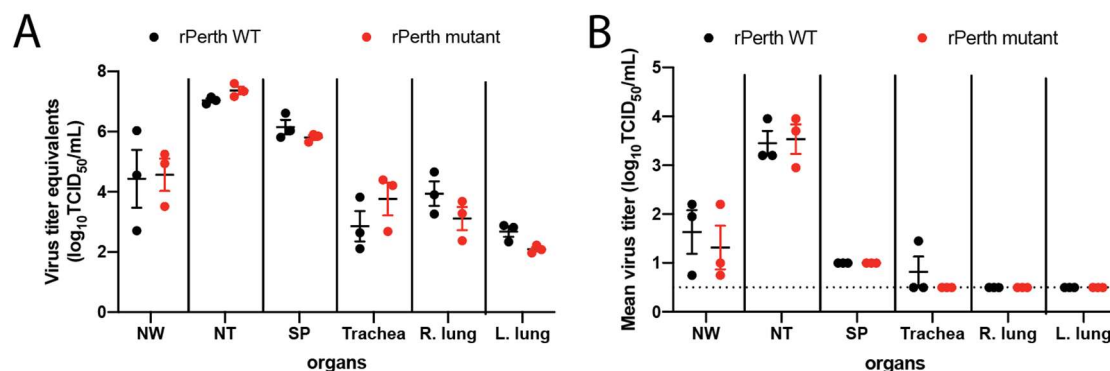


**Figure 3. rPerth mutant has decreased HA stability.** (A) rPerth WT (black line) and mutant (red line) were treated in pH-adjusted PBS for 1 hour at 37 °C. Remaining virus titers were determined by TCID<sub>50</sub> assay. The experiment was performed in triplicate, and representative data is shown. The data was fit with an asymmetric sigmoidal curve to determine the EC<sub>50</sub>. (B) Seasonal and swine H3N2 viruses were incubated in PBS of different pHs for 1 hour at 37 °C, performed in triplicate. The remaining virus titers were determined by TCID<sub>50</sub> assay and the EC<sub>50</sub> values were plotted using regression analysis of the dose-response curve. The mean ( $\pm$ SD) correspond to two experiments with triplicate samples.

To examine the acid stability of other seasonal human or swine origin H3N2 viruses, we evaluated the pH of inactivation for a range of contemporary seasonal and swine H3N2 viruses. As expected, the biological A/Perth/16/2009 had the same pH of inactivation (EC<sub>50</sub> = 5.57) as the rPerth WT while the seasonal human viruses tested range in pH from 5.5 to 4.9 (Figure 3B). These data are consistent with human seasonal H1N1 viruses, which have been shown to have a pH of inactivation <5.5 [34]. The two H3N2 swine viruses tested had EC<sub>50</sub> values that were higher (EC<sub>50</sub> = 5.88 and 5.68) and more similar to rPerth mutant (Figure 3B).

**Replication of the rPerth mutant in ferrets is similar to WT.** Based on the replication fitness in the various cell culture systems, we wanted to compare the replication capacity *in vivo*. Ferrets are an established model for the study of influenza pathogenicity and transmissibility because their lung physiology, receptor expression patterns, clinical signs, and transmissibility patterns resemble those of humans [35]. To determine the infectivity of the rPerth mutant in ferrets, we intranasally inoculated three ferrets with either rPerth WT or rPerth mutant virus.

On day 3 post-infection, the ferrets were sacrificed and respiratory tract tissues (nasal turbinates, soft palate, trachea, right middle lung and left cranial lung) were collected. The amount of viral RNA present in the tissue homogenates was assessed by quantitative PCR and infectious virus. Based on viral RNA quantification, no difference was observed between the recovery of rPerth WT and mutant viruses in each of respiratory tract tissues collected (Figure 4A). Infectious virus was determined on MDCK cells and was primarily observed in the upper respiratory tract (nasal wash, nasal turbinate and soft palate) (Figure 4B). No significant difference was observed between infectious virus titers from rPerth WT and rPerth mutant infected ferrets. Discrepancy in detection of viral RNA and infectious virus may be due to issues with freeze-thaw of the homogenates and titration on MDCK cells rather than MDCK-SIAT cells. Taken together, these observations suggest that cell-adapted H3N2 viruses replicate similarly in ferrets, in line with replication data from HBE cells rather than MDCK cells.



