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1 **Role of the amino acid mutations in the HA gene of H9N2 avian**
2 **influenza virus under selective pressure in escape vaccine antibodies**
3
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30

31 **Abstract**

32 It has been well-documented that some amino acid mutations in hemagglutinin
33 (HA) of H9N2 avian influenza virus (H9N2 virus) alter the viral antigenicity, but little
34 is reported about the role of antibody escape mutations in escape vaccine antibodies.
35 In this study, we found that the evolution of F/98 strain in chicken embryos or
36 chickens resulted in significant differences in immune escape, and identify the
37 contribution of HA mutations to the antigenic variation and immune escape of H9N2
38 virus. Among amino acid mutations in the HA of the antigen variant viruses occurring
39 in embryonated chicken eggs and/or chickens with or without the selection pressure of
40 vaccine antibodies, the mutations, S145N, Q164L, A168T, A198V, M224K and
41 Q234L, affect the antigen drift of H9N2 virus. Specially, the A198V mutation, located
42 at the receptor-binding site on the head domain of HA, significantly contributed the
43 antigenic variation of H9N2 virus. The mutation A198V or Q234L significantly
44 improved the receptor binding activity, while S145N mutation decreased the receptor
45 binding activity. Single S145N mutation could promote viral escape from polyclonal
46 antibodies (pAbs) by preventing Ab binding physically, and single A198V mutation
47 could promote viral escape from pAbs by enhancing the receptor binding activity.
48 Additionally, either the mutation S145N or A198V did interfere with the
49 immunogenicity of the inactivated vaccine, resulting in reduction of the protective
50 efficiency of H9N2 inactivated vaccine, which contributed escape from the
51 antibody-based immunity. Our findings provided an important reference for the
52 accurate evaluation of the role of the amino acids mutation in HA affecting the
53 antigenicity of H9N2 virus on immune escape, and delivered a new perspective for
54 monitoring the adaptive evolution of H9N2 virus.

55

56

57 **Importance**

58 In this study, the role of the HA mutations of H9N2 virus occurring with and
59 without antibody selective pressure on escaping from the antibody-based immune
60 response in host was analyzed. The results demonstrated that (i) the HA mutations

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61 S145N, Q164L, A168T, A198V, M224K, and Q234L occurring in the process of the
62 adaptive evolution of H9N2 virus in embryonated chicken eggs and/or chickens could
63 affect the antigenic variation of H9N2 virus. Among these mutations, the HA mutation
64 A198V had the most significant effect on the antigenic variation; (ii) S145N mutation
65 promoted viral escape from pAbs by preventing Abs binding physically; (iii) A198V
66 mutation did promote viral escape from pAbs by enhancing the receptor binding
67 activity; (iv) neither the HA mutation S145N or A198V interfered with the
68 immunogenicity of the inactivated vaccine, resulting in reduction of the protective
69 efficiency of H9N2 inactivated vaccine.

70

71 **Keywords**

72 H9N2, avian influenza virus, antibody selective pressure, immune escape,
73 haemagglutinin, mutations

74

75

76 Introduction

77 H9N2 avian influenza virus (AIV) spread rapidly and infected more than 90% of
78 chicken flocks since its breakout in Hebei province, China in 1998. It became one of
79 the most important epidemics in poultry industry in China (1). Since then, vaccination
80 strategy of inactivated vaccine for control of H9N2 avian influenza had been
81 extensively executed, and worked well for a long period (2). However, H9N2 virus is
82 undergoing adaptive evolution under the vaccine immune pressure. As a major
83 antigen and receptor binding protein of H9N2 virus, the haemagglutinin (HA) from
84 the circulating field strains were clustered into three lineages before 2007,
85 A/Chicken/Beijing/1/94-like (BJ/94-like), A/Quail/Hong Kong/G1/97-like (G1-like),
86 and A/Duck/Hong Kong/Y439/97-like (Y439/97-like) (3). In 2013, G57 strains were
87 emerged as a predominant genotype of H9N2 virus. A new genotype G118 was
88 discovered in 2015 (4). With the evolution of H9N2 virus, the specific antibodies
89 induced by inactivated vaccine could not effectively block the attachment of HA of
90 the circulating virus to the target cells (5). This resulted in the decrease in the

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91 protection efficacy of the existing vaccines and isolation of breakthrough H9N2
92 viruses isolated in vaccinated chicken flocks with high antibody titer (6). Therefore, it
93 is important to monitor antigenic mutation of the HA of H9N2 virus.

94 Currently, over 30 antigenic sites of H9N2 virus have been reported (7-13). In
95 these studies, most of mutations on the antigenic sites of HA were gained by
96 monoclonal antibody (mAb) precisely, which promoted virus to escape from mAb
97 neutralization by changing virus-Ab binding. Naturally, the occurrence of antigenic
98 drift of HA is determined by several factors, such as environment, genetic background,
99 and immune status. The immune selective pressure and natural selection could also
100 drive HA mutation on antigenic sites to escape neutralizing antibody by adding
101 N-linked glycosylation (NLG) for shielding the antigenic sites (14, 15), changing
102 virus-antibody binding property (16), or altering receptor-binding specificity (17-20).

103 We previously reported that the H9N2 vaccine representative strain
104 A/Chicken/Shanghai/F/1998 (F/98, H9N2), which belonged to BJ/94-like lineage,
105 occurred antigenic variation continually when passaged in specific pathogen-free
106 (SPF) chicken embryos or SPF chickens with or without homologous vaccine
107 antibodies (21, 22). In order to identify the contribution of HA mutations to the
108 antigenic variation and immune escape of H9N2 virus, recombinant viruses
109 containing single HA mutation or multiple HA mutations which might affect the
110 antigenic variants of F/98 strains in F/98 backbone were generated to define the role
111 of HA mutations of F/98 strain passaged under the selection pressure with or without
112 homologous vaccine antibodies in immune escape. We found that the evolution of
113 F/98 strain in chicken embryos or chickens resulted in significant differences in
114 immune escape. The results showed that the HA mutations under the selection
115 pressure with or without vaccine antibodies, including S145N, Q164L, A168T,
116 M224K and Q234L, had some effect on the antigenic drift of H9N2 virus, and the HA
117 mutation A198V significantly affected the antigenic variation. Although the virus
118 possessing the HA mutation S145N or A198V escaped from the pAbs-neutralization
119 reaction, the molecular mechanism of antibody neutralization was different between
120 the mutations S145N and A198V, S145N mutation promoted viral escape from pAbs

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121 by preventing Abs binding physically, whereas A198V mutation by enhancing the
122 receptor binding activity. Additionally, each of the mutations S145N and A198V
123 interfered with the immunogenicity of the inactivated vaccine, resulting in reduction
124 of the protective efficiency of H9N2 inactivated vaccine, which contributed escape
125 from the antibody-based immunity.
126
127 **Results**

128 **The adaptive evolution of H9N2 virus in SPF embryonated chicken eggs or SPF**
129 **chickens drove different immune escape.**

130 We previously reported that antigenic drift of the passaged virus occurred when
131 the F/98 strain passaged continuously in the 47th generation under selective pressure
132 from vaccine antibodies and in the 52nd generation without selective pressure from
133 vaccine antibodies in SPF embryonated chicken eggs (21). The second-generation
134 quasispecies of F/98 strain under selection pressure from vaccine antibodies had
135 undergone 100% antigenic variation in SPF chickens, while after passaging to the
136 fifth generation without selection pressure from vaccine antibodies, only 30-40% of
137 the quasispecies displayed antigen drift (22). The antigenic variant evF47, the 47th
138 generation under vaccine antibodies in SPF chicken embryonated eggs, has mutations
139 K131R, S145N, G181E and A198V in HA. The antigenic variant enF52, the 52nd SPF
140 embryonated chicken eggs SPF embryonated chicken eggs generation without vaccine
141 antibodies in SPF embryonated chicken eggs, also has same mutations K131R, S145N,
142 G181E and A198V in HA. The antigenic variant cvF20, the 20th generation with
143 vaccine antibodies in SPF chicken, has mutations K131R, A198V and Q234L in HA.
144 The antigenic variant cnF20, the 20th generation without vaccine antibodies in SPF
145 chicken, has mutations A168T, A198V and M224K in HA. The above 4 strains were
146 the first antigenic variants, whose genome were still stable after 3 generations of
147 embryos blind passage, and used to determine the role of HA mutations in escaping
148 from antibody-based immune responses with different selection pressures or different
149 models with F/98 strain as a control. SPF chickens immunized by oil-emulsion of
150 inactive whole virus vaccine of F/98 strain were challenged with each of antigenic

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151 variants. The virus shedding was detected in tracheal swabs from chickens to analyze
152 the differences in immune escape. The results showed that 100% of SPF chickens (6/6)
153 immunized with F/98 vaccine shed viruses at 3 days after challenge with the virus
154 evF47 or enF52; 66.7% of chickens (4/6) shed virus cvF20; 16.7% of chickens (1/6)
155 shed virus cnF20 (Figure 1). These results revealed that the adaptive evolution of
156 H9N2 virus between embryonated chicken eggs and chickens drove different immune
157 escape, and indicated that the contribution of HA mutations from the above antigenic
158 variants to antigenic drift and immune escape might also be different.

