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

Meng Hu[1], Jeremy C. Jones[1], Balaji Banoth[1], Chet Raj Ojha[1], Jeri Carol Crumpton[1], Lisa Kercher[1], Robert G. Webster[1], Richard J. Webby[1, 2], and Charles J. Russell[1, 2]

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

Running Title: Polymerase 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 in ferrets the replication and transmission 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 airborne-transmitted and propagated in one ferret. 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, HA stabilization played a more prominent role than polymerase enhancement in the replication and transmission of these viruses in ferrets. The results suggest pandemic risk-assessment studies may benefit from deep sequencing to identify minor variants with human-adapted traits.

35 **IMPORTANCE**

36 Diverse IAVs circulate in animals, yet few acquire the viral traits needed to start a human pandemic.
37 A stabilized HA and mammalian-adapted polymerase have been shown to promote the adaptation
38 of IAVs to humans and ferrets (the gold-standard model for IAV replication, pathogenicity, and
39 transmissibility). Here, we used swine IAV isolates of the gamma lineage as a model to investigate
40 the importance of HA stability and polymerase activity in promoting replication and transmission
41 in ferrets. These are emerging viruses that bind to both α-2,6- and α-2,3-linked receptors. Using
42 isolates containing mixed populations, a stabilized HA was selected within days in inoculated
43 ferrets. An enhanced polymerase was also selected and propagated after airborne transmission to
44 a ferret. Thus, HA stabilization was a stricter requirement, yet both traits promoted transmissibility.
45 Knowing the viral traits needed for pandemic potential, and the relative importance of each, will
46 help identify emerging 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, 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 delta2 (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), which may result in increased transmissibility in swine or humans. 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 can be taken.

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

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

94 Influenza pandemic risk assessment algorithms by the US Centers for Disease Control and
95 Prevention (CDC) and World Health Organization (WHO) explicitly consider HA receptor-
96 binding specificity and indirectly account for other human-adaptive viral traits through airborne
97 transmission studies (47, 48). Ferrets are currently considered the gold-standard animal model to
98 study transmission of influenza viruses (20, 49).

99 Previously, we studied the replication and transmission of gamma-clade H1N1 isolates
100 G15-HA1-mixed and P4-PA-mixed in groups of 3 donor, contact, and airborne ferrets (39). These
101 isolates are A/swine/Illinois/2A-1213-G15/2013 (H1N1) and A/swine/Illinois/2B-0314-P4/2014
102 (H1N1), respectively. They both contain swine gamma genes in the PB2, PB1, PA, HA, NA, and
103 NS segments and H1N1pdm09 genes in the NP and M segments (39). In the polymerase genes,
104 G15-HA1-mixed and P4-PA-mixed differed at PB2-648 and PA-271; additionally, P4-PA-mixed
105 contained minor variants at PA-321 and PA-386 (Fig. 1A). With respect to HA, P4-PA-mixed
106 contained an HA1-S210 residue (H3 numbering) and had an HA activation pH of 5.5. G15-HA1-
107 mixed contained a mixed population of 85% HA1-N210 and 15% HA1-S210 and had an HA
108 activation pH of 5.8. G15-HA1-mixed had 3/3 airborne transmission events ferrets, while P4-PA-
109 mixed had only 1/3 airborne transmission. As these viruses contained variations and mixed
110 populations in the HA and polymerase proteins and the number of animals per group (n=3) was
111 low, the relative importance of HA stability and polymerase activity in the airborne transmissibility
112 of these viruses was unclear.

113 In the present work, we isolated the minor variant G15-HA1-S210 and compared its
114 properties to G15-HA1-mixed and P4-PA-mixed in cultured cells and in ferrets. We also
115 characterized the genotypes and phenotypes of viruses recovered from ferret nasal washes. Overall,
116 enhanced HA stability and polymerase activity were associated with airborne transmissibility, and
117 a stabilized HA appeared to be more stringently required for replication and transmission.

118 **RESULTS**

119 **Impact of HA and polymerase variations on HA stability and virus replication in**
120 **MDCK cells.** Whole-genome sequencing analyses showed variations at only five amino-acid
121 positions between P4-PA-mixed and G15-HA1-mixed in their entire genomes (Fig. 1A). In the
122 polymerase genes, P4-PA-mixed contained PA-T271, 92% PA-N321 (8% PA-S321), 86% PA-
123 D386 (14% PA-N386), and PB2-M648, while G15-HA1-mixed contained PA-P271, PA-N321,
124 PA-D386, and PB2-L648. In HA, the two isolates differed at HA1-210 with P4-PA-mixed
125 containing HA1-S210 and G15-HA1-mixed containing 85% HA1-N210 (15% HA1-S210). From
126 G15-HA1-mixed, we isolated the majority population (G15-HA1-N210) and the minor variant
127 (G15-HA1-S210). Residue HA1-210 interacts with residues across the trimer interface in the head
128 domain, so the HA1-S210 variation most likely stabilizes the HA protein by resisting dissociation
129 of the heads (39).

130 We measured the pH of HA activation of the four viruses by syncytia formation assay (Fig.
131 1B). Vero cells infected with G15-HA1-S210 were triggered to cause syncytia at a highest value
132 of pH 5.6, while G15-HA1-N210 was activated at pH 5.8. Thus, HA1-S210 was more stable than
133 HA1-N210. P4-PA-mixed contained HA1-S210 and induced syncytia at pH 5.5. As 85% of G15-
134 HA1-mixed contained the less-stable HA1-N210 variation, infection with this isolate resulted in
135 syncytia formation at pH 5.8.

136 The pH of virus inactivation was measured by exposing virus aliquots to media of varying
137 pH, neutralizing, and measuring residual infectivity. G15-HA1-S210 and P4-PA-mixed (which
138 contained HA1-S210) had midpoints of inactivation of pH 5.6 (Fig. 1C), similar to their activation
139 pH values. G15-HA1-N210 had an inactivation pH of 5.8, the same as its activation pH. G15-
140 HA1-mixed had an inactivation pH of 5.75; therefore, this mix of unstable (85%) and stable (15%)

141 components had just a small shift in overall inactivation pH due to the presence of the minority,
142 more-stable HA1-S210.

143 Previous reports showed differences in polymerase activity and/or HA stability may affect
144 virus one-step growth (50-52). To explore this, we inoculated viruses into MDCK cells at an MOI
145 of 2 PFU/cell and measured the virus titers of the supernatants at 6- and 8-hours post-infection
146 (hpi). Compared to P4-PA-mixed, the three other viruses had higher titers at 6 hpi (Fig. 1D). As
147 P4-PA-mixed and G15-HA1-S210 have identical HA proteins, enhanced replication at 6 hpi was
148 mapped to differences in the polymerase genes. At 8 hpi, G15-HA-N210 had higher titers than the
149 other three viruses. G15-HA1-N210 and G15-HA1-S210 were identical except for HA1 residue
150 210. Therefore, the HA1-N210 variation (HA activation pH 5.8) enhanced replication at 8 hpi in
151 MDCK cells compared to HA1-S210 (pH 5.6). Overall, both the HA variation and the polymerase
152 variations were found to modulate the production of infectious virus in MDCK cells at relatively
153 early time points when inoculated at an MOI of 2 PFU/cell.

154 **G15-HA1-S210 had higher polymerase activity than P4-PA-mixed**. Virus polymerase
155 activity can be assessed by the accumulation of viral mRNA, cRNA, and vRNA, which are
156 produced by virus transcription and replication (53). To study the impact of the PB2 and PA
157 variations on polymerase activity, we compared G15-HA1-S210, P4-PA-mixed, and P4-PA-
158 purified. G15-HA1-S210 and P4-PA-mixed had identical HA genes, differed at PA-271 and PB2-
159 648, and P4-PA-mixed also had minor variants at PA-321 and PA-386 (Fig. 1A). To exclude the
160 effect of P4-PA-mixed minor variants at PA-321 and PA-386, we obtained purified P4 virus
161 without the above minor mixed populations. Thus, P4-PA-purified and G15-HA1-S210 differed
162 only at PA-271 and PB2-648. The three viruses were inoculated into MDCK cells at an MOI of 2
163 PFU/cell, and the resulting cell lysates and supernatants were harvested at 4 and 6 hpi. P4-PA-
164 mixed and P4-PA-purified yielded similar levels of mRNA, cRNA, vRNA, and viral titers (Fig.

165 2); therefore, the minor populations in P4-PA-mixed did not alter virus transcription and
166 replication. Compared to P4-PA-mixed and P4-PA-purified, G15-HA1-S210 had higher mRNA,
167 cRNA, and vRNA levels at 4 and 6 hpi (Fig. 2A-B) and higher viral loads in cell supernatants at 6
168 hpi (Fig. 2C). In summary, the presence of PA-P271 and/or PB2-L648 in G15-HA1-S210 was
169 associated with higher polymerase activity. These two variations were also present in G15-HA1-
170 mixed and G15-HA1-N210.

171 **Infection of donor ferrets inoculated with P4-PA-mixed, G15-HA1-mixed, and G15-**
172 **HA1-S210.** G15-HA1-S210 had relatively high polymerase activity and a stabilized HA protein.
173 Compared to G15-HA1-S210, P4-PA-mixed had decreased polymerase activity (due to the
174 presence of PA-P271 and/or PB2-L648) and G15-HA1-mixed had higher HA activation pH and
175 virus inactivation pH (due to 85% HA1-N210) (Fig. 3A). We previously studied the infection of
176 P4-PA-mixed and G15-HA1-mixed in ferrets, using a total of 3 donor, 3 contact, and 3 airborne
177 ferrets for each virus (39). Here, we repeated the experiment and report the combined data for a
178 total of 6 donor, 6 contact, and 6 airborne ferrets, in part to comply with the USDA's policy on
179 appropriate use of ferrets. The present data is referred to as cubicle 1 and the previous data as
180 cubicle 2. In the present work, we also included two cubicles of ferrets infected with the newly
181 purified virus G15-HA1-S210, thus the ferrets in this group totaled 6 donor, 6 contact, and 6
182 airborne.

183 In each cubicle, three ferrets were directly inoculated with 10^6 PFU of viruses, and one day
184 later we introduced three naïve contact and three naïve airborne ferrets (Fig. 3B). Each virus group
185 was evaluated in an isolated cubicle and personal protective equipment was changed before
186 moving between cubicles to avoid cross contamination of viruses. Each cubicle was set to a
187 temperature of approximately 22 °C and relative humidity of 40-60%. All ferrets were monitored

188 for body weight and temperature daily, and ferret nasal washes were collected every other day
189 until day 14. Ferret sera were collected on day 21.

190 On day 1, G15-HA1-S210 donor ferrets had an average nasal wash titer of 2.18 $\times 10^7$
191 $TCID_{50}$/ml, a value more than 10-fold higher than that of P4-PA-mixed (6.88 $\times 10^5$ $TCID_{50}$/ml, P
192 = 0.0065) and 100-fold higher than G15-HA1-mixed (1.08 $\times 10^5$ $TCID_{50}$/ml, $P < 0.0001$) (Fig. 3C
193 and Table 1). G15-HA1-S210 had greater virus growth after 1 day infection in ferrets than G15-
194 HA1-mixed, while virus replication was similar for the two viruses after 6-8h infection in MDCK
195 cells. This was consistent with previous results showing that HA stabilizing mutations enhanced
196 replication by resisting extracellular inactivation in the mildly acidic upper respiratory tract,
197 whereas MDCK extracellular media were buffered at neutral pH (6, 34). G15-HA1-S210 had peak
198 titers on day 1, while G15-HA1-mixed and P4-PA-mixed had mean peak titers on day 3 and 2.7,
199 respectively (Fig. 3D). Additionally, the average peak titer of G15-HA1-S210 in donor ferrets
200 (2.18 $\times 10^7$ $TCID_{50}$/ml) was significantly higher than that of G15-HA1-mixed (1.48 $\times 10^6$
201 $TCID_{50}$/ml, $P < 0.5$) (Fig. 3E). Overall, G15-HA1-S210, which had a stabilized HA and enhanced
202 polymerase activity, had earlier and higher growth in the nasal cavities of inoculated donor ferrets.

203 All ferrets seroconverted without significant differences between groups (Fig. 3F). No
204 significant differences in body temperature were detected between the donor ferret groups. Three
205 of six ferrets in the G15-HA1-S210 group had >10% body weight loss for at least two days,
206 compared to only one of six ferrets inoculated with P4-PA-mixed and G15-HA1-mixed (P = 0.04
207 and 0.0055, respectively, Fig. 3G).

208 **Contact and airborne transmission by P4-PA-mixed.** P4-PA-mixed had 6/6 contact
209 transmission and 4/6 airborne transmission as assessed by nasal viral loads and HAI assay (Fig.
210 4A-B). Airborne transmission events were 3/3 in cubicle one and 1/3 in cubicle two (Table 1). To
211 determine if variants emerged during the experiment, we used next-generation sequencing to

212 determine the whole genomes of viruses from ferret nasal washes. Single-nucleotide variants
213 (SNVs) resulting in amino acid changes were observed for each sample (Fig. 4C). Minor variant
214 PA-S321 represented approximately 8% of the inoculum (Fig. 4D). In cubicle one, PA-S321 was
215 measured at frequencies greater than 10% in all three donors, one contact, and two airborne ferrets
216 (Fig. 4C-D). In airborne ferret A1, PA-S321 was 100% abundant when the virus was first isolated
217 on day 8 and remained at 100% on day 10. On day 8, ferret A1 also had over a dozen minor variants
218 (abundance 5-30%) in HA2, NP, NS1, and NEP. These variants and PA-S321 were also present in
219 airborne ferret A2 on the first day of isolation (day 10), suggesting transmission to A2 may have
220 originated from A1. In both ferret A1 and A2, the minor variants disappeared in the following days.
221 In ferret A2, PA-S321 was approximately 50% abundant on day 10 but was not detectable on day
222 14. Other variants in PB2, PB1, HA2, NP, NS1, and NEP were either found only in a single ferret
223 or were observed at low percentages. In cubicle two, the frequency of PA-S321 was reduced to an
224 undetectable level in donor and contact ferrets by days 5 and 6, respectively, and PA-S321 was
225 not detected after the single airborne transmission event, which occurred later (by day 12).

