

1 **Plasticity of amino acid residue 145 near the receptor binding site of H3 swine**
2 **influenza A viruses and its impact on receptor binding and antibody**
3 **recognition.**

4 **Short Title: Significance of aa 145 in the HA of H3N2 swine influenza A virus**

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23 **Abstract**

24 The hemagglutinin (HA), a glycoprotein on the surface of influenza A virus (IAV),
25 initiates the virus life cycle by binding to terminal sialic acid (SA) residues on host cells. The HA
26 gradually accumulates amino acid (aa) substitutions that allow IAV to escape immunity through
27 a mechanism known as antigenic drift. We recently confirmed that a small set of aa residues are
28 largely responsible for driving antigenic drift in swine-origin H3 IAV. All identified residues are
29 located adjacent to the HA receptor binding site (RBS), suggesting that substitutions associated
30 with antigenic drift may also influence receptor binding. Among those substitutions, residue 145
31 was shown to be a major determinant of antigenic evolution. To determine whether there are
32 functional constraints to substitutions near the RBS and their impact on receptor binding and
33 antigenic properties, we carried out site-directed mutagenesis experiments at the single aa level.
34 We generated a panel of viruses carrying substitutions at residue 145 representing all 20 amino
35 acids. Despite limited amino acid usage in nature, most substitutions at residue 145 were well
36 tolerated without major impact on virus replication *in vitro*. All substitutions retained receptor
37 binding specificity, but frequently led to decreased receptor binding. Glycan microarray analysis
38 showed that substitutions at residue 145 modulate binding to a broad range of glycans.
39 Furthermore, antigenic characterization identified specific substitutions at residue 145 that
40 altered antibody recognition. This work provides a better understanding of the functional effects
41 of aa substitutions near the RBS and the interplay between receptor binding and antigenic drift.

42 **Importance**

43 The complex and continuous antigenic evolution of IAVs remains a major hurdle for vaccine
44 selection and effective vaccination. On the virus' hemagglutinin (HA) of the H3N2 IAVs, the aa
45 substitution N145K causes significant antigenic changes. We show that aa 145 displays
46 remarkable amino acid plasticity *in vitro* tolerating multiple aa substitutions, many of which have
47 not yet been observed in nature. Mutant viruses carrying substitutions at residue 145 showed no
48 major impairment on virus replication in the presence of lower receptor binding avidity.
49 However, their antigenic characterization confirmed the impact of the 145K substitution in
50 antibody immunodominance. We provide a better understanding of the functional effects of aa
51 substitutions implicated in antigenic drift and its consequences on receptor binding and
52 antigenicity. The mutation analyses presented in this report represent a significant dataset to aid
53 and test computational approaches' ability to predict binding of glycans and in antigenic
54 cartography analyses.

55 **Introduction**

56 The surface hemagglutinin (HA) glycoprotein of influenza A virus (IAV) has a pivotal
57 role in initiating the virus life cycle by binding to the virus receptor on target cells. HA binds to
58 sialic acid (SA) residues that occur as terminal monosaccharides in glycoproteins and glycolipids
59 on the cell surface. SA receptors engaged by IAV are linked to galactose (Gal) in an $\alpha 2$ -3 (SA $\alpha 2$ -
60 3Gal) or $\alpha 2$ -6 (SA $\alpha 2$ -6Gal) linkage configuration (1). Located in a small depression on the
61 globular head of HA, the receptor-binding site (RBS) is composed of the 130-loop, the 150-loop,
62 the 190-helix and the 220-loop. A series of conserved residues, including Tyr98, Ser136, Trp153,
63 and His183 (H3 numbering), forms the base of the RBS and are important for SA interaction (2,
64 3). Although some HA residues on the RBS are critical for receptor specificity (4-6), other
65 residues may influence binding by modulating virus-receptor binding avidity (7, 8).

66 IAV remains an important pathogen for humans and swine (9). While influenza vaccines
67 are commercially available, the relative effectiveness of these vaccines are heavily dependent on
68 the antigenic match of vaccine strains to circulating virus strains (10, 11). Most of the humoral
69 immune response elicited by influenza vaccination or natural exposure is directed against HA to
70 block virus infection (12, 13). Through a mechanism known as antigenic drift, IAV can
71 circumvent the preexisting antibody response by generating genetic variants during replication
72 with amino acid (aa) substitutions in key HA epitopes on the globular head, allowing for the
73 emergence of escape mutant viruses (14). Antigenic drift is a frequent cause of reduced vaccine
74 effectiveness, especially for H3N2 IAVs (15).

75 Defining the molecular basis of antigenic drift has important implications for
76 understanding IAV evolution and has been facilitated by methodological advances, such as
77 antigenic cartography (16). A recent study identified seven residues (145, 155, 156, 158, 159,
78 189 and 193) on the globular head of the HA as the major determinants of antigenic drift during
79 the evolution of human H3N2 IAVs (17). Interestingly, all seven residues were located adjacent
80 to the RBS (17), and therefore these residues may also influence receptor binding. The
81 importance of this small set of HA residues as major drivers of antigenic evolution has been
82 demonstrated for IAVs circulating in other hosts, including swine H3N2 IAVs (18).

83 Antigenic changes in human H3N2 IAVs over the course of time were shown to be
84 frequently caused by a single aa substitution in one of the seven residues, with specific
85 substitutions involved in antigenic change more than once (17). One of those substitutions,
86 N145K, was shown in two separate instances to be the major determinant of antigenic drift
87 during the evolution of human H3N2 IAVs (16, 17). While there is evidence that N145K caused
88 large antigenic changes (7, 16-18) and also altered receptor binding avidity (7), it remains

89 unclear whether there are functional constraints to substitutions at residue 145 or how alternative
90 aa substitutions at this residue may affect receptor binding and antigenic properties.

91 In the present study, we carried out site-directed mutagenesis to prepare a panel of H3
92 HA mutant viruses carrying a single aa substitution at residue 145 and subsequently evaluated
93 the impact of substitutions at this key HA residue on receptor binding and antibody recognition.
94 Our data indicates that residue 145 displayed remarkable amino acid plasticity *in vitro* tolerating
95 multiple aa substitutions, many of which have not yet been observed in nature. Mutant viruses
96 carrying substitutions at residue 145 showed no major impairment on virus replication. While all
97 substitutions retained binding to SA α 2-6Gal glycans compared to wild type, mutant viruses with
98 substitutions not commonly found in nature displayed diminished receptor binding capacity.
99 Antigenic characterization confirmed the impact of HA residue 145K in antibody
100 immunodominance. These findings have important implications for understanding virus
101 evolution and aiding the development of novel vaccine design approaches.

102 **Results**

103 ***HA residue 145 displayed remarkable aa plasticity in vitro.*** The HA N145K substitution results
104 in significant antigenic changes in both human and swine H3N2 IAVs (7, 16-18). Asparagine
105 (Asn or N) was the most prevalent aa found at residue 145 across all analyzed hosts (**Fig. 1A**). In
106 swine-origin IAVs, around 64% of isolates possessed Asn at residue 145, followed by lysine
107 (Lys or K) at ~33% and serine (Ser or S) at ~2.5%. In human-origin IAVs, about 42% of isolates
108 carried Asn, followed by Ser at ~39% and Lys at ~18%. In avian-origin IAVs, the frequency was
109 ~49% for Asn, ~45% for Ser and ~4.6% for glycine (Gly or G). In canine-origin IAVs, around
110 ~73% of isolates possessed Asn, followed by aspartic acid (Asp or D) at ~24%. In equine-origin
111 IAVs, 99% of isolates carried Asn. Isolates possessing arginine (Arg or R), histidine (His or H),

112 isoleucine (Ile or I), cysteine (Cys or C), methionine (Met or M), glutamine (Gln or Q), or
113 threonine (Thr or T) at residue 145 were found at a frequency below 1%. Alanine (Ala or A),
114 glutamic acid (Glu or E), leucine (Leu or L), phenylalanine (Phe or F), proline (Pro or P),
115 tryptophan (Trp or W), tyrosine (Tyr or Y) and valine (Val or V) were not observed in any of the
116 isolates from analyzed hosts.

