Swine H1N1 influenza virus variants with enhanced polymerase activity and HA stability promote airborne transmission in ferrets

Meng Hu¹, Jeremy C. Jones¹, Balaji Banoth¹, Chet Raj Ojha¹, Jeri Carol Crumpton¹, Lisa Kercher¹, Robert G. Webster¹, Richard J. Webby^{1,2}, and Charles J. Russell^{1,2}

¹Department of Infectious Diseases, St. Jude Children's Research Hospital, 262 Danny Thomas Place, Memphis, Tennessee 38105-3678, ²Department of Microbiology, Immunology & Biochemistry, College of Medicine, The University of Tennessee Health Science Center, Memphis, Tennessee 38163.

Running Title: Polymerase activity and HA stability in influenza transmission

*** Corresponding author at:** Department of Infectious Diseases, MS 330, St. Jude Children's Research Hospital, 262 Danny Thomas Place, Memphis, TN 38105-3678, USA. Phone: 901-595-5648. Fax: 901-595-8559. Email: charles.russell@stjude.org.

Keywords: Influenza A virus / swine viruses / polymerase / viral fusion protein / transmissibility / virus adaptation

ABSTRACT

Understanding how animal influenza A viruses (IAVs) acquire airborne transmissibility in humans and ferrets is needed to prepare for and respond to pandemics. Here, we investigated the replication and transmission in ferrets of swine H1N1 isolates P4 and G15, whose majority population had decreased polymerase activity and poor HA stability, respectively. For both isolates, a minor variant was selected and transmitted in ferrets. Polymerase-enhancing variant PA-S321 was selected in half of P4 isolates that airborne-transmitted in ferrets. HA-stabilizing variant HA1-S210 was selected in all G15-inoculated ferrets and was transmitted by contact and airborne routes. With an efficient polymerase and a stable HA, the purified minor variant G15-HA1-S210 had earlier and higher peak titers in inoculated ferrets and was recovered at a higher frequency after airborne transmission than P4 and G15. Overall, a combination of enhanced polymerase activity and a stabilized HA protein promoted airborne transmission of swine H1N1 viruses in ferrets, and minor variants containing both properties were selected. The results suggest pandemic risk-assessment studies may benefit from deep sequencing to identify minor variants with human-adapted traits.

IMPORTANCE

Diverse IAVs circulate in avians, swine, and other species; however, few acquire the viral traits needed to start a human pandemic. A receptor-binding preference for α -2,6-linked sialic acid receptors is necessary but not sufficient for pandemic potential. Other viral traits are needed but are less well understood and are not explicitly considered in current pandemic risk-assessment algorithms. A stabilized HA and mammalian-adapted polymerase have been shown to promote the adaptation of IAVs to humans and ferrets (the gold-standard model for IAV replication, pathogenicity, and transmissibility). Here, we used swine IAV isolates of the gamma lineage as a model to investigate the importance of HA stability and polymerase activity in promoting replication and transmission in ferrets. These are emerging viruses that bind to both α -2,6- and α -2,3-linked receptors. Using isolates containing mixed populations, a stabilized HA was selected within days in inoculated ferrets. An enhanced polymerase was selected later in half of transmission events. Thus, HA stabilization was a stricter requirement, yet both traits promoted transmissibility in ferrets. Knowing the combination of viral traits needed for pandemic potential, and the relative importance of each, will help identify viruses of greatest concern.

INTRODUCTION

IAVs circulate in numerous species, and it remains challenging to identify animal-origin IAVs at greatest risk to cause a pandemic in humans. In addition to bats (1-3), IAVs originate from a reservoir of wild aquatic birds (4). From wild birds, avian IAVs can infect and either directly or indirectly become endemic in wild and domestic birds, swine, humans, and other domestic or aquatic mammals (5, 6). IAVs have eight RNA gene segments that encode for over a dozen proteins including an RNA-dependent RNA polymerase (RdRp) complex (PB1, PB2, and PA) and the surface glycoproteins hemagglutinin (HA) and neuraminidase (NA) (7, 8). Antigenically diverse HA and NA subtypes have been identified and currently number from H1-H18 and N1-N11. H1N1 caused the 1918 Spanish influenza pandemic, circulated in humans until 1957, became endemic in swine, reemerged in humans in 1977, and caused the 2009 pandemic (pH1N1) (8, 9). In 1957, H1N1 was supplanted in humans by the H2N2 Asian influenza pandemic. H2N2 was supplanted by the H3N2 Hong Kong influenza pandemic in 1968. H3N2 viruses, along with pH1N1 and influenza B viruses, cause seasonal influenza in humans. H1N1, H1N2, and H3N2 viruses are currently endemic in swine (10-12).

H1 swine viruses have been divided into six clades: alpha (1A.1), beta (1A.2), gamma (1A.3.3.3), H1pandemic (1A.3.3.2), delta1 (1B.2.2), and delta 2 (1B.2.1) (13, 14). Swine gamma and pandemic clades diverged approximately twenty years ago and have caused numerous human infections (15). Currently, H1 gamma clade is endemic in swine and frequently reassorts with human pandemic H1N1 isolates (16). Preparing for and responding to future human pandemics requires an understanding of viral traits and host markers associated with human adaptation so that high-risk emerging viruses can be identified, and countermeasures taken.

IAV adaptations that have been associated with human-to-human transmission include HA receptor-binding specificity, HA stability, polymerase complex efficiency, HA-NA balance,

NA stalk length, interferon antagonism, and filamentous virus morphology (5, 17). During viral entry, the HA protein binds sialic acid-containing receptors. This triggers virion endocytosis and acidification, which induces irreversible HA structural changes that cause membrane fusion. Human-adapted IAVs contain HA proteins that preferentially bind α -2,6-linked sialic acid (SA) receptors over α -2,3-linked ones, which are preferred by avian IAVs (8, 18-22). In human- and ferret-adapted IAVs, the avidity of the HA protein for cell-surface glycan receptors is balanced with the substrate selectivity and catalytic rate of the NA protein (17, 23-26). Decreased HA activation pH in the context of H5N1 viruses reduces replication and transmission in avian species but increases nasal replication and is required for airborne transmission in ferrets (27-34). Similarly, HA stabilization occurred during the adaptation of pH1N1 to humans (35, 36). HA stabilization has been shown to be necessary for airborne transmission of pH1N1 and swine H1N1 gamma viruses in ferrets, in part by increasing longevity of expelled virions (37-39).