159 **HA mutations of F/98 strain under selection pressure played different roles in the**
160 **antigenic variation**

161 The passaged viruses evF47 and enF52 possessed the same mutations in HA.
162 The S145N mutation located adjacent to the receptor-binding sites, the A198V
163 mutation located at the receptor-binding sites, and the mutations K131R and G181E
164 located in the HA globular domain (Figure 2A). In order to evaluate the role of these
165 mutations, 9 recombinant viruses containing single or multiple HA mutations from the
166 viruses evF47 and enF52 in F/98 backbone were generated, respectively, including
167 rF/HA_{K131R}, rF/HA_{S145N}, rF/HA_{G181E}, rF/HA_{A198V}, rF/HA_{348} (K131R+S145N+G181E),
168 rF/HA_{349} (K131R+S145N+A198V), rF/HA_{389} (K131R+ G181E +A198V), rF/HA_{489}
169 (S145N+G181E+A198V), and rF/HA47 (K131R+S145N+G181E+A198V) (Table 1).
170 The serum against the paternal virus F/98 in chickens was used as the reference serum
171 to analyze the antigenicity of the recombinant viruses by HI assay. Compared with the
172 paternal virus F/98, the mutations K131R and G181E did not affect the readouts of HI
173 titer, the viruses rF/HA_{S145N} and rF/HA_{348} exhibited 1.67-fold lower HI titers, and the
174 viruses rF/HA_{A198V}, rF/HA_{349}, rF/HA_{389}, rF/HA_{489} and rF/HA47 displayed 6.67-fold
175 lower HI titers, which were antigenically distinct from F/98 (Figure 2B). These results
176 suggested that the mutations S145N and A198V were related to the change of the
177 antigenicity of H9N2 virus, and the contribution of mutation A198V to the antigenic
178 drift of F/98 strain was significantly more than that of the mutation S145N.

179 In the process of F/98 strain continuously passaged in chickens, 390
180 quasispecies were isolated when F/98 strain was passaged for 20 generations in

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181 chickens, and 13 HA mutations were identified, including K131R, Q133H, Q164L,
182 A168T, A198V, M224K, Q234L, Y264H, G270R, G274R, K278E, I386V and
183 K399N (22). The mutation A198V was located at receptor-binding sites, Q164L was
184 next to receptor-binding sites, Q234L was located in the right edge of the
185 receptor-binding pocket, the mutations A168T and M224K were around the
186 receptor-binding sites, and the other HA mutations except for K278E, I386V and
187 K399N were all located in the HA globular domain (Figure. 2A). Then, 13
188 recombinant viruses containing single HA mutation in F/98 backbone were generated
189 (Table 1) and were tested for their antigenicity against anti-F/98 serum using HI assay.
190 Eight recombinant viruses, rF/HA_{K131R}, rF/HA_{Q133H}, rF/HA_{Y264H}, rF/HA_{G270R},
191 rF/HA_{G274R}, rF/HA_{K278E}, rF/HA_{I386V} and rF/HA_{K399N}, had similar antigenicity to the
192 paternal virus F/98. Three recombinant viruses, rF/HA_{Q164L}, rF/HA_{A168T} and
193 rF/HA_{M224K}, exhibited 1.67-fold lower HI titers to the anti-F/98 serum. The virus
194 rF/HA_{A198V} exhibited 6.67-fold lower HI titers (a change of more than 4-fold) to the
195 anti-F/98 serum, which is an indication of antigenically distinct from F/98. The virus
196 rF/HA_{Q234L} displayed 1.2-fold higher HI titer to the anti-F/98 serum (Figure. 2C).
197 These results suggested that the mutations Q164L, A168T, A198V, M224K and
198 Q234L were related to antigenic variation.

199 In conclusion, the mutations S145N, Q164L, A168T, A198V, M224K and
200 Q234L in the HA occurred in embryonated chicken eggs or chickens with or without
201 the selection pressure resulted in antigenic drift of F/98 strain. Among these mutations,
202 the mutation A198V at the receptor binding site of HA significantly promoted the
203 antigenic drift.

204 **HA mutations have unequal effects on the receptor binding avidity**

205 Because the mutations S145N, Q164L, A168T, A198V, M224K and Q234L
206 affecting the antigenicity of F/98 strain were all around the receptor binding sites of
207 HA, we hypothesized that these mutations might alter the receptor binding avidity or
208 the interaction between the virus and the receptor on the surface of the chicken red
209 blood cells. The results showed that the virus cvF20, rF/HA_{A198V} and rF/HA_{Q234L}
210 bound to chicken erythrocytes treated with 32-fold higher α2-3,6,8 neuraminidase

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211 concentrations than the F/98 strain. The virus evF47 and rF/HA47 bound to chicken
212 red blood cells treated with 4-fold higher α2-3,6,8 neuraminidase concentrations than
213 F/98 strain. Compared to F/98 strain, the virus rF/HA_{M224K} bound to red blood cells
214 treated with 2-fold higher α2-3,6,8 neuraminidase concentrations, while the virus
215 rF/HA_{S145N} bound to chicken erythrocytes less avidly (Figure 3). In order to further
216 confirm that S145N mutation decreased receptor binding avidity in these HA
217 mutations, a recombinant "7+1" influenza virus rF/HA47 containing HA from the
218 virus evF47 in F/98 backbone was generated. rF/HA47 possesses K131R, S145N,
219 G181E, and A198V mutations in HA. Four recombinant viruses in rF/HA47 backbone
220 were generated by introducing single HA mutation R131K, N145S, E181G and
221 V198A, respectively, namely rF/HA_{489} (S145N+G181E+A198V) (R131K in HA),
222 rF/HA_{389} (K131R+G181E+A198V) (N145S in HA), rF/HA_{349}
223 (K131R+S145N+A198V) (E181G in HA), and rF/HA_{348} (K131R+S145N+G181E)
224 (V198A in HA) (Table 1). As shown in Figure 4, the receptor binding avidity of the
225 virus rF/HA_{389}, rF/HA47 introducing the HA mutation N145S, was increased by
226 8-fold compared with that of the virus rF/HA47, which was consistent with the result
227 that S145N HA mutation caused the decrease of the receptor binding avidity, and the
228 receptor binding avidity of the virus rF/HA_{348}, V198A HA mutation introduced in
229 rF/HA47, was decreased by 4-fold compared with that of the virus rF/HA47 (Figure
230 3). These results indicated that the antigenic variants passaged in embryonated
231 chicken eggs or chickens affected receptor binding avidity. The mutations A198V and
232 Q234L significantly improved the receptor binding avidity, while the S145N mutation
233 decreased the receptor binding avidity.

234 Although the mutation S145N or A198V affected the antigenicity of H9N2 virus,
235 the A198V mutation increased the receptor binding avidity while the S145N mutation
236 was the opposite to the A198V mutation for receptor binding avidity, which suggested
237 the mechanism of S145N mutation for antigenic drift might be different from that of
238 A198V mutation. Therefore, we selected the mutations S145N and A198V to further
239 study on the escape mechanism from selective pressure exerted by inactivated vaccine
240 induced antibodies.