117 To evaluate the impact of substitutions at residue 145, a panel of viruses carrying single
118 substitutions representing each of the 20 amino acids was generated by reverse genetics (19, 20)
119 (**Fig. 1B**). Since these viruses were generated in the context of a swine-origin H3N2 IAV (**Fig.**
120 **1A**), 145N, 145K and 145S are referred as naturally occurring substitutions whereas the
121 remaining substitutions are termed alternative or non-naturally occurring. Substitutions were
122 introduced into the HA of A/turkey/Ohio/313053/2004 (H3N2) – herein referred to as OH/04 wt
123 virus that naturally carries 145N (**Fig. 1C**). Of the 19 OH/04 HA 145 single aa mutant viruses
124 rescued, next generation sequencing analysis revealed that 12 substitutions (145A, 145C, 145G,
125 145H, 145K, 145L, 145M, 145P, 145Q, 145R, 145S and 145T) were well tolerated (98-100% of
126 sequenced reads possessed the expected codon at residue 145) after 3 passages in MDCK cells.
127 For substitutions 145F, 145I, 145V and 145Y, the percentage of reads bearing the mutated codon
128 was around 90% while the remaining 10% of reads showed a partial reversion to the wt codon
129 (I145N, V145N and Y145N) or a partial transition for a codon specifying Ser (F145S). None of
130 these HA substitutions led to compensatory substitutions on the HA or neuraminidase (NA)
131 segments. Three substitutions (145D, 145E and 145W) were not well tolerated, leading to partial
132 reversion to the wt codon (D145N) or partial transition to a codon specifying either Gly (E145G)
133 or Leu (W145L). Moreover, additional substitutions emerged on the HA for 145E and 145W

134 (T128A) or on the NA for 145E (T148I). For these reasons 145D, 145E and 145W viruses were
135 excluded from further analysis.

136 Viruses were assayed for their ability to agglutinate red blood cells (RBCs) from different
137 species (turkey, chicken, and horse) by standard hemagglutination (HA) assay. Turkey and
138 chicken RBCs are known to carry both SA α 2-3Gal and SA α 2-6Gal receptors on their cell surface
139 while horse RBCs mainly display SA α 2-3Gal (21-23). Similar to 145N (wt) virus, nearly all
140 mutant viruses were found to agglutinate turkey RBCs efficiently, with the exception of 145C
141 virus that displayed low HA titers (**Table 2**). In contrast, agglutination of chicken RBCs was less
142 consistent with HA titers of some mutant viruses comparable to that of the 145N (wt) virus while
143 other mutant viruses exhibited an 8 to 16-fold decrease in HA titers (145F, 145G, 145V and
144 145Y viruses) or no agglutination (145C virus) (**Table 2**). Only the 145F virus showed
145 detectable, albeit low, HA titers using horse RBCs (**Table 2**). There was no correlation between
146 the ability to agglutinate RBCs and virus titers. Due the decreased ability to agglutinate RBCs,
147 the 145C virus was not tested in further assays.

148 Viruses were then compared in a multiple-step infection cycle *in vitro*. MDCK cells were
149 infected at a low multiplicity of infection (MOI of 0.01). All viruses grew to high titers and
150 displayed similar growth kinetics (Fig. 2A). There was no discernable difference in peak titers
151 (~6.5 to 7.0 log₁₀ TCID₅₀/ml equivalents) at 72 hpi, with the exception of 145P and 145H viruses
152 that showed slightly lower peak titers (~5.5 log₁₀ TCID₅₀/ml equivalents, *P* <0.001). Taken
153 together, these results indicate that residue 145 shows high levels of plasticity *in vitro* despite of
154 the limited detection of aa variation in nature. Furthermore, single aa substitutions did not have a
155 major impact in growth kinetics *in vitro* but modulated the ability of mutant viruses to
156 agglutinate RBCs from particular hosts.

157 *Viruses with alternative substitutions at residue 145 retain SA α 2-6Gal binding, but frequently*
158 *displayed decreased receptor binding avidity.* To further expand on the receptor binding
159 characterization of the 145 mutants, we analyzed whether these substitutions were involved in
160 modulating receptor avidity or receptor specificity by measuring agglutination of turkey RBCs
161 previously treated with different concentrations of bacterial neuraminidase (**Fig. 2B-C**). All of
162 the mutant viruses bound to desialylated RBCs to a varying degree. Naturally occurring
163 substitutions showed the highest avidity to receptors. Binding of 145N (wt) virus was
164 indistinguishable from either the 145K (both neuraminidases) or 145S (*C. perfringens*
165 neuraminidase) viruses. Nearly all mutant viruses carrying alternative substitutions at residue
166 145 displayed decreased receptor binding avidity, with the notable exception of 145M virus that
167 showed no discernable difference in binding compared to 145N (wt) virus. Overall, the results
168 suggest differences in avidity regardless of the bacterial neuraminidases tested, although
169 specificity cannot be completely ruled out.

170 Next, we performed a glycan-based ELISA using monospecific preparations of Fet-HRP
171 as surrogates of binding to SA α 2-3Gal (3-Fet-HRP) or SA α 2-6Gal (6-Fet-HRP) (24) glycans
172 (**Fig. 3A-P**). The assay reliably discriminated receptor binding specificity as evidenced by the
173 viruses used as controls, with human pH1N1 showing a preference to SA α 2-6Gal (**Fig. 3Q**)
174 while avian Δ H5N1 displayed restricted binding to SA α 2-3Gal (**Fig. 3R**). All of the mutant
175 viruses retained binding to SA α 2-6Gal with no residual binding to SA α 2-3Gal. Naturally
176 occurring substitutions [145N (wt), 145K and 145S viruses] showed the greatest binding to
177 SA α 2-6Gal (**Fig. 3A, G and M**). Consistent with differences in avidity from previous results,
178 mutant viruses carrying alternative substitutions at residue 145 displayed weaker binding to
179 SA α 2-6Gal (**Fig. 3B-F, H-L and N-P**) compared to viruses possessing naturally occurring

180 substitutions. Among mutant viruses carrying alternative substitutions, 145M and 145P viruses
181 demonstrated the highest binding (**Fig. 3I and J**) while 145F and 145G showed substantial
182 decrease in binding to SA α 2-6Gal (**Fig. 3C and D**). Overall, these results suggest substitutions in
183 HA at residue 145 do not affect receptor specificity but modulate receptor avidity. Nearly all the
184 alternative substitutions led to decreased receptor binding avidity.

185 *Substitutions at residue 145 modulate binding to a broad range of SA α 2-6Gal glycans.* To
186 further compare the impact of substitutions at residue 145 on receptor binding specificity, glycan
187 array analysis was performed for all viruses using a glycan microarray containing linear, O-
188 linked, and N-linked glycans with extended poly-N-acetyl-lactosamine (poly-LacNAc) repeats
189 found in human, swine and ferret airway tissues (25). The array provides a broad semi-
190 quantitative view of the binding preference for specific viruses. All of the viruses retained
191 specificity to SA α 2-6Gal glycans (**Fig. 4A-P**), corroborating the results from the glycan-based
192 ELISA (**Fig. 3**). Viruses carrying naturally occurring substitutions showed expanded binding to
193 nearly all SA α 2-6Gal glycan types in the array. OH/04 wt (145N) virus had slightly preferred
194 binding to O-glycans (**Fig. 4A**) while 145K virus appeared to favor binding to N-glycans (**Fig.**
195 **4G**). 145S virus binding showed a slight reduction in binding to linear and some of the smaller
196 O-glycans (**Fig. 4M**) compared to OH/04 wt (145N) virus (**Fig. 4A**). There were distinct patterns
197 of binding for viruses carrying alternative substitutions. 145F, 145L, 145M, 145P 145Q, 145T
198 and 145V viruses demonstrated binding to a broader range of glycans that was comparable to
199 OH/04 wt (145N) virus (**Fig. 4C, H, I-K, N and O**). The 145A, 145G, 145H, 145I, 145R and
200 145Y mutant viruses displayed a more restricted binding to glycans in the array, although they
201 appear to maintain a preference to O-linked glycans (**Fig. 4B, D, E, F, L and P**). Taken together
202 with the glycan-based ELISA results, these data indicated that while certain substitutions in HA

203 at residue 145 lead to broad reductions in binding to SA α 2-6Gal glycans, many non-natural
204 variants appear to be well-tolerated, with only minor variance in receptor specificity detected.

205 ***Substitutions at residue 145 modulate sera reactivity.*** For human H3N2 IAVs, it has been
206 proposed that the emergence of the HA N145K substitution led to a change in antibody
207 immunodominance (7). To assess whether a similar phenomenon occurs in swine-origin H3
208 IAVs and how alternative substitutions at residue 145 affect antigenicity, the sera reactivity of
209 each mutant virus in the panel was tested by ELISA (**Fig. 5A-C**) and HI assays (**Fig. 6A and B**).

210 For these experiments, swine antisera against NY/11 or IA/14 wild type viruses were tested. The
211 HA1 domain of these viruses differs by only two amino acids: NY/11 virus possesses 145N/289P
212 while IA/14 carries 145K/289S. Based on its location at the base of the HA1 globular head and
213 no prior evidence of an antigenic role, we assume that position 289 is antigenically irrelevant.

214 Additionally, swine antisera against OH/04 wt virus were tested. To prevent confounding effects,
215 the HA gene segment of the NY/11 and IA/14 viruses were rescued by reverse genetics in the
216 background of 7 gene segments from the OH/04 wt virus. The 1+7 NY/11 and IA/14 reverse
217 genetics viruses were used as controls in ELISA and HI assays.

