



Title	Antigenic diversity of H5 highly pathogenic avian influenza viruses of clade 2.3.4.4 isolated in Asia
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1 **Original article**

2 **Antigenic diversity of H5 highly pathogenic avian influenza viruses of clade 2.3.4.4 isolated**  
3 **in Asia**

4

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28  
29 **Running title:** Antigenicity of H5 influenza viruses

**30 Abstract**

31 H5 highly pathogenic avian influenza viruses (HPAIVs) have spread in both poultry and  
32 wild birds since the late 2003. The continued circulation of HPAIVs in poultry in several regions  
33 of the world has led to antigenic drift. In this study, we analyzed the antigenic properties of H5  
34 HPAIVs isolated in Asia using four neutralizing monoclonal antibodies (MAbs) recognizing the  
35 hemagglutinin, which were established using A/chicken/Kumamoto/1-7/2014 (H5N8), belonging  
36 to clade 2.3.4.4 and also using polyclonal antibodies. Viruses of clades 1.1, 2.3.2.1, 2.3.4, and  
37 2.3.4.4 had different reactivity patterns to the panel of MAbs, thereby indicating that the  
38 antigenicity of the viruses of clade 2.3.4.4 were similar but differed from other clades. In  
39 particular, the antigenicity of the viruses of clade 2.3.4.4 differed from those of the viruses of  
40 clades 2.3.4 and 2.3.2.1, which suggests that the recent H5 HPAIVs have further evolved  
41 antigenically divergent. In addition, reactivity of antiserum suggest that the antigenicity of  
42 viruses of clade 2.3.4.4 differed slightly among group A, B, and C. Vaccines are still used in  
43 poultry in the endemic countries, so the antigenicity of H5 HPAIVs should be monitored  
44 continually to facilitate the control of avian influenza. The panel of MAbs established in the  
45 present study will be useful for detecting antigenic drift in the H5 viruses that emerge from the  
46 current strains.

47

**48 Keywords**

49 antigenicity; H5N8; hemagglutinin; highly pathogenic avian influenza virus

50

## 51 **Introduction**

52        Since the late 2003, H5N1 highly pathogenic avian influenza viruses (HPAIVs) have spread  
53 in both poultry and wild birds throughout the world (1, 2). The continued evolution of H5N1  
54 viruses has led to the periodic emergence of new phylogenetic groups of H5 HPAIVs in several  
55 regions of the world (3). In 2014, H5N6 and H5N8 reassortant viruses that shared H5  
56 hemagglutinin (HA) genes originating from H5N1 viruses of clade 2.3.4 were isolated from  
57 poultry and wild birds in 14 countries across East Asia (China, South Korea, Japan, Russia, Laos,  
58 Taiwan, and Vietnam), Europe (Germany, the Netherlands, the United Kingdom, Hungary, and  
59 Italy), and North America (Canada and the United States of America) (3). The HA genes of  
60 these emerging H5 viruses are classified in clade 2.3.4.4 (3). HPAIVs belonging to clade 2.3.4.4  
61 with different neuraminidase subtypes, including N1, N2, N3, N5, N6, and N8, continued to  
62 spread by the end of 2015 (2). Due to genetic divergence via evolution, the HA genes of clade  
63 2.3.4.4 are phylogenetically divided into three subgroups: those found in the isolates from Europe  
64 (group A), North America (group B), and Kyusyu, Japan (group C) (4).

65        The HA is a surface glycoprotein of influenza A viruses and a major target for neutralizing  
66 antibodies (5). Amino acid substitutions in the HA, especially in the globular head domain, may  
67 result in antigenic drift in viruses, thereby allowing viruses to escape from host humoral immunity.  
68 Therefore, H5 viruses that are phylogenetically clustered into different clades exhibit significant  
69 differences in their antigenicity (6, 7). According to epitope mappings of the H1 and H3 HAs  
70 obtained using neutralizing monoclonal antibodies (MAbs), the antigenic sites responsible for the  
71 antigenic differences between HA subtypes are located mainly in the globular head domain (8, 9).

72 In brief, the antigenic sites Sa, Sb, Ca1, Ca2, and Cb are defined in H1 HA, and sites A, B, C, D,  
73 and E in H3 HA (8, 9). Sites 1 and 2 were identified in H5 HA, where site 1 corresponds to site  
74 Ca in H1 HA and site A in H3 HA, whereas site 2 corresponds to site Sa in H1 HA and site B in  
75 H3 HA (10, 11). In addition, the fusion subdomain F' (F' domain) (12) is one of the antigenic  
76 sites in H5 HA (10). Conserved epitopes among the clades of H5 HA were also identified in the  
77 head domain and stalk region of the HA (13, 14, 15).