The polymerases from avian IAVs are relatively inefficient at genome replication in mammalian cells (40, 41). Therefore, they require adaptative mutations in their polymerase complexes to replicate and transmit in mammals (42-44). Mammalian-adaptive PB2 mutations include T271A, G590S, Q591R, E627K, and D701N (5). PB2-E627K, the best-characterized mutation, was present in the 1918 H1N1, 1957 H2N2, and 1968 H3N2 pandemic viruses and has been associated with avian H7N9 and H5N1 influenza virus adaptation in humans and other mammalian hosts (45, 46). The 2009 pH1N1 virus lacked the PB2-E627K mutation but had phenotypically similar mutations PB2-G590S/Q591R, which were located near residue 627. The swine H1N1 gamma viruses in the present study have mammalian-preferred PB2-G590S/Q591R mutations and avian-like PB2-E627 and PB2-D701.

Influenza pandemic risk assessment algorithms by the US Centers for Disease Control and Prevention (CDC) and World Health Organization (WHO) explicitly consider HA receptor-

binding specificity and indirectly account for other human-adaptive viral traits through airborne transmission studies (47, 48). Ferrets are currently considered the gold-standard animal model to study transmission of influenza viruses (20, 49).

In a previous study, we found HA stabilization promoted the replication and transmission of swine H1N1 IAVs in ferrets (39). Related isolates A/swine/Illinois/2A-1213-G15/2013 (H1N1) and A/swine/Illinois/2B-0314-P4/2014 (H1N1) were found to have a single HA variation at residue 210 (H3 numbering) located between the receptor-binding domain heads, which resulted in HA activation pH values of 5.8 and 5.5, respectively. The G15 group had 3/3 airborne-transmission events in ferrets after selection of a stabilized variant (HA1-S210, HA activation pH 5.5). Despite also having an HA activation pH of 5.5, the P4 group had only 1/3 airborne transmission. The G15 and P4 viruses also contained polymerase gene variations at PB2-648 and PA-271, and P4 had minor variants at PA-321 and PA-386. Therefore, we hypothesized that P4 and G15 differed in polymerase activity in addition to HA stability and that enhanced polymerase activity contributed to increased airborne transmissibility of P4 over G15. Meanwhile, we could not rule out whether having an initial mixed population of HA stability in the case of P4 was responsible for increased airborne transmissibility. In the present work, we isolated the G15 virus containing the stabilizing HA1-S210 variation and studied replication and transmission of G15-HA1-S210, G15, and P4 viruses in ferrets. Overall, we found that both enhanced HA stability and polymerase activity are associated with airborne transmissibility in ferrets and that a mixed population of HA-stabilized and HA-destabilized fractions was not needed for replication and transmission in ferrets.

RESULTS

Impact of HA and polymerase variations on gamma virus replication in MDCK

cells. Whole-genome sequencing analyses showed that five amino acid variations existed between swine H1N1 gamma viruses P4 and G15 (Fig. 1A). P4 contained 92% PA-N321, 8% PA-S321, 86% PA-D386, and 14% PA-N386, and we designated this virus isolate as P4-PA-mixed. We designated the G15 virus as G15-HA1-mixed because this virus isolate contained 85% HA1-N210 and 15% HA1-S210 (Fig. 1A). Aside from the minor variants, P4-PA-mixed and G15-HA1-mixed differed from each other at HA1-210, PA-271, and PB2-648 (Fig. 1A). From G15-HA1-mixed, we isolated the majority population (G15-HA1-N210) and the minor variant (G15-HA1-S210). Residue HA1-210 interacts with residues across the trimer interface in the head domain, so the HA1-S210 variation most likely stabilizes the HA protein by resisting dissociation of the heads (39).

After infection in Vero cells, the highest pH at which G15-HA1-mixed and G15-HA1-N210 were triggered to cause syncytium formation (cell-to-cell fusion) was 5.8 (Fig. 1B). P4-PA-mixed and G15-HA1-S210 had more stable HA proteins that were activated at pH 5.5 and 5.6, respectively. The pH of virus inactivation was measured by exposing virus aliquots to media of varying pH, neutralizing, and measuring residual infectivity. The pH at the midpoint of inactivation of G15-HA1-mixed and G15-HA1-N210 were 5.75 and 5.8, respectively, while those of P4-PA-mixed and G15-HA1-S210 were 5.6 (Fig. 1C). Overall, HA activation pH values were similar to virus inactivation values, G15-HA1-mixed and G15-HA1-N210 had relatively unstable HA proteins, and P4-PA-mixed and G15-HA1-S210 had stabilized HA proteins.

Previous reports showed differences in polymerase activity and/or HA stability may affect virus one-step growth (50-52). To explore this, we inoculated viruses into MDCK cells at an MOI of 2 PFU/cell and measured the virus titers of the supernatants at 6- and 8-hours post-

infection (hpi). Compared to P4-PA-mixed, the three other viruses had higher titers at 6 hpi (Fig. 1D). As P4-PA-mixed and G15-HA1-S210 have identical HA proteins, enhanced replication at 6 hpi was mapped to PA-P271 and/or PB2-L648. At 8 hpi, G15-HA-N210 had higher titers than the other three viruses. G15-HA1-N210 and G15-HA1-S210 were identical except for HA1 residue 210. Therefore, the HA1-N210 variation (HA activation pH 5.8) enhanced replication at 8 hpi in MDCK cells compared to HA1-S210 (pH 5.6). Overall, both the HA variation and the polymerase variations were found to modulate the production of infectious virus in MDCK cells at relatively early time points.