218 Reactivity of sera generated against the OH/04 wt virus was similar across the entire
219 panel of OH/04 145 single aa mutant viruses (**Fig. 5A**), indicating substitutions at residue 145 in
220 the context of OH/04 HA do not change antigenicity against wt homologous sera. Consistent
221 with amino acid divergence between OH/04 wt and control viruses, OH/04 wt virus antisera had
222 reduced reactivity to both NY/11 and IA/14 control viruses. Surprisingly, sera raised against the
223 NY/11 virus displayed no discernible difference in reactivity among all of the virus evaluated
224 regardless of the 145 aa substitution, including the NY/11 and IA/14 viruses (**Fig. 5B**). As
225 observed for human H3N2 IAVs, the presence of 145K in a swine-origin IAV results in an

226 immunodominant epitope targeted by the antibody response (**Fig. 5C**). Sera generated against the
227 IA/14 virus showed decreased reactivity to the NY/11 virus (possessing HA residue 145N)
228 compared to the IA/14 virus (carrying HA residue 145K). Nearly all of the OH/04 145 single aa
229 mutant viruses, with the exception of 145K virus, exhibited diminished reactivity to the IA/14
230 virus antisera. The 145K virus was the only mutant virus to consistently show reactivity more
231 similar to homologous IA/14 sera generated against the IA/14 virus, confirming the impact of
232 HA residue 145K in modulating antibody immunodominance.

233 Antigenic characterization by HI assay was mostly consistent with the ELISA data. The
234 OH/04 wt virus antisera reacted to all OH/04 145 single aa mutant viruses. Relative to the 145N
235 (wt) virus, changes in HI titers were almost indistinguishable (**Fig. 6A and B**). The OH/04 wt
236 virus antisera had a large reduction in cross-HI reactivity against the IA/14 virus and only a
237 modest reduction against the NY/11 virus. Similar to the ELISA data, most substitutions at
238 residue 145 minimally affected NY/11 virus antisera reactivity, with the 145K virus causing the
239 largest reduction in cross-HI titers. Reactivity of the IA/14 virus antisera was drastically reduced
240 against nearly all of the OH/04 145 single aa mutant viruses. In agreement with previous results,
241 the 145K virus was the only mutant virus to show minimal reduction in sera cross-reactivity
242 compared to IA/14 virus. Collectively, these results indicate that residue 145K has a profound
243 impact in modulating antibody immunodominance and that sera raised against a 145K bearing
244 virus can recognize the epitope bearing such substitution in the context of a distinct H3 HA.

245 **Discussion**

246 HA engagement with terminal SA residues on host cells is an essential step in the IAV's
247 replication cycle. Not surprisingly, receptor-binding specificity is a major host range restriction
248 factor. In a simplistic view, IAVs of avian origin prefer binding to SA α 2-3Gal glycans whereas

249 those that circulate in humans favor binding to SA α 2-6Gal receptors (5, 26-28). During human
250 adaptation of avian-origin IAVs, receptor specificity switch is accompanied by specific
251 substitutions on the HA (E190D/G225D in the H1 subtype and Q226L/G228S in the H2 and H3
252 subtypes) (5, 26, 29, 30). While these residues are critical to receptor binding specificity, other
253 residues on the HA have the potential to affect receptor binding functions, including receptor
254 binding avidity (7, 8, 23, 31). The identification of up to 7 residues (145, 155, 156, 158, 159, 189
255 and 193) near the RBS as the major determinants of antigenic drift during the evolution of
256 human and swine H3N2 IAVs (17, 18, 32) has led to the hypothesis that emerging substitutions
257 in these residues must drive antigenic change and immune escape without disrupting receptor
258 binding properties, potentially limiting the flexibility of aa residues in these positions (17, 33). In
259 agreement with this hypothesis, analysis of HA sequences showed the occurrence of only a small
260 subset of aa substitutions at these residues that are recycled sporadically (17, 18, 32, 34, 35).

261 Here we examined the amino acid plasticity at residue 145, one of the identified key
262 determinants of antigenic drift, by mutating this residue to create a panel of H3 HA mutant
263 viruses carrying a single aa substitution representing every possible amino acid in a swine H3N2
264 backbone. HA residue 145 showed extraordinary plasticity with 16 out of 19 substitutions
265 (145A, 145C, 145F, 145G, 145H, 145I, 145K, 145L, 145M, 145P, 145Q, 145R, 145S, 145T,
266 145V and 145Y) being well tolerated *in vitro* (90-100% of sequenced reads possessing the
267 mutated codon at residue 145). Whether these substitutions are tolerated *in vivo* is beyond the
268 scope of the present report and remain to be determined. Consistent with the intricate balance
269 between immune escape and receptor binding, three substitutions (145D, 145E and 145W) were
270 not tolerated and led to the emergence of partial reversion at residue 145 and additional
271 substitutions in either HA (T128A) and/or NA (T148I). HA T128A disrupted a potential

272 glycosylation site (36, 37) and was shown to emerge along with a substitution at residue 145 in
273 human H3N2 IAVs during the 2013-2014 season (34). NA T148I has been associated with
274 reduced NA activity, decreased susceptibility to neuraminidase inhibitors, and NA-mediated
275 binding and agglutination (38, 39). The previously reported role of these substitutions suggests
276 they may be compensatory.

277 With the exception of 145C virus, there was no discernible difference in the ability of
278 tested mutant viruses to agglutinate turkey RBCs. Turkey and chicken RBCs carry both SA α 2-
279 3Gal and SA α 2-6Gal glycans on their cell surface (21-23), and some of tested mutant viruses
280 exhibited impaired ability to agglutinate chicken RBCs. Loss of the ability to agglutinate RBCs
281 has plagued the antigenic characterization of recent human H3N2 IAVs (40). As previously
282 reported for chicken RBCs (41), an in-depth analysis of major glycan structures present on RBCs
283 from other hosts will help shed light to current issues on antigenic characterization of IAVs.

284 Using independent assays, we demonstrated that all tested mutant viruses retained some
285 binding to SA α 2-6Gal glycans. However, viruses possessing naturally occurring substitutions
286 [145N (wt), 145K and 145S] showed the strongest receptor binding. With the exception of 145M
287 virus, all viruses carrying alternative substitutions (145A, 145C, 145F, 145G, 145H, 145I, 145L,
288 145P, 145Q, 145R, 145T, 145V and 145Y) displayed decreased ability to bind SA α 2-6Gal
289 receptors. In contrast to other reports, no compensatory substitutions were identified on the HA
290 or NA by next-generation sequencing (42, 43). This indicates that the observed changes in
291 receptor binding reported here are directly related to substitutions at residue 145. It is important
292 to emphasize that although viruses carrying alternative substitutions exhibited decreased receptor
293 binding avidity *in vitro*, the lower threshold for biologically relevant avidity *in vivo* is unknown.
294 Glycan array analysis revealed a broad range of SA α 2-6Gal glycan interactions that were

295 modulated by substitutions at residue 145. Interestingly, one of the viruses with the lowest
296 binding to 6-modified fetuin glycans in the panel (145F virus) displayed extensive binding to
297 SA α 2-6Gal glycans, including extended glycans (25). While the expanded glycan array provides
298 important insights on the binding preference, which receptors are relevant for IAV attachment
299 and transmission *in vivo* remain to be elucidated. Furthermore, the density, distribution and
300 organization of glycans on host tissues are poorly defined.

301 As observed in human H3N2 IAVs, the emergence of the HA N145K substitution in
302 swine H3N2 IAVs led to a change in the antibody immunodominance. It is astonishing that
303 reactivity to sera raised against a virus possessing HA 145N was indistinguishable among all of
304 the viruses carrying substitutions at residue 145, but reactivity to sera raised against a virus
305 possessing HA 145K was highly skewed to recognize an epitope bearing HA residue 145K even
306 in the context of a distantly related H3 HA, demonstrating the dramatic impact N145K mutants
307 can have in population immunity. Antibody immunodominance may be key for understanding
308 antigenic drift and refers to the immunological phenomenon in which the immune system
309 preferentially mounts a response to complex antigens in a dynamic hierarchical order (14).
310 Immunodominance hierarchy can occur at the level of viruses within multivalent immunogens,
311 proteins within viruses, antigenic sites within proteins, epitopes within antigenic sites and, as
312 showed in the present report and by others, single aa substitutions within epitopes (7, 14, 16, 17,
313 44, 45). The impact of substitutions at residue 145 on immunogenicity and antibody
314 immunodominance remains to be fully characterized. While there may be a fitness cost
315 associated with the emergence of such substitutions in nature, the ability to manipulate the aa
316 plasticity near the RBS may offer an alternative approach to induce broader protection and,
317 perhaps, potentially elicit receptor mimicry, broad-spectrum neutralizing antibodies (46).

318 The complex and continuous antigenic evolution of IAVs remains a major hurdle for
319 vaccine selection and effective vaccination. We provide a better understanding of the functional
320 effects of aa substitutions near the RBS implicated in antigenic drift and its consequences on
321 receptor binding. The mutation analyses presented in this report represent a significant dataset to
322 aid and test computational approaches' ability to predict binding of glycans.

323 **Materials and Methods**

324 *Cell culture.* Madin-Darby canine kidney (MDCK) and human embryonic kidney 293T cells
325 were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10%
326 fetal bovine serum (FBS). Cells were propagated at 37°C in a humidified incubator under 5%
327 CO₂ atmosphere.

