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 α2-3 (SAα2-

60   3Gal) or α2-6 (SAα2-6Gal) 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 SAα2-6Gal 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

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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 SAα2-3Gal and SAα2-6Gal receptors on their cell surface

139   while horse RBCs mainly display SAα2-3Gal (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 $\log_{10}$ TCID$_{50}$/ml equivalents) at 72 hpi, with the exception of 145P and 145H viruses

152   that showed slightly lower peak titers (~5.5 $\log_{10}$ TCID$_{50}$/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 SAα2-6Gal 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 SAα2-3Gal (3-Fet-HRP) or SAα2-6Gal (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 SAα2-6Gal (**Fig. 3Q**)

174   while avian ΔH5N1 displayed restricted binding to SAα2-3Gal (**Fig. 3R**). All of the mutant

175   viruses retained binding to SAα2-6Gal with no residual binding to SAα2-3Gal. Naturally

176   occurring substitutions [145N (wt), 145K and 145S viruses] showed the greatest binding to

177   SAα2-6Gal (**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   SAα2-6Gal (**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 SAα2-6Gal (**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 SAα2-6Gal 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 SAα2-6Gal 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 SAα2-6Gal 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 SAα2-6Gal 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

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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 SAα2-3Gal glycans whereas

249   those that circulate in humans favor binding to SAα2-6Gal 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 SAα2-

279   3Gal and SAα2-6Gal 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 SAα2-6Gal 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 SAα2-6Gal

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 SAα2-6Gal glycan interactions that were

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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   SAα2-6Gal 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).

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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 CO_2 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

15

341 3' portion of HA followed by polI 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 ($TCID_{50}$) 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 TPCK-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 $\log_{10}$ TCID$_{50}$/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 µl of

17

387   RBC preparations was added to 50 µl 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 µl of 1% RBCs treated with the different

396   neuraminidase concentrations were added to 50 µl 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 α2-3-sialyltransferase from *Pasteurella multocida* (Sigma, St. Louis, MO) for 3-modified

404   fetuin (3-fet-HRP) or α2-6-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 (SAα2-3Gal) or 6-Fet-HRP (SAα2-6Gal) in reaction solution (RS, PBS containing

413   0.02% Tween-80, 0.1% BSA-NA and 2 μM oseltamivir carboxylate) for 1 h at 4ºC. 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% H_2O_2). Reactions were allowed to proceed at room temperature for 30 min unless

417   otherwise stated. Reactions were stopped with 3% (vol/vol in ddH_2O) H_2SO_4. 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

19

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 dH$_2$O, 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), SAα2-3Gal (11-76; Yellow) and SAα2-6Gal (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 4ºC with 16 HAU of each virus in 1X

20

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 ddH$_2$O) H$_2$SO$_4$ 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

483   legend.

484   **Acknowledgements**

494   **References**

495   1.   **Nicholls JM, Chan RW, Russell RJ, Air GM, Peiris JS.** 2008. Evolving complexities

496   of influenza virus and its receptors. Trends Microbiol **16:**149-157.

497   2.   **Wilson IA, Skehel JJ, Wiley DC.** 1981. Structure of the haemagglutinin membrane

498   glycoprotein of influenza virus at 3 A resolution. Nature **289:**366-373.

499   3.   **Skehel JJ, Wiley DC.** 2000. Receptor binding and membrane fusion in virus entry: the

500   influenza hemagglutinin. Annu Rev Biochem **69:**531-569.

501 4. **Matrosovich M, Tuzikov A, Bovin N, Gambaryan A, Klimov A, Castrucci MR,**

502   **Donatelli I, Kawaoka Y.** 2000. Early alterations of the receptor-binding properties of

503   H1, H2, and H3 avian influenza virus hemagglutinins after their introduction into

504   mammals. J Virol **74:**8502-8512.

505 5. **Connor RJ, Kawaoka Y, Webster RG, Paulson JC.** 1994. Receptor specificity in

506   human, avian, and equine H2 and H3 influenza virus isolates. Virology **205:**17-23.