G15-HA1-S210 had higher polymerase activity than P4-PA-mixed. Virus polymerase activity can be assessed by the accumulation of viral mRNA, cRNA, and vRNA, which are produced in virus transcription and replication (53). To study the impact of the PB2 and PA variations on polymerase activity, we compared G15-HA1-S210 and P4-PA-mixed, which had identical HA genes. The viruses were inoculated into MDCK cells at an MOI of 2 PFU/cell and the resulting cell lysates and supernatants were harvested at 4 and 6 hpi. Compared to P4-PA-mixed, G15-HA1-S210 had higher mRNA, cRNA, and vRNA levels at 4 and 6 hpi (Fig. 2A-B). G15-HA1-S210 also had higher viral loads in cell supernatants at 6 hpi (Fig. 2C). Overall, the presence of PA-P271 and/or PB2-L648 in G15-HA1-S210 was associated with higher polymerase activity. These two variations were also present in G15-HA1-mixed and G15-HA1-N210.

Infection of donor ferrets inoculated with P4-PA-mixed, G15-HA1-mixed, and G15-HA1-S210. While G15-HA1-S210 had relatively efficient polymerase activity and a stabilized HA protein, P4-PA-mixed had decreased polymerase activity and G15-HA1-mixed had a relatively unstable HA protein (Fig. 3A). In each cubicle, three ferrets were directly inoculated with 10^6 PFU of virus, and one day later we introduced three naïve contact and three naïve

airborne ferrets (Fig. 3B). Each virus group was evaluated in an isolated cubicle and personal protective equipment was changed before moving between cubicles to avoid cross contamination of viruses. Each cubicle was set to a temperature of approximately 22 °C and relative humidity 40-60 (Fig. S1). Here, we infected two cubicles of ferrets with G15-HA1-S210, so that this group had a total of n=18. We previously infected one cubicle of ferrets with P4-PA-mixed and G15-HA1-mixed each (n=9), which were the same as the present setting in Fig. 3B (39). We chose to continue to infect another cubicle of ferrets with P4-PA-mixed and G15-HA1-mixed each for the present study (n=9). Therefore, all three virus groups had a total of 18 ferrets (6 donor, 6 contact, and 6 airborne). All ferrets were monitored for body weight and temperature daily, and ferret nasal washes were collected every other day until day 14. Ferret sera were collected on day 21.

On day 1, G15-HA1-S210 donor ferrets had an average nasal wash titer of 2.18×10^7 TCID₅₀/ml, a value more than 10-fold higher than that of P4-PA-mixed (6.46×10^5 TCID₅₀/ml, $P = 0.039$) and 100-fold higher than G15-HA1-mixed (9.48×10^4 TCID₅₀/ml, $P = 0.0007$) (Fig. 3C and Table 1). G15-HA1-S210 had peak titers on day 1, while G15-HA1-mixed and P4-PA-mixed had mean peak titers on day 3 (Fig. 3D). Additionally, the average peak titer of G15-HA1-S210 in donor ferrets (2.18×10^7 TCID₅₀/ml) was significantly higher than that of G15-HA1-mixed (8.34×10^5 TCID₅₀/ml) (Fig. 3E). Overall, G15-HA1-S210, which had a stabilized HA and enhanced polymerase activity, had earlier and higher growth in the nasal cavities of inoculated ferrets.

The average HAI titers of donor ferret sera of G15-HA1-S210 were higher than those of P4-PA-mixed and G15-HA1-mixed (Fig. 3F). One donor ferret in cubicle 1 and two in cubicle 2 of G15-HA1-S210 had >10% body weight loss for at least two days, compared to no ferrets inoculated with P4-PA-mixed and G15-HA1-mixed ($P < 0.05$, Fig. 3G). No significant differences in body temperature were observed between the donor ferret groups (Fig. S2).

Contact and airborne transmission in P4-PA-mixed. P4-PA-mixed had 3/3 contact and 3/3 airborne transmission events (Fig. 4A and 4B). To determine if variants emerged during the experiment, we used next-generation sequencing to determine the whole genomes of viruses from ferret nasal washes. Single-nucleotide variants (SNVs) resulting in amino acid changes were observed for each sample. Minor variant PA-S321 represented approximately 8% of the inoculum (Fig. 1A and 4D). Interestingly, we found PA-S321 consistently appeared in donor, contact, and airborne ferrets with dominant percentages in the initial days of 2/3 airborne-transmitted ferret samples (Fig. 4C and 4D). Other PB1, HA, NP, and NS SNVs were detected in the first days of airborne transmitted samples in A1 and A2 ferrets. However, these variants did not enrich to higher percentages than PA-S321 and disappeared in both airborne-infected ferrets in the following days. We also detected other variants in PB2, HA, NP, and NS genes. However, these variants were either found only in a single ferret or were observed at low percentages. In our previous study, the P4 virus achieved 1/3 airborne transmission in ferrets without the selection of PA-S321 (39). Overall, minor variant PA-S321 played the most important role in airborne transmission and was associated with 2/4 airborne transmission events.

PA-S321 had enhanced polymerase activity. PA-N321S has been identified as an important human-adaptive mutation for avian H5 influenza A viruses (54, 55). Therefore, efficient airborne transmission in P4-PA-mixed was most likely due to propagation of the PA-S321 variant. We plaque purified P4-PA-S321 and compared this virus *in vitro* to P4-PA-mixed and G15-HA1-S210. P4-PA-S321 had higher levels of mRNA, cRNA, and vRNA at 4 and 6 hpi in MDCK cells than P4-PA-mixed, elevating polymerase activity to a level similar to G15-HA1-S210 (Fig. 5A-B). Compared to P4-PA-mixed, P4-PA-S321 also yielded higher virions released from MDCK cells at 6 hpi (Fig. 5C). P4-PA-S321 yielded RNA levels and virus titers similar to G15-HA1-S210 (Fig. 5A-C). Therefore, PA-S321 increased the polymerase activity to a level

similar to that of the G15 isolates, which had the alternative polymerase-enhancing variations PA-P271/PB2-L648.