226 **PA-S321 enhanced polymerase activity.** PA-N321S has been identified as an important
227 human-adaptive mutation for avian H5 influenza A viruses (54, 55). Therefore, to assess the effect
228 of the PA-S321 variation on polymerase activity, we plaque purified P4-PA-S321. Compared to
229 P4-PA-mixed, which contained approximately 92% PA-N321 and 8% PA-S321, P4-PA-S321 had
230 higher levels of mRNA, cRNA, vRNA, and viral titers at 4 and 6 hpi in MDCK cells (Fig. 5). This
231 showed that the PA-S321 variant had enhanced polymerase activity. P4-PA-S321 lacked PA-P271
232 and/or PB2-L648, which were previously shown to enhance the polymerase activity of G15-HA1-
233 S210 (Fig. 2). However, P4-PA-S321 had similar mRNA, cRNA, vRNA, and viral titers at 4 and
234 6 hpi in MDCK cells as G15-HA1-S210 (Fig. 5), showing that the PA-S321 variation compensated
235 for the lack of PA-P271 and/or PB2-L648.

236 **Contact and airborne transmission of G15-HA1-mixed and G15-HA1-S210.** For G15-
237 HA1-mixed, contact transmission occurred in 6/6 ferrets (100% in both cubicles), and airborne
238 transmission occurred in 5/6 ferrets (2/3 in cubicle one and 3/3 in cubicle two) as detected by
239 seroconversion (Fig. 6B). However, virus was only recovered from 2 airborne ferrets (both in
240 cubicle two) (Fig. 6A). The inoculum of G15-HA1-mixed contained approximately 15% HA1-
241 S210, yet HA1-S210 became enriched to a level greater than 90% in all donor ferrets within three
242 days and was transmitted to all six contact animals (Fig. 6 C-D). We were only able to perform
243 next-generation sequencing on the nasal washes from one airborne ferret (A1), and the virus
244 isolated from this ferret contained 100% G15-HA1-S210, the stabilizing variant.

245 Transmission studies were next performed with the G15-HA1-S210 virus. For G15-HA1-
246 S210, contact transmission occurred in 6/6 ferrets (100% in both cubicles), and airborne
247 transmission occurred in 4/6 ferrets (3/3 in cubicle one and 1/3 in cubicle two) (Fig. 7A-B). The
248 HA1-S210 variation remained at 100% in all virus samples sequenced. Virus was recovered from
249 one airborne ferret in cubicle two on days 2-6 and three airborne ferrets in cubicle one between
250 days 8-14 (Fig. 7A). The G15-HA1-S210 group contained a number of minor variants in donor,
251 contact, and airborne ferrets (Fig. 7C). PB1-E751STOP was the most abundant variant isolated
252 from airborne ferrets but was only detected in 2/4, in one of those was not present upon first
253 detectable infection (ferret A1, day 8, cubicle one), and was not detected in nasal washes from the
254 donor and contact ferrets (Fig. 7C). Therefore, this variant was not likely associated with
255 transmission. Ferret A2 in cubicle one contained NP-V352M, NP-E443G, and NA-D311N
256 variations, although these were not detected in the nasal washes of the other airborne viruses or
257 those from donor and contact animals. There were no significant differences in body weight and
258 temperature among the contact and airborne groups.

259 **HA activation pH values of viruses isolated from ferrets**. P4-PA-mixed contained 100%
260 HA1-S210, had an inoculum with an activation pH of 5.5, and all recovered virus samples from
261 this group (donor, contact, and airborne) had activation pH values of 5.5-5.6 (Fig. 8A). Similarly,
262 G15-HA1-S210 had a syncytia formation value of pH 5.6, and all recovered virus samples had
263 syncytia formation pH values of 5.5-5.6 (Fig. 8B). Thus, the phenotype of a relatively stable HA
264 protein was maintained for P4-PA-mixed and G15-HA1-S210 after transmission in ferrets. On the
265 other hand, the G15-HA-mixed inoculum contained approximately 85% HA1-N210 and 15%
266 HA1-S210, and the relatively unstable majority component (HA1-N210) contributed to syncytia
267 formation occurring at the relatively high pH of 5.8 (Fig. 1B). Virus isolates obtained from the
268 G15-HA1-mixed donor ferrets, which had mixed populations of HA1-N210 and HA1-S210, had
269 syncytia formation pH values ranging from pH 5.55 to 5.85 (Fig. 8C). Virus isolates from contact
270 ferrets in the G15-HA1-mixed group contained nearly 100% HA1-S210 (Fig. 6D). The three
271 contact ferrets from cubicle one yielded nasal wash samples with HA activation pH values of
272 approximately 5.6-5.7 (Fig. 8C). The single contact ferret from which virus samples were obtained
273 in cubicle two had higher HA activation pH values despite containing HA1-S210 (Fig. 8C). This
274 was most likely due to the presence of a minor population of HA2-N117T, which was previously
275 shown to destabilize the HA protein (39). Airborne-transmitted viruses recovered from G15-HA1-
276 mixed ferret A1 had an HA activation pH of approximately 5.6 (Fig. 8C).

277 In summary, the G15-HA1-mixed inoculum contained 15% HA1-S210 and this HA-
278 stabilized variant was selected within inoculated ferrets within days and was then transmitted by
279 contact and airborne routes. For all groups studied, all detectable airborne-transmitted viruses had
280 HA activation pH values of approximately 5.5-5.6 and none retained an HA activation pH of 5.8.
281 The P4-PA-mixed inoculum contained 8% PA-S321. This polymerase-enhancing variant remained
282 as a minor population in donor and contact ferrets. PA-S321 was detected in 2/4 airborne-

283 transmitted ferrets. Overall, a stable HA and an efficient polymerase were associated with airborne
284 transmission, and a stabilized HA protein was more stringently selected in ferrets than enhanced
285 polymerase activity.

286 **DISCUSSION**

287 Three viral traits thought to be necessary for human-to-human and ferret-to-ferret
288 transmissibility by influenza A viruses are HA receptor-binding specificity for α-2,6-linked sialic-
289 acid receptors, an HA protein relatively resistant to acid-induced activation/inactivation, and a
290 polymerase complex capable of efficient replication in mammalian cells (5, 17). The swine H1N1
291 gamma viruses studied here have been shown to bind α-2,6-linked receptors with high affinity in
292 addition to having lower-level binding to α-2,3-linked receptors (39). P4-PA-mixed had a
293 stabilized HA protein but lacked an efficient polymerase, and G15-HA1-mixed had an efficient
294 polymerase but contained a majority of relatively unstable HA proteins. Both virus isolates
295 contained minority variants at a level of 8-15% abundance that overcame the respective deficiency.
296 Polymerase-enhancing variants were observed in nasal washes from half of recoverable airborne-
297 transmitted P4-PA-mixed, and the HA-stabilizing variant in G15-HA1-mixed was rapidly selected
298 in inoculated donor ferrets and transmitted. Compared to P4-PA-mixed and G15-HA1-mixed,
299 variant G15-HA1-S210, which contained both a stabilized HA protein and an efficient polymerase,
300 had increased early growth in the nasal cavities of inoculated ferrets. Overall, the data
301 demonstrated that the combination of HA stability and polymerase efficiency promotes efficient
302 airborne transmissibility of swine H1N1 gamma viruses in ferrets and that minor variants
303 containing both traits can be selected and airborne transmitted when the majority of a virus sample
304 lacks a stabilized HA or an efficient polymerase.

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

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

312 With respect to pathway A, several other studies showed that an IAV containing α-2,6
313 receptor-binding specificity and an efficient polymerase, but having a relatively unstable HA
314 protein, can acquire airborne transmissibility in ferrets by acquiring an HA-stabilizing mutation.
315 Wild-type 2009 pH1N1 has an HA activation pH of 5.5, and a destabilizing HA1-Y17H mutation
316 increased this value to 6.0 (37, 52, 56). The HA1-Y17H mutant was attenuated in ferrets and did
317 not airborne transmit until acquiring stabilizing HA2-R106K and HA1-H17Y mutations, which
318 decreased the HA activation pH to 5.3 (37). Mechanistically, HA stabilization was shown to
319 increase replication in human airway cells (57), dampen Type I interferon responses in dendritic
320 cells (52), and increase the survivability of infectious virions after respiratory expulsion in ferrets
321 (38). In another study, a recombinant virus was generated that contained the HA from
322 A/Vietnam/1203/2004 (H5N1) with two HA mutations that conferred α2,6 receptor-binding
323 specificity, and the other seven genes from A/California/04/2009 (H1N1) with a PB2-E627K
324 mutation that enhanced polymerase activity (27). This virus and an adaptive mutant containing an
325 HA1-N158D mutation that deleted a glycosylation on the HA head were incapable of airborne
326 transmissibility in ferrets. Instead, a final mutation needed for the acquisition of airborne
327 transmissibility was HA1-T318I in the stalk, which decreased the HA activation pH to 5.3. When
328 HA-stabilizing mutations were introduced into other H5N1 viruses lacking α2,6 receptor-binding
329 specificity, virus growth in the nasal cavities of ferrets was enhanced but airborne transmission did
330 not occur (29, 30, 58).

331 With respect to pathway B, P4-PA-mixed had a stabilized HA protein and its airborne
332 transmission in 2/4 cases was associated, at least initially, with the presence of the PA-S321
333 variation that enhanced polymerase activity. A/Indonesia/5/2005 (H5N1) acquired airborne

334 transmissibility in ferrets by a combination of mutagenesis and adaptation that enhanced receptor-
335 binding activity (HA-T156A and HA-Q222L or HA-G224S), polymerase activity (PB2-E627K
336 and PB1-H99Y), and HA stability (HA-H103Y) (28, 59). These mutations arose at similar steps
337 during adaptation, and viruses lacking any of the single mutations HA-T156A, PB2-E627K, PB1-
338 H99Y, or HA-H103Y, or both the HA-Q222L or HA-G224S mutations, were loss-of-function for
339 airborne transmission in ferrets. In the case of reassortant IAVs containing genes from the human
340 1918 virus and avian H1N1 viruses, the human HA gene enabled efficient contact transmission
341 but did not allow airborne transmission in ferrets (20). Instead, the human 1918 PB2 protein was
342 found to be both necessary and sufficient to enable ferret airborne transmission in the context of a
343 virus that contained the human 1918 HA and NA genes. Thus, while selection of enhanced
344 polymerase activity in the present work was less stringent than selection of a stabilized HA, the
345 adaptation of IAVs for airborne transmission in ferrets, and presumably in humans, has been
346 associated with both HA stability and polymerase activity and both evolutionary pathways (A and
347 B) appear viable.

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

358 proceed through linear accumulation of adaptative mutations (65). Overall, as next-generation
359 sequencing becomes cheaper, faster, and more efficient, real-time monitoring of human-adaptive
360 variations may enable better preparedness for future outbreaks.

361 Numerous studies have been performed to identify and characterize human- and ferret-
362 adaptive mutations in IAVs. Examples include those focusing on the polymerase complex (42, 51,
363 66-72) and the HA protein (38, 56, 58, 73-81). In the present study, we found G15 isolates
364 containing PB2-L648/PA-P271 had higher polymerase activities than P4-PA-mixed, which
365 contained PB2-M648/PA-T271. These mutations may lead to increased production of viral
366 pathogen-associated molecular patterns (PAMPs) or defective interfering particles as well (82-84).
367 PB2-L648 was previously shown to increase polymerase activity of an H10N8 virus (85). The
368 present study also showed that PA-S321 increased polymerase activity. PA-S321 has previously
369 been identified as an adaptative marker for H5N1 influenza A viruses in humans (54, 55, 86). The
370 HA1-S210 mutation was shown to decrease the pH of HA activation and the pH of virus
371 inactivation here and in our previous work (39).

372 In summary, a combination of efficient polymerase activity and a stabilized HA were found
373 to enhance airborne transmission of swine H1N1 gamma viruses in ferrets, and these two
374 properties may be acquired in either order. Human infections of seasonal influenza viruses have
375 been far less frequent since the start of the SARS-CoV-2 pandemic, mask mandates, and social
376 distancing. Reemergence of seasonal influenza has begun to occur. Ultimately, exotic IAVs in
377 other species, especially swine, and the potential for cross-species transmission make it essential
378 to monitor potential pandemic IAV strains and to assess their pandemic risk. Swine H1N1 gamma
379 viruses are one of the most prevalent lineages in swine (87), have caused sporadic human infections
380 in recent years (15, 88), and have been shown here to acquire increased transmissibility in ferrets
381 by adaptive HA and polymerase mutations. A recent study has identified a swine Eurasian avian-

382 like influenza virus with pandemic traits (89). Surveillance and risk assessment studies are needed
383 for IAV pandemic preparedness in addition to studies investigating the evolutionary pathways by
384 which pandemic potential may arise and genetic and phenotypic traits associated therewith.
385

386 **MATERIALS AND METHODS**

387 **Cells and viruses**

388 Madin–Darby canine kidney (MDCK) cells were maintained in minimum essential
389 medium (MEM, Thermo Fisher Scientific). African green monkey kidney (Vero) cells were
390 maintained in Dulbecco's Modified Eagle Medium (DMEM, Life Technologies™). Both culture
391 media were supplemented with 10% HyClone® standard fetal bovine serum (FBS, Life
392 Technologies) and 1% penicillin/streptomycin (P/S, Thermo Fisher Scientific). The cells were
393 grown at 37 °C with 5% CO_2 (50, 51).