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241 **The S145N and A196V mutations used distinct mechanisms to escape from**
242 **neutralizing-antibodies**

243 In order to study the roles of the mutations S145N and A198V in escape from
244 neutralizing antibodies, microneutralization (MN) assay was performed, which was
245 more sensitive than HI assay (23). In the cross-MN assay between the F/98 strain and
246 the virus rF/HA_{S145N}, the virus rF/HA_{S145N} had 4-fold lower MN titer to the anti-F/98
247 serum (Figure 4A), or even to the anti-rF/HA_{S145N} serum (Figure 4B). The result
248 suggested that the S145N mutation not only caused the virus rF/HA_{S145N} escape from
249 anti-F/98 serum, but also escape from anti-rF/HA_{S145N} serum against itself. Antibody
250 binding ELISA confirmed that the area under the curve (AUC) of anti-F/98 serum
251 binding to the F/98 strain was 3.2-fold higher than that of anti-F/98 serum binding to
252 the rF/HA_{S145N} virus ($P<0.01$) (Figure 5A). The AUC of anti-rF/HA_{S145N} serum
253 binding to the rF/HA_{S145N} virus was 2.3-fold higher than that of anti-rF/HA_{S145N}
254 serum binding to the F/98 virus ($P<0.001$) (Figure 5B). These results revealed that
255 the anti-F/98 serum or anti-rF/HA_{S145N} serum bound less efficiently to the rF/HA_{S145N}
256 virus than those to the F/98 virus. These data indicated that the S145N mutation
257 promoted virus escape from pAbs by physically preventing virus-Ab binding.

258 In comparation to the F/98 strain, the rF/HA_{A198V} virus exhibited 8-fold
259 reduction of MN titers to anti-F/98 serum (Figure 4A), and 5.33-fold reduction of MN
260 titers to homologous anti-rF/HA_{A198V} serum (Figure 4C), which suggested that the
261 A198V mutation promoted the rF/HA_{A198V} virus escape from anti-F/98 and
262 anti-rF/HA_{A198V} serum. Antibody binding ELISA confirmed that the anti-F/98 serum
263 bound similarly to either the F/98 virus or the rF/HA_{A198V} virus (Figure. 5C), and
264 anti-rF/HA_{A198V} serum also bound similarly to either the F/98 virus or the rF/HA_{A198V}
265 virus (Figure 5D). Taken together, these data indicated that the A198V mutation
266 promoted escape from pAb pressure by increasing viral receptor binding avidity, but
267 not by preventing antibody binding physically.

268 **The mutations S145N and A198V reduced the protection efficiency of the**
269 **corresponding inactivated vaccine**

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270 The anti-sera against the whole inactivated vaccine of the virus F/98 or
271 rF/HA_{S145N} were generated in SPF chickens, and the HI assay was performed. The
272 results showed that the average HI titer of the anti-F/98 serum against the F/98 strain
273 was 1.83-fold higher than that against the rF/HA_{S145N} virus ($P<0.05$). Average HI
274 titer of anti-rF/HA_{S145N} serum against the F/98 strain was 1.46-fold higher than that
275 against the rF/HA_{S145N} virus. The HI titers of serum from the chickens vaccinated with
276 the rF/HA_{S145N} virus were slightly lower than that from the chickens vaccinated with
277 the F/98 virus (Figure 6A). These data suggested that the neutralizing antibody in
278 serum induced by the inactivated vaccine of the rF/HA_{S145N} virus was slightly lower
279 than that induced by the inactivated vaccine of the paternal virus F/98. The protection
280 test showed that the antibody induced by the F/98 vaccine could provide 100%
281 protection against the challenge by either the F/98 virus or the rF/HA_{S145N} virus. The
282 antibody induced by the rF/HA_{S145N} vaccine could provide 100% protection against
283 the challenge by the F/98 virus, while 83.3% protection against the challenge by the
284 rF/HA_{S145N} virus (Table 2).

285 Additionally, the anti-F/98 serum against the F/98 virus was 15.3-fold higher
286 than that against the rF/HA_{A198V} virus ($P<0.001$); the anti-rF/HA_{A198V} serum against
287 the F/98 virus was 14.4-fold higher than that against the rF/HA_{A198V} virus ($P<0.001$);
288 the HI titer of serum from the chickens vaccinated with rF/HA_{A198V} virus were lower
289 than that from the chickens vaccinated with F/98 virus (Figure 6B). These data
290 demonstrated that the neutralizing-antibody in serum induced by the inactivated
291 vaccine of the rF/HA_{A198V} virus was lower than that induced by the inactivated
292 vaccine of the F/98 virus. The immunogenic test showed that the antibody induced by
293 the F/98 vaccine could provide 100% protection against the challenge by the F/98
294 virus, and 83.3% protection against the challenge by the rF/HA_{A198V} virus; while the
295 antibody induced by the rF/HA_{A198V} vaccine could provide 83.3% protection against
296 the challenge by either the F/98 virus or the rF/HA_{A198V} virus (Table 2).

297 Taken together, these results indicated that the mutations S145N and A198V
298 both reduced the protective efficacy of H9N2 inactivated vaccine, and promoted
299 H9N2 virus escape from antibody-based immune response.

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300 **Discussion**

301 The adaptive evolution of H9N2 virus is determined by environment, genetic
302 background and immune status, et al., resulting in introducing mutations into the viral
303 genome of the H9N2 virus. As reported previously (21, 22), we found that the same
304 four HA mutations occurred in the antigenic variants passaged in the 47th generation
305 under selective pressure with vaccine antibodies, or in the 52nd generation under
306 selective pressure without vaccine antibodies in SPF embryonated chicken eggs,
307 respectively. Given that the lack of a strong immune system in embryonated chicken
308 eggs and the presence of maternal antibodies mainly in the yolk and weakly in
309 allantoic fluids led to the lack of sufficient immune pressure in the evolution of H9N2
310 virus, which ensured that the evolution of H9N2 virus in embryonated chicken eggs
311 almost independent and free from the selection pressure, and antigenic variants
312 occurring in embryonated chicken eggs could completely escape neutralizing
313 antibodies induced by the paternal virus F/98 in host. Additionally, 66.67% of the
314 antigenic variants occurring in chickens under the selection pressure of vaccine
315 antibody could escape from antibody-based selection *in vivo*, whereas only 16.7% of
316 the antigenic variants occurring in chickens without the selection pressure of vaccine
317 antibody. We speculated that the adaptive evolution of H9N2 virus reflected the
318 results of the virus-host interaction process. H9N2 virus undergoing the selection of
319 immune pressure in chickens would develop a symbiotic relationship with an
320 appropriate escape strategy in the process of adaptive evolution. The presence of
321 additional selection pressure of vaccine antibody would promote the host to restrict
322 the infection and replication of H9N2 virus, while H9N2 virus would increase the
323 immune escape to counteract specific antibodies binding.

324 HA is the most important antigenic protein of H9N2 virus, which stimulates host
325 chicken to product the HA-specific neutralizing antibodies. HA mutations in antigenic
326 sites promoted the virus to escape from antibody-based immune responses in host.
327 Several studies reported HA mutations that affect the antigenic variation of H9N2
328 virus. In these studies, HA mutations from different H9N2 antigenic variants are
329 mainly located at or near the receptor binding sites in HA, most of which were

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330 selected with HA-specific mAbs *in vitro* (7-13). Few studies on the HA mutations
331 selected with pAbs driving from inactivated vaccine *in vivo* and the contribution of
332 single HA mutation in H9N2 virus to antigenic variation or immune escape were
333 reported. Here, the contribution of 15 HA mutations, occurring *in vivo*, to antigenic
334 variation and immune escape were studied using HA genes from antigenic variants
335 passaged with or without selection pressure of H9N2 inactivated vaccine as our
336 previously reported (21, 22). We found that the HA mutations S145N, Q164L, A168T,
337 A198V, M224K, and Q234L were responsible for the antigenic variation of H9N2
338 virus. The A198V and Q234L mutations are at the receptor binding sites of HA. The
339 mutations S145N, Q164L, A168T and M224K are near the receptor binding sites. The
340 mutations S145N, Q164L and A168T have been reported when H9N2 virus were
341 selected by either pAbs or mAbs *in vitro* (9-12). Our results also indicated that these
342 mutations played an important role in the antigenic variation of H9N2 virus. The
343 amino acid residues at the position 234 in HA from different H9N2 strains could be
344 mutated under the selection pressures of either pAbs or mAbs *in vitro*, and the residue
345 L234 was responsible to bind to human type α2,6 linked sialic acid receptors (24),
346 suggesting the Q234L mutation occurred under selection pressure of vaccine
347 antibodies increased the potential of F/98 strain to infect human. Although the HA
348 mutation A198V has not been reported in antigenic mapping of HA of H9N2 virus
349 using selection with HA-specific mAbs, about 90% of H9N2 wild viruses possess V
350 or T at position 198 in HA (25), and the A198V mutation occurred in all of the
351 passaged virus in this study, suggesting that the HA mutation A198V played a key role
352 in the process of adaptive evolution of F/98 strain.