Contact and airborne transmission of G15-HA1-mixed and G15-HA1-S210. The inoculum of G15-HA1-mixed contained approximately 15% HA1-S210 (Fig. 1A and 6D). HA1-S210 was present at a level greater than 90% in all donor ferrets within three days and was transmitted to all of the contact animals in the present study (Fig. 6 C-D). Thus, the stabilizing HA1-S210 variation was rapidly selected in inoculated ferrets and was exclusively transmitted. The inocula of the P4-PA-mixed and G15-HA1-S210 groups contained 100% HA1-S210, and this residue remained HA1-S210 during replication and transmission in ferrets (Fig. 4C and Fig. 8C). Therefore, all contact ferrets in G15-HA1-mixed, P4-PA-mixed, and G15-HA1-S210 contained the stabilizing HA1-S210 variation and had HA activation pH values of approximately 5.6 (Fig. 7A-D).

Airborne transmission of G15-HA1-mixed occurred in 2/3 ferrets, as detected by seroconversion (Fig. 6B). We were unable to isolate G15-HA1-mixed virus from the nasal washes of two seroconverted airborne ferrets in the present study (Fig. 6A). In our previous study (39), virus was recovered from 2/3 of airborne ferrets in the G15-HA1-mixed group and the recovered virus was exclusively G15-HA1-S210.

G15-HA1-S210 had a seroconversion rate of 4/6 after airborne transmission (Fig. 8A-B and Table 1). Airborne transmission occurred in 3/3 ferrets in cubicle one and 1/3 ferrets in cubicle two (Fig. 8A-B). The airborne ferret in cubicle two had early nasal virus growth on days 2-6, while all the other three airborne ferrets in cubicle one shed nasal virus between days 8 and 14 (Fig. 8A). G15-HA1-S210 also contained a variety of minor variants in the donor, contact, and airborne ferrets (Fig. 8C). PB1-E751STOP was the most-abundant variant in this group. However, PB1-E751STOP did not emerge on the initial day in airborne ferret A1, and it emerged

with a much lower percentage in ferret A3 in cubicle one (Fig. 8C). Additionally, we didn't detect this variant in the donor and contact ferrets. Therefore, this variant may not be associated with transmission. We re-cultured ferret nasal washes in MDCK cells and found more isolates growing successfully from the G15-HA1-S210 airborne group than from the airborne ferrets in the other two groups (Fig. 8D). There were no significant differences in body weight and temperature among the contact and airborne groups (Figs. S3 and S4).

In summary, the G15-HA1-mixed inoculum contained 15% HA1-S210 and this HA-stabilized variant was selected within inoculated ferrets within days and was exclusively transmitted by contact and airborne routes. For all groups studied, all airborne-transmitted viruses had HA activation pH values of 5.5-5.6 and none retained an HA activation pH of 5.8 (Fig. 9). The P4-PA-mixed inoculum contained 8% PA-S321, this polymerase-enhancing variant remained as a minor population in donor and contact ferrets, and PA-S321 was detected in 2/4 airborne ferrets. Thus, for all groups studied, 11/13 viruses in the airborne ferrets had the polymerase-enhancing variation while 2/13 retained lower polymerase activity (Fig. 9). Overall, a stabilized HA protein was more stringently selected than enhanced polymerase activity, yet both a stable HA and an efficient polymerase were associated with airborne transmission.

DISCUSSION

Three viral traits thought to be necessary for human-to-human and ferret-to-ferret transmissibility by influenza A viruses are HA receptor-binding specificity for α -2,6-linked sialic-acid receptors, an HA protein relatively resistant to acid-induced activation/inactivation, and a polymerase complex capable of efficient replication in mammalian cells (5, 17). The swine H1N1 gamma viruses studied here have been shown to bind α -2,6-linked receptors with high affinity in addition to having lower-level binding to α -2,3-linked receptors (39). P4-PA-mixed had a stabilized HA protein but lacked an efficient polymerase, and G15-HA1-mixed had an efficient polymerase but a relatively unstable HA protein. Both virus isolates contained minority variants at a level of 8-15% abundance that overcame the respective deficiency. These variants were selected in ferrets and accounted for 2/3 of P4-PA-mixed and all of G15-HA1-mixed airborne transmission events. Compared to P4-PA-mixed and G15-HA1-mixed, variant G15-HA1-S210, which contained both a stabilized HA protein and an efficient polymerase, had increased early growth in the nasal cavities of inoculated ferrets and was recovered in greater abundance from the nasal cavities of airborne-exposed ferrets. Overall, the data demonstrated that the combination of HA stability and polymerase efficiency promotes efficient airborne transmissibility of swine H1N1 gamma viruses in ferrets and that minor variants containing both traits can be selected and airborne transmitted when the majority of a virus sample lacks a stabilized HA or an efficient polymerase.

Multiple traits are required for IAV airborne transmissibility in ferrets, and it is important to understand if these traits must be acquired in a particular order or if they may evolve in any order. For IAVs already possessing α -2,6 receptor-binding specificity, the present study revealed two evolutionary pathways for the enhancement of ferret airborne transmissibility: (A) from a virus quasispecies that has an efficient polymerase, a minor variant with a stabilized HA protein

may be selected, or (B) from a virus quasispecies with a stabilized HA protein, a minor variant with an enhanced polymerase may be selected.