328 *Molecular cloning and virus rescue.* The OH/04 wt strain is a prototypic swine-origin virus
329 amenable to genetic manipulation by an established reverse genetics (RG) system (18, 47, 48).
330 Single aa substitutions representing each of the 20 naturally occurring amino acids (**Table 1**)
331 were inserted by site-directed mutagenesis at the codon corresponding to HA residue 145 (H3
332 numbering). To prevent the carryover of OH/04 wt HA plasmid DNA during PCR amplification,
333 the cloned OH/04 wt HA segment was split into two overlapping plasmids: pDP-SD1 and pDP-
334 SD2. The pDP-SD1 plasmid carries the mouse RNA polymerase I terminator (t1) (20) followed
335 by nucleotides 1-522 of OH/04 wt HA segment. The pDP-SD2 plasmid contains nucleotides
336 500-1762 of OH/04 wt HA segment followed by the human RNA polymerase I promoter (polI)
337 (20). To introduce the desired mutations into OH/04 wt HA, a PCR fragment containing t1
338 followed by the 5' portion of the HA was generated with forward primer 5'-ACC GGA GTA
339 CTG GTC GAC CTC CGA AGT TGG GGG GGA GCA AAA GCA GG-3' and the respective
340 reverse primer (**Table 1**) using pDP-SD1 as template. Similarly, a PCR fragment comprising the

341 3' portion of HA followed by polyI was amplified from pDP-SD2 using reverse primer 5'-ATG
342 CTG ACA ACG TCC CCG GCC CGG CGC TGC T-3' and the respective forward primer
343 (**Table 1**). All PCR products were purified by gel extraction using QIAquick Gel Extraction Kit
344 (Qiagen, Valencia, CA) and combined to produce RG-ready PCR-based HA segments for
345 individual OH/04 HA 145 single aa mutants by overlapping PCR as previously described (19).
346 All PCR reactions were performed with Phusion High Fidelity DNA polymerase (New England
347 Biolabs, Ipswich, MA) and confirmed to be free of unwanted mutations by sequencing. Viruses
348 were rescued by PCR-based RG using a co-culture of 293T/MDCK cells as previously described
349 (19, 20). To generate OH/04 HA 145 single aa mutant viruses, the respective RG PCR-based HA
350 segment was paired with the seven plasmids representing the remaining OH/04 wt gene
351 segments. Following transfection, cells were incubated at 35°C. After 24 h incubation, media
352 was replaced with Opti-MEM I (Life Technologies, Carlsbad, CA) containing 1 µg/mL TPCK-
353 trypsin (Worthington Biochemicals, Lakewood, NJ) and 1% antibiotics/antimycotic solution
354 (Sigma-Aldrich, St. Louis, MO). Following virus rescue, virus stocks were amplified in MDCK
355 cells. Virus stocks were titrated by tissue culture infectious dose 50 (TCID₅₀) and virus titers
356 were determined by the Reed and Muench method (49).

357 *Whole-genome sequencing.* Virus RNA from tissue culture supernatant virus stocks were
358 purified using the RNeasy mini kit (Qiagen, Valencia, CA) or MagNA Pure LC RNA Isolation
359 Kit (Roche Life Science, Mannheim, Germany). Isolated virus RNA served as template in a one-
360 step reverse transcriptase PCR (RT-PCR) reaction for multi-segment, whole genome
361 amplification (50). Amplicon sequence libraries were prepared as previously described (50) or
362 using Nextera XT DNA Library Prep Kit (Illumina, San Diego, CA) according to the
363 manufacturer's protocol. Barcoded libraries were multiplexed and sequenced on the high-

364 throughput Illumina MiSeq sequencing platform in a paired-end 150 nt run format. De novo
365 genome assembly was performed as described previously (50) and HA and NA-specific reads
366 were mapped to OH/04 reference sequences using Geneious 10.1.3 (51).

367 *In vitro growth kinetics.* Confluent monolayers of MDCK cells were inoculated at 0.01
368 multiplicity of infection (MOI) for each virus. After 1 h incubation, virus inoculum was
369 removed, and cells washed twice with 1X phosphate-buffered saline (PBS). Then, Opti-MEM I
370 (Life Technologies, Carlsbad, CA) containing TPCCK-trypsin (Worthington Biochemicals,
371 Lakewood, NJ) and antibiotics/antimycotic solution (Sigma-Aldrich, St. Louis, MO) was added
372 to the cells. At indicated time points, tissue culture supernatant from inoculated cells was
373 collected for virus titer quantification. Virus RNA from tissue culture supernatant was isolated
374 using the MagMAX-96 AI/ND Viral RNA Isolation Kit (Thermo Fisher Scientific, Waltham,
375 MA). Virus titers were determined using a real-time reverse transcriptase PCR (rRT-PCR) assay
376 based on the influenza A matrix gene (52). The rRT-PCR was performed in a LightCycler 480
377 Real Time PCR instrument (Roche Diagnostics, Rotkreuz, Switzerland) using the LightCycler
378 480 RNA Master Hydrolysis Probes kit (Roche Life Science, Mannheim, Germany). A standard
379 curve was generated using 10-fold serial dilutions from an OH/04 wt virus stock of known titer
380 to correlate qPCR crossing point (Cp) values with virus titers, as previously described (53). Virus
381 titers were expressed as \log_{10} TCID₅₀/ml equivalents.

382 *Hemagglutination (HA) assay.* Chicken (Poultry Diagnostic and Research Center, Athens, GA),
383 turkey (Poultry Diagnostic and Research Center, Athens, GA) and horse (Lampire Biologicals,
384 Pipersville, PA) red blood cells (RBCs) were prepared from whole blood preparations using
385 standard techniques (54). Virus HA assays were carried out using 0.5% (vol/vol in PBS) chicken
386 RBCs, 0.5% (vol/vol in PBS) turkey RBCs or 1% (vol/vol in PBS) horse RBCs. Briefly, 50 μ l of

387 RBC preparations was added to 50 μ l of two-fold serial dilutions of virus stocks allowed to
388 incubate at room temperature for 45 min. After incubation agglutination was measured, and data
389 expressed as the inverse of the highest dilution that allowed full agglutination.

390 *Receptor cell binding assay.* The receptor cell binding assay was performed as previously
391 described (8, 18, 55). Briefly, 10% (vol/vol in PBS) turkey RBCs were pretreated for 1 h at 37°C
392 with two-fold serial dilutions of bacterial neuraminidase from *Arthrobacter ureafaciens* (New
393 England BioLabs, Ipswich, MA) or *Clostridium perfringens* (New England BioLabs, Ipswich,
394 MA). Following neuraminidase treatment, treated RBCs were then washed twice with cold PBS,
395 and then resuspended to 1% (vol/vol in PBS). 50 μ l of 1% RBCs treated with the different
396 neuraminidase concentrations were added to 50 μ l of each virus (8 HAU, as determined on
397 untreated RBCs) and allowed to incubate at room temperature for 45 min. After incubation
398 agglutination was measured, and data expressed as the maximal concentration of neuraminidase
399 that allowed full agglutination.

400 *Solid-phase assay of receptor binding specificity.* The receptor-binding specificity was
401 determined in a solid phase direct binding assay using monospecific preparations of peroxidase
402 (HRP)-conjugated fetuin (fet-HRP). Monospecific preparations of fet-HRP were synthesized
403 using α 2-3-sialyltransferase from *Pasteurella multocida* (Sigma, St. Louis, MO) for 3-modified
404 fetuin (3-fet-HRP) or α 2-6-sialyltransferase from *Photobacterium damsela* (Sigma, St. Louis,
405 MO) for 6-modified fetuin (6-fet-HRP), essentially as described previously (24). 96-well native
406 fetuin-coated flat-bottom plates (Greiner Bio-One, Monroe, NC) were incubated overnight at 4°C
407 with 128 HAU of each virus in 0.02 M tris-buffered saline (TBS), pH 7.2–7.4. Virus samples
408 were run in duplicate. Plates were washed three times with PBS and blocked with blocking
409 solution [BS, PBS containing 0.1% neuraminidase-treated bovine serum albumin (BSA-NA)] for

410 2 h at room temperature. After blocking, plates were washed twice with ice-cold washing
411 solution (WS, PBS containing 0.02% Tween 80) and incubated with two-fold serial dilution of 3-
412 Fet-HRP (SA α 2-3Gal) or 6-Fet-HRP (SA α 2-6Gal) in reaction solution (RS, PBS containing
413 0.02% Tween-80, 0.1% BSA-NA and 2 μ M oseltamivir carboxylate) for 1 h at 4°C. After
414 incubation, plates were washed five times with ice-cold WS before adding freshly prepared
415 substrate solution (SS, 0.01% 3,3',5,5'-tetramethylbenzidine in 0.05 M sodium acetate with
416 0.03% H₂O₂). Reactions were allowed to proceed at room temperature for 30 min unless
417 otherwise stated. Reactions were stopped with 3% (vol/vol in ddH₂O) H₂SO₄. Absorbance
418 readings were obtained at 450 nm using a Victor x3 Multilabel Plate Reader (PerkinElmer,
419 Waltham, MA).