78 The pathogenicity and phylogenetic characteristics of the H5 HPAIVs of clade 2.3.4.4 have  
79 been analyzed intensively (16, 17, 18, 19, 20), but the antigenicity of these viruses is not well  
80 understood (7, 21). In particular, the antigenic structure of each antigenic site in the HA of clade  
81 2.3.4.4 is still unclear. To determine whether the antigenicity of these viruses has evolved  
82 further, we established four MAbs for the viruses in clade 2.3.4.4 and characterized the  
83 antigenicity of the H5 viruses that have been isolated recently in Asia.

84

## 85 **Materials and methods**

### 86 **Viruses and cells**

87 Influenza viruses A/chicken/Kumamoto/1-7/2014 (Kum/1-7) (H5N8) and  
88 A/chicken/Miyazaki/7/2014 (H5N8) were kindly provided by Dr. T. Saito at the National Institute  
89 of Animal Health, Japan (7, 20). Kum/1-7 (H5N8) and the other H5 viruses isolated from birds  
90 and humans were grown in 10-day-old embryonated chicken eggs and the allantoic fluid  
91 containing the virus was stored at -80°C until use. Madin-Darby canine kidney (MDCK) cells  
92 were maintained in minimal essential medium (MEM) (Nissui, Japan) supplemented with 10%

93 calf serum and antibiotics.

94

#### 95 **MAbs**

96 MAbs against Kum/1-7 (H5N8) designated as A32/2, A262/2, B3/2, and B157/1 were  
97 prepared as described by Kida et al. (22). Briefly, BALB/c mice (Japan SLC, Shizuoka, Japan)  
98 were immunized with formalin-inactivated Kum/1-7 (H5N8) virus and splenocytes were fused  
99 with Sp<sub>2</sub>O-Ag14 myeloma cells (23). The hybridoma cells that secreted MAbs specific to the  
100 virus antigen were selected using an enzyme-linked immunosorbent assay (ELISA), as follows:  
101 50 µl of the cell culture supernatant was added to each well of a 96-well plate coated with the  
102 virus antigens and the specific MAbs were detected by horseradish peroxidase-conjugated goat  
103 IgG to mouse IgG (MP Biomedicals, Santa Ana, CA, USA). The hybridoma cells were then  
104 cloned in 0.4% bacto-agar (Becton, Dickinson and Company, New Jersey, USA). The isotypes  
105 of the MAbs were determined using Mouse Monoclonal Antibody Isotyping Reagents (Sigma  
106 Aldrich, St Louis, MO, USA). Ascitic fluid of mice that contains each MAb were obtained and  
107 the aliquots were used for characterization of MAbs and antigenic analysis of H5 HPAIVs.  
108 Neutralizing MAbs that recognized the HA of A/duck/Pennsylvania/10218/1984 (Dk/Penn)  
109 (H5N2) (24) were also used in the present study.

110

#### 111 **Hyperimmune sera and single immunized sera**

112 Hyperimmune sera used in the present study has been previously prepared (6, 24). To  
113 prepare single immunized sera for analysis of slight antigenic change (25), chickens were

114 immunized intramuscularly with 500  $\mu$ l of inactivated allantoic fluid containing viruses,  
115 A/chicken/Kumamoto/1/7/2014 (H5N8) or escape mutant namely mtA32/2, with FCA (Thermo  
116 Fisher Scientific, Santa Clara, CA, USA).

117

### 118 **Serological tests**

119 The biological properties of the MAbs and antigenic characteristics of the H5 HA were  
120 determined using a hemagglutination-inhibition (HI) test, neutralization (NT) test, and  
121 immunofluorescent antibody assay (IFA), as described by Sakabe et al. (26).

122 The HI test was performed as follows. The MAbs and antisera were serially diluted with  
123 PBS, mixed with 8 hemagglutination unit of virus, and incubated for 30 min at room temperature.  
124 The HI titers were expressed as reciprocals of the highest serum sample dilution that inhibited  
125 hemagglutination. For NT test, the test serum and 100 times the 50% tissue culture infectious  
126 dose (TCID<sub>50</sub>) of virus were mixed and incubated for 1 h at room temperature. The mixture was  
127 used to inoculate MDCK cells, which were incubated for 1 h at 35°C. Then, the cells were rinsed  
128 and incubated for 3 days in MEM without serum. NT titers were determined as reciprocals of  
129 the highest MAbs dilution that the cells did not show cytopathic effect. For IFA, cells were fixed  
130 with cold acetone 12 h after infection with the viruses. Antigens were captured by the MAbs  
131 established in the present study and then detected using fluorescein isothiocyanate (FITC)-  
132 conjugated goat IgG to mouse IgG (MP Biomedicals, Santa Ana, CA, USA).