**Figure 4. Quantification of H3N2 virus in respiratory tissues of infected ferrets.** Ferrets were intranasally infected with  $10^6$  TCID<sub>50</sub> in 0.5 mL of rPerth WT (black circles) or rPerth mutant (red circles) and sacrificed on day 3 post-infection. NW – nasal wash, NT- nasal turbinate, SP- soft palate. (A) RNA was isolated from the indicated respiratory tract organ homogenates and qPCR for a region of the M segment was performed to quantify the relative amounts of influenza virus, normalized to RNA isolated from a virus stock with a known titer. (B) Titration of tissue homogenates to quantify infectious viral titers. Each dot represents a single animal and mean line +/- SEM are depicted.

**H3N2 mutant virus with a higher pH of fusion has reduced airborne transmission to naïve ferrets.** HA stability is an important factor that impacts

transmission efficiency [18, 20, 21]. To determine whether pH stability of H3N2 confers an airborne transmission disadvantage the impact of prolonged MDCK cell passage on the transmission efficiency of the rPerth mutant, we performed two sequential transmission studies with each virus. Three donor ferrets were infected intranasally with either rPerth WT or rPerth mutant and 24 hours later a naïve recipient ferret was placed in the adjacent cage, which has directional air flow from the donor to recipient [27, 29]. The naïve recipient was exposed for 14 days. Viral titers in nasal washes were collected every other day and seroconversion was determined on day 14 post-infection. rPerth WT transmitted to 3/3 recipients while the transmission efficiency of the rPerth mutant was reduced to 1/3 recipients (Table 1). All donors and recipients that shed virus in their nasal washes also seroconverted (Table 1). These results indicate that the cell-adapted rPerth mutant virus has reduced airborne transmission as compared to WT.

Table 1: Airborne transmission of rPerth WT and mutant viruses

Virus	Exposure time	Status	Transmission efficiency	Temperature increase*	Weight loss*	H3N2 microneutralization titers^	HAI titers^^
rPerth WT	14 days	INF		0/3	0/3	2560, 250, 2560	2560, 250, 2560
		Naïve	3/3	1/3	0/3	1280, 2560, 1280	1280, 2560, 1280
rPerth mutant	14 days	INF		1/3	0/3	2260, 1010, 2260	1280, 1280, 690
		Naïve	1/3	1/3	0/3	<20, <20, 1010	<10, <10, 2560

\* Temperature increase is defined as >1.5deg from day 0 temperature. Weight loss determined as > 10% of day 0 weight

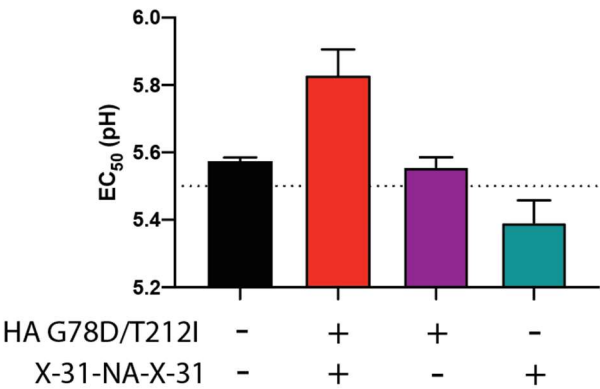
^ Antibody titers of day 14 are presented. All day 0 sera had a titer <20.

^^ Antibody titers of day 14 are presented. All day 0 sera had a titer <10.

**NA segment noncoding regions can influence the virus pH of fusion.** To determine the contribution of the X-31 UTRs on the pH of inactivation, we generated H3N2 viruses with different combinations of HA and NA (WT versus X-31 UTRs). Retention of the rPerth HA X-31 UTRs with the head mutations (G78D and T212I) along with the WT NA segment produced a pH of inactivation (EC<sub>50</sub> = 5.56), which was similar to rPerth WT (EC<sub>50</sub> = 5.57) (Figure 5). However, a rPerth virus strain containing the WT HA segment and the X-31 UTR NA segment had a lower pH of inactivation (EC<sub>50</sub> = 5.39) as compared to rPerth WT (Figure 5), indicating that X-31 UTR flanked NA segment is necessary but not sufficient to



alter the pH of inactivation. These results show that both HA (G78D and T212I) and NA X-31 UTR segments are required but not sufficient alone to increase the pH of inactivation for the rPerth virus.



**Figure 5. Increased pH of inactivation requires X-31 UTRs in NA segment and HA mutations.** The indicated mutant H3N2 viruses were incubated in PBS of different pHs for 1 hour at 37 °C, performed in triplicate. The remaining virus titers were determined by TCID<sub>50</sub> assay and the EC<sub>50</sub> values were plotted using regression analysis of the dose-response curve. The mean (±SD) correspond to at least two experiments with triplicate samples.