507 6. **Wan H, Perez DR.** 2007. Amino acid 226 in the hemagglutinin of H9N2 influenza

508   viruses determines cell tropism and replication in human airway epithelial cells. J Virol

509   **81:**5181-5191.

510 7. **Li Y, Bostick DL, Sullivan CB, Myers JL, Griesemer SB, Stgeorge K, Plotkin JB,**

511   **Hensley SE.** 2013. Single hemagglutinin mutations that alter both antigenicity and

512   receptor binding avidity influence influenza virus antigenic clustering. J Virol **87:**9904-

513   9910.

514 8. **Hensley SE, Das SR, Bailey AL, Schmidt LM, Hickman HD, Jayaraman A,**

515   **Viswanathan K, Raman R, Sasisekharan R, Bennink JR, Yewdell JW.** 2009.

516   Hemagglutinin receptor binding avidity drives influenza A virus antigenic drift. Science

517   **326:**734-736.

518 9. **Nelson MI, Vincent AL.** 2015. Reverse zoonosis of influenza to swine: new perspectives

519   on the human-animal interface. Trends Microbiol **23:**142-153.

520 10. **Belongia EA, Simpson MD, King JP, Sundaram ME, Kelley NS, Osterholm MT,**

521   **McLean HQ.** 2016. Variable influenza vaccine effectiveness by subtype: a systematic

522   review and meta-analysis of test-negative design studies. Lancet Infect Dis **16:**942-951.

23

523   11.   **Rajao DS, Perez DR.** 2018. Universal Vaccines and Vaccine Platforms to Protect

524   against Influenza Viruses in Humans and Agriculture. Front Microbiol **9:**123.

525   12.   **Altman MO, Bennink JR, Yewdell JW, Herrin BR.** 2015. Lamprey VLRB response to

526   influenza virus supports universal rules of immunogenicity and antigenicity. Elife **4**.

527   13.   **Virelizier JL.** 1975. Host defenses against influenza virus: the role of anti-hemagglutinin

528   antibody. J Immunol **115:**434-439.

529   14.   **Altman MO, Angeletti D, Yewdell JW.** 2018. Antibody Immunodominance: The Key

530   to Understanding Influenza Virus Antigenic Drift. Viral Immunol **31:**142-149.

531   15.   **Chambers BS, Parkhouse K, Ross TM, Alby K, Hensley SE.** 2015. Identification of

532   Hemagglutinin Residues Responsible for H3N2 Antigenic Drift during the 2014-2015

533   Influenza Season. Cell Rep **12:**1-6.

534   16.   **Smith DJ, Lapedes AS, de Jong JC, Bestebroer TM, Rimmelzwaan GF, Osterhaus**

535   **AD, Fouchier RA.** 2004. Mapping the antigenic and genetic evolution of influenza virus.

536   Science **305:**371-376.

537   17.   **Koel BF, Burke DF, Bestebroer TM, van der Vliet S, Zondag GC, Vervaet G,**

538   **Skepner E, Lewis NS, Spronken MI, Russell CA, Eropkin MY, Hurt AC, Barr IG,**

539   **de Jong JC, Rimmelzwaan GF, Osterhaus AD, Fouchier RA, Smith DJ.** 2013.

540   Substitutions near the receptor binding site determine major antigenic change during

541   influenza virus evolution. Science **342:**976-979.

542   18.   **Abente EJ, Santos J, Lewis NS, Gauger PC, Stratton J, Skepner E, Anderson TK,**

543   **Rajao DS, Perez DR, Vincent AL.** 2016. The Molecular Determinants of Antibody

544   Recognition and Antigenic Drift in the H3 Hemagglutinin of Swine Influenza A Virus. J

545   Virol **90:**8266-8280.

24

546   19.   **Chen H, Ye J, Xu K, Angel M, Shao H, Ferrero A, Sutton T, Perez DR.** 2012. Partial

547   and full PCR-based reverse genetics strategy for influenza viruses. PLoS One **7:**e46378.