133

### 134 **Selection of escape mutants**



135           The antigenic variants were selected as follows. Each virus was incubated with equivalent  
136 volume of ascitic fluid containing MAbs that were diluted 10 times with PBS. MAbs for 1 h at  
137 room temperature and the mixture was then used to inoculate 10-day-old embryonated chicken  
138 eggs or MDCK cells. The viruses obtained were detected using the hemagglutination test after  
139 incubation for 48 h at 35°C and cloned by limiting dilution in embryonated chicken eggs.  
140 Escape from MAbs was confirmed by the failure to detect by the IFA method and the nucleotide  
141 sequences of the HA genes of the mutants were determined.

142

#### 143 **Sequence analysis of virus genes**

144           Virus RNA was extracted from the allantoic fluid of virus-infected chicken embryos using  
145 TRIzol LS Reagent (Thermo Fisher Scientific, Santa Clara, CA, USA) according to the  
146 manufacturer's protocol. The extracted RNA was reverse-transcribed with Uni 12 primer and  
147 M-MLV Reverse Transcriptase (Thermo Fisher Scientific, Santa Clara, CA, USA), and the HA  
148 segments were then amplified by gene-specific primers (27). The nucleotide sequences of the  
149 amplified HA segments were determined directly or after cloning in pGEM-T Easy vector  
150 (Promega, Madison, WI, USA) using an Auto-sequencer 3500 Genetic Analyzer (Thermo Fisher  
151 Scientific, Santa Clara, CA, USA). The positions of the amino acid substitutions in the HA  
152 molecule were visualized in the three-dimensional structure obtained from the Protein Databank  
153 (PDB accession number: 4k62) (28) with Accelrys DS Visualizer v4.0 (BIOVIA, La Jolla, CA,  
154 USA).

155

156 **Ethics statement**

157       All *in vivo* experiments were authorized by the Institutional Animal Care and Use Committee  
158 of the Graduate School of Veterinary Medicine, Hokkaido University (approval number: 13-  
159 0093), and performed according to the guidelines of this committee.

## 160 **Results**

### 161 **Characterization of MAbs that recognized the HA of Kum/1-7 (H5N8)**

162 To characterize the antigenic structure of H5 HPAIVs, we established four MAbs against the  
163 HA of Kum/1-7 (H5N8) belonging to clade 2.3.4.4 (Table 1). All four MAbs exhibited  
164 neutralizing activity (i.e., 40,960 NT titer by A32/2, 2,560 NT titer by A262/2 and 640 NT titer  
165 by B3/2 and B157/1). Among these, only MAb A32/2 showed HI activity of 80 HI titer. To  
166 determine the epitope of each MAb, escape mutants were selected in the presence of MAbs and  
167 the amino acid sequences of the HA molecules were compared with that of the parental Kum/1-7  
168 (H5N8). All of the escape mutants had one or two amino acid changes in the HA1 region (Table  
169 1). The mutants, mtA32/2 and mtB157/1, which selected by MAbs A32/2 and B157/1,  
170 respectively, had single amino acid substitution in the receptor subdomain R (R domain) (12).  
171 An amino acid substitution of mtA32/2 was located in position 160 (H3 numbering is used  
172 throughout the present study) (29), which corresponds to site B in H3 HA. Besides, an amino  
173 acid substitution of mtB157/1 was located in position 124, which corresponds to site A in H3 HA.  
174 The mutant mtA262/2, which was prepared from A262/2, had single amino acid substitutions in  
175 position 50, which locates on the F' domain, and corresponds at site C in H3 HA. The mutant  
176 mtB3/2-D47N-H287N was selected by MAb B3/2 and carried double amino acid substitutions in  
177 position 47 and 287. These substitutions on the HA of mtB3/2-D47N-H287N are on the the F'  
178 domain and site C in H3 HA similar to that of mtA262/2. To exclude the possibility that mtB3/2-  
179 D47N-H287N is a mixture of viruses with two independent substitutions, the HA gene from  
180 mtB3/2-D47-H287 was cloned and we confirmed that these substitutions are on the same HA.

181 We also confirmed that MAb B3/2 is not derived from the mixture of two hybridoma cells by  
182 similar reactivity of Mab B3/2/1, which were further cloned from the hybridoma cells of MAb  
183 B3/ 2.