353 N-linked glycosylation (NLG) is a specific posttranslational modification of HA.
354 Both NLG pattern and HA protein sequence determine the antigenic property of AIV
355 (26). The NLG near the antigen epitope may shield the antigenic sites on the HA,
356 causing immune escape by disturbing Abs recognition or blocking Abs binding (27,
357 28), and the NLG near the receptor binding sites may change its receptor-binding
358 properties and maintain viral fitness of the receptor binding activity (29-31). For
359 example, the mutation K144N of PR8/H1N1 virus introduced a glycosylation site in

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360 HA, and followed by the compensatory mutations D225G, N193K, or P186S that
361 increased the receptor binding avidity (32). Naturally, the NLG at the position 145 in
362 HA, which is present in about 10% of H9N2 isolates, is an important glycosylation
363 site for H9N2 virus (33). In this study, the mutation S145N of the F/98 strain near the
364 receptor binding sites of HA resulted in the addition of a glycosylation site, which
365 shielded or interfered with the receptor sites and blocked Abs binding (9). The single
366 S145N mutation decreased receptor binding activity, and promoted viral escape in
367 MN or HI assays by preventing Ab binding. This finding revealed the molecular
368 mechanism of the S145N mutation escaping from pAbs deriving from inactivated
369 vaccine and its role in viral antigenic variation, which was not reported in the
370 previous studies on the antigenic mutation in HA. In addition, the inactivated vaccine
371 of the F/98 virus introduced single S145N mutation induced lower antibody level in
372 serum, and could not provide 100% protection efficiency for its own virus rF/HA_{S145N}.
373 This is a new discovery of the immune escape strategy of the S145N mutation.

374 The receptor binding sites in HA of H9N2 virus include the residues at the
375 position 109, 161, 163, 191, 198, 202 and 203, of which all are conservative except
376 the residues at the position 198 (33). Sealy et al. investigated 55 H9N2 wild strains in
377 Pakistan from 2014 to 2016, and found that the mutation A198V/T enhanced the
378 receptor binding avidity of H9N2 virus (34). Herein, we demonstrated that the A198V
379 mutation had the greatest effect on the antigenic variation of the F/98 virus among 15
380 HA mutations from the passaged viruses. It increased the receptor binding avidity
381 significantly and facilitated the virus rF/HA_{A198V} to escape from the antibodies
382 induced by the vaccine of the paternal virus F/98 or its own. Additionally, the
383 inactivated vaccine of the rF/HA_{A198V}, only introduced the A198V mutation in F/98
384 virus, induced lower antibody levels in serum, and could not provide 100% protection
385 efficiency against rF/HA_{A198V}. These results might explain the phenomenon that
386 homologous vaccine antibodies could not provide acceptable protection for
387 vaccinated chicken flocks in recent years (6).

388 In summary, our finding revealed that the HA mutations occurring in the
389 selection pressure with or without vaccine antibody affected antigenic variation of

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390 H9N2 virus, including S145N, Q164L, A168T, A198V, M224K and Q234L, of which
391 the mutation S145N or A198V significantly affected antigenic variation through
392 different mechanisms. Further data showed that S145N substitution promoted viral
393 escape from pAbs driving from vaccine by preventing Abs binding physically, while
394 A198V substitution did promote H9N2 virus escape from pAbs-neutralizing reaction
395 by enhancing the receptor binding activity. Additionally, both S145N and A198V
396 mutations interfered with the immunogenicity of the inactivated vaccine, resulting in
397 reduction of the protective efficiency of H9N2 inactivated vaccine, which contributed
398 escape from the antibody-based immunity.
399
400
401 **Materials and methods**
402 *Ethical compliance*
403 The SPF chickens and chicken embryos used in this study were purchased from
404 Nanjing Biology Medical Factory, Qian Yuan-hao Biological Co, Ltd.. Procedures
405 involving the care and use of animals were approved by the Jiangsu Administrative
406 Committee for Laboratory Animals (permission number SYXK 2016-0020) and
407 performed in accordance with the Jiangsu Laboratory Animal Welfare and Ethics
408 guidelines of the Jiangsu Administrative Committee of Laboratory Animals.
409 *Viruses and cells*
410 The H9N2 virus F/98 was isolated in Shanghai in 1998, stored at - 70 °C at the
411 Animal Infectious Disease Laboratory, School of Veterinary Medicine, Yangzhou
412 University. The GenBank accession numbers of the sequence of the F/98 strain are
413 AY253750-AY253756 and AF461532 (35). Human embryonic kidney cells (293T)
414 and Madin-Darby canine kidney (MDCK) cells, purchased from ATCC (Manassas,
415 VA, USA), were maintained in Dulbecco's modified Eagle's medium (DMEM)
416 (Sigma, St. Louis, MO, USA) supplemented with 10% fetal calf serum (Hyclone,
417 South Logan, UT, USA) and were incubated at 37 °C with 5% CO_2.
418 *Generation of H9N2 AIVs by reverse genetics*

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419 The primers, synthesized by Tsingke Biological Technology (Nanjing, China),
420 used to amplify the DNA sequence to add the single mutation in HA protein of F/98
421 virus were designed using Primer 5.0 software (Primer-E Ltd., Plymouth, UK) based
422 on the HA gene sequence of the F/98 H9N2 avian influenza virus. The full-length HA
423 genes containing the single mutation was amplified by PCR, and inserted into a
424 transcriptional/expression vector pHW2000 by using ClonExpress II One Step
425 Cloning Kit (Vazyme Biotech Co., Ltd., Nanjing, China), resulting in the plasmids
426 pHW204-HA145N. Seven dual-promoter plasmids, including pHW201-PB2,
427 pHW202-PB1, pHW203-PA, pHW205-NP, pHW206-NA, pHW207-M, and
428 pHW208-NS, from the F/98 virus strain possessing S145 HA, were stocked in our lab
429 at -70 °C (36). The recombinant viruses were rescued by transfection in the 293T cell
430 as previously described (37). Briefly, a total weight of 2.4 ng of the eight plasmids
431 mixture with a rate of 1:1 was mixed with 100 μL Opti-MEM medium (GIBCO, BRL,
432 Grand Island, USA). Next, 7 μL of PolyFect transfection reagent (QIAGEN,
433 Duesseldorf, Germany) was added. The samples were incubated at room temperature
434 for 10 min and then added to the 70-80% confluent monolayers of 293T cell in
435 24-well plates. After incubation at 37°C with 5% CO_2 for 6 h, 2 μg/mL of
436 TPCK-trypsin (Sigma, St. Louis, MO, USA) was added to the wells. Thirty hours
437 after transfection, the supernatants were harvested and inoculated into 10-day-old SPF
438 embryonated chicken eggs for virus propagation. The rescued virus was analyzed with
439 a hemagglutinin assay, and the HA genes from the rescued virus was sequenced by
440 Tsingke Biological Technology (Nanjing, China) to confirm the accuracy of the
441 designed mutation.