420 *Glycan array analysis.* Virus stocks were grown in MDCK cells, clarified by low-speed
421 centrifugation, and inactivated by treatment with 0.1% β -propiolactone (BPL) for 1 day at 4°C.
422 Inactivated virus stocks were concentrated as described previously (56). Concentrated virus
423 stocks were resuspended in PBS with 5% glycerol to HAU, aliquoted and stored at -80°C. HA
424 titers of concentrated virus stocks were determined by HA assay using 0.5% (vol/vol in PBS)
425 turkey RBCs. Glycan array analysis was performed using an NHS ester-coated glass microarray
426 slide containing six replicates of 128 diverse synthetic sialic acid-containing glycans, including
427 terminal sequences as well as N-linked and O-linked glycans found on mammalian and avian
428 glycoproteins and glycolipids (25). Whole influenza virus samples were diluted to 256 HAU in
429 PBS containing 3% BSA (PBS-BSA) and incubated on the array surface for 1 h at room
430 temperature in a humidity-controlled chamber. After incubation, slides were washed in PBS and
431 incubated with OH/04 wt swine antisera (18) diluted 1:200 in PBS-BSA for 1 h. Slides were
432 washed in PBS and incubated for 1 h in goat anti-pig IgG conjugated to fluorescein

433 isothiocyanate (FITC; Thermo Fisher Scientific, Waltham, MA) diluted in PBS-BSA (20 µg/ml
434 final concentration). Slides were washed twice in PBS, and in dH₂O, then dried prior to
435 detection. Slide scanning to detect bound virus was conducted using an InnoScan 1100AL
436 (Innopsys, Carbonne, France) fluorescent microarray scanner. Fluorescent signal intensity was
437 measured using Mapix (Innopsys, Carbonne, France) and mean intensity minus mean
438 background of 4 replicate spots was calculated. A complete list of the glycans present in the
439 array is presented in **Supplementary Table 1**. The array is comprised of non-sialoside control
440 (1-10; Grey), SA α 2-3Gal (11-76; Yellow) and SA α 2-6Gal (77-128; Green) glycans. Glycans are
441 grouped by structure type: L, linear; O, O-linked; N, N-linked and L^x, sialyl Le^x.

442 *Antisera*. Swine antisera against A/swine/New York/A01104005/2011 (H3N2) [NY/11],
443 A/swine/Iowa/A01480656/2014 (H3N2) [IA/14] and A/turkey/Ohio/313053/2004 (H3N2)
444 [OH/04] were generated in previous studies (18, 32). For each virus, two pigs were primed and
445 boosted intramuscularly with UV-inactivated whole virus vaccine prepared with commercial oil-
446 in-water adjuvant (1:5 ratio; Emulsigen D, MVP Laboratories, Inc., Ralston, NE). The pigs were
447 humanely euthanized for blood collection.

448 *Hemagglutination inhibition (HI) assay*. HI assays were performed as previously described (54).
449 Prior to HI testing, swine antisera were treated overnight with receptor-destroying enzyme
450 (Denka Seiken, Tokyo, Japan) and heat inactivated at 56°C for 30 min. Serial two-fold dilutions
451 starting at 1:10 were tested for the ability to inhibit the agglutination of 0.5% turkey RBCs with 4
452 HAU of each virus. HI titers were recorded as the inverse of the highest dilution that inhibited
453 hemagglutination.

454 *Enzyme-linked immunosorbent assay (ELISA)*. For ELISA, 96-well flat-bottom plates (Greiner
455 Bio-One, Monroe, NC) were incubated overnight at 4°C with 16 HAU of each virus in 1X

456 coating solution (Seracare, Milford, MA). Virus samples were run in duplicate. Plates were
457 blocked with StartingBlock (PBS) blocking buffer (Thermo Fisher Scientific, Waltham, MA) for
458 1 h at room temperature. After blocking, plates were washed three times with PBS containing
459 0.05% tween 20 (PBS-T). Two-fold serial dilutions of swine antisera were added and allowed to
460 incubate for 1 h at room temperature. After incubation, plates were washed three times with
461 PBS-T before adding the secondary goat anti-swine IgG-HRP HRP-conjugated polyclonal
462 antibody (Seracare, Milford, MA). Plates were incubated at room temperature for 1 h. After
463 incubation, plates were washed three times with PBS-T before adding freshly prepared SS buffer
464 or 2, 2'-azino-di(3-ethylbenzthiazoline-6-sulfonate) [ABTS] 1-component microwell peroxidase
465 substrate (Seracare, Milford, MA) for 1 h at room temperature. The reaction was stopped by
466 adding 3% (vol/vol in ddH₂O) H₂SO₄ or ABTS peroxidase stop solution (Seracare, Milford,
467 MA), respectively. Absorbance readings were obtained at 405 nm using a Victor x3 Multilabel
468 Plate Reader (PerkinElmer, Waltham, MA).

469 *Statistical analysis.* All statistical analyses were performed using the GraphPad Prism Software
470 Version 7 (GraphPad Software Inc., San Diego, CA). For multiple comparisons, one-way or two-
471 way ANOVA followed by a post-hoc Tukey test were performed. When indicated, a *P* value
472 below 0.05 ($P < 0.05$) was considered significant.

473 *Structure modeling.* A model of the structure of the HA of A/turkey/Ohio/313053/2004 (H3N2)
474 was built by homology modeling using Modeller v9.16 (57) based upon the crystal structure of
475 multiple H3 HA proteins [Protein Data Bank (PDB) codes 2YP7, 1HA0, 2YP2, 4WE8 and
476 4WE5). The generated model was subsequently rendered with PyMOL v2.1 (58).

477 *Computational analysis of HA sequences.* The frequency distribution of aa identities at residue
478 145 (H3 numbering) was computed using the sequence variation analysis tool in the Influenza

479 Research Database (IRD) (59). HA aa sequences from IAVs of the H3 subtype isolated from
480 swine, human, avian, canine and equine hosts and publicly available in the IRD as of September
481 06, 2017 were analyzed. For humans, the frequency was calculated from precomputed data in the
482 IRD database. Only amino acids that reached a frequency of at least 1% are labeled in the plot
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- 691

692 **Figure legends**

693 **Figure 1. Amino acid plasticity at residue 145.** (A) Publicly available H3 HA sequences were
694 retrieved and the relative frequency of identified amino acids at residue 145 were calculated for
695 swine, human, avian, canine and equine AIVs. Amino acids present at frequency below 1% are
696 not labeled in the figure. (B) Schematic representation of the HA gene segment of
697 A/turkey/Ohio/313053/2004 (H3N2) depicting the codon corresponding to residue 145 and
698 respective amino acid substitutions introduced by site-directed mutagenesis. (C) HA monomeric
699 structure of A/turkey/Ohio/313053/2004 (H3N2) indicating the location of HA residue 145.

700 **Figure 2. Substitutions at residue 145 show no major impact on virus growth but decrease**
701 **receptor binding avidity.** A) Confluent monolayers of MDCK cells were inoculated with H3
702 viruses carrying amino acid substitutions at residue 145 at a MOI of 0.01 and incubated at 37°C.
703 At 6, 12, 24, 48, and 72 hpi, tissue culture supernatants from inoculated cells were collected for
704 virus RNA quantification by rRT-PCR and expressed as log₁₀ TCID₅₀/ml equivalents. Plotted
705 data represent means ± standard errors (SD). Turkey red blood cells pretreated with different
706 amounts of neuraminidase from either (B) *Clostridium perfringens* or (C) *Arthrobacter*
707 *ureafaciens* were mixed with H3 viruses carrying amino acid substitutions at residue 145 to
708 quantify virus agglutination as measure of virus binding avidity. Data are expressed as the
709 maximal amount of neuraminidase that allowed full agglutination. (B) and (C) are representative
710 data of one out of 2 and 3 independent experiments, respectively, with samples run in duplicates
711 in each experiment. Plotted data represent means ± standard errors (SD).

712 **Figure 3. Substitutions at residue 145 retain binding to SA α 2-6Gal.** H3 viruses carrying
713 amino acid substitutions at residue 145 were tested for receptor binding specificity with varying
714 concentrations of SA α 2-3Gal (α 2-3-linked SA) or SA α 2-6Gal (α 2-6-linked SA). (A) 145N (wt),

715 (B) 145A, (C) 145F, (D) 145G, (E) 145H, (F) 145I, (G) 145K, (H) 145L, (I) 145M, (J) 145P, (K)
716 145Q, (L) 145R, (M) 145S, (N) 145T, (O) 145V and (P) 145Y viruses. (Q) Human pH1N1 and
717 (R) avian Δ H5N1 were used as binding control to SA α 2-6Gal and SA α 2-3Gal, respectively.
718 Glycan concentration is expressed as arbitrary units (AU). Plotted data represent means \pm
719 standard errors (SD). Data is representative of 2 independent experiments with 2 replicates per
720 experiment.