184 The loss of reactivity of each mutant with each MAb was confirmed by IFA (Table 2).  
185 None of the mutants exhibited loss of reactivity with the other three MAbs, which were not used  
186 to select the mutants. Each of the MAbs recognized independent epitopes and the recognition  
187 sites of MAb A262/2 and B3/2 were close to each other in the HA structure. The mutants,  
188 mtB3/2-D47N and mtB3/2-H287N, were carried a single substitution in position 47 or 287,  
189 respectively, however, they does not escape from MAb B3/2 according to IFA, thereby indicating  
190 that at least the asparagine residue in position 47 or the histidine residue in position 287 is  
191 necessary for binding with MAb B3/2.

192

### 193 **Antigenic analysis of H5 viruses with MAbs**

194 To compare the antigenicity of the H5 viruses, we compared the reactivity to the MAbs by  
195 H5 viruses that have been isolated recently in East and Southeast Asia by NT titers (Table 3).  
196 Viruses of clades 1.1, 2.3.2.1, 2.3.4, and 2.3.4.4 had different reactivity patterns and titers to the  
197 panel of MAbs, thereby indicating that the antigenicity of the viruses differed among these clades.  
198 MAbs against the HA of Dk/Penn (H5N2) (25), which was isolated from a wild water bird, were  
199 also used in addition to the MAbs established in the present study. All of the MAbs against the  
200 HA of Kum/1-7 (H5N8) reacted with high titer to the viruses belonging to clade 2.3.4.4. This  
201 reaction was supported by amino acid sequences of epitopes which proposed by amino acid

202 change (Table 4). These amino acids are highly conserved in viruses in clade 2.3.4.4. MAb  
203 25/2/5 prepared with Dk/Penn (H5N2) did not react with the Eurasian H5 HPAIVs of clades 1.1,  
204 2.3.2.1, 2.3.4, and 2.3.4.4, which were circulating recently among poultry in Asia, whereas MAbs  
205 A262/2 and B3/2 against the HA of Kum/1-7 (H5N8) reacted with these viruses. However,  
206 MAbs A32/2 and B157/1 did not react with A/peregrine falcon/Hong Kong/810/2009 (Pf/HK)  
207 (H5N1), which suggests that the epitopes recognized by these MAbs were responsible for the  
208 differences in antigenicity among the viruses of clades 2.3.4.4 and 2.3.4, such as glycosylation of  
209 HA (Table 4). MAb A262/2 cross-reacted with H5 viruses in the Eurasian lineage as well as  
210 those in the North American lineage.

211

#### 212 **Antigenic analysis of H5 viruses using antiserum**

213 The antigenicity of the H5 viruses was further analyzed using polyclonal antibodies with the  
214 HI test (Table 5). Criteria for antigenicity was based on the HI titer of tested viruses with  
215 antisera was 8-fold lower than homologous titers; it consider that these viruses were antigenically  
216 different each other. In agreement with our previous study, the antigenicity of the viruses of  
217 clade 2.3.4.4 differed significantly from that of the viruses of other clades (6). Kum/1-7 (H5N8)  
218 of clade 2.3.4.4 reacted with the antiserum against Pf/HK (H5N1) of clade 2.3.4 at an eight-fold  
219 lower titer compared with the homologous titer (i.e., HI titer of 2,560). Pf/HK (H5N1) reacted  
220 with the antiserum against Kum/1-7 (H5N8) at a 32-fold lower titer compared with the  
221 homologous titer, thereby indicating that the antigenicity of Kum/1-7 (H5N8) of clade 2.3.4.4  
222 was different from that of Pf/HK (H5N1) of clade 2.3.4. In addition, the reactivity of the viruses

223 of clade 2.3.4.4 with antisera against Kum/1-7 (H5N8) and Pf/HK (H5N1) differed slightly among  
224 the subgroups. The HI titers of A/environment/Kagoshima/KU-ngr-H/2014 (H5N8) in group A  
225 (Fig. S1) with antiserum against Kum/1-7 (H5N8) were comparable to the homologous titers.  
226 The HI titers of viruses in group B with antiserum against Kum/1-7 (H5N8) were also comparable  
227 to the homologous titer, whereas the HI titers of these viruses with antiserum against Pf/HK  
228 (H5N1) were 16-fold lower than the homologous titer. Moreover, in group C, the HI titer of  
229 A/crane/Kagoshima/KU41/2014 (H5N8) with antiserum against Kum/1-7 (H5N8) was slightly  
230 lower compared with the viruses of other subgroups. These results suggest that the antigenicity  
231 of the viruses of clade 2.3.4.4 differed slightly among groups A, B, and C.

232 Interestingly, the antiserum against Kum/1-7 (H5N8) also reacted with  
233 A/mallard/Hokkaido/24/2009 (Mal/Hok) (H5N1), which is a non-pathogenic virus that circulates  
234 among wild birds, at a higher titer compared with that of the homologous strain. However, the  
235 antiserum against Mal/Hok (H5N1) reacted with Kum/1-7 (H5N8) at a significantly lower titer  
236 compared with that of the homologous strain.