548   20.   **Perez DR, Angel M, Gonzalez-Reiche AS, Santos J, Obadan A, Martinez-Sobrido L.**

549   2017. Plasmid-Based Reverse Genetics of Influenza A Virus. Methods Mol Biol

550   **1602:**251-273.

551   21.   **Ito T, Suzuki Y, Mitnaul L, Vines A, Kida H, Kawaoka Y.** 1997. Receptor specificity

552   of influenza A viruses correlates with the agglutination of erythrocytes from different

553   animal species. Virology **227:**493-499.

554   22.   **Medeiros R, Escriou N, Naffakh N, Manuguerra JC, van der Werf S.** 2001.

555   Hemagglutinin residues of recent human A(H3N2) influenza viruses that contribute to the

556   inability to agglutinate chicken erythrocytes. Virology **289:**74-85.

557   23.   **Bradley KC, Galloway SE, Lasanajak Y, Song X, Heimburg-Molinaro J, Yu H,**

558   **Chen X, Talekar GR, Smith DF, Cummings RD, Steinhauer DA.** 2011. Analysis of

559   influenza virus hemagglutinin receptor binding mutants with limited receptor recognition

560   properties and conditional replication characteristics. J Virol **85:**12387-12398.

561   24.   **Matrosovich MN, Gambaryan AS.** 2012. Solid-phase assays of receptor-binding

562   specificity. Methods Mol Biol **865:**71-94.

563   25.   **Peng W, de Vries RP, Grant OC, Thompson AJ, McBride R, Tsogtbaatar B, Lee PS,**

564   **Razi N, Wilson IA, Woods RJ, Paulson JC.** 2017. Recent H3N2 Viruses Have Evolved

565   Specificity for Extended, Branched Human-type Receptors, Conferring Potential for

566   Increased Avidity. Cell Host Microbe **21:**23-34.

567   26.   **Matrosovich MN, Gambaryan AS, Teneberg S, Piskarev VE, Yamnikova SS, Lvov**

568   **DK, Robertson JS, Karlsson KA.** 1997. Avian influenza A viruses differ from human

25

569   viruses by recognition of sialyloligosaccharides and gangliosides and by a higher

570   conservation of the HA receptor-binding site. Virology **233:**224-234.

571   27.   **Rogers GN, Paulson JC.** 1983. Receptor determinants of human and animal influenza

572   virus isolates: differences in receptor specificity of the H3 hemagglutinin based on

573   species of origin. Virology **127:**361-373.

574   28.   **Yamada S, Suzuki Y, Suzuki T, Le MQ, Nidom CA, Sakai-Tagawa Y, Muramoto Y,**

575   **Ito M, Kiso M, Horimoto T, Shinya K, Sawada T, Kiso M, Usui T, Murata T, Lin Y,**

576   **Hay A, Haire LF, Stevens DJ, Russell RJ, Gamblin SJ, Skehel JJ, Kawaoka Y.** 2006.

577   Haemagglutinin mutations responsible for the binding of H5N1 influenza A viruses to

578   human-type receptors. Nature **444:**378-382.

579   29.   **Gamblin SJ, Haire LF, Russell RJ, Stevens DJ, Xiao B, Ha Y, Vasisht N, Steinhauer**

580   **DA, Daniels RS, Elliot A, Wiley DC, Skehel JJ.** 2004. The structure and receptor

581   binding properties of the 1918 influenza hemagglutinin. Science **303:**1838-1842.

582   30.   **Rogers GN, Paulson JC, Daniels RS, Skehel JJ, Wilson IA, Wiley DC.** 1983. Single

583   amino acid substitutions in influenza haemagglutinin change receptor binding specificity.

584   Nature **304:**76-78.

585   31.   **Wu NC, Thompson AJ, Xie J, Lin CW, Nycholat CM, Zhu X, Lerner RA, Paulson**

586   **JC, Wilson IA.** 2018. A complex epistatic network limits the mutational reversibility in

587   the influenza hemagglutinin receptor-binding site. Nat Commun **9:**1264.