442 *Determination of the 50% tissue cell infectious dose ($TCID_{50}$)*

443 The $TCID_{50}$ assay was performed as we described previously (37). Briefly, the
444 viruses were diluted in DMEM without serum to a concentration of 10^{-1} to 10^{-11} and
445 then added to MDCK cells in 96-well plates, respectively. After incubation at 37 °C
446 with 5% CO_2 for 1 h, the supernatants were removed. The plates were washed twice
447 with PBS, and then 100 μL of DMEM was added to each well. After incubation at

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448 37°C with 5% CO_2 for 72 h, the HA titers of the cell supernatants were analyzed. The
449 virus titers were calculated according to the Reed-Muench formula.
450 *Anti-sera*
451 As described previously (25), six three-week-old SPF chickens were immunized
452 twice by subcutaneous injection of 0.3 mL of oil-emulsion of inactivated whole virus
453 vaccines of the viruses F/98, rF/HA_{S145N} and rF/HA_{A198V}, which were inactivated by
454 adding 0.2% formalin (*v*/v) for 24 h at 37 °C, respectively. The antisera were collected
455 and pooled from the vaccinated SPF chickens at three weeks after the vaccination.
456 *Hemagglutinin-inhibition (HI) assay and Microneutralization (MN) assay*
457 Antisera were treated with cholera filtrate (Sigma-Aldrich, St. Louis, MO, USA)
458 to remove nonspecific hemagglutination inhibitors before HI assay. HI assay was
459 performed using 4 hemagglutination units (HAU) of H9N2 and 1% (*v*/v) chicken
460 erythrocytes as we described previously (37).
461 MN assay was performed as previously described (38). Briefly, the sera were
462 serially diluted with 100 $TCID_{50}$ virus and incubated at 37 °C with 5% CO_2 for 1 h.
463 The serum-virus mixtures were added to MDCK cells and incubated for 1 h. After
464 incubation, the serum-virus mixtures were removed. Serum-free DMEM containing
465 2 μg/mL TPCK- trypsin was added to each cell and incubated at 37°C and 5% CO_2.
466 After 72 h of incubation, culture supernatant was mixed with equal volume of 1% (*v*/v)
467 chicken erythrocytes to confirm the existence of hemagglutination by virus. The MN
468 titer was defined as the highest dilution of serum with absence of hemagglutination.
469 *Enzyme-linked immunosorbent assay (ELISA)*
470 ELISA assay was performed as we described previously (37). Sucrose
471 gradient-purified viruses were diluted in PBS and added to Nunc-Immuno MaxiSop
472 96-well plates (Corning, NY, USA) at 16 HAU per well. After incubation overnight at
473 4°C, samples in wells were blocked with PBS-nonfat dry milk. Antisera against the
474 F/98 virus, the rF/HA_{S145N} virus or the rF/HA_{A198V} virus in chickens were then added
475 in serial twofold dilutions with PBS containing 0.05% Tween 20, respectively, and
476 incubated for 3 h at 37 °C. After washing, goat anti chicken horseradish peroxidase
477 antibody (Abcam, Cambridge, MA) was added and allowed to incubate for 1.5 h at

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478 37 °C. After washing, TMB (3,3′,5,5′ Tetramethylbenzidine) (Sigma, St. Louis, MO,
479 USA) substrate was added, and the reaction was stopped by adding H_2SO_4.
480 Absorbance was recorded at 450 nm using an automated ELISA plate reader (model
481 EL311SX; Biotek, Winooski, VT). The area under curve (AUC) of either virus was
482 assessed for virus-Abs binding with GraphPad Prism 8 software (San Diego, CA)
483 above that of the corresponding negative control.

484 *Receptor binding assay*

485 Receptor binding assay was performed as previously described (39). Briefly, the
486 chicken erythrocytes were pretreated with different amounts of α2-3,6,8
487 neuraminidase (New England Biolabs, Beverly, MA, USA) for 1 h at 37 °C. The
488 chicken erythrocytes were washed with PBS and added (as 1% (*v*/v) solutions) to 4
489 HAU of each virus (as determined using nontreated chicken erythrocytes).
490 Agglutination was measured after incubation for 1 hour. Virus with higher receptor
491 binding avidity is able to bind to chicken erythrocytes that are treated with high
492 amounts of α2-3,6,8 neuraminidase.

493 *Chicken experiments*

494 i) A total of 30 three-week-old SPF chickens vaccinated with the whole
495 inactivated F/98 virus were divided into five groups: the F/98 challenge group, the
496 evF47 challenge group, the enF52 challenge group, the cvF20 challenge group, and
497 the cnF20 challenge group. Each group has 6 chickens. At day 21 post-vaccination,
498 each group was challenged intranasally and intratracheally with 10^6 EID_{50} of the
499 corresponding virus. ii) A total of 42 three-week-old SPF chickens were divided into
500 three groups: the F/98 vaccine group including 18 chickens, the rF/HA_{S145N} vaccine
501 group including 12 chickens, and the rF/HA_{A198V} vaccine group including 12 chickens,
502 which were immunized with the emulsion vaccine of the F/98 virus, the rF/HA_{S145N}
503 virus, and the rF/HA_{A198V} virus, respectively. At day 21 post-vaccination, chickens
504 were bled from the wing vein for sera, and HI reactions against the F/98 virus, the
505 rF/HA_{S145N} virus or the rF/HA_{A198V} virus was performed, respectively. Then, six
506 chickens from each group were challenged intranasally and intratracheally with 10^6
507 EID_{50} of the F/98 virus, the rF/HA_{S145N} virus or the rF/HA_{A198V} virus. Chickens were

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508 monitored daily for morbidity and mortality after challenge. At days 3 and 5
509 post-challenge, tracheal and cloacal swabs from challenged chickens were collected in
510 1 mL of PBS containing antibiotics. After one freeze-thaw cycle, the swabs were
511 centrifuged at 3000 rpm for 10 min. A 0.2 mL supernatant was taken to inoculate
512 10-day-old SPF chicken eggs. Viral shedding in the trachea and cloacal was evaluated
513 via HA titers of the allantoic cavity of SPF chicken eggs at day 5 post-inoculated
514 according to the standard of HA$\geq 2^3$.

515 *Statistics analysis*

516 Data were shown as the mean ± *SD* for all assays. The Student's t test analysis
517 was used to compare between different groups and analyzed with GraphPad Prism 8
518 software. Differences were considered statistically significant when a *P* value was
519 <0.05.

520

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658 Table 1 The characterization and HA mutations of the H9N2 viruses used in this study

659

Strain	Characterization	Mutations in HA
F/98	Wild type	
evF47	47th generation under vaccine antibodies in SPF chicken embryonated eggs	K131R, S145N, G181E, A198V
enF52	52nd generation without vaccine antibodies in SPF embryonated chicken eggs	K131R, S145N, G181E, A198V
cvF20	20th generation with vaccine antibodies in SPF chicken	K131R, A198V, Q234L
cnF20	20th generation without vaccine antibodies in SPF chicken	A168T, A198V, M224K
rF/HA$_{K131R}$	The recombinant virus containing single HA mutation from evF47 or cvF20 in F/98 backbone	K131R
rF/HA$_{S145N}$	The recombinant virus containing single HA mutation from evF47 in F/98 backbone	S145N
rF/HA$_{G181E}$		G181E
rF/HA$_{A198V}$	The recombinant virus containing single HA mutation from enF52, evF47, cnF20 or cvF20 in F/98 backbone	A198V
rF/HA$_{348}$	The recombinant virus containing multiple HA mutation from evF47 in F/98 backbone	K131R, S145N, G181E,
rF/HA$_{349}$		K131R, S145N, A198V
rF/HA$_{389}$		K131R, G181E, A198V
rF/HA$_{489}$		S145N, G181E, A198V
rF/HA47		K131R, S145N, G181E, A198V
rF/HA$_{A168T}$	The recombinant virus containing single HA mutation from enF20 in F/98 backbone	A168T
rF/HA$_{M224K}$		M224K
rF/HA$_{Q234L}$	The recombinant virus containing single HA mutation from evF20 in F/98 backbone	Q234L
rF/HA$_{Q133H}$	The recombinant virus containing single HA mutation from the other quasispecies passaged in SPF chicken in F/98 backbone	Q133H
rF/HA$_{Q164L}$		Q164L
rF/HA$_{Y264H}$		Y264H
rF/HA$_{G270R}$		G270R
rF/HA$_{G274R}$		G274R
rF/HA$_{K278E}$		K278E
rF/HA$_{I386V}$		I386V
rF/HA$_{K399N}$		K399N

660

661

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662

663 Table 2 Virus shedding from the swabs on days 3 and 5 post challenged with F/98,

664 rF/HA_{S145N} or rF/HA_{A198V} viruses

Group	Virus shedding#		Protection (%)
	D3	D5	
Chickens vaccinated the F/98 inactive vaccine			
Challenged with F/98 virus	0/6[a]	0/6[a]	100
Challenged with rF/HA_{A198V} virus	1/6[a]	0/6[a]	83.3
Challenged with rF/HA_{S145N} virus	0/6[a]	0/6[a]	100
Chickens vaccinated the rF/HA_{A198V} inactive vaccine			
Challenged with F/98 virus	1/6[a]	0/6[a]	83.3
Challenged with rF/HA_{A198V} virus	1/6[a]	0/6[a]	83.3
Chickens vaccinated the rF/HA_{S145N} inactive vaccine			
Challenged with F/98 virus	0/6[a]	0/6[a]	100
Challenged with rF/HA_{S145N} virus	1/6[a]	0/6[a]	83.3

665 # Data are numbers of chickens shedding virus/total number of chickens at days 3 and 5.
666 Positive sample of chickens shedding virus indicated higher than the detection limit of 2^2 HA titer.
667 Protection was calculated with data on day 3. The appearance of the same letter means that there is
668 no marked difference among the groups under the condition of $P > 0.05$.