237 The amino acid substitution of alanine 160 to threonine carried by mtA32/2 was found in  
238 several H5 strains. Among the viruses that we tested in the present study, Pf/HK (H5N1) and  
239 A/Muscovy duck/Vietnam/OIE-559/2011 (H5N1) has this substitution (Table 4). The viruses  
240 that carried threonine 160 were predicted to possess an N-linked oligosaccharide chain at position  
241 158 (30). The acquisition of oligosaccharide chain contributes to antigenic drift, so single  
242 immunized antisera against Kum/1-7 (H5N8) and mtA32/2 were prepared to observe slight  
243 antigenic difference and to evaluate the importance of amino acid position 160 in the HA for the

244 antigenicity of Kum/1-7 (H5N8). We found that mtA32/2 had an eight-fold lower titer with the  
245 antiserum against Kum/1-7 (H5N8) compared with the homologous titer (Table 6). Therefore,  
246 position 160 in the HA plays a critical role in the antigenic differentiation of H5 HPAIVs of clade  
247 2.3.4.4.

248

## 249 **Discussion**

250 H5 HPAIVs have spread in both poultry and wild birds since the late 2003 (1, 2). The  
251 continued circulation of HPAIVs in poultry in several regions of the world has led to antigenic  
252 drift (6, 7). To analyze the antigenic properties of H5 HPAIVs isolated in Asia, we established  
253 neutralizing MAbs that recognized the HA using Kum/1-7 (H5N8). Each of the MAbs  
254 established in the present study recognized independent epitopes according to the reactivity of  
255 escape mutants with MAbs (Table 2). Two of the four MAbs recognized the R domain of the  
256 HA molecule and the other two recognized the F' domain (Fig. 1).

257 Antigenic difference between Kum/1-7 (H5N8) of clade 2.3.4.4 and Pf/HK (H5N1) of clade  
258 2.3.4 was clearly detected by MAbs in a similar manner to our previous study (6) and that of  
259 Kanehira et al. (7). H5 viruses belonging to clade 2.3.4.4 reacted with all the MAbs established  
260 using Kum/1-7 (H5N8) in the present study, which indicates that the epitopes recognized by these  
261 MAbs are conserved within the H5 viruses of clade 2.3.4.4 that we tested. However, the  
262 reactivity pattern of these viruses with chicken hyperimmune sera against Kum/1-7 (H5N8) and  
263 Pf/HK (H5N1) indicated that the antigenicity of the H5 viruses belonging to each subgroups of  
264 clade 2.3.4.4 differed slightly. In particular, A/crane/Kagoshima/KU41/2014 (H5N8) in group

265 C had the lowest titer with the antiserum against Kum/1-7 (H5N8) and with that against Pf/HK  
266 (H5N1) compared with the viruses belonging to other subgroups of clade 2.3.4.4. These results  
267 are consistent with the report that the HA genes of group C viruses are phylogenetically distinct  
268 from those of the group A and group B viruses (Fig. S1) (4), thereby indicating that the H5  
269 HPAIVs of clade 2.3.4.4 have evolved divergent antigenically and phylogenetically. Further  
270 study should be conducted to clarify the antigenic variation of viruses in subgroup of clade 2.3.4.4.

271 We also demonstrated the importance of position 160 in the HA for the antigenic structure  
272 of Kum/1-7 (H5N8) (Table 4). In particular, the H5 HA possesses a relatively conserved  
273 asparagine residue in position 158; therefore threonine 160 contributes to the glycosylation on  
274 position 158 in several H5 viruses. The mutant selected by MAb A32/2, mtA32/2 carried  
275 threonine 160 and it was predicted to possess an N-linked oligosaccharide chain on position 158  
276 (30). This oligosaccharide chain should shield the antigenic site, so the acquisition of  
277 glycosylation sites in the HA may allow the escape from antibodies (31). Thus, the amino acid  
278 substitution of alanine 160 to threonine in mtA32/2 causes the acquisition of an N-linked glycan  
279 in the head domain of the HA, thereby leading to the antigenic change from Kum/1-7 (H5N8) by  
280 one amino acid substitution. Furthermore, the acquisition or loss of a putative glycosylation site  
281 due to an amino acid substitution at position 160 in the HA occurred in several clusters of H5  
282 HAs, where those of clade 2.3.4 had a putative glycosylation site on position 158 (threonine 160),  
283 whereas the H5 HAs of clade 2.3.4.4 did not (alanine 160). This evidence indicates that position  
284 160 in H5 HA plays a critical role in the emergence of antigenic variants. Antiserum against  
285 Kum/1-7 (H5N8) cross-reacted with Mal/Hok (H5N1), which is a non-pathogenic virus that



286 circulates among wild water birds (32). Similar to Kum/1-7 (H5N8), Mal/Hok (H5N1) does not  
287 have a putative glycosylation site at position 158 (alanine 160), which could partially explain the  
288 cross-reaction with antiserum against Kum/1-7 (H5N8).