Discussion

Cross-species host adaptation is common in influenza viral infections and is the result of selective pressures at the site of replication, cell receptor availability, and compatibility with transcription and translation factors. Only H1N1, H2N2, and H3N2 influenza virus subtypes have become established within the human population and transmit person-to-person, while avian H5 and H7 influenza viruses will sporadically infect humans without efficient transmission between individuals. Gain-of-function studies with H5N1 influenza viruses indicate that mutation of HA to confer α2,6-linkage sialic acid binding, loss of glycosylation within the HA head domain and increasing HA stability improves airborne transmissibility in the ferret model [20, 21]. Evolution of HA is an important player in interspecies transmission and host range expansion. Mutations that stabilize the H5 HA can enhance replication in the upper respiratory tract of mice and ferrets [36-38], but result in a concomitant decrease in replication, virulence and transmissibility in its natural avian host [39, 40]. Conversely, adaptation of human influenza viruses to a murine host for use as an animal model for influenza virus research, requires adaptive



changes in the HA protein to alter receptor preference and decreased acid stability from 5.2-5.3 to 5.6-5.8 [41-43].

In this study, we have demonstrated that MDCK cell culture adaptation of an H3N2 influenza virus results in a virus with lower acid stability than the WT virus. The HA pH of activation is a known determinant of host adaptation. Consistent with this, we found that human seasonal viruses have an HA pH of fusion of 4.9-5.5 while swine H3N2 viruses have a pH of fusion >5.5. Previous work has shown a similar phenotype with human and gamma clade swine H1N1 viruses [34]. Additionally, gamma clade swine H1 viruses replicate to higher titers in MDCK cells [34], which is consistent with our observations of rPerth mutant. Interestingly, the observation of increased replication capacity was not recapitulated in HBE cells and ferrets, suggesting that MDCK replication capacity is not a strong correlate to results in relevant organoid cultures or *in vivo*.

HA is an important determinant of influenza virus transmissibility, which needs to remain stable as it travels in the environment between hosts [18, 44]. HA proteins that are stable at acidic pH have an advantage because they are less prone to being inactivated within the environment [45-47]. Yet, striking a fine pH balance for influenza virus is important because within the host cell a replicative advantage is conferred to viruses that encode a less acid stable HA as this facilitates efficient viral uncoating in endosomes [48]. However, avian virus HA proteins with a higher activation pH value have some advantages over their more acid stable human-adapted HA counterparts. During macrophage infection, less acid stable HA proteins release avian viruses earlier from the endosome to escape lysosomal degradation and allow continued replication [49]. Furthermore, a higher membrane fusion pH has been shown to help avian virus HA proteins avoid detection by the interferon-inducible transmembrane proteins IFITM2 and IFITM3, which restrict virus fusion [50]. Mutations in the head region of rPerth mutant, which arose during repeated passage in MDCK cells, lead to destabilization of HA and raised its activation pH. Similarly, MDCK cell culture adaption of egg-grown H3N2 X-31 virus

caused mutations in or near the fusion peptide, which resulted in higher pH of fusion mutants within a few passages [51]. Improving virus growth in cell culture is important for producing high yields of virus for vaccines. This enhanced growth is often associated with a broader pH range of virus-host fusion. Site-directed mutagenesis indicates that mutations in HA2 fusion peptide [32, 33, 52] and the transmembrane domain [31] can stabilize HA to produce a virus that replicates to higher titers in cell culture. Taken together, these data suggest that cell-adaptation correlates with decreased acid stability.

HA receptor binding and stability are important determinants of transmission but others including polymerase activity, resistance to host countermeasures that restrict influenza virus replication and virus morphology [53]. Influenza viruses are pleiomorphic structures with the two major surface glycoproteins HA and NA being packed closely but irregularly distributed on the surface of the virus particle [54-56]. Typically, NA is present in much smaller quantities than HA and it has been estimated that influenza virus particles have roughly 300 HA and 20-40 NA proteins [55, 57]. HA and NA have opposite functions and a fine balance is required for efficient virus replication as HA binds to sialic acid containing receptors and NA removes sialic acid from host cells. Mutations that alter the NA enzymatic active site or stalk length have been linked to unbalancing the HA/NA relationship [58-60]. The activation pH of the rPerth mutant, which carries mutation G78D and T212I in HA and X-31 UTRs flanking the NA segment (Figure 1A) was altered, however, the HA mutations alone were insufficient to raise the pH of activation. Similarly, the X-31 UTRs flanking NA segment was insufficient on its own to increase the pH of fusion and needed to be expressed in combination with the mutant HA. The viral gene segment UTRs are essential promoter elements required for initiation of viral replication and transcription. Decreased incorporation of NA in virions has been observed to negatively impact NA activity and compensate for functional differences in HA [61, 62]. The balance between HA and NA is critical for influenza virus fitness and a future avenue of research will be determining how this balance is affected by different UTRs.