669

670

671 Figure legends:

672 Figure 1. Protection efficiency of the whole inactivated F/98 vaccine against the antigen variant
673 viruses passaged in SPF embryonated chicken eggs or in the chickens with or without homologous
674 vaccine antibodies. The negative sample of chickens shedding virus from the swabs on 3 days post
675 challenged with a specific virus indicated that the HA titer lower than the detection limit of 2^2.
676 Each group had six chickens.The appearance of the same letter means that there is no marked
677 difference among the groups under the condition of $P > 0.05$.

678

679

680 Figure 2. (A) The location of amino acid mutations in the three-dimensional structure of HA
681 protein of H9N2 subtype avian influenza virus. A. Yellow color indicates the locations of the HA
682 receptor binding sites including the positions 146-150, 109, 161, 163, 191, 198, 202, 203, and
683 232-237. Pink color indicates mutations on antigenic sites that have been reported. Red color
684 indicates mutations that have not been previously reported as antigenic sites. (B) HI titers of F/98
685 immune sera from chickens (n=8) to each recombinant virus with single or multiple mutants in
686 HA from the passaged viruses occurred in the 47th generation in embryonated chicken eggs under

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687 selective pressure on antibodies. (C) HI titers of F/98 immune sera from chickens (n=8) to the
688 recombinant virus with each single mutant in HA from the quasispecies occurred in the chickens
689 under the selection pressure of vaccine antibodies. A ≥4-fold change in HI titers of standard
690 antiserum was considered as significant antigenic change.
691
692 Figure 3. HA mutations A198V, M224K, Q234L increase receptor binding avidity of H9N2 virus
693 F/98, whereas S145N decreases receptor binding avidity. And the evolution of F/98 strain in
694 chicken embryos or chickens with homologous vaccine antibodies resulted in the increase of
695 receptor binding avidity. Relative viral receptor binding avidities were determined by
696 hemagglutination of red blood cells pretreated with increasing amounts of α2-3,6,8
697 neuraminidase. Data are expressed as the maximal amount of neuraminidase that allowed full
698 agglutination. The data are representative of three independent experiments.
699
700
701 Figure 4. HA S145N or A198V mutation results in a decrease in MN titer. (A) MN titers of F/98
702 immune sera from chickens (n=8) to the viruses F/98, rF/HA_{S145N}, and rF/HA_{A198V}. (B) MN titers
703 of rF/HA_{S145N} immune sera from chickens (n=8) to the viruses F/98 and rF/HA_{S145N}. (C) MN titers
704 of rF/HA_{A198V} immune sera from chickens (n=8) to the viruses F/98 and rF/HA_{A198V}. A ≥4-fold
705 change in MN titers of standard antiserum was considered as significant antigenic change.
706
707
708 Figure 5. Single S145N mutation physically prevents Ab binding, whereas single A198V mutation
709 does not affect Ab binding. Direct antibody binding to F/98 or rF/HA_{A198V} viruses were
710 determined by ELISA using sera collected from chickens vaccinated with inactivated F/98 (A) or
711 rF/HA_{A198V} (B). Direct antibody binding to F/98 or rF/HA_{S145N} viruses were determined by ELISA
712 using sera collected from chickens vaccinated with inactivated F/98 (C) or rF/HA_{S145N} (D). The
713 AUC of ELISA was calculated for virus-Abs binding by GraphPad Prism 8 software above the
714 value of the corresponding negative control, which was performed under the same conditions.
715 Means and SD from three independent experiments. Statistical significance was based on student's
716 t test (**$P < 0.01$; *** $P < 0.001$). O.D., optical density.
717

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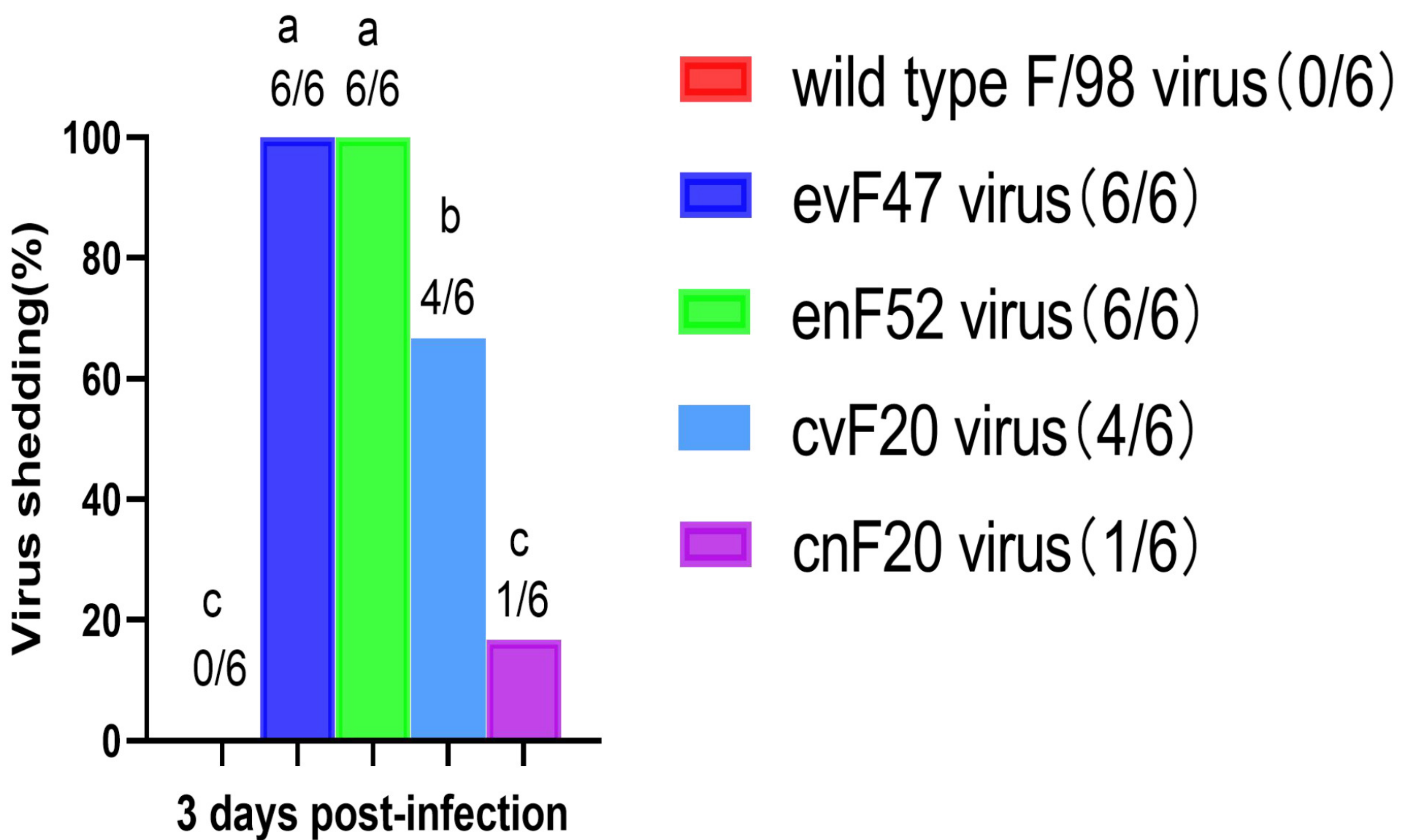
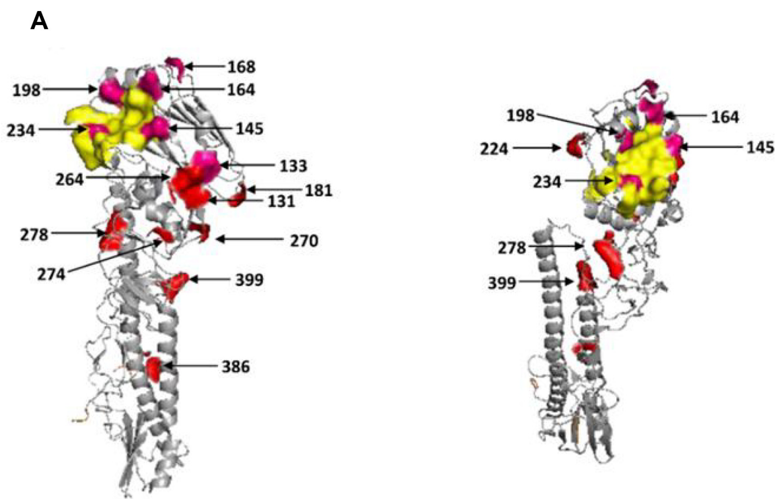
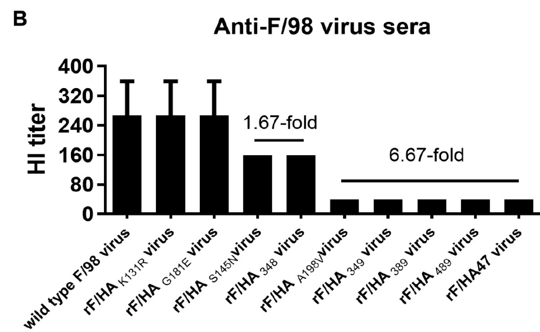
718