289 MAb A262/2 exhibited broad cross-clade reactivity and this MAb reacted with H5 HPAIVs  
290 of clades 0, 1.1, 2.2, 2.3.2.1, 2.3.4, 2.3.4.4, and 2.5, as well as viruses in the North American  
291 lineage (Table 3). The mutant selected by MAb A262/2 had an amino acid substitution at  
292 position 50 in the HA. The glycine residue at position 50 in the HA, which is carried by Kum/1-  
293 7 (H5N8), is highly conserved in H5 HAs used in the present study, although amino acid  
294 difference was observed at position 49 or 51 (Table 4). This suggests that MAb A262/2 might  
295 be useful to detect H5 HPAIVs which are circulating in the world. Further study using a lot of  
296 H5 HPAIVs is necessary to confirm this broad reactivity of MAb A262/2. Interestingly, MAb  
297 B3/2, which recognizes the F' domain exhibited exclusive reactivity with Eurasian H5 HPAIV.  
298 The mutant mtB3/2-D47N-H287N had double amino acid mutations to escape from single MAb  
299 same as previously reported in escape mutant of H5N1 virus and influenza B virus (33, 34).  
300 Previous studies have shown that the F' domain of H5 HA contains highly conserved epitopes  
301 compared with the R domain (35, 36). However, Yi et al. (37) reported MAbs that recognize  
302 the F' domain of HA of H1N1pdm09, but they did not recognize 1918 H1N1 strains. Thus,  
303 MAb B3/2 might recognize the epitope that varies among H5 HAs, unlike MAb A262/2.

304 H5 HPAIVs are still endemic in poultry and they are associated with vaccination programs  
305 in some countries (38, 39). The reactivity of the MAbs established in the present study as well  
306 as representative antisera against H5 viruses clearly demonstrated the antigenic divergence of

307 the H5 HPAIVs that have been isolated recently in Asia, especially those of clade 2.3.4.4. The  
308 panels of MAbs established in the present study should be useful for monitoring and detecting  
309 the emergence of further antigenic variants. Moreover, our results suggest that the continued  
310 monitoring of H5 viruses is required for the control of avian influenza.

311

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### 324 **Disclosure**

325 The authors have no conflicts of interest to declare.

326 **References**

- 327 1 Xu, X., Subbarao, Cox, N.J. & Guo, Y. (1999) Genetic characterization of the pathogenic  
328 influenza A/Goose/Guangdong/1/96 (H5N1) virus: similarity of its hemagglutinin gene  
329 to those of H5N1 viruses from the 1997 outbreaks in Hong Kong. *Virology*, 261: 15-9.
- 330 2 Web portal on Avian Influenza: OIE - World Organisation for Animal Health UPDATE ON  
331 HIGHLY PATHOGENIC AVIAN INFLUENZA IN ANIMALS (TYPE H5 and H7). (6  
332 June 2016) Available from: <http://www.oie.int/animal-health-in-the-world/>
- 333 3 Smith, G.J., Donis, R.O., World Health Organization/World Organization for Animal  
334 Health/Food and Agriculture Organization (WHO/OIE/FAO) H5 Evolution Working  
335 Group. (2015) Nomenclature updates resulting from the evolution of avian influenza  
336 A(H5) virus clades 2.1.3.2a, 2.2.1, and 2.3.4 during 2013-2014. *Influenza Other Respir*  
337 *Viruses*, 9: 271-6.
- 338 4 Ozawa, M., Matsuu, A., Tokorozaki, K., Horie, M., Masatani, T., Nakagawa, H., Okuya, K.,  
339 Kawabata, T. & Toda, S. (2015) Genetic diversity of highly pathogenic H5N8 avian  
340 influenza viruses at a single overwintering site of migratory birds in Japan, 2014/15. *Euro*  
341 *Surveill*, 20.
- 342 5 Wright, P.F., Neumann, G. & Kawaoka, Y. (2013) Orthomyxoviruses. In: Knipe M.D., Howley  
343 P. Fields *Virology*, sixth edn. Philadelphia: Wolters Kluwer Health/Lippincott Williams  
344 & Wilkins, pp. 1190-1191.
- 345 6 Hiono, T., Ohkawara, A., Ogasawara, K., Okamatsu, M., Tamura, T., Chu, D.H., Suzuki, M.,  
346 Kuribayashi, S., Shichinohe, S., Takada, A., Ogawa, H., Yoshida, R., Miyamoto, H., Nao,