**Acknowledgments**

This work was supported by the National Institute of Allergy and Infectious Diseases (HHSN272201400007C, SSL; 1R01AI139063-01A1, SSL; R01AI127893, JDB). Additional funding for SSL includes the American Lung Association Biomedical Research grant, and a New Initiative Award from the Charles E. Kaufman Foundation, a supporting organization of The Pittsburgh Foundation. SAF and MMM are funded through the Cystic Fibrosis Foundation Research Development Program to the University of Pittsburgh. We would like to thank the University of Pittsburgh DLAR technicians and veterinarians for their hard work and dedication as well as the members of Lakdawala lab for constructive discussions. JDB is an Investigator of the Howard Hughes Medical Institute.

**Author Contributions**

V.L. and S.S.L. conceptualized the project and wrote the manuscript. V.L., J.D.B. and S.S.L. designed the experiments. V.L. and S.S.L. analyzed and interpreted the data. V.L., K.A.K., E.N., J.M.L. performed the experiments. M.M.M. and S.A.F. contributed to resources and sample preparation. All authors edited and approved the manuscript.

**Conflict of interest**

The authors declare that they have no conflict of interest

## References

1. Connor, R.J., et al., *Receptor specificity in human, avian, and equine H2 and H3 influenza virus isolates*. Virology, 1994. **205**(1): p. 17-23.
2. Shinya, K., et al., *Avian flu: influenza virus receptors in the human airway*. Nature, 2006. **440**(7083): p. 435-6.
3. van Riel, D., et al., *H5N1 Virus Attachment to Lower Respiratory Tract*. Science, 2006. **312**(5772): p. 399.
4. Basak, S., M. Tomana, and R.W. Compans, *Sialic acid is incorporated into influenza hemagglutinin glycoproteins in the absence of viral neuraminidase*. Virus Res, 1985. **2**(1): p. 61-8.
5. Palese, P., et al., *Characterization of temperature sensitive influenza virus mutants defective in neuraminidase*. Virology, 1974. **61**(2): p. 397-410.
6. de Vries, E., et al., *Influenza A Virus Hemagglutinin-Neuraminidase-Receptor Balance: Preserving Virus Motility*. Trends Microbiol, 2020. **28**(1): p. 57-67.
7. Bottcher, E., et al., *Proteolytic activation of influenza viruses by serine proteases TMPRSS2 and HAT from human airway epithelium*. J Virol, 2006. **80**(19): p. 9896-8.
8. Steinhauer, D.A., *Role of hemagglutinin cleavage for the pathogenicity of influenza virus*. Virology, 1999. **258**(1): p. 1-20.
9. Skehel, J.J. and D.C. Wiley, *Receptor binding and membrane fusion in virus entry: the influenza hemagglutinin*. Annu Rev Biochem, 2000. **69**: p. 531-69.
10. Gabbard, J.D., et al., *Novel H7N9 influenza virus shows low infectious dose, high growth rate, and efficient contact transmission in the guinea pig model*. J Virol, 2014. **88**(3): p. 1502-12.
11. Hanson, A., et al., *Identification of Stabilizing Mutations in an H5 Hemagglutinin Influenza Virus Protein*. J Virol, 2015. **90**(6): p. 2981-92.
12. Herfst, S., et al., *Human Clade 2.3.4.4 A/H5N6 Influenza Virus Lacks Mammalian Adaptation Markers and Does Not Transmit via the Airborne Route between Ferrets*. mSphere, 2018. **3**(1).
13. Linster, M., et al., *Identification, characterization, and natural selection of mutations driving airborne transmission of A/H5N1 virus*. Cell, 2014. **157**(2): p. 329-339.
14. Richard, M., et al., *Limited airborne transmission of H7N9 influenza A virus between ferrets*. Nature, 2013. **501**(7468): p. 560-3.
15. Watanabe, Y., et al., *Characterization of H5N1 influenza virus variants with hemagglutinin mutations isolated from patients*. mBio, 2015. **6**(2).
16. Costello, D.A., G.R. Whittaker, and S. Daniel, *Variations in pH sensitivity, acid stability, and fusogenicity of three influenza virus H3 subtypes*. J Virol, 2015. **89**(1): p. 350-60.
17. Galloway, S.E., et al., *Influenza HA subtypes demonstrate divergent phenotypes for cleavage activation and pH of fusion: implications for host range and adaptation*. PLoS Pathog, 2013. **9**(2): p. e1003151.