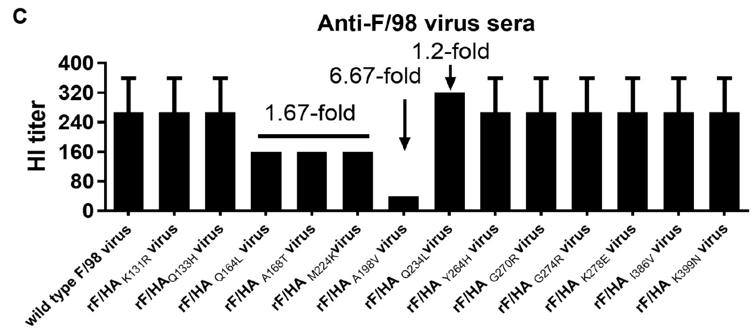
719 Figure 6. Single S145N mutation or single A198V mutation resulted in low HI reaction for
720 chicken serum. Three-week-old SPF chickens were vaccinated once by subcutaneous injection of
721 0.3 mL of oil-emulsion of inactivated whole virus vaccines of the viruses F/98, rF/HA_{S145N}, and
722 rF/HA_{A198V}, respectively. At 21 d.p.v., twelve chickens from each group (the F/98 vaccine group,
723 the rF/HA_{S145N} vaccine group, and the rF/HA_{A198V} vaccine group) were bled to analyze cross-HI
724 titers against the F/98 and rF/HA_{S145N} viruses (A), or against the F/98 and rF/HA_{A198V} viruses (B).
725 Statistical significance was based on student's t test (*$p < 0.05$).
726
727

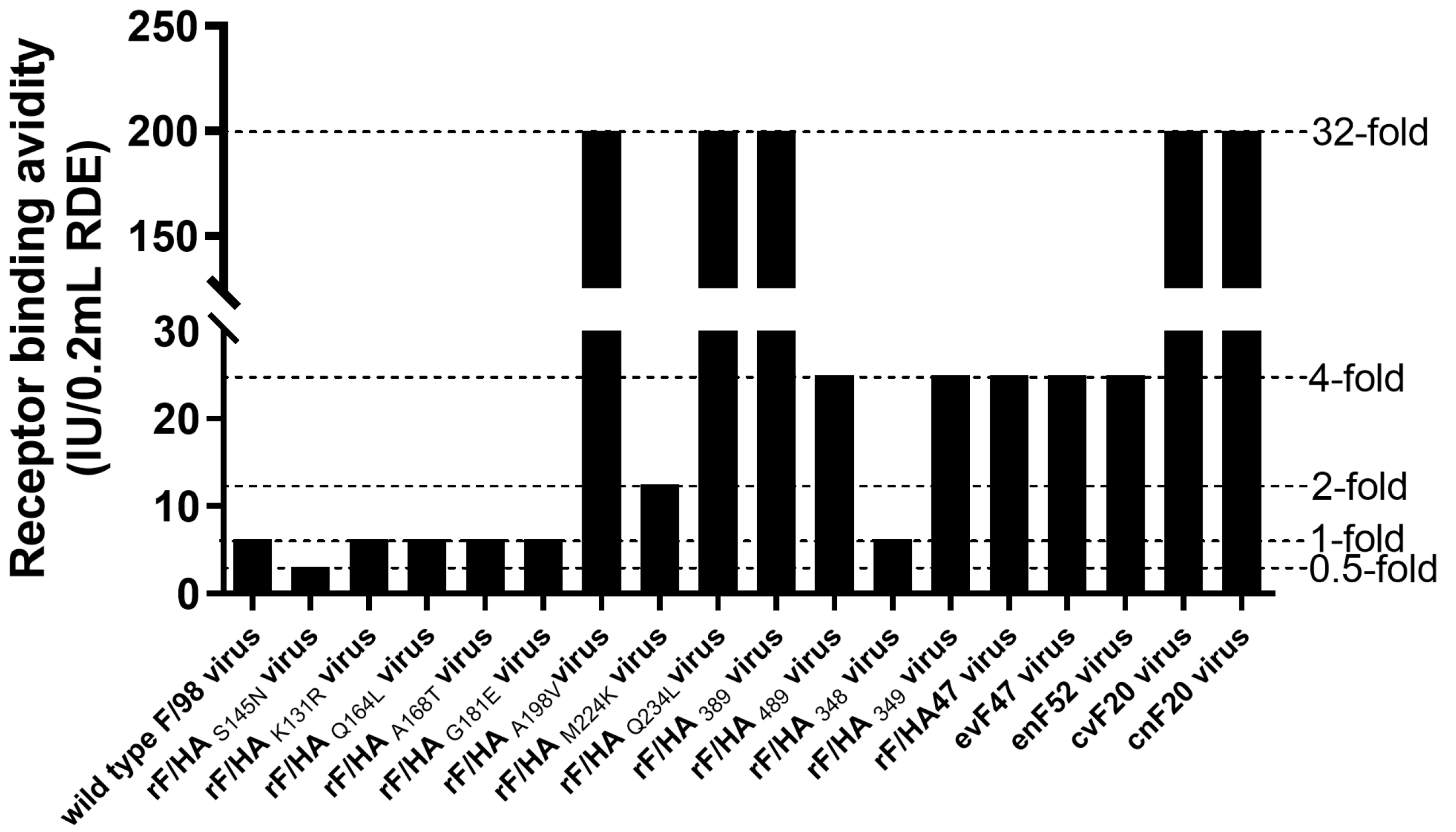


Virus shedding(%)
100
80
60
40
20
0
3 days post-infection
c
0/6
a
6/6
a
6/6
b
4/6
c
1/6
wild type F/98 virus（0/6）
evF47 virus（6/6）
enF52 virus（6/6）
cvF20 virus（4/6）
cnF20 virus（1/6）