With respect to pathway A, several other studies showed that an IAV containing α -2,6 receptor-binding specificity and an efficient polymerase, but having a relatively unstable HA protein, can acquire airborne transmissibility in ferrets by acquiring an HA-stabilizing mutation. Wild-type 2009 pH1N1 has an HA activation pH of 5.5, and a destabilizing HA1-Y17H mutation increased this value to 6.0 (37, 52, 56). The HA1-Y17H mutant was attenuated in ferrets and did not airborne transmit until acquiring stabilizing HA2-R106K and HA1-H17Y mutations, which decreased the HA activation pH to 5.3 (37). Mechanistically, HA stabilization was shown to increase replication in human airway cells (57), dampen Type I interferon responses in dendritic cells (52), and increase the survivability of infectious virions after respiratory expulsion in ferrets (38). In another study, a recombinant virus was generated that contained the HA from A/Vietnam/1203/2004 (H5N1) with two HA mutations that conferred α 2,6 receptor-binding specificity, and the other seven genes from A/California/04/2009 (H1N1) with a PB2-E627K mutation that enhanced polymerase activity (27). This virus and an adaptive mutant containing an HA1-N158D mutation that deleted a glycosylation on the HA head were incapable of airborne transmissibility in ferrets. Instead, a final mutation needed for the acquisition of airborne transmissibility was HA1-T318I in the stalk, which decreased the HA activation pH to 5.3. When HA-stabilizing mutations were introduced into other H5N1 viruses lacking α 2,6 receptor-binding specificity, virus growth in the nasal cavities of ferrets was enhanced but airborne transmission did not occur (29, 30, 58).

With respect to pathway B, P4-PA-mixed had a stabilized HA protein and its airborne transmission in 2/4 cases was associated with selection of the PA-S321 mutation that enhanced polymerase activity. A/Indonesia/5/2005 (H5N1) acquired airborne transmissibility in ferrets by

a combination of mutagenesis and adaptation that enhanced receptor-binding activity (HA-T156A and HA-Q222L or HA-G224S), polymerase activity (PB2-E627K and PB1-H99Y), and HA stability (HA-H103Y) (28, 59). These mutations arose at similar steps during adaptation, and viruses lacking any of the single mutations HA-T156A, PB2-E627K, PB1-H99Y, or HA-H103Y, or both the HA-Q222L or HA-G224S mutations, were loss-of-function for airborne transmission in ferrets. In the case of reassortant IAVs containing genes from the human 1918 virus and avian H1N1 viruses, the human HA gene enabled efficient contact transmission but did not allow airborne transmission in ferrets (20). Instead, the human 1918 PB2 protein was found to be both necessary and sufficient to enable ferret airborne transmission in the context of a virus that contained the human 1918 HA and NA genes. Thus, while selection of enhanced polymerase activity in the present work was less stringent than selection of a stabilized HA, the adaptation of IAVs for airborne transmission in ferrets, and presumably in humans, has been associated with both HA stability and polymerase activity and both pathways A and B appear viable.

Quasispecies have been associated with IAV fitness and virulence in various hosts (60, 61). In this study, minor variants of swine H1N1 viruses with enhanced polymerase activity or HA stability were selected in ferrets and associated with airborne transmissibility. The transmission of minor variants has been previously observed for IAVs in humans (62), equines (63), and guinea pigs (64). This suggests minor variants, in addition to the majority population of a virus, need to be considered when assessing the risk of IAVs for human pandemic potential. Next-generation sequencing has been used to track the outgrowth of HA-stabilizing variants in ferrets in this study and others (37-39, 56). Deep sequencing of samples from H7N9-infected patients and their surrounding poultry/environment showed acquisition of the polymerase-enhancing PB2-E627K variation in humans (45). H3N2 and H1N1 growth in mice has been shown to not necessarily proceed through linear accumulation of adaptative mutations (65).

Overall, as next-generation sequencing becomes cheaper, faster, and more efficient, real-time monitoring of human-adaptive variations may enable better preparedness for future outbreaks.

Numerous studies have been performed to identify and characterize human- and ferret-adaptive mutations in IAVs. Examples include those focusing on the polymerase complex (42, 51, 66-72) and the HA protein (38, 56, 58, 73-81). In the present study, we found G15 isolates containing PB2-L648/PA-P271 had higher polymerase activities than P4-PA-mixed, which contained PB2-M648/PA-T271. PB2-L648 was previously shown to increase polymerase activity of an H10N8 virus (82). The present study also showed that PA-S321 increased polymerase activity. PA-S321 has previously been identified as an adaptive marker for H5N1 influenza A viruses in humans (54, 55, 83). The HA1-S210 mutation was shown to decrease the pH of HA activation and the pH of virus inactivation here and in our previous work (39).

In summary, a combination of efficient polymerase activity and a stabilized HA were found to enhance airborne transmission of swine H1N1 gamma viruses in ferrets, and these two properties may be acquired in either order. Human infections of seasonal influenza viruses have been far less frequent since the start of the SARS-CoV-2 pandemic, mask mandates, and social distancing. Widespread reemergence of seasonal influenza is expected to occur in the post-SARS-CoV-2-pandemic era. Ultimately, exotic IAVs in other species, especially swine, and the potential for cross-species transmission make it essential to monitor potential pandemic IAV strains and to assess their pandemic risk. Swine H1N1 gamma viruses are one of the most prevalent lineages in swine (84), have caused sporadic human infections in recent years (15, 85), and have been shown here to acquire increased transmissibility in ferrets by adaptive HA and polymerase mutations. A recent study has identified a swine Eurasian avian-like influenza virus with pandemic traits (86). Surveillance and risk assessment studies are needed for IAV pandemic

353 preparedness in addition to studies investigating the evolutionary pathways by which pandemic
354 potential may arise and genetic and phenotypic traits associated therewith.
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MATERIALS AND METHODS

Cells and viruses

Madin–Darby canine kidney (MDCK) cells were maintained in minimum essential medium (MEM, Thermo Fisher Scientific). African green monkey kidney (Vero) cells were maintained in Dulbecco’s Modified Eagle Medium (DMEM, Life Technologies™). Both culture media were supplemented with 10% HyClone® standard fetal bovine serum (FBS, Life Technologies) and 1% penicillin/streptomycin (P/S, Thermo Fisher Scientific). The cells were grown at 37 °C with 5% CO₂ (50, 51).