588   32.   **Lewis NS, Anderson TK, Kitikoon P, Skepner E, Burke DF, Vincent AL.** 2014.

589   Substitutions near the hemagglutinin receptor-binding site determine the antigenic

590   evolution of influenza A H3N2 viruses in U.S. swine. J Virol **88:**4752-4763.

26

591   33.   **Petrova VN, Russell CA.** 2018. The evolution of seasonal influenza viruses. Nat Rev

592   Microbiol **16:**47-60.

593   34.   **Barr IG, Russell C, Besselaar TG, Cox NJ, Daniels RS, Donis R, Engelhardt OG,**

594   **Grohmann G, Itamura S, Kelso A, McCauley J, Odagiri T, Schultz-Cherry S, Shu**

595   **Y, Smith D, Tashiro M, Wang D, Webby R, Xu X, Ye Z, Zhang W, Writing**

596   **Committee of the World Health Organization Consultation on Northern**

597   **Hemisphere Influenza Vaccine Composition f.** 2014. WHO recommendations for the

598   viruses used in the 2013-2014 Northern Hemisphere influenza vaccine: Epidemiology,

599   antigenic and genetic characteristics of influenza A(H1N1)pdm09, A(H3N2) and B

600   influenza viruses collected from October 2012 to January 2013. Vaccine **32:**4713-4725.

601   35.   **Klimov AI, Garten R, Russell C, Barr IG, Besselaar TG, Daniels R, Engelhardt OG,**

602   **Grohmann G, Itamura S, Kelso A, McCauley J, Odagiri T, Smith D, Tashiro M, Xu**

603   **X, Webby R, Wang D, Ye Z, Yuelong S, Zhang W, Cox N, Writing Committee of the**

604   **World Health Organization Consultation on Southern Hemisphere Influenza**

605   **Vaccine Composition f.** 2012. WHO recommendations for the viruses to be used in the

606   2012 Southern Hemisphere Influenza Vaccine: epidemiology, antigenic and genetic

607   characteristics of influenza A(H1N1)pdm09, A(H3N2) and B influenza viruses collected

608   from February to September 2011. Vaccine **30:**6461-6471.

609   36.   **Govorkova EA, Matrosovich MN, Tuzikov AB, Bovin NV, Gerdil C, Fanget B,**

610   **Webster RG.** 1999. Selection of receptor-binding variants of human influenza A and B

611   viruses in baby hamster kidney cells. Virology **262:**31-38.

612   37.   **Lin Y, Wharton SA, Whittaker L, Dai M, Ermetal B, Lo J, Pontoriero A,**

613   **Baumeister E, Daniels RS, McCauley JW.** 2017. The characteristics and antigenic

27

614 properties of recently emerged subclade 3C.3a and 3C.2a human influenza A(H3N2)

615 viruses passaged in MDCK cells. Influenza Other Respir Viruses **11:**263-274.

616 38. **Tamura D, Nguyen HT, Sleeman K, Levine M, Mishin VP, Yang H, Guo Z, Okomo-**

617 **Adhiambo M, Xu X, Stevens J, Gubareva LV.** 2013. Cell culture-selected substitutions

618 in influenza A(H3N2) neuraminidase affect drug susceptibility assessment. Antimicrob

619 Agents Chemother **57:**6141-6146.

620 39. **Mohr PG, Deng YM, McKimm-Breschkin JL.** 2015. The neuraminidases of MDCK

621 grown human influenza A(H3N2) viruses isolated since 1994 can demonstrate receptor

622 binding. Virol J **12:**67.

623 40. **Zost SJ, Parkhouse K, Gumina ME, Kim K, Diaz Perez S, Wilson PC, Treanor JJ,**

624 **Sant AJ, Cobey S, Hensley SE.** 2017. Contemporary H3N2 influenza viruses have a

625 glycosylation site that alters binding of antibodies elicited by egg-adapted vaccine strains.