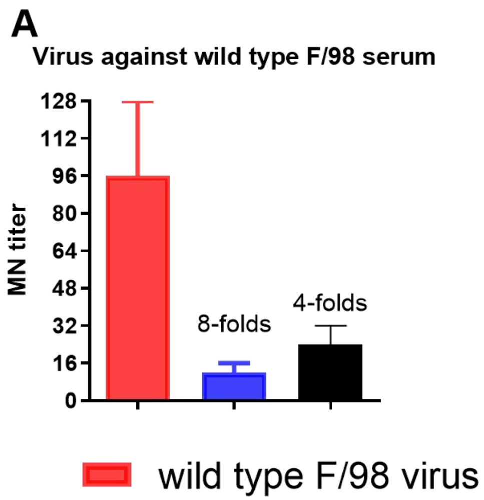

A
168
198
164
234
145
133
264
181
131
278
270
274
399
386
198
164
224
145
234
278
399

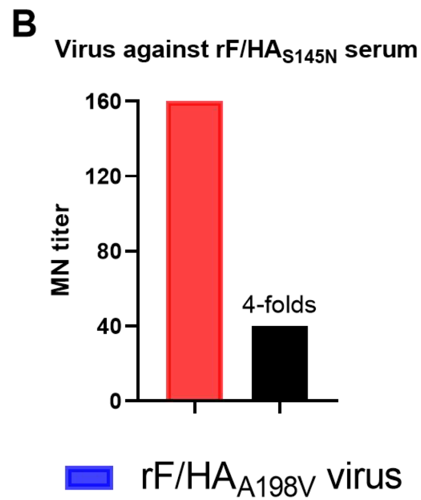

B
Anti-F/98 virus sera
HI titer
400
320
240
160
80
0
1.67-fold
6.67-fold
wild type F/98 virus
rF/HA K131R virus
rF/HA G181E virus
rF/HA S145N virus
rF/HA 348 virus
rF/HA A198V virus
rF/HA 349 virus
rF/HA 389 virus
rF/HA 489 virus
rF/HA47 virus

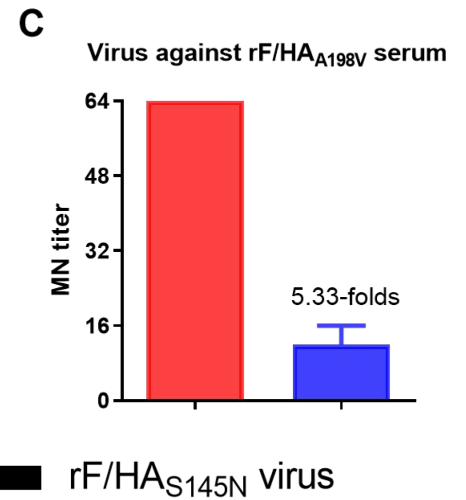

C
Anti-F/98 virus sera
HI titer
400
320
240
160
80
0
1.67-fold
6.67-fold
1.2-fold
wild type F/98 virus
rF/HA K131R virus
rF/HA Q133H virus
rF/HA Q164L virus
rF/HA A168T virus
rF/HA M224K virus
rF/HA A198V virus
rF/HA Q234L virus
rF/HA Y264H virus
rF/HA G270R virus
rF/HA G274R virus
rF/HA K278E virus
rF/HA I386V virus
rF/HA K399N virus

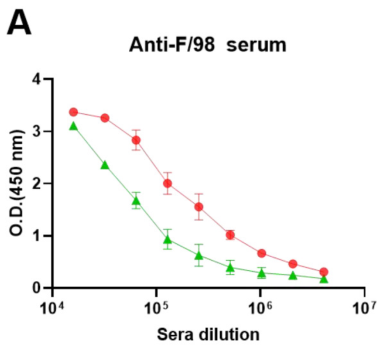


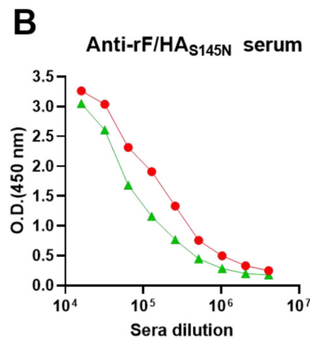
Receptor binding avidity (IU/0.2mL RDE)
250
200
150
30
20
10
0
32-fold
4-fold
2-fold
1-fold
0.5-fold
wild type F/98 virus
rF/HA S145N virus
rF/HA K131R virus
rF/HA Q164L virus
rF/HA A168T virus
rF/HA G181E virus
rF/HA A198V virus
rF/HA M224K virus
rF/HA Q234L virus
rF/HA 389 virus
rF/HA 489 virus
rF/HA 348 virus
rF/HA 349 virus
rF/HA47 virus
evF47 virus
enF52 virus
cvF20 virus
cnF20 virus

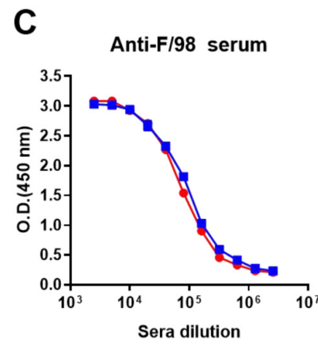

A
Virus against wild type F/98 serum
MN titer
0
16
32
48
64
80
96
112
128
8-folds
4-folds
wild type F/98 virus

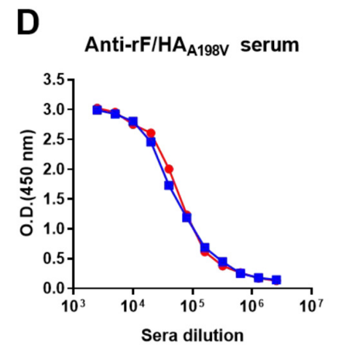

B
Virus against rF/HA$_{S145N}$ serum
MN titer
0
40
80
120
160
4-folds
rF/HA$_{A198V}$ virus

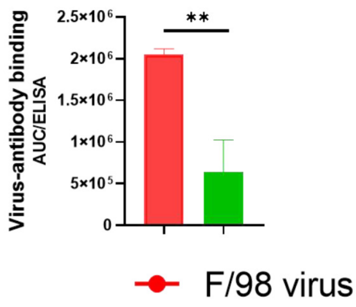

C
Virus against rF/HA$_{A198V}$ serum
MN titer
0
16
32
48
64
5.33-folds
rF/HA$_{S145N}$ virus

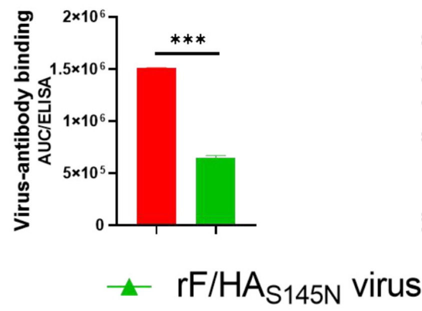


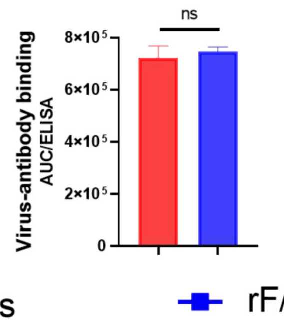


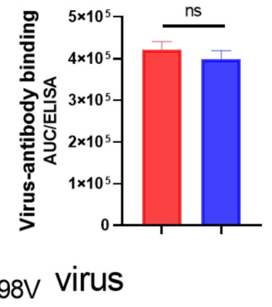




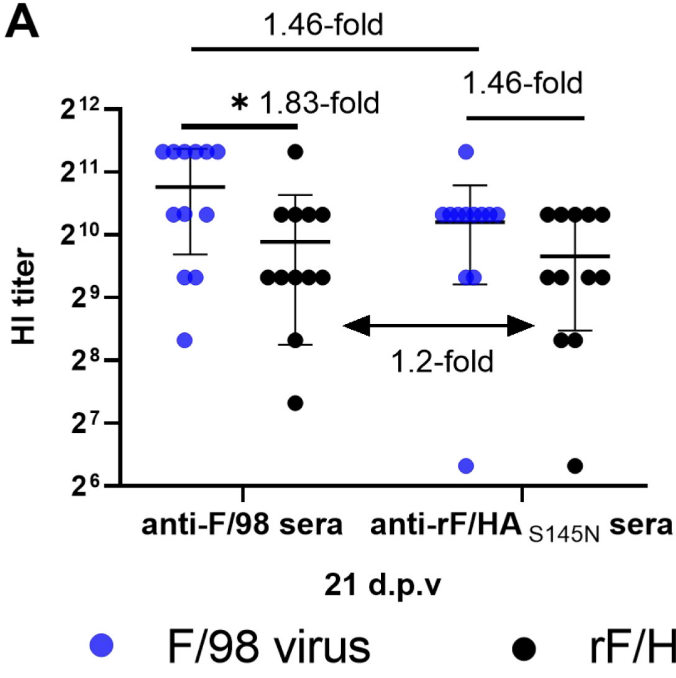








F/98 virus   rF/HA$_{S145N}$ virus   rF/HA$_{A198V}$ virus



rF/HA$_{S145N}$ virus