- 347 N., Furuyama, W., Maruyama, J., Eguchi, N., Ulziibat, G., Enkhbold, B., Shatar, M.,  
348 Jargalsaikhan, T., Byambadorj, S., Damdinjav, B., Sakoda, Y. & Kida, H. (2015) Genetic  
349 and antigenic characterization of H5 and H7 influenza viruses isolated from migratory  
350 water birds in Hokkaido, Japan and Mongolia from 2010 to 2014. *Virus Genes*, 51: 57-  
351 68.
- 352 7 Kanehira, K., Uchida, Y., Takemae, N., Hikono, H., Tsunekuni, R. & Saito, T. (2015)  
353 Characterization of an H5N8 influenza A virus isolated from chickens during an outbreak  
354 of severe avian influenza in Japan in April 2014. *Arch Virol*, 160: 1629-43.
- 355 8 Caton, A.J., Brownlee, G.G., Yewdell, J.W. & Gerhard, W. (1982) The antigenic structure of  
356 the influenza virus A/PR/8/34 hemagglutinin (H1 subtype). *Cell*, 31: 417-27.
- 357 9 Wiley, D.C., Wilson, I.A. & Skehel, J.J. (1981) Structural identification of the antibody-binding  
358 sites of Hong Kong influenza haemagglutinin and their involvement in antigenic  
359 variation. *Nature*, 289: 373-8.
- 360 10 Kaverin, N.V., Rudneva, I.A., Ilyushina, N.A., Varich, N.L., Lipatov, A.S., Smirnov, Y.A.,  
361 Govorkova, E.A., Gitelman, A.K., Lvov, D.K. & Webster, R.G. (2002) Structure of  
362 antigenic sites on the haemagglutinin molecule of H5 avian influenza virus and  
363 phenotypic variation of escape mutants. *J Gen Virol*, 83: 2497-505.
- 364 11 Stevens, J., Blixt, O., Tumpey, T.M., Taubenberger, J.K., Paulson, J.C. & Wilson, I.A. (2006)  
365 Structure and receptor specificity of the hemagglutinin from an H5N1 influenza virus.  
366 *Science*, 312: 404-10.
- 367 12 Ha, Y., Stevens, D.J., Skehel, J.J. & Wiley, D.C. (2002) H5 avian and H9 swine influenza

- 368 virus haemagglutinin structures: possible origin of influenza subtypes. *EMBO J*, 21: 865-  
369 75.
- 370 13 Chen, Y., Qin, K., Wu, W.L., Li, G., Zhang, J., Du, H., Ng, M.H., Shih, J.W., Peiris, J.S., Guan,  
371 Y., Chen, H. & Xia, N. (2009) Broad cross-protection against H5N1 avian influenza  
372 virus infection by means of monoclonal antibodies that map to conserved viral epitopes.  
373 *J Infect Dis*, 199: 49-58.
- 374 14 Ho, H.T., Qian, H.L., He, F., Meng, T., Szyporta, M., Prabhu, N., Prabakaran, M., Chan, K.P.  
375 & Kwang, J. (2009) Rapid detection of H5N1 subtype influenza viruses by antigen  
376 capture enzyme-linked immunosorbent assay using H5- and N1-specific monoclonal  
377 antibodies. *Clin Vaccine Immunol*, 16: 726-32.
- 378 15 Sui, J., Hwang, W.C., Perez, S., Wei, G., Aird, D., Chen, L.M., Santelli, E., Stec, B., Cadwell,  
379 G., Ali, M., Wan, H., Murakami, A., Yammanuru, A., Han, T., Cox, N.J., Bankston, L.A.,  
380 Donis, R.O., Liddington, R.C. & Marasco, W.A. (2009) Structural and functional bases  
381 for broad-spectrum neutralization of avian and human influenza A viruses. *Nat Struct*  
382 *Mol Biol*, 16: 265-73.
- 383 16 Saito, T., Tanikawa, T., Uchida, Y., Takemae, N., Kanehira, K. & Tsunekuni, R. (2015)  
384 Intracontinental and intercontinental dissemination of Asian H5 highly pathogenic avian  
385 influenza virus (clade 2.3.4.4) in the winter of 2014-2015. *Rev Med Virol*, 25: 388-405.
- 386 17 Jiao, P., Cui, J., Song, Y., Song, H., Zhao, Z., Wu, S., Qu, N., Wang, N., Ouyang, G. & Liao,  
387 M. (2016) New Reassortant H5N6 Highly Pathogenic Avian Influenza Viruses in  
388 Southern China, 2014. *Front Microbiol*, 7: 754.