A/swine/Illinois/2A-1213-G15/2013 (H1N1) (G15-HA1-mixed) and A/swine/Illinois/2B-0314-P4/2014 (H1N1) (P4-PA-mixed) were originally isolated from nasal swabs from pigs in commercial swine herds in the United States that showed symptoms of influenza-like illness, as described previously (39, 87). G15-HA1-S210 and G15-HA1-N210 were plaque purified twice from MDCK cells using G15-HA1-mixed. The whole-genome sequences of G15-HA1-S210 and G15-HA1-N210 were verified by next-generation sequencing. All viruses were propagated in MDCK cells with 1 µg/ml tosylsulfonyl phenylalanyl chloromethyl ketone (TPCK)–treated trypsin, as described previously (39, 50).

Syncytium assay

Viruses were inoculated into Vero cells in 24–well plates at a multiplicity (MOI) of 3 PFU/cell. After maintaining at 37 °C with 5% CO₂ for 16 hours (h), the infected cells were treated with DMEM supplemented with 5 µg/ml TPCK–treated trypsin for 15 minutes (m) and then pH–adjusted PBS buffers for 15 m. Subsequently, the infected cells were maintained in DMEM supplemented with 5% FBS for 3 h at 37 °C. After that, the infected cells were fixed and stained using a Hema 3™ Fixative and Solutions (Fisher Scientific). Syncytia were recorded using a light microscope. The cutoff values for virus HA activation pH were determined at the

treated pH that the stained cells in 24-well plates containing more than two syncytia with at least five nuclei, as described previously (39, 52, 88).

HA inactivation pH assay

Tested viruses were treated with a series of pH-adjusted PBS buffers in a ratio of 1:100 for 1 h at 37 °C. The resulting infectious viruses were titrated by TCID₅₀ on MDCK cells (30, 37). After obtaining virus titers for treatments, HA inactivation pH values were calculated by using GraphPad Prism version 7 software (Graph-Pad Software, San Diego, CA). The parameter LogEC₅₀ was generated using the model Nonlinear Regression [Equation: log (agonist) vs. response-Variable slope (four parameters)] followed by the least squares fitting method.

Virus growth assay

Tested viruses were inoculated into MDCK cells in 24-well plates at an MOI of 2 PFU/cell. After incubation for 1 h at 37 °C, the infected cells were washed by PBS twice, and continued to be maintained at 37 °C with MEM culture media (1 ml/well) supplemented with 1 µg/ml TPCK-treated trypsin. At 6- and 8-hours post-infection (hpi), cell culture supernatant was collected and titrated by TCID₅₀ on MDCK cells (37, 89).

Virus mRNA, cRNA and vRNA quantification

Production of virus RNAs (mRNA, cRNA, and vRNA) during influenza A virus transcription and replication processes were quantified using a two-step real time RT-qPCR, modified from the method described previously (51, 68, 90). Briefly, viruses were inoculated into confluent MDCK cells in 24-well plates at an MOI of 2. The infected cells were incubated at 37 °C for 1 h, washed by PBS twice, and cultured with MEM supplemented with 1 µg/ml TPCK-treated trypsin (1 ml/well). At 4 and 6 hpi, culture supernatants and infected cells were harvested separately.

To quantify virus mRNA, cRNA, and vRNA, total RNAs were extracted from the infected cells using RNeasy Mini Kit (Qiagen). The corresponding cDNAs of mRNA, cRNA, and vRNA were generated by reverse-transcription PCR (RT-PCR) using Transcriptor First Strand cDNA Synthesis Kit (Roche). Primers with specific tags for differentiations of virus mRNA, cRNA, and RNA were used (mRNA-RT-PCR primer: CCAGATCGTTCGAGTCGTTTTTTTTTTTTTTTTTCTCATGTTTCT; cRNA-RT-PCR primer: GCTAGCTTCAGCTAGGCATCAGTAGAAACAAGGGTGTTTTTCTC; vRNA-RT-PCR primer: GGCCGTCATGGTGGCGAATTGGCCACAGGATTAAGGAATATC).

Real-time quantitation PCR were performed to quantify these three RNA levels with specific primers targeting virus HA gene (mRNA-real time-forward primer (FP): CCAGTTCATTGGTACTGGTAGTC, mRNA-real time-reverse primer (RP): CCAGATCGTTCGAGTCGT; cRNA-real time-FP: CCAGTTCATTGGTACTGGTAGTC, cRNA-real time-RP: GCTAGCTTCAGCTAGGCATC; vRNA-real time-FP: GGCCGTCATGGTGGCGAAT, vRNA-real time-RP: ACCGTACCACCCATCTATCA;).

Internal control GAPDH mRNA was also reverse-transcribed and quantified using oligo (dT)₂₀ as RT-PCR primer and the following as real-time PCR primers (FP: ATTCCACCCATGGCAAATTC, RP: CGCTCCTGGAAGATGGTGAT).

To quantify viral copy number released in cell-free supernatant, total RNAs were extracted using QIAamp Viral RNA kits (Qiagen). A two-step quantitative real-time reverse-transcription (RT) PCR was applied to quantify viral copy number. The primers used were listed as the following, RT-PCR-primer: AGCAAAAGCAGG; Real-time-FP (targeting NP gene): CTGCTTGTGTGTATGGGCTT; Real-time-RP (targeting NP gene): TGAGCTGGATTTTCATTTGGT. Plasmid PHW2000-NP (G15-HA1-mixed) was used as a standard for quantitative real-time PCR.

Ferret transmission experiments

Thirty-six male Fitch ferrets (five- to six-month-old, purchased from Triple F Farms, Sayre, PA) were verified to be serologically negative for currently circulating influenza A and B viruses and the tested swine H1N1 gamma influenza viruses by HAI assay. At the same time, these ferrets were identified to be serologically negative for currently circulating SARS-CoV-2 by ELISA. The nasal swabs of these ferrets were verified to be negative before the experiments by performing real-time PCR reactions.

The transmission study was performed as described in previous work (39). Ferret body weight and temperature were monitored daily until day 14. Ferret nasal washes were collected every other day until day 14. Additionally, all ferrets were euthanized on day 21 to collect whole blood. Ferret sera were examined for seroconversion using HAI assay (37, 39, 88). Animal studies were performed in compliance with St. Jude Children's Research Hospital Animal Care and Use Committee guidelines under protocol 459.