394 A/swine/Illinois/2A-1213-G15/2013 (H1N1) (G15-HA1-mixed) and A/swine/Illinois/2B-
395 0314-P4/2014 (H1N1) (P4-PA-mixed) were originally isolated from nasal swabs from pigs in
396 commercial swine herds in the United States that showed symptoms of influenza-like illness, as
397 described previously (39, 90). G15-HA1-S210 and G15-HA1-N210 were plaque purified twice
398 from MDCK cells using G15-HA1-mixed. The whole-genome sequences of G15-HA1-S210 and
399 G15-HA1-N210 were verified by next-generation sequencing. P4-PA-purified was plaque purified
400 from P4-PA-mixed and verified by next-generation sequencing to not contain minor mixed
401 populations at PA-321 and PA-386. All viruses were propagated in MDCK cells with 1 μg/ml
402 tosylsulfonyl phenylalanyl chloromethyl ketone (TPCK)–treated trypsin, as described previously
403 (39, 50).

404 **Syncytium assay**

405 Viruses were inoculated into Vero cells in 24–well plates at a multiplicity (MOI) of 3
406 PFU/cell. After maintaining at 37 °C with 5% CO_2 for 16 hours (h), the infected cells were treated
407 with DMEM supplemented with 5 μg/ml TPCK–treated trypsin for 15 minutes (m) and then pH–
408 adjusted PBS buffers for 15 m. Subsequently, the infected cells were maintained in DMEM
409 supplemented with 5% FBS for 3 h at 37 °C. After that, the infected cells were fixed and stained

410 using a Hema 3™ Fixative and Solutions (Fisher Scientific). Syncytia were recorded using a light
411 microscope. The cutoff values for virus HA activation pH were determined at the highest treated
412 pH for which the stained cells in 24-well plates contained more than two syncytia with at least five
413 nuclei, as described previously (39, 52, 91).

414 **Virus inactivation pH assay**

415 Viruses were treated with a series of pH-adjusted PBS buffers in a ratio of 1:100 for 1 h at
416 37 °C. The resulting infectious viruses were titrated by $TCID_{50}$ on MDCK cells (30, 37). After
417 obtaining virus titers as a function of exposure pH, virus inactivation pH values were calculated
418 by using GraphPad Prism version 7 software (Graph-Pad Software, San Diego, CA). The
419 parameter LogEC50 was generated using the model Nonlinear Regression [Equation: log (agonist)
420 vs. response-Variable slope (four parameters)] followed by the least squares fitting method.

421 **Virus growth assay**

422 Viruses were inoculated into MDCK cells in 24–well plates at an MOI of 2 PFU/cell. After
423 incubation for 1 h at 37 ºC, the infected cells were washed by PBS twice and then maintained at
424 37 ºC with MEM culture media (1 ml/well) supplemented with 1 μg/ml TPCK-treated trypsin. At
425 6- and 8- hpi, cell culture supernatant was collected and titrated by $TCID_{50}$ on MDCK cells (37,
426 92).

427 **Virus mRNA, cRNA and vRNA quantification**

428 Production of virus RNAs (mRNA, cRNA, and vRNA) during influenza A virus
429 transcription and replication was quantified using a two-step real time RT-qPCR, modified from
430 the method described previously (51, 68, 93). Briefly, viruses were inoculated into confluent
431 MDCK cells in 24–well plates at an MOI of 2. The infected cells were incubated at 37 °C for 1 h,
432 washed by PBS twice, and cultured with MEM supplemented with 1 ug/ml TPCK-treated trypsin
433 (1 ml/well). At 4 and 6 hpi, culture supernatants and infected cells were harvested separately.

434 To quantify virus mRNA, cRNA, and vRNA, total RNAs were extracted from the infected
435 cells using RNeasy Mini Kit (Qiagen). The corresponding cDNAs of mRNA, cRNA, and vRNA
436 were generated by reverse-transcription PCR (RT-PCR) using Transcriptor First Strand cDNA
437 Synthesis Kit (Roche). Primers with specific tags for differentiations of virus mRNA, cRNA, and
438 RNA were used (mRNA-RT-PCR primer:
439 CCAGATCGTTCGAGTCGTTTTTTTTTTTTTTTTTCTCATGTTTCT; cRNA-RT-PCR primer:
440 GCTAGCTTCAGCTAGGCATCAGTAGAAACAAGGGTGTTTTTTCTC; vRNA-RT-PCR
441 primer: GGCCGTCATGGTGGCGAATTGGCCACAGGATTAAGGAATATC)
442 Real-time quantitation PCR were performed to quantify these three RNA levels with specific
443 primers targeting virus HA gene (mRNA-real time-forward primer (FP):
444 CCAGTTCATTGGTACTGGTAGTC, mRNA-real time-reverse primer (RP):
445 CCAGATCGTTCGAGTCGT; cRNA-real time-FP: CCAGTTCATTGGTACTGGTAGTC,
446 cRNA-real time-RP: GCTAGCTTCAGCTAGGCATC; vRNA-real time-FP:
447 GGCCGTCATGGTGGCGAAT, vRNA-real time-RP: ACCGTACCACCCATCTATCA;).
448 Internal control GAPDH mRNA was also reverse-transcribed and quantified using oligo $(dT)_{20}$ as
449 RT-PCR primer and the following as real-time PCR primers (FP:
450 ATTCCACCCATGGCAAATTC, RP: CGCTCCTGGAAGATGGTGAT).
451 To quantify viral copy number released in cell-free supernatant, total RNAs were extracted
452 using QIAamp Viral RNA kits (Qiagen). A two-step quantitative real-time reverse-transcription
453 (RT) PCR was applied to quantify viral copy number. The primers used were listed as the
454 following, RT-PCR-primer: AGCAAAAGCAGG; Real-time-FP (targeting NP gene):
455 CTGCTTGTGTGTATGGGCTT; Real-time-RP (targeting NP gene):
456 TGAGCTGGATTTTCATTTGGT. Plasmid PHW2000-NP (G15-HA1-mixed) was used as a
457 standard for quantitative real-time PCR.

458 **Ferret transmission experiments**

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

465 The transmission study was performed as described in previous work (39). Ferret body
466 weight and temperature were monitored daily until day 14. Ferret nasal washes were collected
467 every other day until day 14. Additionally, all ferrets were euthanized on day 21 to collect whole
468 blood. HAI assays were performed to examine seroconversion of ferret sera using the
469 corresponding inoculated virus in each group (37, 39, 91). The presence or absence of the
470 stabilizing mutation HA1-N210S did not alter the results of HAI assays in control experiments.
471 Animal studies were performed in compliance with St. Jude Children's Research Hospital Animal
472 Care and Use Committee guidelines under protocol 459.

473 **Virus whole-genome sequencing and analyses**

474 Virus whole genomes were obtained by next-generation sequencing (39, 56, 91). Viral
475 RNAs were extracted from ferret nasal wash samples or cell-free supernatants using QIAamp Viral
476 RNA kits (Qiagen). The corresponding cDNAs were achieved by reverse–transcription PCR using
477 SuperScript III First-strand Synthesis System (Thermo Fisher Scientific). DNA library was
478 prepared by PCR amplification using Phusion High-Fidelity PCR Master Mix with HF Buffer
479 (New England BioLabs). The resulting PCR products were purified by QIAquick Gel Extraction
480 Kit (Qiagen) and submitted to St Jude Harwell Center for virus whole-genome sequencing.

481 Sequencing data were analyzed using CLC Genomics Workbench version 11.0.1. SNVs
482 resulting amino-acid variations were reported when the predefined quality scores were met and
483 present in forward and reverse reads at an equal ratio. All variants presented in this study were
484 supported by at least 10 reads with a minimum frequency of 5% (unless otherwise stated). Heat
485 maps and parts of whole were generated by using GraphPad Prism version 7 software (GraphPad
486 Software, San Diego, CA). The frequency of a SNV is calculated by the percent of read counts of
487 a nucleotide variant to the whole nucleotide read counts at a specific position.

488 **Statistical analysis**

489 All data analyses were performed using GraphPad Prism version 7. One-way ANOVA
490 followed by a Tukey's multiple comparisons test, or Mann-Whitney U test were used to determine
491 statistical significances. P value < 0.05 was considered significant.

492

ACKNOWLEDGMENTS

We thank Guohua Yang, Ashley Webb, and Melissa Penaflor for help with ferret experiments. We also thank St. Jude Animal Resources Center (ARC) 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.

508 **References**

509 1. Tong S, Li Y, Rivailler P, Conrardy C, Castillo DA, Chen LM, Recuenco S, Ellison JA,
510 Davis CT, York IA, Turmelle AS, Moran D, Rogers S, Shi M, Tao Y, Weil MR, Tang K,
511 Rowe LA, Sammons S, Xu X, Frace M, Lindblade KA, Cox NJ, Anderson LJ, Rupprecht
512 CE, Donis RO. 2012. A distinct lineage of influenza A virus from bats. Proc Natl Acad
513 Sci U S A 109:4269-74.

514 2. Tong S, Zhu X, Li Y, Shi M, Zhang J, Bourgeois M, Yang H, Chen X, Recuenco S,
515 Gomez J, Chen LM, Johnson A, Tao Y, Dreyfus C, Yu W, McBride R, Carney PJ,
516 Gilbert AT, Chang J, Guo Z, Davis CT, Paulson JC, Stevens J, Rupprecht CE, Holmes
517 EC, Wilson IA, Donis RO. 2013. New world bats harbor diverse influenza A viruses.
518 PLoS Pathog 9:e1003657.

519 3. Kandeil A, Gomaa MR, Shehata MM, El Taweel AN, Mahmoud SH, Bagato O,
520 Moatasim Y, Kutkat O, Kayed AS, Dawson P, Qiu X, Bahl J, Webby RJ, Karesh WB,
521 Kayali G, Ali MA. 2018. Isolation and characterization of a distinct influenza A virus
522 from Egyptian bats. J Virol doi:10.1128/JVI.01059-18.

523 4. Webster RG, Bean WJ, Gorman OT, Chambers TM, Kawaoka Y. 1992. Evolution and
524 ecology of influenza A viruses. Microbiol Rev 56:152-79.

525 5. Long JS, Mistry B, Haslam SM, Barclay WS. 2019. Host and viral determinants of
526 influenza A virus species specificity. Nat Rev Microbiol 17:67-81.

527 6. Russell CJ, Hu M, Okda FA. 2018. Influenza Hemagglutinin Protein Stability,
528 Activation, and Pandemic Risk. Trends Microbiol doi:10.1016/j.tim.2018.03.005.

529 7. Shi Y, Wu Y, Zhang W, Qi J, Gao GF. 2014. Enabling the 'host jump': structural
530 determinants of receptor-binding specificity in influenza A viruses. Nat Rev Microbiol
531 12:822-31.

532 8. Schrauwen EJ, Fouchier RA. 2014. Host adaptation and transmission of influenza A
533 viruses in mammals. Emerg Microbes Infect 3:e9.
534 9. Webster RG, Govorkova EA. 2014. Continuing challenges in influenza. Ann N Y Acad
535 Sci 1323:115-39.
536 10. Webby RJ, Rossow K, Erickson G, Sims Y, Webster R. 2004. Multiple lineages of
537 antigenically and genetically diverse influenza A virus co-circulate in the United States
538 swine population. Virus Res 103:67-73.
539 11. Gao S, Anderson TK, Walia RR, Dorman KS, Janas-Martindale A, Vincent AL. 2017.
540 The genomic evolution of H1 influenza A viruses from swine detected in the United
541 States between 2009 and 2016. J Gen Virol 98:2001-2010.
542 12. Walia RR, Anderson TK, Vincent AL. 2019. Regional patterns of genetic diversity in
543 swine influenza A viruses in the United States from 2010 to 2016. Influenza Other Respir
544 Viruses 13:262-273.
545 13. Anderson TK, Nelson MI, Kitikoon P, Swenson SL, Korslund JA, Vincent AL. 2013.
546 Population dynamics of cocirculating swine influenza A viruses in the United States from
547 2009 to 2012. Influenza Other Respir Viruses 7 Suppl 4:42-51.
548 14. Anderson TK, Macken CA, Lewis NS, Scheuermann RH, Van Reeth K, Brown IH,
549 Swenson SL, Simon G, Saito T, Berhane Y, Ciacci-Zanella J, Pereda A, Davis CT, Donis
550 RO, Webby RJ, Vincent AL. 2016. A Phylogeny-Based Global Nomenclature System
551 and Automated Annotation Tool for H1 Hemagglutinin Genes from Swine Influenza A
552 Viruses. mSphere 1.
553 15. Pulit-Penaloza JA, Belser JA, Tumpey TM, Maines TR. 2019. Sowing the Seeds of a
554 Pandemic? Mammalian Pathogenicity and Transmissibility of H1 Variant Influenza
555 Viruses from the Swine Reservoir. Trop Med Infect Dis 4.