721 **Figure 4. Substitutions at residue 145 modulate binding to a broad range of SA α 2-6Gal**
722 **glycans.** Glycan microarray analysis of H3 viruses carrying amino acid substitutions at residue
723 145. The array is comprised of non-sialoside control (1-10; Grey), SA α 2-3Gal (11-76; Yellow)
724 and SA α 2-6Gal (77-128; Green) glycans. Glycans are grouped by structure type: L, linear; O, O-
725 linked; N, N-linked and L^x, sialyl Le^x. (A) 145N (wt), (B) 145A, (C) 145F, (D) 145G, (E) 145H,
726 (F) 145I, (G) 145K, (H) 145L, (I) 145M, (J) 145P, (K) 145Q, (L) 145R, (M) 145S, (N) 145T, (O)
727 145V and (P) 145Y viruses. Plotted data represent means \pm standard errors (SD) using four
728 replicates per virus. RFU, relative fluorescent units.

729 **Figure 5. Substitutions at residue 145 modulate sera reactivity.** Antibody responses to H3
730 viruses carrying amino acid substitutions at residue 145 was determined by ELISA using swine
731 antisera generated against (A) OH/04, (B) NY/11 possessing HA residue 145N or (C) IA/14
732 possessing HA residue 145K. Two sets of sera were tested independently. The first set of sera
733 was tested 2 times in duplicates. The second set of sera was tested only once in duplicate. Plotted
734 data represent means \pm standard errors (SD). O.D., optical density.

735 **Figure 6. Substitutions at residue 145 impact HI titers.** HI titers were measured against H3
736 viruses carrying amino acid substitutions at residue 145 using swine antisera generated against
737 OH/04 (cyan), NY/11 possessing HA residue 145N (red) or IA/14 possessing HA residue 145K

738 (light green). (A and B) two sets of sera were tested independently. The first set of sera was
739 tested 3 times in duplicates. Confirmatory tests were run on a second set of sera, which tested
740 once in duplicates. Sera reactivity of all H3 mutant viruses to the respective swine antisera are
741 depicted as fold change $[(\log_2 \text{ HI titer mutant virus})/(\log_2 \text{ HI titer homologous virus})]$. Plotted
742 data represent means \pm standard errors (SD). Colors are based on the antigenic cluster
743 designation for swine H3N2 IAVs.

Table 1. Primers used to introduce amino acid substitutions at residue 145

aa at position 145	Forward primer sequence (5'-3')*	Reverse primer sequence (5'-3')*
A	GGAATCTGTT G CTAGTTTCTTTAGTAGATT	TAAAGAAACTAGCAACAGATTCCTTCT
C	GGAATCTGTT TG TAGTTTCTTTAGTAGATT	TAAAGAAACTACAAACAGATTCCTTCT
D	GGAATCTGTT GAT AGTTTCTTTAGTAGATT	TAAAGAAACTATCAACAGATTCCTTCT
E	GGAATCTGTT GAA AGTTTCTTTAGTAGATT	TAAAGAAACTTTCAACAGATTCCTTCT
F	GGAATCTGTT TTT TAGTTTCTTTAGTAGATT	TAAAGAAACTAAAAACAGATTCCTTCT
G	GGAATCTGTT GGA AGTTTCTTTAGTAGATT	TAAAGAAACTTCCAACAGATTCCTTCT
H	GGAATCTGTT CAT AGTTTCTTTAGTAGATT	TAAAGAAACTATGAACAGATTCCTTCT
I	GGAATCTGTT TATA AGTTTCTTTAGTAGATT	TAAAGAAACTTATAACAGATTCCTTCT
K	GGAATCTGTT AAA AGTTTCTTTAGTAGATT	TAAAGAAACTTTTAACAGATTCCTTCT
L	GGAATCTGTT CTC AGTTTCTTTAGTAGATT	TAAAGAAACTGAGAACAGATTCCTTCT
M	GGAATCTGTT ATG AGTTTCTTTAGTAGATT	TAAAGAAACTCATAACAGATTCCTTCT
P	GGAATCTGTT CCA AGTTTCTTTAGTAGATT	TAAAGAAACTTGGAACAGATTCCTTCT
Q	GGAATCTGTT CAA AGTTTCTTTAGTAGATT	TAAAGAAACTTTGAACAGATTCCTTCT
R	GGAATCTGTT CGG AGTTTCTTTAGTAGATT	TAAAGAAACTCCGAACAGATTCCTTCT
S	GGAATCTGTT AGC AGTTTCTTTAGTAGATT	TAAAGAAACTGCTAACAGATTCCTTCT
T	GGAATCTGTT ACA AGTTTCTTTAGTAGATT	TAAAGAAACTTGTAACAGATTCCTTCT
V	GGAATCTGTT GTC AGTTTCTTTAGTAGATT	TAAAGAAACTGACAACAGATTCCTTCT
W	GGAATCTGTT TGG AGTTTCTTTAGTAGATT	TAAAGAAACTCCAAACAGATTCCTTCT
Y	GGAATCTGTT TAT AGTTTCTTTAGTAGATT	TAAAGAAACTATAAACAGATTCCTTCT

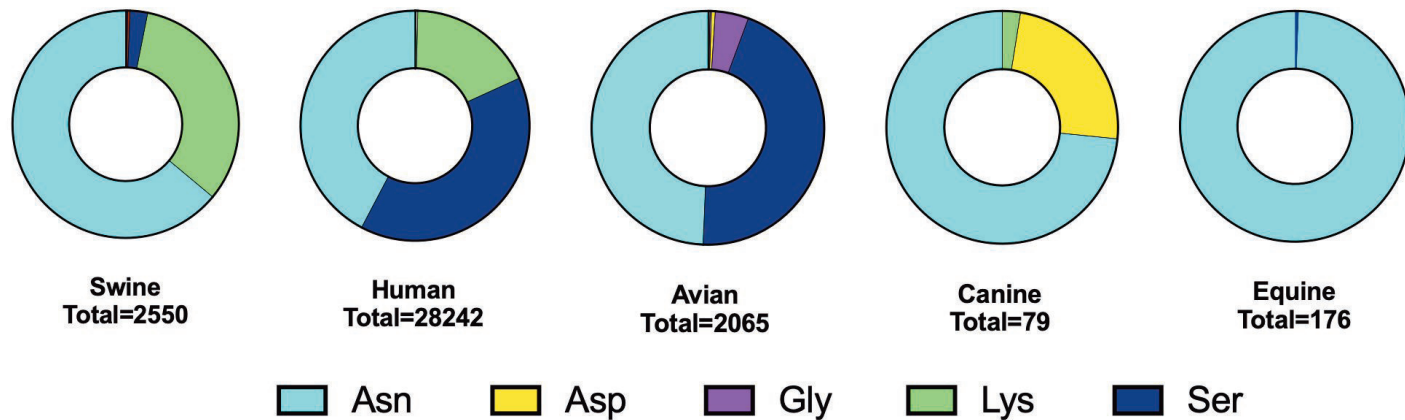
*Bold nucleotides indicate the mutated codon associated to HA residue 145.

745

746

Table 2. Agglutination of erythrocytes by OH/04 145 single aa mutant viruses

aa at position 145	HA titer (HAU)		
	0.5% turkey RBCs	0.5% chicken RBCs	1% horse RBCs
N (wt)	128	256	<2
A	128	64	<2
C	4	<2	<2
F	128	16	8
G	128	32	<2
H	256	128	<2
I	128	64	<2
K	128	128	<2
L	128	128	<2
M	128	128	<2
P	128	64	<2
Q	256	128	<2
R	128	64	<2
S	128	128	<2
T	128	64	<2
V	128	32	<2
Y	128	32	<2