Virus whole-genome sequencing and analyses

Virus whole genomes were obtained by next-generation sequencing (39, 56, 88). Viral RNAs were extracted from ferret nasal wash samples or cell-free supernatants using QIAamp Viral RNA kits (Qiagen). The corresponding cDNAs were achieved by reverse-transcription PCR using SuperScript III First-strand Synthesis System (Thermo Fisher Scientific). DNA library was prepared by PCR amplification using Phusion High-Fidelity PCR Master Mix with HF Buffer (New England BioLabs). The resulting PCR products were purified by QIAquick Gel Extraction Kit (Qiagen) and submitted to St Jude Harwell Center for virus whole-genome sequencing.

Sequencing data were analyzed using CLC Genomics Workbench version 11.0.1. SNVs resulting amino-acid variations were reported when the predefined quality scores were met and

present in forward and reverse reads at an equal ratio. All variants presented in this study were supported by at least 10 reads with a minimum frequency of 5% (unless otherwise stated). Heat maps and parts of whole were generated by using GraphPad Prism version 7 software (GraphPad Software, San Diego, CA).

Statistical analysis

All data analyses were performed using GraphPad Prism version 7. One-way ANOVA followed by a Tukey's multiple comparisons test, Welch's *t* test or Mann-Whitney U test were used to determine statistical significances. *P* value < 0.05 was considered significant.

ACKNOWLEDGMENTS

We thank Guohua Yang, Ashley Webb, and Melissa Penaflor for help with ferret experiments. We also thank St. Jude Animal Resources Center for help with ferret experiments. We thank the Hartwell Center DNA Sequencing & Genotyping, Hartwell Center Functional Genomics, and the Hartwell Center Genome Sequencing Facility. This work was funded, in part, by the National Institute of Allergy and Infectious Diseases under Centers of Excellence for Influenza Research and Surveillance (CEIRS) contract no. HHSN272201400006C, St. Jude Children's Research Hospital, and the American Lebanese Syrian Associated Charities (ALSAC). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

CONFLICT OF INTEREST

The authors declare no competing financial interests.

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Figure Legends

Fig. 1. Virus characterization *in vitro*. (A) Sequence variations. Whole genomes of gamma viruses P4-PA-mixed, G15-HA1-mixed, G15-HA1-S210, and G15-HA1-N210 were obtained by next-generation sequencing. Amino-acid variations with frequencies $\geq 5\%$ and reads ≥ 10 are shown in colored pie charts. H3 numbering was used for the HA protein. (B) HA activation pH values measured by syncytium assay. Viruses were inoculated into Vero cells at an MOI of 3 PFU/cell. Representative images are shown with the HA activation pH in bold. (C) Virus inactivation pH values. Viruses were treated with pH-adjusted PBS, re-neutralized, and subjected to measurement of residual virus infectivity by TCID₅₀. (D) Virus growth in MDCK cells. Viruses were inoculated into MDCK cells at an MOI of 2 PFU/cell. Cell-free culture supernatants were harvested at 6- and 8-hours post-infection (hpi) and titrated by TCID₅₀. All experiments were independently performed at least twice. *P* values were determined according to one-way ANOVA followed by a Tukey's multiple comparisons test. * and ** represent *P* < 0.05 and 0.01, respectively.

Fig. 2. *In vitro* transcription and replication of P4-PA-mixed and G15-HA1-S210. (A) Virus mRNA, cRNA, and vRNA accumulation after 4 hpi. (B) Virus mRNA, cRNA, and vRNA accumulation after 6 hpi. (C) Virus copies released into culture media. G15-HA1-S210 and P4-PA-mixed viruses were inoculated into MDCK cells at an MOI of 2 PFU/cell. At 4 and 6 hpi, the infected MDCK cells were lysed and harvested for total viral RNA. Virus specific mRNA, cRNA, and vRNA were quantified by two-step real-time reverse-transcription PCR using specific primers and normalized to GAPDH mRNA levels. Meanwhile, culture supernatants were harvested for quantification of virions released. Virions released from the cells were

quantified by real-time quantitative PCR using plasmid PHW2000-NP (G15-HA1-mixed) as a standard. Virus copies were quantified. *P* values were determined according to Welch's *t* test. *** represents $P < 0.001$.

Fig. 3. Infection of P4-PA-mixed, G15-HA1-mixed, and G15-HA1-S210 in inoculated donor ferrets. (A) Viral properties. Relative polymerase activity and HA stability for viruses are shown. (B) Ferret experimental caging. Viruses were intranasally inoculated into 3 donor ferrets per group on day 0. After one day, 3 naïve ferrets were introduced into the same cages and 3 into the adjacent cages. These ferrets were designated contact and airborne ferrets, respectively. Overall, there were 9 ferrets for each cubicle. (C) TCID₅₀ titers of virus in nasal washes from donor ferrets. Ferret nasal washes were collected every other day until day 14. Each symbol represents the virus titer of an individual sample. The bottom dashed lines represent the limit of detection. (D) Day of virus peak titer in donor ferrets. Each symbol represents the day that virus peak titer was observed in an individual donor ferret. (E) Virus peak titers in donor ferrets. Each symbol represents the virus peak titer in an individual donor ferret. (F) HAI titers of day-21 sera of donor ferrets. (G) Percent body weight of donor ferrets. Ferret body weight was monitored daily until day 14. *P* values were calculated using Mann-Whitney U test.

Fig. 4. P4-PA-mixed transmission, seroconversion, and frequency of single-nucleotide variants (SNVs) for nasal washes collected from transmitted ferrets. The ferret experiment was carried out as described in Fig. 3A. (A) Virus TCID₅₀ titers of nasal washes from contact and airborne ferrets. (B) HAI titers of day-21 sera of contact and airborne ferrets. (C) Frequencies of SNVs for nasal washes. The whole-genomes of nasal washes collected from contact and airborne ferrets infected or exposed with P4-PA-mixed were obtained by next-

generation sequencing. SNVs with frequencies $\geq 5\%$ and reads ≥ 10 were read out. The listed SNVs met the following criteria: (1) resulted in protein sequence changes, and (2) appeared in nasal washes in each cubicle ≥ 2 times and/or frequencies greater than 30%. The frequencies of SNVs are represented by the colored boxes. Each column represents the SNVs for one ferret nasal wash. (D) The proportions of PA-S321 in P4-PA-mixed from the original inoculum and infected/exposed ferrets.