556 16. Ducatez MF, Hause B, Stigger-Rosser E, Darnell D, Corzo C, Juleen K, Simonson R,
557 Brockwell-Staats C, Rubrum A, Wang D, Webb A, Crumpton JC, Lowe J, Gramer M,
558 Webby RJ. 2011. Multiple reassortment between pandemic (H1N1) 2009 and endemic
559 influenza viruses in pigs, United States. Emerg Infect Dis 17:1624-9.
560 17. Lipsitch M, Barclay W, Raman R, Russell CJ, Belser JA, Cobey S, Kasson PM, Lloyd-
561 Smith JO, Maurer-Stroh S, Riley S, Beauchemin CA, Bedford T, Friedrich TC, Handel
562 A, Herfst S, Murcia PR, Roche B, Wilke CO, Russell CA. 2016. Viral factors in
563 influenza pandemic risk assessment. Elife 5.
564 18. Russell RJ, Stevens DJ, Haire LF, Gamblin SJ, Skehel JJ. 2006. Avian and human
565 receptor binding by hemagglutinins of influenza A viruses. Glycoconj J 23:85-92.
566 19. Rajao DS, Anderson TK, Kitikoon P, Stratton J, Lewis NS, Vincent AL. 2018. Antigenic
567 and genetic evolution of contemporary swine H1 influenza viruses in the United States.
568 Virology 518:45-54.
569 20. Van Hoeven N, Pappas C, Belser JA, Maines TR, Zeng H, Garcia-Sastre A, Sasisekharan
570 R, Katz JM, Tumpey TM. 2009. Human HA and polymerase subunit PB2 proteins confer
571 transmission of an avian influenza virus through the air. Proc Natl Acad Sci U S A
572 106:3366-71.
573 21. Peacock TP, Sealy JE, Harvey WT, Benton DJ, Reeve R, Iqbal M. 2020. Genetic
574 determinants of receptor-binding preference and zoonotic potential of H9N2 avian
575 influenza viruses. J Virol doi:10.1128/JVI.01651-20.
576 22. Herfst S, Zhang J, Richard M, McBride R, Lexmond P, Bestebroer TM, Spronken MIJ,
577 de Meulder D, van den Brand JM, Rosu ME, Martin SR, Gamblin SJ, Xiong X, Peng W,
578 Bodewes R, van der Vries E, Osterhaus A, Paulson JC, Skehel JJ, Fouchier RAM. 2020.

579 Hemagglutinin Traits Determine Transmission of Avian A/H10N7 Influenza Virus
580 between Mammals. Cell Host Microbe 28:602-613 e7.
581 23. Zanin M, Marathe B, Wong SS, Yoon SW, Collin E, Oshansky C, Jones J, Hause B,
582 Webby R. 2015. Pandemic Swine H1N1 Influenza Viruses with Almost Undetectable
583 Neuraminidase Activity Are Not Transmitted via Aerosols in Ferrets and Are Inhibited
584 by Human Mucus but Not Swine Mucus. J Virol 89:5935-48.
585 24. Baum LG, Paulson JC. 1991. The N2 neuraminidase of human influenza virus has
586 acquired a substrate specificity complementary to the hemagglutinin receptor specificity.
587 Virology 180:10-5.
588 25. Yen HL, Liang CH, Wu CY, Forrest HL, Ferguson A, Choy KT, Jones J, Wong DD,
589 Cheung PP, Hsu CH, Li OT, Yuen KM, Chan RW, Poon LL, Chan MC, Nicholls JM,
590 Krauss S, Wong CH, Guan Y, Webster RG, Webby RJ, Peiris M. 2011. Hemagglutinin-
591 neuraminidase balance confers respiratory-droplet transmissibility of the pandemic H1N1
592 influenza virus in ferrets. Proc Natl Acad Sci U S A 108:14264-9.
593 26. Handel A, Akin V, Pilyugin SS, Zarnitsyna V, Antia R. 2014. How sticky should a virus
594 be? The impact of virus binding and release on transmission fitness using influenza as an
595 example. J R Soc Interface 11:20131083.
596 27. Imai M, Watanabe T, Hatta M, Das SC, Ozawa M, Shinya K, Zhong G, Hanson A,
597 Katsura H, Watanabe S, Li C, Kawakami E, Yamada S, Kiso M, Suzuki Y, Maher EA,
598 Neumann G, Kawaoka Y. 2012. Experimental adaptation of an influenza H5 HA confers
599 respiratory droplet transmission to a reassortant H5 HA/H1N1 virus in ferrets. Nature
600 486:420-8.
601 28. Herfst S, Schrauwen EJ, Linster M, Chutinimitkul S, de Wit E, Munster VJ, Sorrell EM,
602 Bestebroer TM, Burke DF, Smith DJ, Rimmelzwaan GF, Osterhaus AD, Fouchier RA.

603 2012. Airborne transmission of influenza A/H5N1 virus between ferrets. Science
604 336:1534-41.
605 29. Shelton H, Roberts KL, Molesti E, Temperton N, Barclay WS. 2013. Mutations in
606 haemagglutinin that affect receptor binding and pH stability increase replication of a PR8
607 influenza virus with H5 HA in the upper respiratory tract of ferrets and may contribute to
608 transmissibility. J Gen Virol 94:1220-9.
609 30. Zaraket H, Bridges OA, Duan S, Baranovich T, Yoon SW, Reed ML, Salomon R, Webby
610 RJ, Webster RG, Russell CJ. 2013. Increased acid stability of the hemagglutinin protein
611 enhances H5N1 influenza virus growth in the upper respiratory tract but is insufficient for
612 transmission in ferrets. J Virol 87:9911-22.
613 31. Reed ML, Bridges OA, Seiler P, Kim JK, Yen HL, Salomon R, Govorkova EA, Webster
614 RG, Russell CJ. 2010. The pH of activation of the hemagglutinin protein regulates H5N1
615 influenza virus pathogenicity and transmissibility in ducks. J Virol 84:1527-35.
616 32. Richard M, Herfst S, van den Brand JMA, de Meulder D, Lexmond P, Bestebroer TM,
617 Fouchier RAM. 2017. Mutations Driving Airborne Transmission of A/H5N1 Virus in
618 Mammals Cause Substantial Attenuation in Chickens only when combined. Sci Rep
619 7:7187.
620 33. Russell CJ. 2014. Acid-induced membrane fusion by the hemagglutinin protein and its
621 role in influenza virus biology. Curr Top Microbiol Immunol 385:93-116.
622 34. Russell CJ. 2021. Hemagglutinin Stability and Its Impact on Influenza A Virus
623 Infectivity, Pathogenicity, and Transmissibility in Avians, Mice, Swine, Seals, Ferrets,
624 and Humans. Viruses 13.

625 35. Maurer-Stroh S, Lee RT, Eisenhaber F, Cui L, Phuah SP, Lin RT. 2010. A new common
626 mutation in the hemagglutinin of the 2009 (H1N1) influenza A virus. PLoS Curr
627 2:RRN1162.
628 36. Klein EY, Blumenkrantz D, Serohijos A, Shakhnovich E, Choi JM, Rodrigues JV, Smith
629 BD, Lane AP, Feldman A, Pekosz A. 2018. Stability of the Influenza Virus
630 Hemagglutinin Protein Correlates with Evolutionary Dynamics. mSphere 3.
631 37. Russier M, Yang G, Rehg JE, Wong SS, Mostafa HH, Fabrizio TP, Barman S, Krauss S,
632 Webster RG, Webby RJ, Russell CJ. 2016. Molecular requirements for a pandemic
633 influenza virus: An acid-stable hemagglutinin protein. Proc Natl Acad Sci U S A
634 113:1636-41.
635 38. Singanayagam A, Zhou J, Elderfield RA, Frise R, Ashcroft J, Galiano M, Miah S,
636 Nicolaou L, Barclay WS. 2020. Characterising viable virus from air exhaled by H1N1
637 influenza-infected ferrets reveals the importance of haemagglutinin stability for airborne
638 infectivity. PLoS Pathog 16:e1008362.
639 39. Hu M, Yang G, DeBeauchamp J, Crumpton JC, Kim H, Li L, Wan XF, Kercher L,
640 Bowman AS, Webster RG, Webby RJ, Russell CJ. 2020. HA stabilization promotes
641 replication and transmission of swine H1N1 gamma influenza viruses in ferrets. Elife 9.
642 40. Manz B, Brunotte L, Reuther P, Schwemmle M. 2012. Adaptive mutations in NEP
643 compensate for defective H5N1 RNA replication in cultured human cells. Nat Commun
644 3:802.
645 41. Cauldwell AV, Long JS, Moncorge O, Barclay WS. 2014. Viral determinants of
646 influenza A virus host range. J Gen Virol 95:1193-1210.
647 42. Mehle A, Doudna JA. 2009. Adaptive strategies of the influenza virus polymerase for
648 replication in humans. Proc Natl Acad Sci U S A 106:21312-6.

649 43. Gabriel G, Klingel K, Otte A, Thiele S, Hudjetz B, Arman-Kalcek G, Sauter M, Shmidt
650 T, Rother F, Baumgarte S, Keiner B, Hartmann E, Bader M, Brownlee GG, Fodor E,
651 Klenk HD. 2011. Differential use of importin-alpha isoforms governs cell tropism and
652 host adaptation of influenza virus. Nat Commun 2:156.
653 44. Gabriel G, Fodor E. 2014. Molecular determinants of pathogenicity in the polymerase
654 complex. Curr Top Microbiol Immunol 385:35-60.
655 45. Liu WJ, Li J, Zou R, Pan J, Jin T, Li L, Liu P, Zhao Y, Yu X, Wang H, Liu G, Jiang H,
656 Bi Y, Liu L, Yuen KY, Liu Y, Gao GF. 2020. Dynamic PB2-E627K substitution of
657 influenza H7N9 virus indicates the in vivo genetic tuning and rapid host adaptation. Proc
658 Natl Acad Sci U S A 117:23807-23814.
659 46. Hatta M, Gao P, Halfmann P, Kawaoka Y. 2001. Molecular basis for high virulence of
660 Hong Kong H5N1 influenza A viruses. Science 293:1840-2.
661 47. Centers for Disease Control and Prevention. 2019, posting date. Influenza Risk
662 Assessment Tool (IRAT). https://www.cdc.gov/flu/pandemic-resources/national-
663 strategy/risk-assessment.htm.
664 48. World Health Organization. 2020, posting date. Tool for Influenza Pandemic Risk
665 Assessment (TIPRA). https://www.who.int/publications/m/item/tool-for-influenza-
666 pandemic-risk-assessment-(tipra).
667 49. Belser JA, Eckert AM, Huynh T, Gary JM, Ritter JM, Tumpey TM, Maines TR. 2020. A
668 Guide for the Use of the Ferret Model for Influenza Virus Infection. Am J Pathol 190:11-
669 24.
670 50. Hu M, Chu H, Zhang K, Singh K, Li C, Yuan S, Chow BK, Song W, Zhou J, Zheng BJ.
671 2016. Amino acid substitutions V63I or A37S/I61T/V63I/V100A in the PA N-terminal
672 domain increase the virulence of H7N7 influenza A virus. Sci Rep 6:37800.

673 51. Hu M, Yuan S, Zhang K, Singh K, Ma Q, Zhou J, Chu H, Zheng BJ. 2017. PB2
674 substitutions V598T/I increase the virulence of H7N9 influenza A virus in mammals.
675 Virology 501:92-101.
676 52. Russier M, Yang G, Briard B, Meliopoulos V, Cherry S, Kanneganti TD, Schultz-Cherry
677 S, Vogel P, Russell CJ. 2020. Hemagglutinin Stability Regulates H1N1 Influenza Virus
678 Replication and Pathogenicity in Mice by Modulating Type I Interferon Responses in
679 Dendritic Cells. J Virol 94.
680 53. Te Velthuis AJW, Long JS, Barclay WS. 2018. Assays to Measure the Activity of
681 Influenza Virus Polymerase. Methods Mol Biol 1836:343-374.
682 54. Miotto O, Heiny AT, Albrecht R, Garcia-Sastre A, Tan TW, August JT, Brusic V. 2010.
683 Complete-proteome mapping of human influenza A adaptive mutations: implications for
684 human transmissibility of zoonotic strains. PLoS One 5:e9025.
685 55. El-Shesheny R, Kandeil A, Bagato O, Maatouq AM, Moatasim Y, Rubrum A, Song MS,
686 Webby RJ, Ali MA, Kayali G. 2014. Molecular characterization of avian influenza H5N1
687 virus in Egypt and the emergence of a novel endemic subclade. J Gen Virol 95:1444-
688 1463.
689 56. Russier M, Yang G, Marinova-Petkova A, Vogel P, Kaplan BS, Webby RJ, Russell CJ.
690 2017. H1N1 influenza viruses varying widely in hemagglutinin stability transmit
691 efficiently from swine to swine and to ferrets. PLoS Pathog 13:e1006276.
692 57. Singanayagam A, Zambon M, Barclay WS. 2019. Influenza Virus with Increased pH of
693 Hemagglutinin Activation Has Improved Replication in Cell Culture but at the Cost of
694 Infectivity in Human Airway Epithelium. J Virol 93.