626 Proc Natl Acad Sci U S A **114:**12578-12583.

627 41. **Aich U, Beckley N, Shriver Z, Raman R, Viswanathan K, Hobbie S, Sasisekharan R.**

628 2011. Glycomics-based analysis of chicken red blood cells provides insight into the

629 selectivity of the viral agglutination assay. FEBS J **278:**1699-1712.

630 42. **Das SR, Hensley SE, David A, Schmidt L, Gibbs JS, Puigbo P, Ince WL, Bennink**

631 **JR, Yewdell JW.** 2011. Fitness costs limit influenza A virus hemagglutinin glycosylation

632 as an immune evasion strategy. Proc Natl Acad Sci U S A **108:**E1417-1422.

633 43. **Hensley SE, Das SR, Gibbs JS, Bailey AL, Schmidt LM, Bennink JR, Yewdell JW.**

634 2011. Influenza A virus hemagglutinin antibody escape promotes neuraminidase

635 antigenic variation and drug resistance. PLoS One **6:**e15190.

28

636   44.   **Huang KY, Rijal P, Schimanski L, Powell TJ, Lin TY, McCauley JW, Daniels RS,**

637   **Townsend AR.** 2015. Focused antibody response to influenza linked to antigenic drift. J

638   Clin Invest **125:**2631-2645.

639   45.   **Stark SE, Caton AJ.** 1991. Antibodies that are specific for a single amino acid

640   interchange in a protein epitope use structurally distinct variable regions. J Exp Med

641   **174:**613-624.

642   46.   **Ekiert DC, Kashyap AK, Steel J, Rubrum A, Bhabha G, Khayat R, Lee JH, Dillon**

643   **MA, O'Neil RE, Faynboym AM, Horowitz M, Horowitz L, Ward AB, Palese P,**

644   **Webby R, Lerner RA, Bhatt RR, Wilson IA.** 2012. Cross-neutralization of influenza A

645   viruses mediated by a single antibody loop. Nature **489:**526-532.

646   47.   **Pena L, Vincent AL, Ye J, Ciacci-Zanella JR, Angel M, Lorusso A, Gauger PC,**

647   **Janke BH, Loving CL, Perez DR.** 2011. Modifications in the polymerase genes of a

648   swine-like triple-reassortant influenza virus to generate live attenuated vaccines against

649   2009 pandemic H1N1 viruses. J Virol **85:**456-469.

650   48.   **Tang Y, Lee CW, Zhang Y, Senne DA, Dearth R, Byrum B, Perez DR, Suarez DL,**

651   **Saif YM.** 2005. Isolation and characterization of H3N2 influenza A virus from turkeys.

652   Avian Dis **49:**207-213.

653   49.   **Reed LJ, Muench H.** 1938. A simple method for estimating fifty percent endpoints. Am

654   J Hyg **27:**493-497.

655   50.   **Mena I, Nelson MI, Quezada-Monroy F, Dutta J, Cortes-Fernandez R, Lara-Puente**

656   **JH, Castro-Peralta F, Cunha LF, Trovao NS, Lozano-Dubernard B, Rambaut A,**

657   **van Bakel H, Garcia-Sastre A.** 2016. Origins of the 2009 H1N1 influenza pandemic in

658   swine in Mexico. Elife **5**.

29

659   51.   **Kearse M, Moir R, Wilson A, Stones-Havas S, Cheung M, Sturrock S, Buxton S,**

660   **Cooper A, Markowitz S, Duran C, Thierer T, Ashton B, Meintjes P, Drummond A.**

661   2012. Geneious Basic: an integrated and extendable desktop software platform for the

662   organization and analysis of sequence data. Bioinformatics **28:**1647-1649.