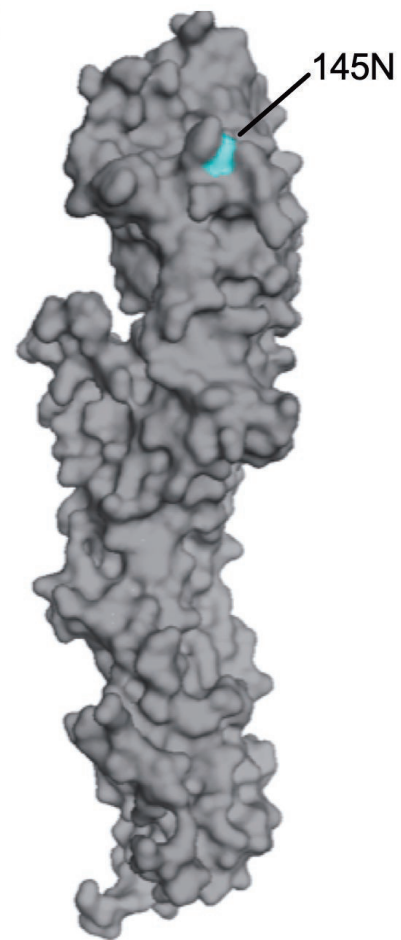
A**B**

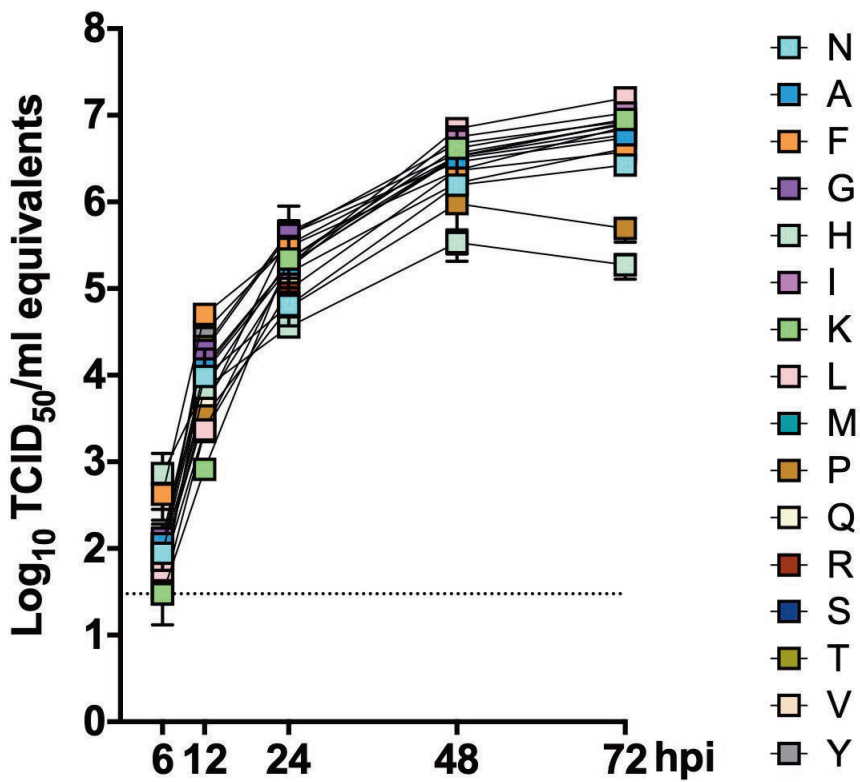
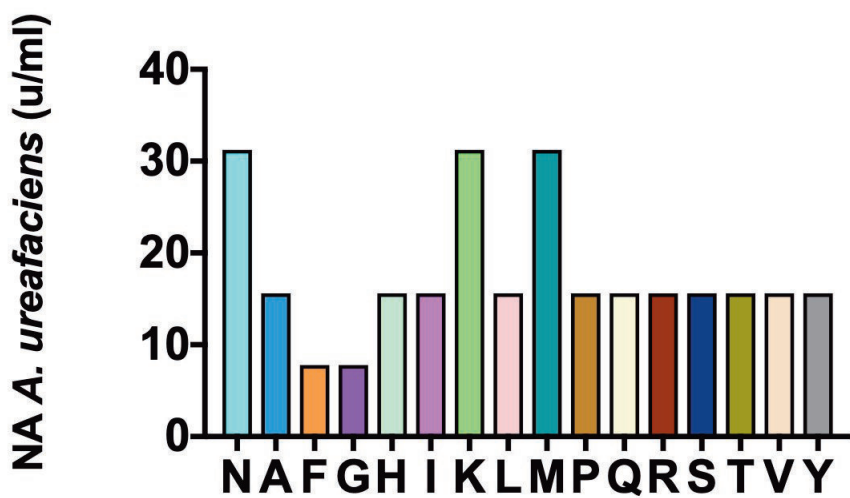
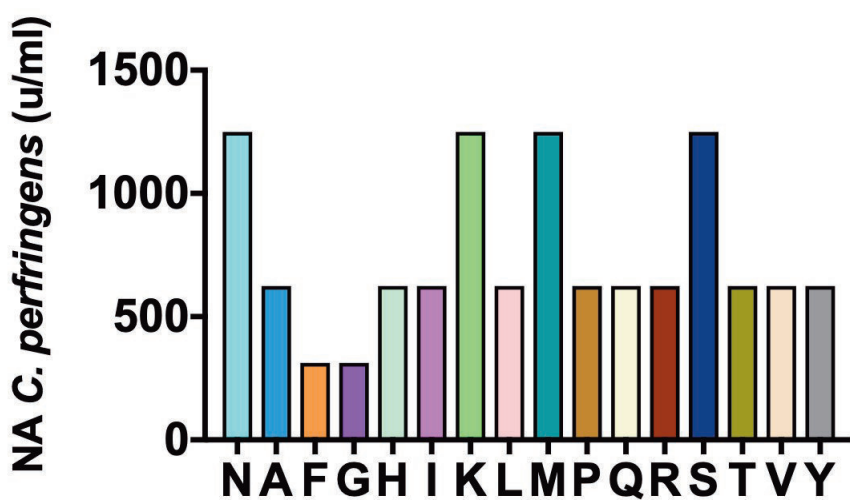
A/turkey/Ohio/303153/2004(H3N2), OH/04