695 58. Zaraket H, Bridges OA, Russell CJ. 2013. The pH of activation of the hemagglutinin
696 protein regulates H5N1 influenza virus replication and pathogenesis in mice. J Virol
697 87:4826-34.
698 59. Linster M, van Boheemen S, de Graaf M, Schrauwen EJA, Lexmond P, Manz B,
699 Bestebroer TM, Baumann J, van Riel D, Rimmelzwaan GF, Osterhaus A, Matrosovich
700 M, Fouchier RAM, Herfst S. 2014. Identification, characterization, and natural selection
701 of mutations driving airborne transmission of A/H5N1 virus. Cell 157:329-339.
702 60. Lauring AS, Andino R. 2010. Quasispecies theory and the behavior of RNA viruses.
703 PLoS Pathog 6:e1001005.
704 61. Honce R, Karlsson EA, Wohlgemuth N, Estrada LD, Meliopoulos VA, Yao J, Schultz-
705 Cherry S. 2020. Obesity-Related Microenvironment Promotes Emergence of Virulent
706 Influenza Virus Strains. mBio 11.
707 62. Poon LL, Song T, Rosenfeld R, Lin X, Rogers MB, Zhou B, Sebra R, Halpin RA, Guan
708 Y, Twaddle A, DePasse JV, Stockwell TB, Wentworth DE, Holmes EC, Greenbaum B,
709 Peiris JS, Cowling BJ, Ghedin E. 2016. Quantifying influenza virus diversity and
710 transmission in humans. Nat Genet 48:195-200.
711 63. Hughes J, Allen RC, Baguelin M, Hampson K, Baillie GJ, Elton D, Newton JR, Kellam
712 P, Wood JL, Holmes EC, Murcia PR. 2012. Transmission of equine influenza virus
713 during an outbreak is characterized by frequent mixed infections and loose transmission
714 bottlenecks. PLoS Pathog 8:e1003081.
715 64. Varble A, Albrecht RA, Backes S, Crumiller M, Bouvier NM, Sachs D, Garcia-Sastre A,
716 tenOever BR. 2014. Influenza A virus transmission bottlenecks are defined by infection
717 route and recipient host. Cell Host Microbe 16:691-700.

718 65. Wasik BR, Voorhees IEH, Barnard KN, Alford-Lawrence BK, Weichert WS, Hood G,
719 Nogales A, Martinez-Sobrido L, Holmes EC, Parrish CR. 2019. Influenza Viruses in
720 Mice: Deep Sequencing Analysis of Serial Passage and Effects of Sialic Acid Structural
721 Variation. J Virol 93.
722 66. Soh YS, Moncla LH, Eguia R, Bedford T, Bloom JD. 2019. Comprehensive mapping of
723 adaptation of the avian influenza polymerase protein PB2 to humans. Elife 8.
724 67. Jones JC, Pascua PNQ, Fabrizio TP, Marathe BM, Seiler P, Barman S, Webby RJ,
725 Webster RG, Govorkova EA. 2020. Influenza A and B viruses with reduced baloxavir
726 susceptibility display attenuated in vitro fitness but retain ferret transmissibility. Proc
727 Natl Acad Sci U S A 117:8593-8601.
728 68. Hu M, Yuan S, Ye ZW, Singh K, Li C, Shuai H, Fai N, Chow BK, Chu H, Zheng BJ.
729 2017. PAN substitutions A37S, A37S/I61T and A37S/V63I attenuate the replication of
730 H7N7 influenza A virus by impairing the polymerase and endonuclease activities. J Gen
731 Virol 98:364-373.
732 69. Lee LY, Zhou J, Koszalka P, Frise R, Farrukee R, Baba K, Miah S, Shishido T, Galiano
733 M, Hashimoto T, Omoto S, Uehara T, Mifsud EJ, Collinson N, Kuhlbusch K, Clinch B,
734 Wildum S, Barclay WS, Hurt AC. 2021. Evaluating the fitness of PA/I38T-substituted
735 influenza A viruses with reduced baloxavir susceptibility in a competitive mixtures ferret
736 model. PLoS Pathog 17:e1009527.
737 70. Goldhill DH, Yan A, Frise R, Zhou J, Shelley J, Gallego Cortes A, Miah S, Akinbami O,
738 Galiano M, Zambon M, Lackenby A, Barclay WS. 2021. Favipiravir-resistant influenza
739 A virus shows potential for transmission. PLoS Pathog 17:e1008937.

740 71. Cheung PP, Watson SJ, Choy KT, Fun Sia S, Wong DD, Poon LL, Kellam P, Guan Y,
741 Malik Peiris JS, Yen HL. 2014. Generation and characterization of influenza A viruses
742 with altered polymerase fidelity. Nat Commun 5:4794.
743 72. Imai M, Yamashita M, Sakai-Tagawa Y, Iwatsuki-Horimoto K, Kiso M, Murakami J,
744 Yasuhara A, Takada K, Ito M, Nakajima N, Takahashi K, Lopes TJS, Dutta J, Khan Z,
745 Kriti D, van Bakel H, Tokita A, Hagiwara H, Izumida N, Kuroki H, Nishino T, Wada N,
746 Koga M, Adachi E, Jubishi D, Hasegawa H, Kawaoka Y. 2020. Influenza A variants with
747 reduced susceptibility to baloxavir isolated from Japanese patients are fit and transmit
748 through respiratory droplets. Nat Microbiol 5:27-33.
749 73. Lee JM, Huddleston J, Doud MB, Hooper KA, Wu NC, Bedford T, Bloom JD. 2018.
750 Deep mutational scanning of hemagglutinin helps predict evolutionary fates of human
751 H3N2 influenza variants. Proc Natl Acad Sci U S A 115:E8276-E8285.
752 74. Lakdawala SS, Jayaraman A, Halpin RA, Lamirande EW, Shih AR, Stockwell TB, Lin
753 X, Simenauer A, Hanson CT, Vogel L, Paskel M, Minai M, Moore I, Orandle M, Das
754 SR, Wentworth DE, Sasisekharan R, Subbarao K. 2015. The soft palate is an important
755 site of adaptation for transmissible influenza viruses. Nature 526:122-5.
756 75. Murakami S, Horimoto T, Ito M, Takano R, Katsura H, Shimojima M, Kawaoka Y. 2012.
757 Enhanced growth of influenza vaccine seed viruses in vero cells mediated by broadening
758 the optimal pH range for virus membrane fusion. J Virol 86:1405-10.
759 76. DuBois RM, Zaraket H, Reddivari M, Heath RJ, White SW, Russell CJ. 2011. Acid
760 stability of the hemagglutinin protein regulates H5N1 influenza virus pathogenicity.
761 PLoS Pathog 7:e1002398.

762 77. Stevens J, Blixt O, Tumpey TM, Taubenberger JK, Paulson JC, Wilson IA. 2006.
763 Structure and receptor specificity of the hemagglutinin from an H5N1 influenza virus.
764 Science 312:404-10.
765 78. Byrd-Leotis L, Galloway SE, Agbogu E, Steinhauer DA. 2015. Influenza hemagglutinin
766 (HA) stem region mutations that stabilize or destabilize the structure of multiple HA
767 subtypes. J Virol 89:4504-16.
768 79. Zhong L, Wang X, Li Q, Liu D, Chen H, Zhao M, Gu X, He L, Liu X, Gu M, Peng D,
769 Liu X. 2014. Molecular mechanism of the airborne transmissibility of H9N2 avian
770 influenza A viruses in chickens. J Virol 88:9568-78.
771 80. Linster M, Schrauwen EJA, van der Vliet S, Burke DF, Lexmond P, Bestebroer TM,
772 Smith DJ, Herfst S, Koel BF, Fouchier RAM. 2019. The Molecular Basis for Antigenic
773 Drift of Human A/H2N2 Influenza Viruses. J Virol 93.
774 81. Reed ML, Yen HL, DuBois RM, Bridges OA, Salomon R, Webster RG, Russell CJ.
775 2009. Amino acid residues in the fusion peptide pocket regulate the pH of activation of
776 the H5N1 influenza virus hemagglutinin protein. J Virol 83:3568-80.
777 82. Killip MJ, Fodor E, Randall RE. 2015. Influenza virus activation of the interferon system.
778 Virus Res 209:11-22.
779 83. Tapia F, Laske T, Wasik MA, Rammhold M, Genzel Y, Reichl U. 2019. Production of
780 Defective Interfering Particles of Influenza A Virus in Parallel Continuous Cultures at
781 Two Residence Times-Insights From qPCR Measurements and Viral Dynamics
782 Modeling. Front Bioeng Biotechnol 7:275.
783 84. Dimmock NJ, Easton AJ. 2014. Defective interfering influenza virus RNAs: time to
784 reevaluate their clinical potential as broad-spectrum antivirals? J Virol 88:5217-27.

785 85. Xiao C, Ma W, Sun N, Huang L, Li Y, Zeng Z, Wen Y, Zhang Z, Li H, Li Q, Yu Y,
786 Zheng Y, Liu S, Hu P, Zhang X, Ning Z, Qi W, Liao M. 2016. PB2-588 V promotes the
787 mammalian adaptation of H10N8, H7N9 and H9N2 avian influenza viruses. Sci Rep
788 6:19474.
789 86. Arai Y, Kawashita N, Elgendy EM, Ibrahim MS, Daidoji T, Ono T, Takagi T, Nakaya T,
790 Matsumoto K, Watanabe Y. 2020. PA Mutations Inherited during Viral Evolution Act
791 Cooperatively To Increase Replication of Contemporary H5N1 Influenza Virus with an
792 Expanded Host Range. J Virol 95.
793 87. Diaz A, Marthaler D, Corzo C, Munoz-Zanzi C, Sreevatsan S, Culhane M, Torremorell
794 M. 2017. Multiple Genome Constellations of Similar and Distinct Influenza A Viruses
795 Co-Circulate in Pigs During Epidemic Events. Sci Rep 7:11886.
796 88. World Health Organization. 2018, posting date. Antigenic and genetic characteristics of
797 zoonotic influenza viruses and candidate vaccine viruses developed for potential use in
798 human vaccines,
799 https://www.who.int/influenza/vaccines/virus/characteristics_virus_vaccines/en/.
800 89. Sun H, Xiao Y, Liu J, Wang D, Li F, Wang C, Li C, Zhu J, Song J, Sun H, Jiang Z, Liu
801 L, Zhang X, Wei K, Hou D, Pu J, Sun Y, Tong Q, Bi Y, Chang KC, Liu S, Gao GF, Liu
802 J. 2020. Prevalent Eurasian avian-like H1N1 swine influenza virus with 2009 pandemic
803 viral genes facilitating human infection. Proc Natl Acad Sci U S A 117:17204-17210.
804 90. Zanin M, Duan S, Wong SS, Kumar G, Baviskar P, Collin E, Russell C, Barman S,
805 Hause B, Webby R. 2017. An Amino Acid in the Stalk Domain of N1 Neuraminidase Is
806 Critical for Enzymatic Activity. J Virol 91.
807 91. Zaraket H, Baranovich T, Kaplan BS, Carter R, Song MS, Paulson JC, Rehg JE, Bahl J,
808 Crumpton JC, Seiler J, Edmonson M, Wu G, Karlsson E, Fabrizio T, Zhu HC, Guan Y,

809 Husain M, Schultz-Cherry S, Krauss S, McBride R, Webster RG, Govorkova EA, Zhang
810 JH, Russell CJ, Webby RJ. 2015. Mammalian adaptation of influenza A(H7N9) virus is
811 limited by a narrow genetic bottleneck. Nature Communications 6.
812 92. Hu M, Yuan S, Ye ZW, Singh K, Li C, Shuai H, Fai N, Chow BKC, Chu H, Zheng BJ.
813 2017. PAN substitutions A37S, A37S/I61T and A37S/V63I attenuate the replication of
814 H7N7 influenza A virus by impairing the polymerase and endonuclease activities. J Gen
815 Virol 98:364-373.
816 93. Kawakami E, Watanabe T, Fujii K, Goto H, Watanabe S, Noda T, Kawaoka Y. 2011.
817 Strand-specific real-time RT-PCR for distinguishing influenza vRNA, cRNA, and
818 mRNA. J Virol Methods 173:1-6.
819

820 **Figure Legends**

821

822 **Fig. 1. Virus characterization *in vitro*.** (A) Sequence variations. Whole genomes of gamma
823 viruses P4-PA-mixed, G15-HA1-mixed, G15-HA1-S210, and G15-HA1-N210 were obtained by
824 next-generation sequencing. Amino-acid variations with frequencies ≥ 5% and reads ≥ 10 are
825 shown in colored pie charts. H3 numbering was used for the HA protein. (B) HA activation pH
826 values measured by syncytium assay. Viruses were inoculated into Vero cells at an MOI of 3
827 PFU/cell. Representative images are shown with the HA activation pH in bold. (C) Virus
828 inactivation pH values. Viruses were treated with pH-adjusted PBS, re-neutralized, and subjected
829 to measurement of residual virus infectivity by $TCID_{50}$. (D) Virus growth in MDCK cells. Viruses
830 were inoculated into MDCK cells at an MOI of 2 PFU/cell. Cell-free culture supernatants were
831 harvested at 6- and 8-hpi and titrated by $TCID_{50}$. All experiments were independently performed
832 at least twice. *P* values were determined according to one-way ANOVA followed by a Tukey's
833 multiple comparisons test. * and ** represent $P < 0.05$ and 0.01, respectively.