Fig. 5. Minor variant PA-S321 had enhanced polymerase activity. (A-C) Evaluation of polymerase activity of P4-PA-S321. The experiment was performed as described in Fig. 2. The transcription and replication of virus containing 100% variant PA-S321 were compared to those of G15-HA1-S210 and P4-PA-mixed. The experiments were independently performed twice. *P* values were determined according to one-way ANOVA followed by a Tukey's multiple comparisons test.

Fig. 6. G15-HA1-mixed transmission, seroconversion, and frequency of SNVs for nasal washes collected from transmitted ferrets. The ferret experiment was carried out as described in Fig. 3A, and frequency of SNVs were achieved and listed as described in Fig. 5C. (A) Virus TCID₅₀ titers of nasal washes from contact and airborne ferrets. (B) HAI titers of day-21 sera of contact and airborne ferrets. (C) Frequencies of SNVs for nasal washes. (D) The proportions of HA1-S210 in G15-HA1-mixed from the original inoculum and infected/exposed ferrets.

Fig. 7. Minor variant HA1-S210 had enhanced HA stability. (A-D) Virus HA activation pH values of isolates from G15-HA1-mixed, P4-PA-mixed, and G15-HA1-S210 infected/exposed

ferrets. Virus isolates from nasal washes were propagated in MDCK cells once. Syncytium assays were performed to measure the HA activation pH values of these isolates.

Fig. 8. G15-HA1-S210 transmission, seroconversion, and frequency of SNVs for nasal washes collected from transmitted ferrets. The ferret experiment was carried out as described in Fig. 3A, and frequency of SNVs were achieved and listed as described in Fig. 5C. (A) Virus TCID₅₀ titers of nasal washes from contact and airborne ferrets. (B) HAI titers of day-21 sera of contact and airborne ferrets. (C) Frequencies of SNVs for nasal washes. (D) The number of virus isolates from ferret nasal washes of donor, contact, and airborne ferrets.

Fig. 9. Relative polymerase activities and HA stabilities in ferrets. Relative polymerase activity and HA activation pH were compared for inoculated virus (left) and after airborne transmission in ferrets (right). Each symbol represents an individual ferret.

Table 1. Virus characteristics before and after infection and transmission in ferrets.

Virus characterization		P4-PA-mixed	G15-HA1-mixed	G15-HA1-S210	G15-HA1-N210
Genotype variations	HA1-210	S	85% N, 15% S	S	N
	PB2-648	M	L	L	L
	PA-271	T	P	P	P
	PA-321	92% N, 8% S	N	N	N
	PA-386	86% D, 14% N	D	D	D
Phenotype variations in vitro	HA activation pH ^a	5.5	5.8	5.6	5.8
	HA inactivation pH ^b	5.6	5.75	5.6	5.8
	Polymerase activity	lower	higher	higher	higher
	One-step growth	lower	higher	higher	highest
Donor ferrets	Virus growth (log ₁₀ TCID ₅₀)	5.8 (± 1.2)	4.98 (± 0.25)	7.3 (± 0.67)	- ^f
	Number of isolates from ferrets ^c	9	9	9; 9 ^d	-
	Seroconversion	3/3	3/3	3/3; 3/3	-
	Day of peak titers	3 (± 2)	3	1	-
	Peak titers (log ₁₀ TCID ₅₀)	6.7 (± 0.43)	6.2 (± 0.54)	7.3 (± 0.67)	-
	Major variants	PA-S321	HA1-S210	none	-
	HA activation pH range	5.5-5.6	5.5-5.9	5.5-5.6	-
Contact ferrets	Number of isolates from ferrets	9	9	9; 10	-
	Seroconversion	3/3	3/3	3/3; 3/3	-
	HA activation pH range	5.5-5.6	5.6-5.9	5.5-5.6	-
	Major variants	PA-S321	HA1-S210	none	-
Airborne ferrets	Number of isolates from ferrets	4	0	7; 3	-
	Days for virus shedding	8-14	-;	8-14; 2-6	-
	Seroconversion	3/3	2/3	3/3; 1/3	-
	Major variants	PA-S321	HA1-S210	sporadic	-
	Polymerase activity	higher & lower	higher	higher	-
	HA activation pH ^e	5.5-5.6	~5.6	5.5-5.6	-

^a HA activation pH measured by syncytia assay.

^b HA inactivation pH measured by treating viruses with indicated pH and titrating resulting virions by TCID₅₀.

^c The Number of isolates is listed for those grown successfully in MDCK cells after recovery from ferrets in cubicle

1 and cubicle 2.

^d Data for each virus is listed for each cubicle with the first X/Y number representing cubicle 1 and the second X/Y

representing cubicle 2.

^e HA activation pH of airborne-transmitted virus on first day of isolation.

^f -, Not measured.

A **B** **C** **D**

A

	Relative polymerase activity	HA activation pH
P4-PA-mixed	Lower	5.5
G15-HA1-mixed	Higher	5.8
G15-HA1-S210	Higher	5.6

B **C** **D** **E** **F** **G**

A **B** **C** **D**

A**4h post-infection** **B****6h post-infection** **C**

A **B** **C** **D**

A**G15-HA1-mixed** **B****P4-PA-mixed** **C****G15-HA1-S210 Cubicle 1** **D****G15-HA1-S210 Cubicle 2**

Time (day after experiments)

B

C

D

Inoculated Viruses:

- G15-HA1-mixed
- P4-PA-mixed
- G15-HA1-S210

Selected Variants:

- G15-HA1-S210
- P4-PA-S321