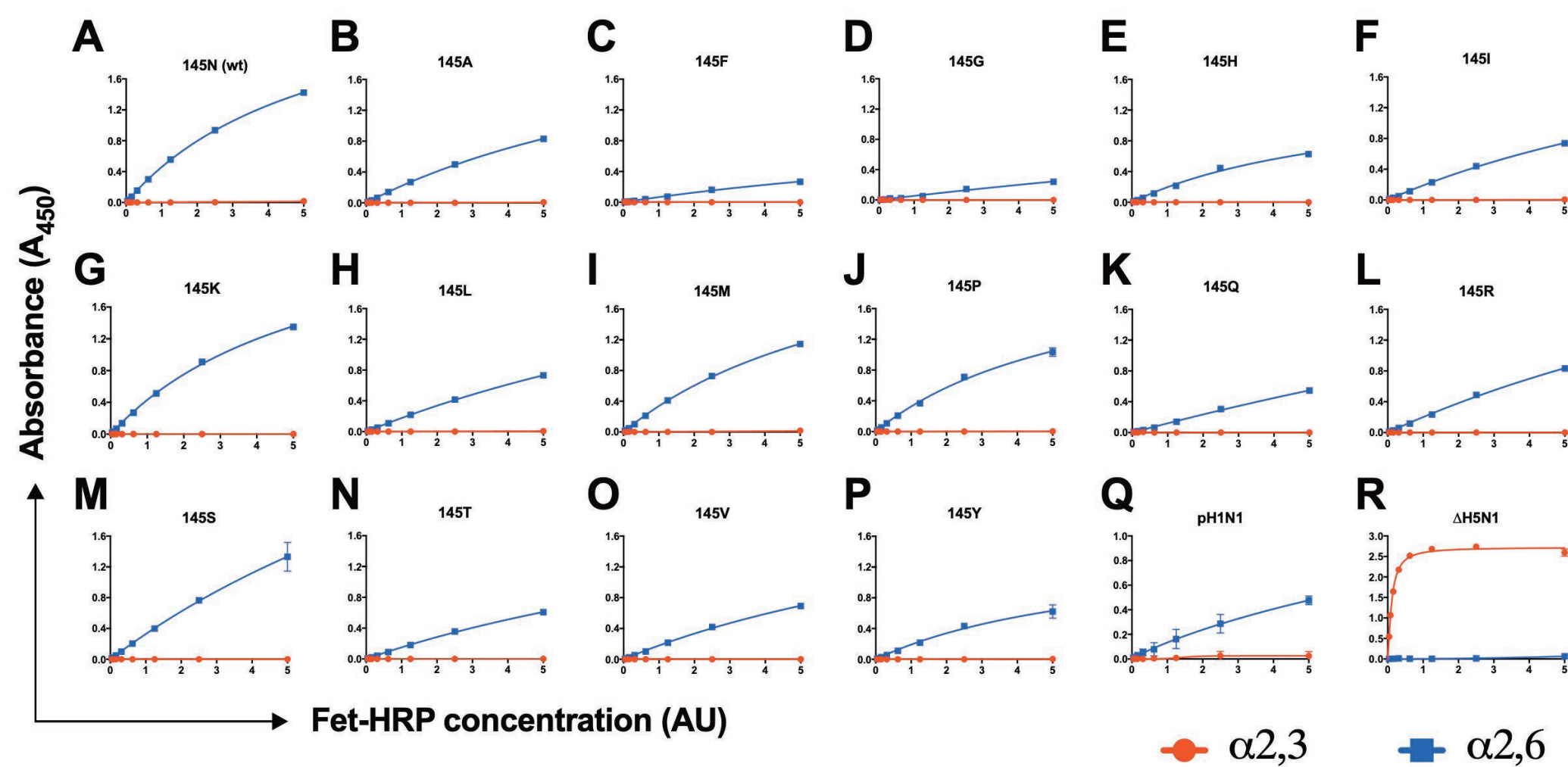


145
5' AAC 3'
Asn

Ala Arg Asp Cys Gln Glu Gly His Ile Leu Lys Met Phe Pro Ser Thr Trp Tyr Val

C

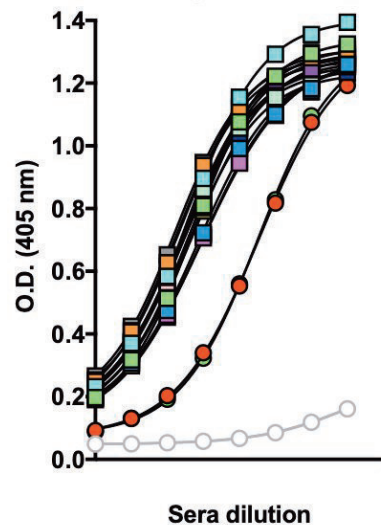
A**B****C**



A

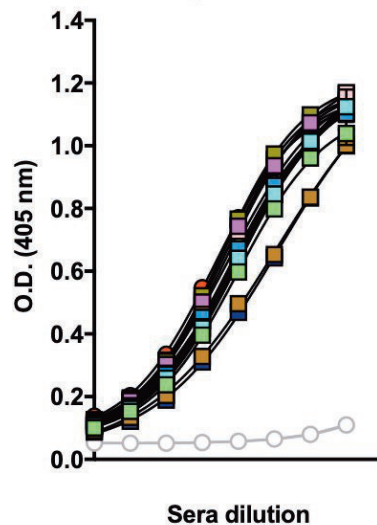
OH/04 wt swine antisera (anti-HA 145N sera)

Pig donor #1

**B**

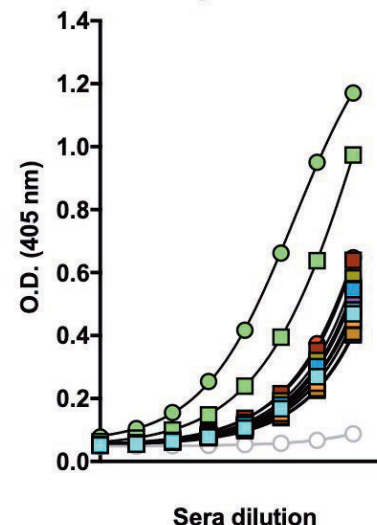
NY/11 swine antisera (anti-HA 145N sera)

Pig donor #2

**C**

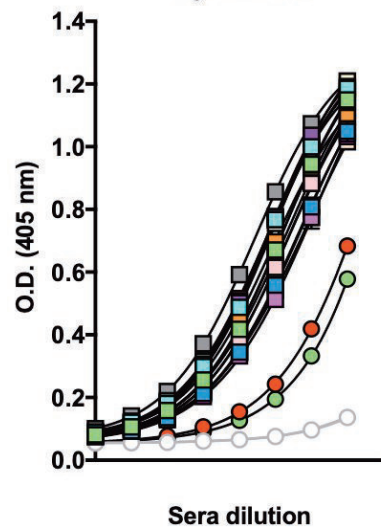
IA/14 swine antisera (anti-HA 145K sera)

Pig donor #3



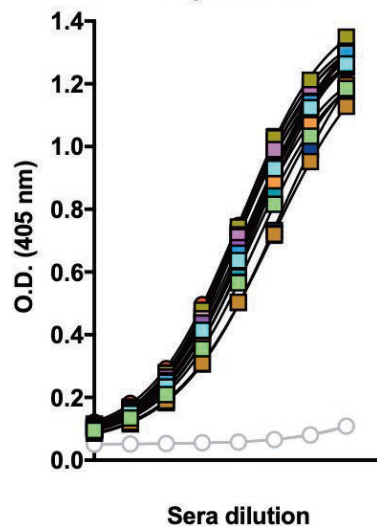
OH/04 wt swine antisera (anti-HA 145N sera)

Pig donor #4



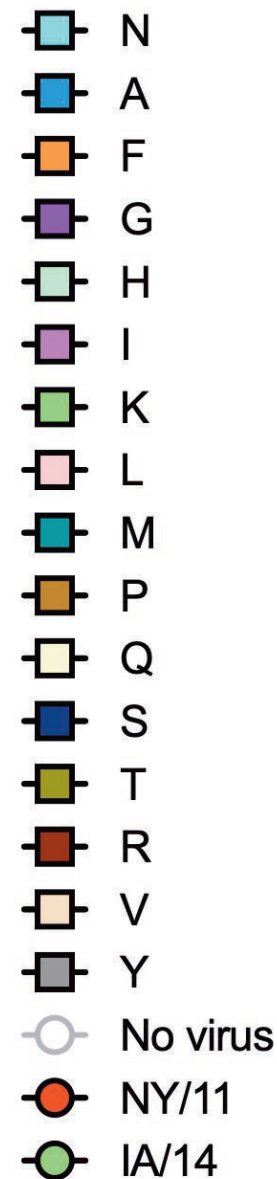
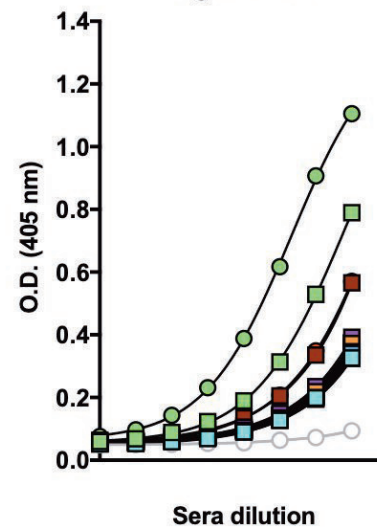
NY/11 swine antisera (anti-HA 145N sera)

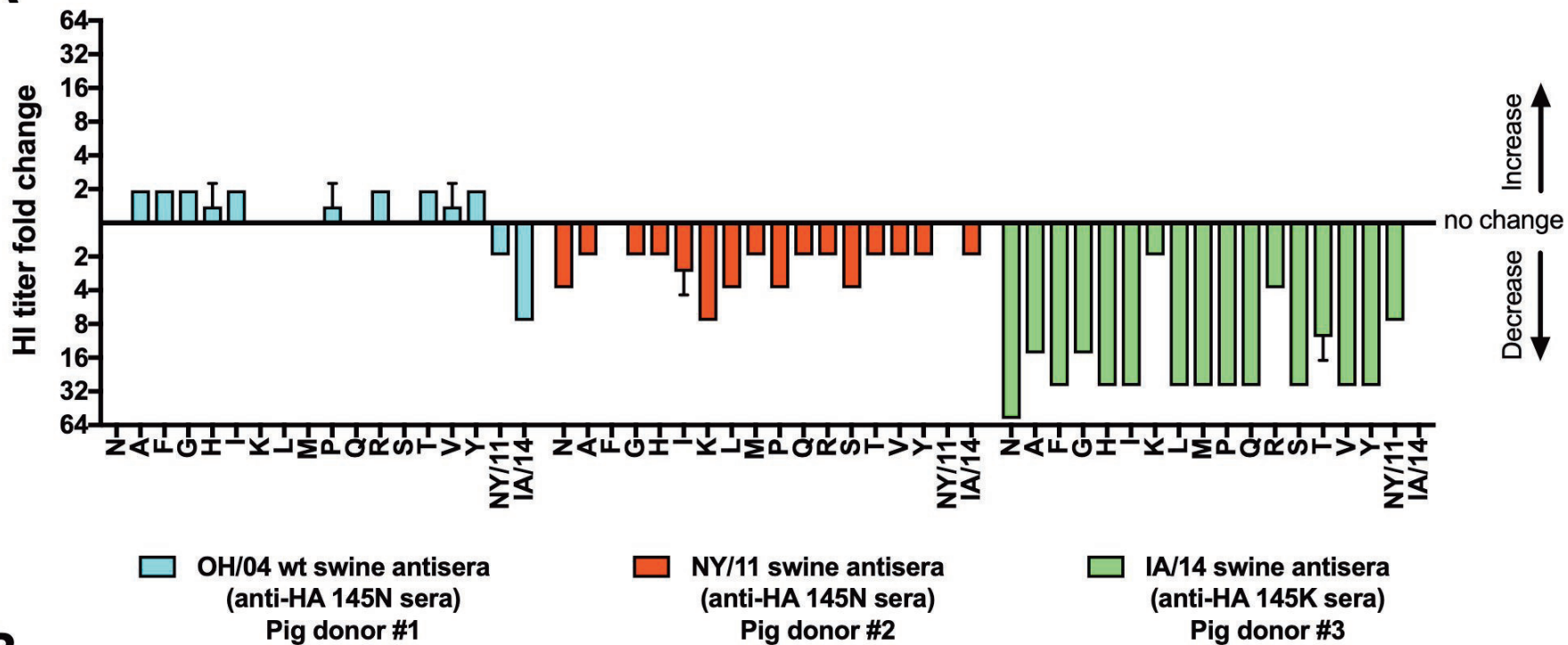
Pig donor #5



IA/14 swine antisera (anti-HA 145K sera)

Pig donor #6



A**B**