834

835 **Fig. 2. *In vitro* transcription and replication of P4-PA-mixed, P4-PA-purified, and G15-HA1-**
836 **S210.** (A) Virus mRNA, cRNA, and vRNA accumulation after 4 hpi. (B) Virus mRNA, cRNA,
837 and vRNA accumulation after 6 hpi. (C) Virus copies released into culture media. Viruses were
838 inoculated into MDCK cells at an MOI of 2 PFU/cell. At 4 and 6 hpi, the infected MDCK cells
839 were lysed and harvested for total viral RNA. Virus specific mRNA, cRNA, and vRNA were
840 quantified by two-step real-time reverse-transcription PCR using specific primers and normalized
841 to GAPDH mRNA levels. Meanwhile, culture supernatants were harvested for quantification of
842 virions released. Virions released from the cells were quantified as virus copies by real-time
843 quantitative PCR using plasmid PHW2000-NP (G15-HA1-mixed) as a standard. *P* values were

844 determined according to one-way ANOVA followed by a Tukey's multiple comparisons test. *
845 and *** represent $P < 0.05$ and 0.001, respectively.
846
847 **Fig. 3. Infection of P4-PA-mixed, G15-HA1-mixed, and G15-HA1-S210 in inoculated donor**
848 **ferrets.** (A) Summary of viral phenotypes. Relative polymerase activity and HA stability for
849 viruses are shown. (B) Ferret experimental caging in each of two cubicles. Viruses were
850 intranasally inoculated into 3 donor ferrets per group on day 0. After one day, 3 naïve ferrets were
851 introduced into the same cages and 3 into the adjacent cages. These ferrets were designated contact
852 and airborne ferrets, respectively. Overall, there were 9 ferrets for each cubicle and two cubicles
853 per virus. (C) $TCID_{50}$ titers of virus in nasal washes from donor ferrets. Ferret nasal washes were
854 collected every other day until day 14. Each bar represents the virus titer of a sample from an
855 individual ferret. For each virus, the first three bars correspond to cubicle one and the next three
856 bars correspond to cubicle two. The bottom dashed lines represent the limit of detection. (D) Day
857 of virus peak titer in donor ferrets. Each symbol represents the day that the virus peak titer was
858 observed in an individual donor ferret. (E) Virus peak titers in donor ferrets. Each symbol
859 represents the virus peak titer in an individual donor ferret. (F) HAI titers of donor ferret sera. (G)
860 Percent body weight of donor ferrets. Ferret body weight was monitored daily until day 14. *P*
861 values were calculated using Mann-Whitney U test. * and ** represent $P < 0.05$ and 0.01,
862 respectively.
863
864 **Fig. 4. P4-PA-mixed transmission, seroconversion, and frequency of SNVs for nasal washes**
865 **collected from ferrets after transmission.** The ferret experiment was carried out as described in
866 Fig. 3 in which all of the corresponding donor ferrets shed detectable viruses and seroconverted.
867 (A) Virus titers ($TCID_{50}$) of nasal washes from contact (solid lines) and airborne ferrets (dashed

868 lines). (B) HAI titers of day-21 sera from contact and airborne ferrets. (C) Frequencies of SNVs
869 for nasal washes. The whole-genomes of nasal washes collected from contact and airborne ferrets
870 were obtained by next-generation sequencing. SNVs with frequencies ≥ 5% and reads ≥ 10 were
871 read out. The listed SNVs met the following criteria: (1) resulted in protein sequence changes, and
872 (2) appeared in nasal washes in each cubicle ≥ 2 times and/or frequencies greater than 30%. The
873 frequencies of SNVs are represented by the colored boxes (scale bar at bottom right). Each column
874 represents the SNVs for one ferret nasal wash. (D) The proportions of PA-S321 in P4-PA-mixed
875 from the original inoculum and infected/exposed ferrets.
876
877 **Fig. 5. Minor variant PA-S321 had enhanced polymerase activity.** (A-C) Evaluation of
878 polymerase activity of P4-PA-S321. The experiment was performed as described in Fig. 2. The
879 transcription and replication of virus containing 100% variant PA-S321 were compared to those
880 of G15-HA1-S210 and P4-PA-mixed. The experiments were independently performed twice. *P*
881 values were determined according to one-way ANOVA followed by a Tukey's multiple
882 comparisons test. *, **, and *** represent $P < 0.05$, 0.01, and 0.001, respectively.
883
884 **Fig. 6. G15-HA1-mixed transmission, seroconversion, and frequency of SNVs for nasal**
885 **washes collected from ferrets after transmission.** The ferret experiment was carried out as
886 described in Fig. 3 in which all of the corresponding donor ferrets shed detectable viruses and
887 seroconverted. The frequency of SNVs were determined and listed as described in Fig. 5C. (A)
888 Virus titers ($TCID_{50}$) of nasal washes from contact and airborne ferrets. (B) HAI titers of day-21
889 sera of contact and airborne ferrets. (C) Frequencies of SNVs for nasal washes. (D) The
890 proportions of HA1-S210 in G15-HA1-mixed from the original inoculum and infected/exposed
891 ferrets.

892

893 **Fig. 7. G15-HA1-S210 transmission, seroconversion, and frequency of SNVs for nasal washes**
894 **collected from transmitted ferrets.** The ferret experiment was carried out as described in Fig.
895 3A, and frequency of SNVs were achieved and listed as described in Fig. 5C. (A) Virus $TCID_{50}$
896 titers of nasal washes from contact and airborne ferrets. (B) HAI titers of day-21 sera of contact
897 and airborne ferrets. (C) Frequencies of SNVs for nasal washes.

898

899 **Fig. 8. HA activation pH values of viruses isolated from ferrets.** (A-C) HA activation pH values
900 of virus-containing isolates from P4-PA-mixed (A), G15-HA1-S210 (B), and G15-HA1-mixed (C).
901 Virus isolates from nasal washes were propagated in MDCK cells once. Syncytium assays were
902 performed to measure the HA activation pH values.

903

904

905 **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)	5.0 (± 0.28)	7.3 (± 0.67)	- [f]
	Seroconversion	3/3; 3/3 [d]	3/3; 3/3	3/3; 3/3	-
	Day of peak titers	2.7 (± 1.5)	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	Seroconversion	3/3; 3/3	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	Days for virus shedding	8-14; 10-14	-; 8-12	8-14; 2-6	-
	Seroconversion	3/3; 1/3	2/3; 3/3	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	-

906 [a] HA activation pH measured by syncytia assay.

907 [b] HA inactivation pH measured by treating viruses with indicated pH and titrating resulting virions by $TCID_{50}$.

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

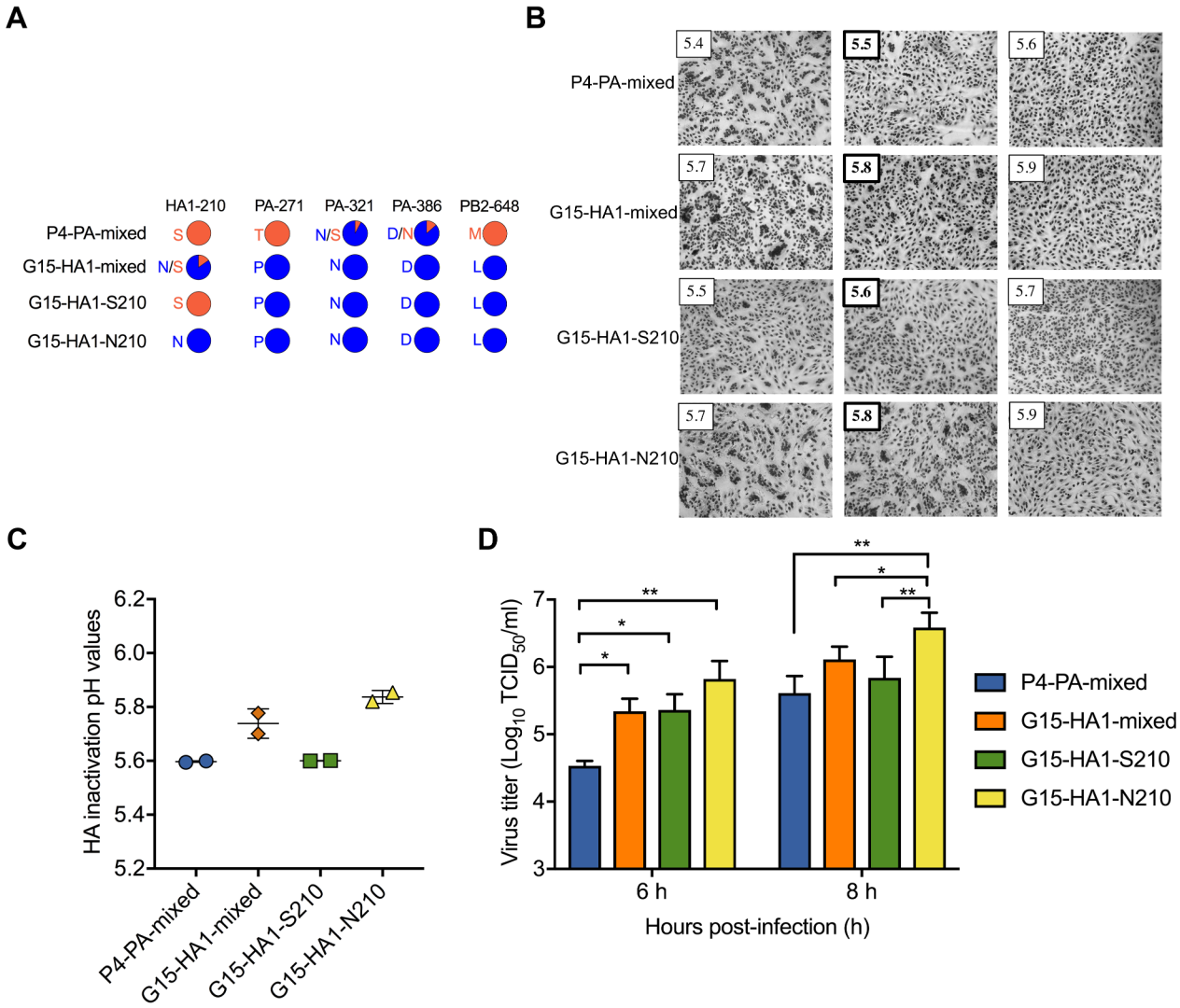
909 one and cubicle two.

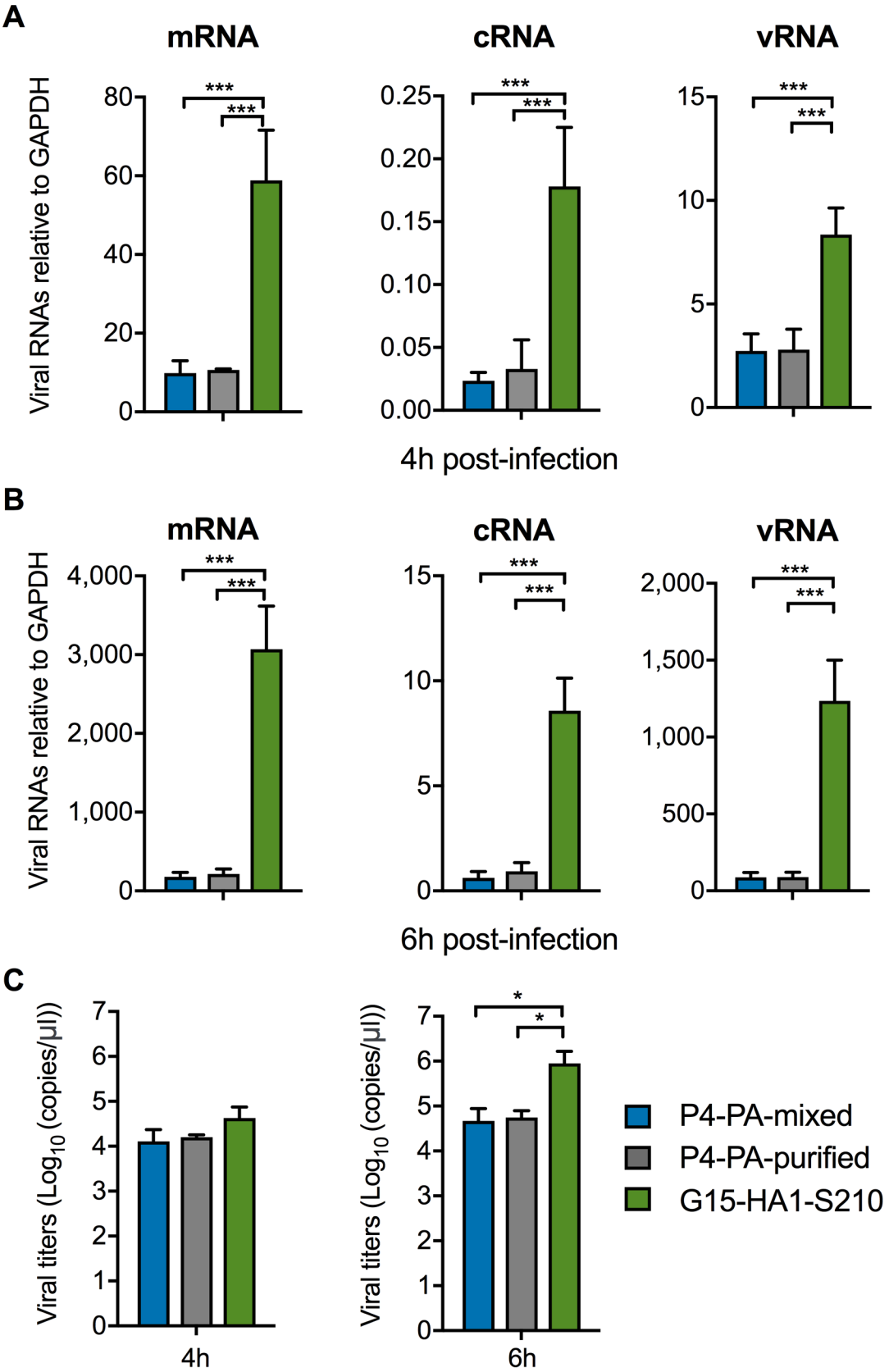
910 [d] Data for each virus is listed for each cubicle with the first X/Y number representing cubicle one and the second

911 X/Y representing cubicle two.

912 [e] HA activation pH of airborne-transmitted virus on first day of isolation.

913 [f] -, Not measured.