663   52.   **Spackman E, Senne DA, Myers TJ, Bulaga LL, Garber LP, Perdue ML, Lohman K,**

664   **Daum LT, Suarez DL.** 2002. Development of a real-time reverse transcriptase PCR

665   assay for type A influenza virus and the avian H5 and H7 hemagglutinin subtypes. J Clin

666   Microbiol **40:**3256-3260.

667   53.   **Santos JJS, Obadan AO, Garcia SC, Carnaccini S, Kapczynski DR, Pantin-**

668   **Jackwood M, Suarez DL, Perez DR.** 2017. Short- and long-term protective efficacy

669   against clade 2.3.4.4 H5N2 highly pathogenic avian influenza virus following prime-

670   boost vaccination in turkeys. Vaccine **35:**5637-5643.

671   54.   **World Health Organization.** 2011. Manual for the laboratory diagnosis and virological

672   surveillance of influenza. World Health Organization, Geneva.

673   55.   **Lakdawala SS, Lamirande EW, Suguitan AL, Jr., Wang W, Santos CP, Vogel L,**

674   **Matsuoka Y, Lindsley WG, Jin H, Subbarao K.** 2011. Eurasian-origin gene segments

675   contribute to the transmissibility, aerosol release, and morphology of the 2009 pandemic

676   H1N1 influenza virus. PLoS Pathog **7:**e1002443.

677   56.   **Stevens J, Chen LM, Carney PJ, Garten R, Foust A, Le J, Pokorny BA,**

678   **Manojkumar R, Silverman J, Devis R, Rhea K, Xu X, Bucher DJ, Paulson JC, Cox**

679   **NJ, Klimov A, Donis RO.** 2010. Receptor specificity of influenza A H3N2 viruses

680   isolated in mammalian cells and embryonated chicken eggs. J Virol **84:**8287-8299.

30

681   57.   **Eswar N, Webb B, Marti-Renom MA, Madhusudhan MS, Eramian D, Shen MY,**

682   **Pieper U, Sali A.** 2006. Comparative protein structure modeling using Modeller. Curr

683   Protoc Bioinformatics **Chapter 5:**Unit-5 6.

684   58.   **Schrodinger, LLC.** 2015. The PyMOL Molecular Graphics System, Version 2.1.

685   59.   **Zhang Y, Aevermann BD, Anderson TK, Burke DF, Dauphin G, Gu Z, He S,**

686   **Kumar S, Larsen CN, Lee AJ, Li X, Macken C, Mahaffey C, Pickett BE, Reardon**

687   **B, Smith T, Stewart L, Suloway C, Sun G, Tong L, Vincent AL, Walters B,**

688   **Zaremba S, Zhao H, Zhou L, Zmasek C, Klem EB, Scheuermann RH.** 2017.

689   Influenza Research Database: An integrated bioinformatics resource for influenza virus

690   research. Nucleic Acids Res **45:**D466-D474.

691

Figure legends

692

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 $\log_{10}$ TCID$_{50}$/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 SAα2-6Gal**. H3 viruses carrying

713 amino acid substitutions at residue 145 were tested for receptor binding specificity with varying

714 concentrations of SAα2-3Gal (α2-3-linked SA) or SAα2-6Gal (α2-6-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 ΔH5N1 were used as binding control to SAα2-6Gal and SAα2-3Gal, respectively.

718   Glycan concentration is expressed as arbitrary units (AU). Plotted data represent means ±

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 SAα2-6Gal**

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), SAα2-3Gal (11-76; Yellow)

724   and SAα2-6Gal (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 ± 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 ± 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

33

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 [(log2 HI titer mutant virus)/(log2 HI titer homologous virus)]. Plotted

742 data represent means ± standard errors (SD). Colors are based on the antigenic cluster

743 designation for swine H3N2 IAVs.