18. Russier, M., et al., *Molecular requirements for a pandemic influenza virus: An acid-stable hemagglutinin protein*. Proc Natl Acad Sci U S A, 2016. **113**(6): p. 1636-41.
19. Russier, M., et al., *H1N1 influenza viruses varying widely in hemagglutinin stability transmit efficiently from swine to swine and to ferrets*. PLoS Pathog, 2017. **13**(3): p. e1006276.
20. Herfst, S., et al., *Airborne transmission of influenza A/H5N1 virus between ferrets*. Science, 2012. **336**(6088): p. 1534-41.
21. Imai, 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**(7403): p. 420-8.
22. Russell, C.J., M. Hu, and F.A. Okda, *Influenza Hemagglutinin Protein Stability, Activation, and Pandemic Risk*. Trends Microbiol, 2018. **26**(10): p. 841-853.
23. Lee, J.M., et al., *Deep mutational scanning of hemagglutinin helps predict evolutionary fates of human H3N2 influenza variants*. Proc Natl Acad Sci U S A, 2018.
24. Myerburg, M.M., et al., *Acute regulation of the epithelial sodium channel in airway epithelia by proteases and trafficking*. Am J Respir Cell Mol Biol, 2010. **43**(6): p. 712-9.
25. Reed LJ, M.H., *A simple method of estimating fifty percent endpoints*. Am J Hyg, 1938. **27**: p. 493-7.
26. Hoffmann, E., et al., *A DNA transfection system for generation of influenza A virus from eight plasmids*. Proc Natl Acad Sci U S A, 2000. **97**(11): p. 6108-13.
27. Lakdawala, S.S., 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**(12): p. e1002443.
28. Lakdawala, S.S., et al., *The soft palate is an important site of adaptation for transmissible influenza viruses*. Nature, 2015. **526**(7571): p. 122-5.
29. Le Sage, V., et al., *Pre-existing heterosubtypic immunity provides a barrier to airborne transmission of influenza viruses*. PLoS Pathog, 2021. **17**(2): p. e1009273.
30. Nturibi, E., et al., *Intracellular Colocalization of Influenza Viral RNA and Rab11A Is Dependent upon Microtubule Filaments*. J Virol, 2017. **91**(19).
31. Kawahara, M., et al., *Cell-Based Influenza A/H1N1pdm09 Vaccine Viruses Containing Chimeric Hemagglutinin with Improved Membrane Fusion Ability*. Vaccines (Basel), 2020. **8**(3).
32. Murakami, S., et al., *Enhanced growth of influenza vaccine seed viruses in vero cells mediated by broadening the optimal pH range for virus membrane fusion*. J Virol, 2012. **86**(3): p. 1405-10.
33. Yang, H., et al., *Structural stability of influenza A(H1N1)pdm09 virus hemagglutinins*. J Virol, 2014. **88**(9): p. 4828-38.
34. Hu, M., et al., *HA stabilization promotes replication and transmission of swine H1N1 gamma influenza viruses in ferrets*. Elife, 2020. **9**.
35. Belser, J.A., J.M. Katz, and T.M. Tumpey, *The ferret as a model organism to study influenza A virus infection*. Dis Model Mech, 2011. **4**(5): p. 575-9.