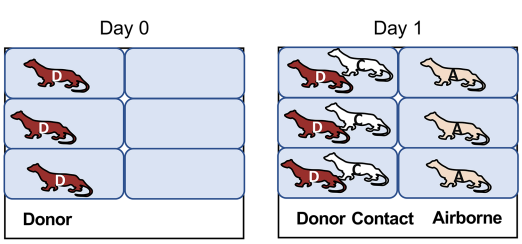

A
HA1-210
PA-271
PA-321
PA-386
PB2-648
P4-PA-mixed
G15-HA1-mixed
G15-HA1-S210
G15-HA1-N210
S
T
N/S
D/N
M
N/S
P
N
D
L
S
P
N
D
L
N
P
N
D
L
B
5.4
5.5
5.6
5.7
5.8
5.9
5.5
5.6
5.7
5.7
5.8
5.9
P4-PA-mixed
G15-HA1-mixed
G15-HA1-S210
G15-HA1-N210
C
HA inactivation pH values
6.2
6.0
5.8
5.6
5.4
5.2
P4-PA-mixed
G15-HA1-mixed
G15-HA1-S210
G15-HA1-N210
D
Virus titer (Log10 TCID50/ml)
7
6
5
4
3
*
*
**
**
*
**
6 h
8 h
Hours post-infection (h)
P4-PA-mixed
G15-HA1-mixed
G15-HA1-S210
G15-HA1-N210


A
mRNA
cRNA
vRNA
Viral RNAs relative to GAPDH
***
4h post-infection
B
6h post-infection
C
Viral titers (Log10 (copies/µl))
*
4h
6h
P4-PA-mixed
P4-PA-purified
G15-HA1-S210