744 **Tables**

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**GCT**AGTTTCTTTAGTAGATT	TAAAGAAACT**AGC**AACAGATTCCCTTCT
C	GGAATCTGTT**TGT**AGTTTCTTTAGTAGATT	TAAAGAAACT**ACA**AACAGATTCCCTTCT
D	GGAATCTGTT**GAT**AGTTTCTTTAGTAGATT	TAAAGAAACT**ATC**AACAGATTCCCTTCT
E	GGAATCTGTT**GAA**AGTTTCTTTAGTAGATT	TAAAGAAACT**TTC**AACAGATTCCCTTCT
F	GGAATCTGTT**TTT**AGTTTCTTTAGTAGATT	TAAAGAAACT**AAA**AACAGATTCCCTTCT
G	GGAATCTGTT**GGA**AGTTTCTTTAGTAGATT	TAAAGAAACT**TCC**AACAGATTCCCTTCT
H	GGAATCTGTT**CAT**AGTTTCTTTAGTAGATT	TAAAGAAACT**ATG**AACAGATTCCCTTCT
I	GGAATCTGTT**ATA**AGTTTCTTTAGTAGATT	TAAAGAAACT**TAT**AACAGATTCCCTTCT
K	GGAATCTGTT**AAA**AGTTTCTTTAGTAGATT	TAAAGAAACT**TTT**AACAGATTCCCTTCT
L	GGAATCTGTT**CTC**AGTTTCTTTAGTAGATT	TAAAGAAACT**GAG**AACAGATTCCCTTCT
M	GGAATCTGTT**ATG**AGTTTCTTTAGTAGATT	TAAAGAAACT**CAT**AACAGATTCCCTTCT
P	GGAATCTGTT**CCA**AGTTTCTTTAGTAGATT	TAAAGAAACT**TGG**AACAGATTCCCTTCT
Q	GGAATCTGTT**CAA**AGTTTCTTTAGTAGATT	TAAAGAAACT**TTG**AACAGATTCCCTTCT
R	GGAATCTGTT**CGG**AGTTTCTTTAGTAGATT	TAAAGAAACT**CCG**AACAGATTCCCTTCT
S	GGAATCTGTT**AGC**AGTTTCTTTAGTAGATT	TAAAGAAACT**GCT**AACAGATTCCCTTCT
T	GGAATCTGTT**ACA**AGTTTCTTTAGTAGATT	TAAAGAAACT**TGT**AACAGATTCCCTTCT
V	GGAATCTGTT**GTC**AGTTTCTTTAGTAGATT	TAAAGAAACT**GAC**AACAGATTCCCTTCT
W	GGAATCTGTT**TGG**AGTTTCTTTAGTAGATT	TAAAGAAACT**CCA**AACAGATTCCCTTCT
Y	GGAATCTGTT**TAT**AGTTTCTTTAGTAGATT	TAAAGAAACT**ATA**AACAGATTCCCTTCT

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

745

746

35

747

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

748

A

Swine
Total=2550

Human
Total=28242

Avian
Total=2065

Canine
Total=79

Equine
Total=176

Asn Asp Gly Lys Ser

B

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

t1   HA   pol1
1   1762

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

145N

A

B

C

Absorbance (A$_{450}$)

A — 145N (wt)
B — 145A
C — 145F
D — 145G
E — 145H
F — 145I
G — 145K
H — 145L
I — 145M
J — 145P
K — 145Q
L — 145R
M — 145S
N — 145T
O — 145V
P — 145Y
Q — pH1N1
R — ΔH5N1

Fet-HRP concentration (AU)

α2,3   α2,6

L: Linear
O: O-linked
N: N-linked
Lˣ: Sialyl Lewis X

Non-sialosides
α2-3 sialosides
α2-6 sialosides

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

Legend: N, A, F, G, H, I, K, L, M, P, Q, S, T, R, V, Y, No virus, NY/11, IA/14

A

HI titer fold change

Increase
no change
Decrease

□ OH/04 wt swine antisera
(anti-HA 145N sera)
Pig donor #1

□ NY/11 swine antisera
(anti-HA 145N sera)
Pig donor #2

□ IA/14 swine antisera
(anti-HA 145K sera)
Pig donor #3

B

HI titer fold change

Increase
no change
Decrease

□ 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