36. Shelton, H., 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**(Pt 6): p. 1220-1229.
37. Zaraket, H., 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**(17): p. 9911-22.
38. Zaraket, H., O.A. Bridges, and C.J. Russell, *The pH of activation of the hemagglutinin protein regulates H5N1 influenza virus replication and pathogenesis in mice.* J Virol, 2013. **87**(9): p. 4826-34.
39. Reed, M.L., et al., *The pH of activation of the hemagglutinin protein regulates H5N1 influenza virus pathogenicity and transmissibility in ducks.* J Virol, 2010. **84**(3): p. 1527-35.
40. Richard, M., et al., *Mutations Driving Airborne Transmission of A/H5N1 Virus in Mammals Cause Substantial Attenuation in Chickens only when combined.* Sci Rep, 2017. **7**(1): p. 7187.
41. Hartley, C.A., et al., *Changes in the hemagglutinin molecule of influenza type A (H3N2) virus associated with increased virulence for mice.* Arch Virol, 1997. **142**(1): p. 75-88.
42. Keleta, L., et al., *Experimental evolution of human influenza virus H3 hemagglutinin in the mouse lung identifies adaptive regions in HA1 and HA2.* J Virol, 2008. **82**(23): p. 11599-608.
43. Koerner, I., et al., *Altered receptor specificity and fusion activity of the haemagglutinin contribute to high virulence of a mouse-adapted influenza A virus.* J Gen Virol, 2012. **93**(Pt 5): p. 970-979.
44. Klein, E.Y., et al., *Stability of the Influenza Virus Hemagglutinin Protein Correlates with Evolutionary Dynamics.* mSphere, 2018. **3**(1).
45. Labadie, T., et al., *Influenza Virus Segment Composition Influences Viral Stability in the Environment.* Front Microbiol, 2018. **9**: p. 1496.
46. Poulson, R.L., et al., *Environmental Stability of Swine and Human Pandemic Influenza Viruses in Water under Variable Conditions of Temperature, Salinity, and pH.* Appl Environ Microbiol, 2016. **82**(13): p. 3721-3726.
47. Russier, M., et al., *Hemagglutinin Stability Regulates H1N1 Influenza Virus Replication and Pathogenicity in Mice by Modulating Type I Interferon Responses in Dendritic Cells.* J Virol, 2020. **94**(3).
48. Singanayagam, A., M. Zambon, and W.S. Barclay, *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**(17).
49. Marvin, S.A., et al., *Influenza Virus Overcomes Cellular Blocks To Productively Replicate, Impacting Macrophage Function.* J Virol, 2017. **91**(2).
50. Gerlach, T., et al., *pH Optimum of Hemagglutinin-Mediated Membrane Fusion Determines Sensitivity of Influenza A Viruses to the Interferon-Induced Antiviral State and IFITMs.* J Virol, 2017. **91**(11).



51. Lin, Y.P., et al., *Adaptation of egg-grown and transfectant influenza viruses for growth in mammalian cells: selection of hemagglutinin mutants with elevated pH of membrane fusion*. Virology, 1997. **233**(2): p. 402-10.
52. Cotter, C.R., H. Jin, and Z. Chen, *A single amino acid in the stalk region of the H1N1pdm influenza virus HA protein affects viral fusion, stability and infectivity*. PLoS Pathog, 2014. **10**(1): p. e1003831.
53. Long, J.S., et al., *Host and viral determinants of influenza A virus species specificity*. Nat Rev Microbiol, 2019. **17**(2): p. 67-81.
54. Calder, L.J., et al., *Structural organization of a filamentous influenza A virus*. Proc Natl Acad Sci U S A, 2010. **107**(23): p. 10685-90.
55. Harris, A., et al., *Influenza virus pleiomorphy characterized by cryoelectron tomography*. Proc Natl Acad Sci U S A, 2006. **103**(50): p. 19123-7.
56. Leser, G.P. and R.A. Lamb, *Lateral Organization of Influenza Virus Proteins in the Budzone Region of the Plasma Membrane*. J Virol, 2017. **91**(9).
57. Hutchinson, E.C., et al., *Conserved and host-specific features of influenza virion architecture*. Nat Commun, 2014. **5**: p. 4816.
58. McKimm-Breschkin, J.L., et al., *Passaging of an influenza A(H1N1)pdm09 virus in a difluoro sialic acid inhibitor selects for a novel, but unfit I106M neuraminidase mutant*. Antiviral Res, 2019. **169**: p. 104542.
59. Mitnaul, L.J., et al., *Balanced hemagglutinin and neuraminidase activities are critical for efficient replication of influenza A virus*. J Virol, 2000. **74**(13): p. 6015-20.
60. Romero-Beltran, L., et al., *Mutations at highly conserved residues in influenza A(H1N1)pdm09 virus affect neuraminidase activity*. Virus Res, 2016. **225**: p. 1-9.
61. Das, S.R., et al., *Defining influenza A virus hemagglutinin antigenic drift by sequential monoclonal antibody selection*. Cell Host Microbe, 2013. **13**(3): p. 314-23.
62. Hensley, S.E., et al., *Influenza A virus hemagglutinin antibody escape promotes neuraminidase antigenic variation and drug resistance*. PLoS One, 2011. **6**(2): p. e15190.