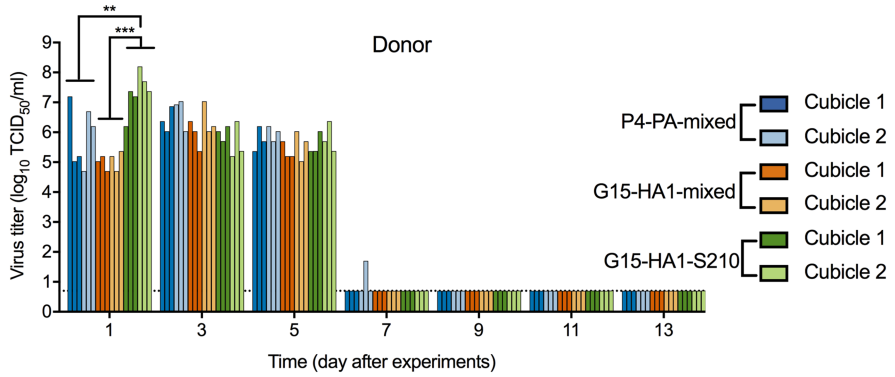
A

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

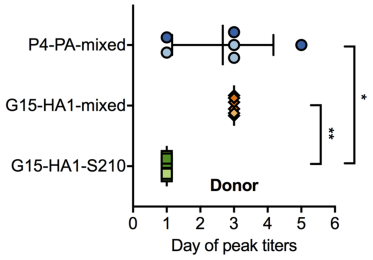
B



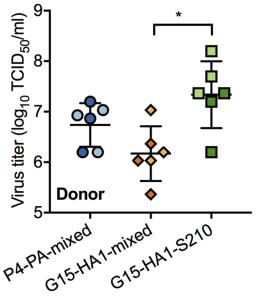
C



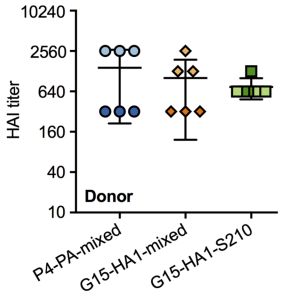
D



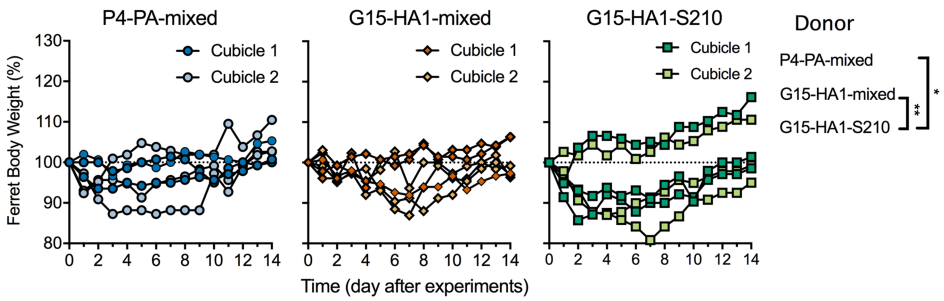
E

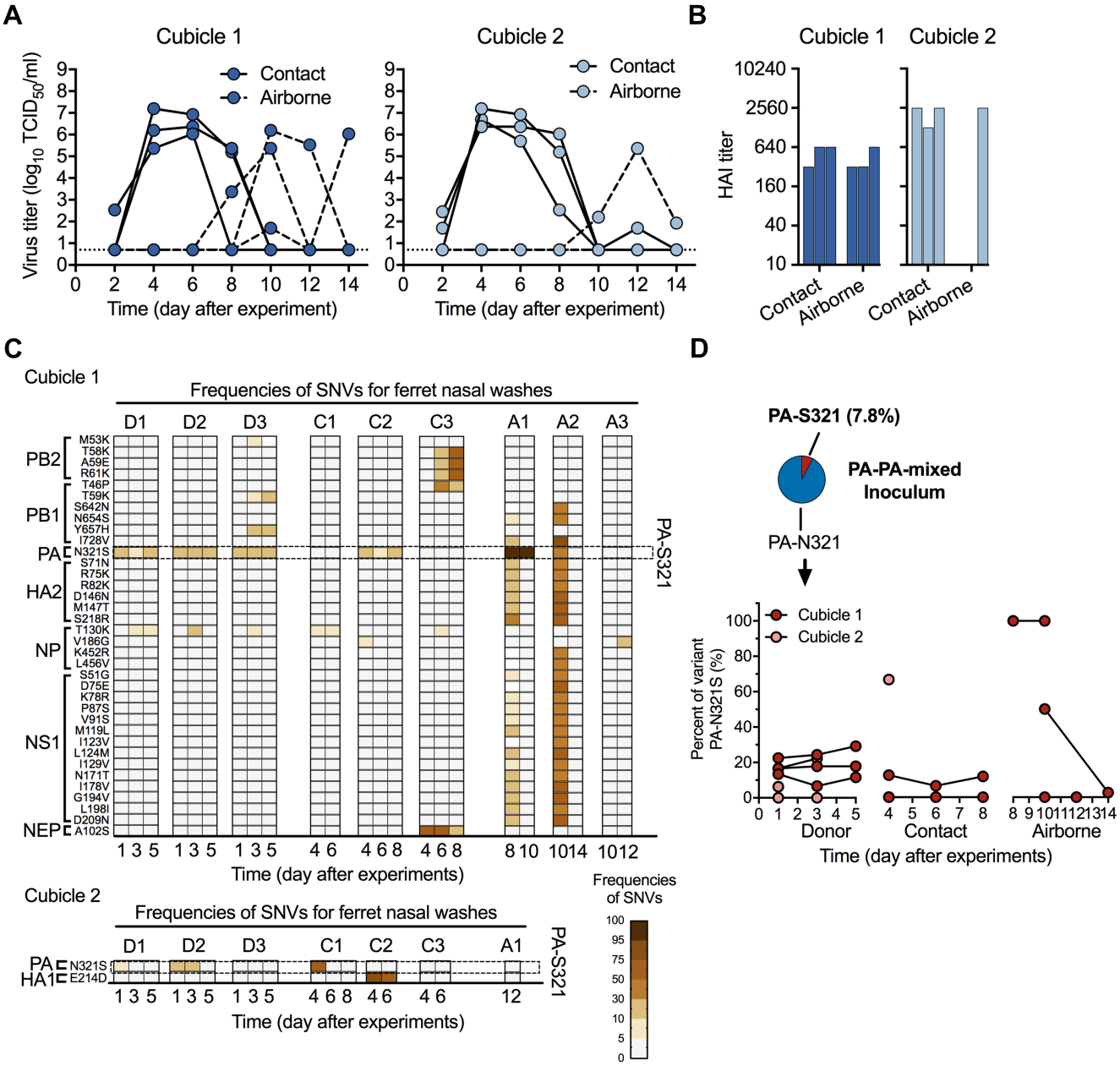


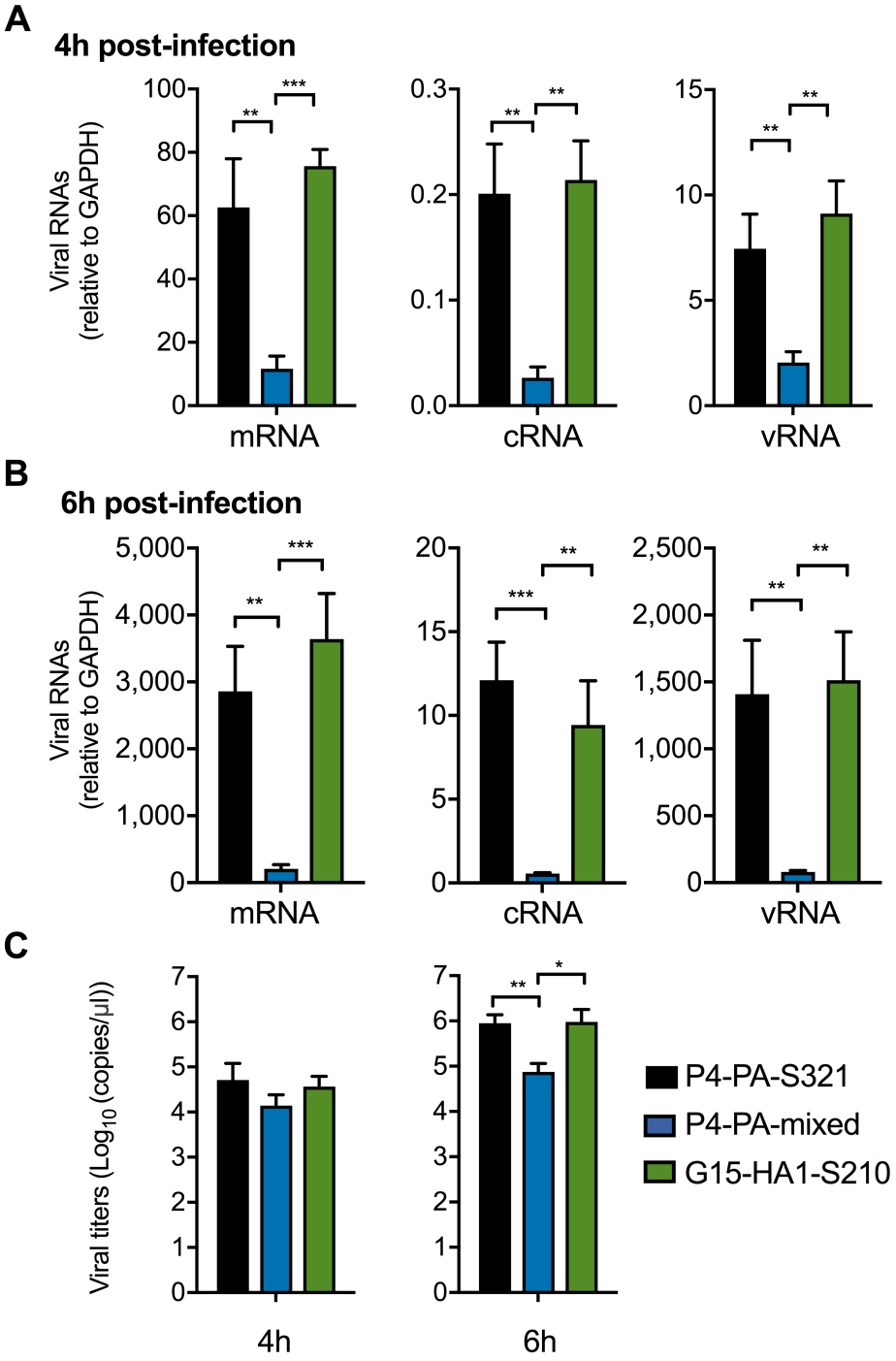
F

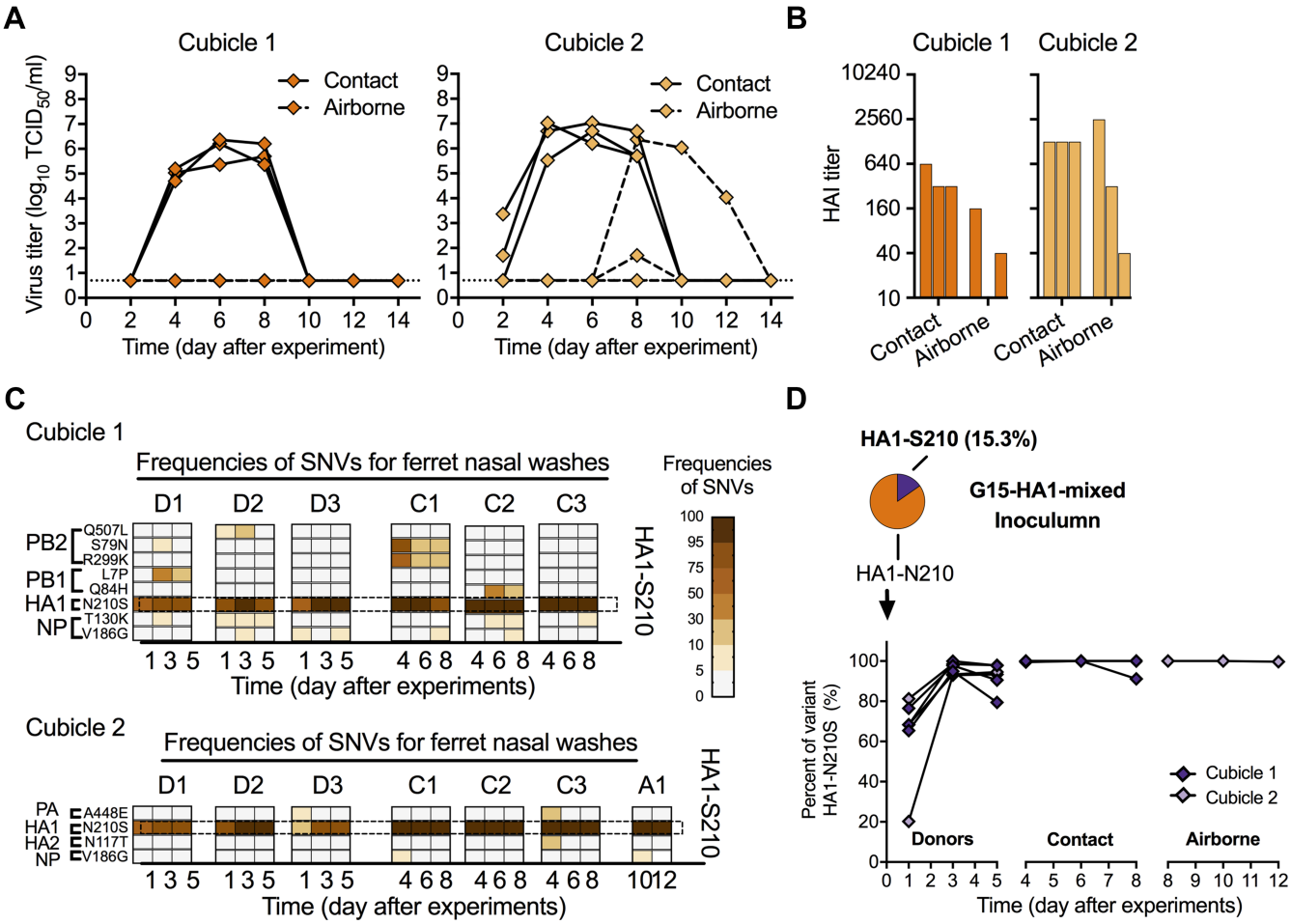


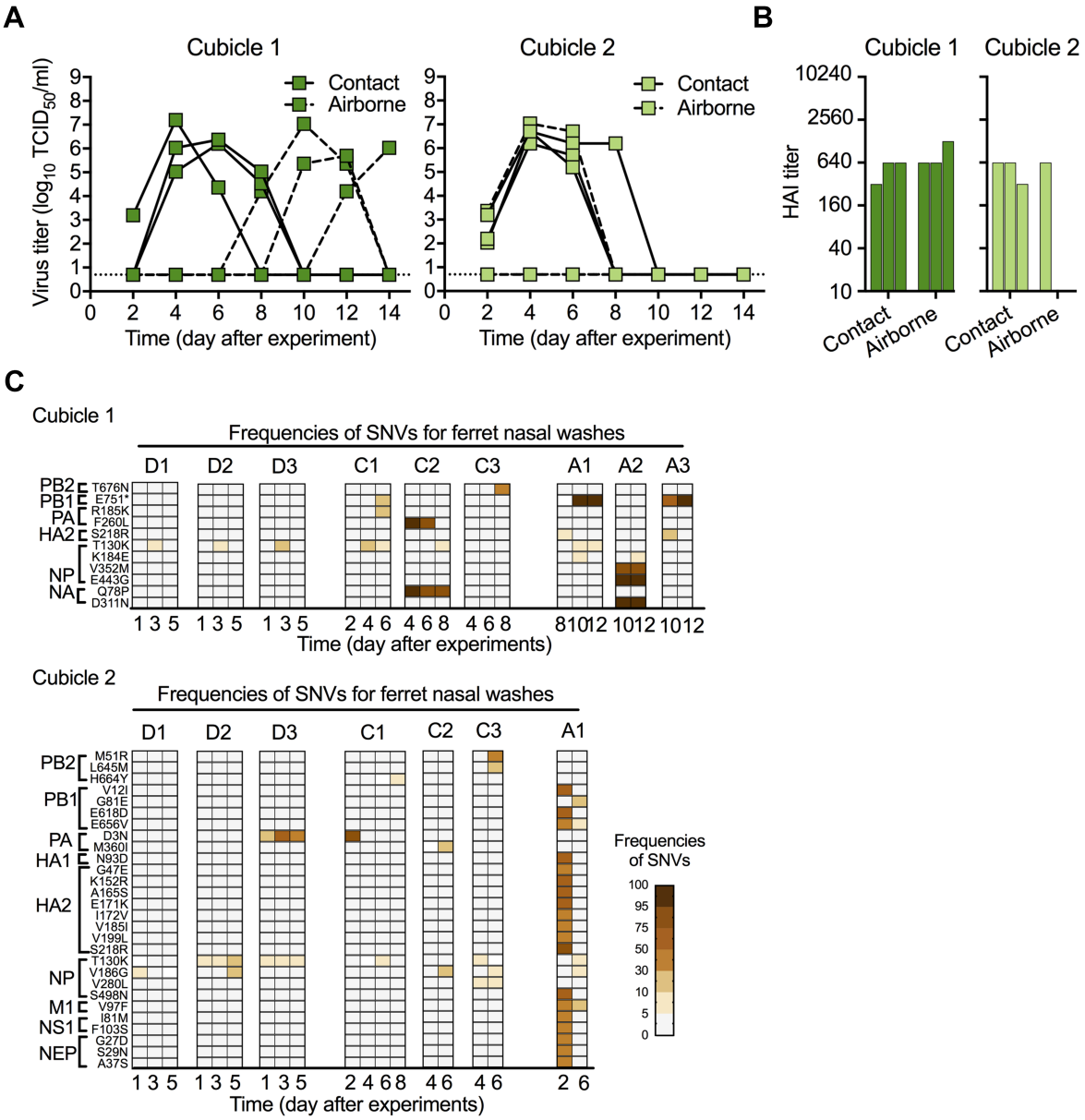
G

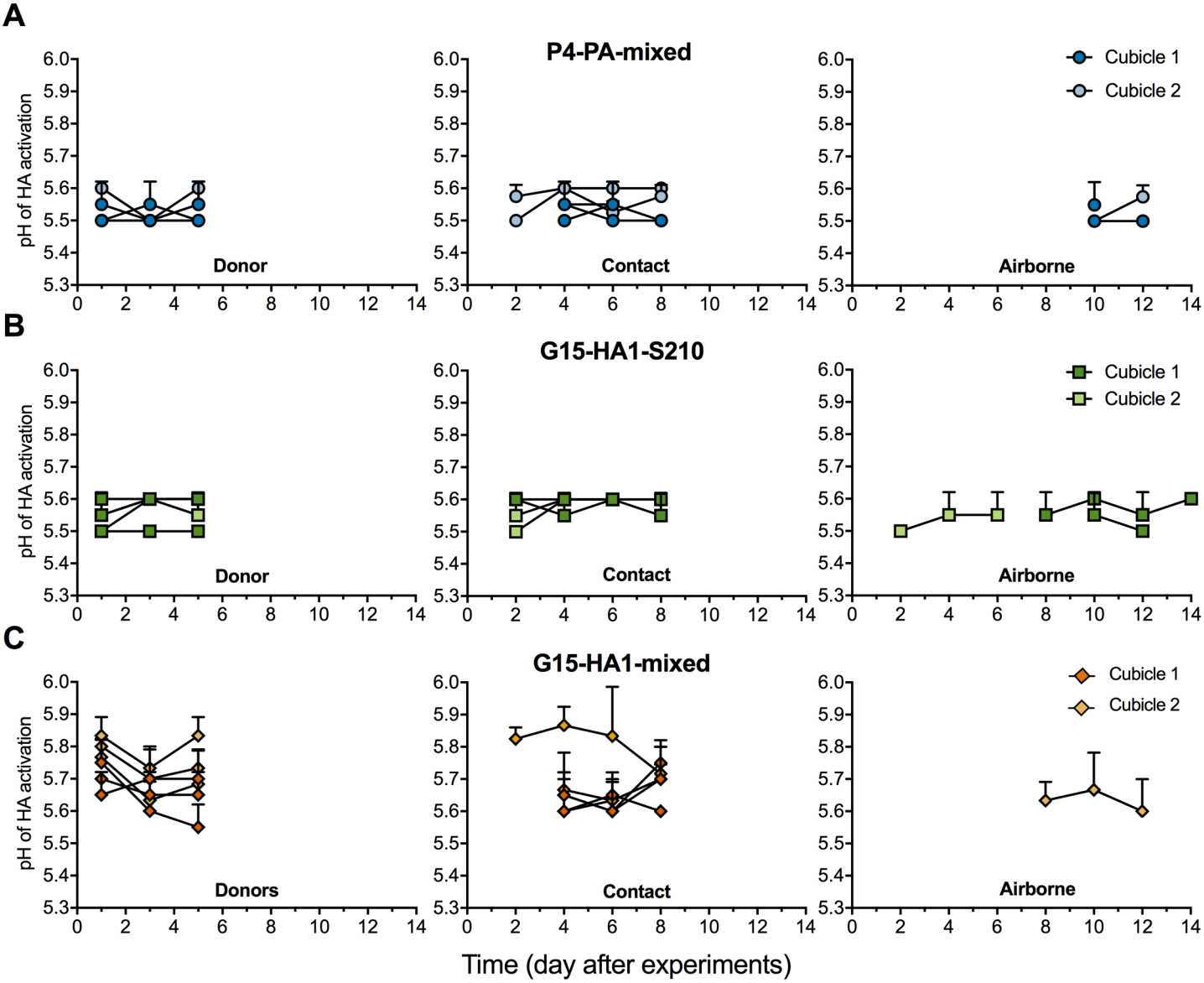



A
Cubicle 1
Cubicle 2
Contact
Airborne
Virus titer (log10 TCID50/ml)
Time (day after experiment)
B
Cubicle 1
Cubicle 2
HAI titer
10240
2560
640
160
40
10
Contact
Airborne
C
Cubicle 1
Frequencies of SNVs for ferret nasal washes
D1 D2 D3 C1 C2 C3 A1 A2 A3
PB2
M53K
T58K
A59E
R61K
PB1
T46P
T59K
S642N
N654S
Y657H
I728V
PA
N321S
PA-S321
HA2
S71N
R75K
R82K
D146N
M147T
S218R
NP
T130K
V186G
K452R
L456V
NS1
S51G
D75E
K78R
P87S
V91S
M119L
I123V
L124M
I129V
N171T
I178V
G194V
L198I
NEP
D209N
A102S
Time (day after experiments)
Cubicle 2
Frequencies of SNVs for ferret nasal washes
D1 D2 D3 C1 C2 C3 A1
PA
N321S
HA1
E214D
PA-S321
Time (day after experiments)
Frequencies of SNVs
100
95
75
50
30
10
5
0
D
PA-S321 (7.8%)
PA-PA-mixed Inoculum
PA-N321
Cubicle 1
Cubicle 2
Percent of variant PA-N321S (%)
Donor
Contact
Airborne
Time (day after experiments)


A
4h post-infection
Viral RNAs (relative to GAPDH)
mRNA
cRNA
vRNA
B
6h post-infection
Viral RNAs (relative to GAPDH)
mRNA
cRNA
vRNA
C
Viral titers (Log10 (copies/µl))
4h
6h
P4-PA-S321
P4-PA-mixed
G15-HA1-S210


A
Cubicle 1
Cubicle 2
Contact
Airborne
Virus titer (log10 TCID50/ml)
Time (day after experiment)
B
Cubicle 1
Cubicle 2
HAI titer
10240
2560
640
160
40
10
Contact
Airborne
C
Cubicle 1
Frequencies of SNVs for ferret nasal washes
D1
D2
D3
C1
C2
C3
PB2
Q507L
S79N
R299K
PB1
L7P
Q84H
HA1
N210S
T130K
NP
V186G
HA1-S210
Frequencies of SNVs
Time (day after experiments)
Cubicle 2
Frequencies of SNVs for ferret nasal washes
A1
PA
A448E
HA1
N210S
HA2
N117T
NP
V186G
HA1-S210
Time (day after experiments)
D
HA1-S210 (15.3%)
G15-HA1-mixed
Inoculumn
HA1-N210
Percent of variant
HA1-N210S (%)
Cubicle 1
Cubicle 2
Donors
Contact
Airborne
Time (day after experiments)


A
Cubicle 1
Cubicle 2
Contact
Airborne
Virus titer (log10 TCID50/ml)
Time (day after experiment)
B
Cubicle 1
Cubicle 2
HAI titer
10240
2560
640
160
40
10
Contact
Airborne
C
Cubicle 1
Frequencies of SNVs for ferret nasal washes
D1 D2 D3 C1 C2 C3 A1 A2 A3
PB2 T676N
PB1 E751*
PA R185K
F260L
HA2 S218R
T130K
K184E
NP V352M
E443G
NA Q78P
D311N
Time (day after experiments)
Cubicle 2
Frequencies of SNVs for ferret nasal washes
D1 D2 D3 C1 C2 C3 A1
PB2 M51R
L645M
H664Y
PB1 V12I
G81E
E618D
E656V
PA D3N
M360I
HA1 N93D
G47E
K152R
A165S
E171K
I172V
V185I
V199L
HA2 S218R
T130K
NP V186G
V280L
S498N
M1 V97F
NS1 I81M
F103S
NEP G27D
S29N
A37S
Time (day after experiments)
Frequencies of SNVs
100
95
75
50
30
10
5
0


A
P4-PA-mixed
Cubicle 1
Cubicle 2
pH of HA activation
Donor
Contact
Airborne
B
G15-HA1-S210
Cubicle 1
Cubicle 2
pH of HA activation
Donor
Contact
Airborne
C
G15-HA1-mixed
Cubicle 1
Cubicle 2
pH of HA activation
Donors
Contact
Airborne
Time (day after experiments)