- 389 18 Huang, P.Y., Lee, C.C., Yip, C.H., Cheung, C.L., Yu, G., Lam, T.T., Smith, D.K., Zhu, H. &  
390 Guan, Y. (2016) Genetic characterization of highly pathogenic H5 influenza viruses from  
391 poultry in Taiwan, 2015. *Infect Genet Evol*, 38: 96-100.
- 392 19 Lee, D.H., Bahl, J., Torchetti, M.K., Killian, M.L., Ip, H.S., Deliberto, T.J. & Swayne, D.E.  
393 (2016) Highly Pathogenic Avian Influenza Viruses and Generation of Novel  
394 Reassortants, United States, 2014-2015. *Emerg Infect Dis*, 22: 1283-5.
- 395 20 Tanikawa, T., Kanehira, K., Tsunekuni, R., Uchida, Y., Takemae, N. & Saito, T. (2016)  
396 Pathogenicity of H5N8 highly pathogenic avian influenza viruses isolated from a wild  
397 bird fecal specimen and a chicken in Japan in 2014. *Microbiol Immunol*, 60: 243-52.
- 398 21 Sun, H., Pu, J., Hu, J., Liu, L., Xu, G., Gao, G.F., Liu, X. & Liu, J. (2016) Characterization of  
399 clade 2.3.4.4 highly pathogenic H5 avian influenza viruses in ducks and chickens. *Vet*  
400 *Microbiol*, 182: 116-22.
- 401 22 Kida, H., Brown, L.E. & Webster, R.G. (1982) Biological activity of monoclonal antibodies  
402 to operationally defined antigenic regions on the hemagglutinin molecule of  
403 A/Seal/Massachusetts/1/80 (H7N7) influenza virus. *Virology*, 122: 38-47.
- 404 23 Köler, G. & Milstein, C. (1975) Continuous cultures of fused cells secreting antibody of  
405 predefined specificity. *Nature*, 256: 495-7.
- 406 24 Soda, K., Ozaki, H., Sakoda, Y., Isoda, N., Haraguchi, Y., Sakabe, S., Kuboki, N., Kishida,  
407 N., Takada, A. & Kida, H. (2008) Antigenic and genetic analysis of H5 influenza viruses  
408 isolated from water birds for the purpose of vaccine use. *Arch Virol*, 153: 2041-8.
- 409 25 Tumová, B., Pereira, HG., (1968) Antigenic relationship between influenza A viruses of human

- 410 and animal origin. *Bull World Health Organ*, 38: 415-420
- 411 26 Sakabe, S., Sakoda, Y., Haraguchi, Y., Isoda, N., Soda, K., Takakuwa, H., Saijo, K., Sawata,  
412 A., Kume, K., Hagiwara, J., Tuchiya, K., Lin, Z., Sakamoto, R., Imamura, T., Sasaki, T.,  
413 Kokumai, N., Kawaoka, Y. & Kida, H. (2008) A vaccine prepared from a non-pathogenic  
414 H7N7 virus isolated from natural reservoir conferred protective immunity against the  
415 challenge with lethal dose of highly pathogenic avian influenza virus in chickens.  
416 *Vaccine*, 26: 2127-34.
- 417 27 Hoffmann, E., Stech, J., Guan, Y., Webster, R.G. & Perez, D.R. (2001) Universal primer set  
418 for the full-length amplification of all influenza A viruses. *Arch Virol*, 146: 2275-89.
- 419 28 Zhang, W., Shi, Y., Lu, X., Shu, Y., Qi, J. & Gao, G.F. (2013) An airborne transmissible avian  
420 influenza H5 hemagglutinin seen at the atomic level. *Science*, 340: 1463-7.
- 421 29 Wilson, I.A., Skehel, J.J. & Wiley, D.C. (1981) Structure of the haemagglutinin membrane  
422 glycoprotein of influenza virus at 3 Å resolution. *Nature*, 289: 366-73.
- 423 30 Hervé P.L., Lorin, V., Jouvion, G., Da Costa, B. & Escriou, N. (2015) Addition of N-  
424 glycosylation sites on the globular head of the H5 hemagglutinin induces the escape of  
425 highly pathogenic avian influenza A H5N1 viruses from vaccine-induced immunity.  
426 *Virology*, 486: 134-145.
- 427 31 Medina, R.A., Stertz, S., Manicassamy, B., Zimmermann, P., Sun, X., Albrecht, R.A., Uusi-  
428 Kerttula, H., Zagordi, O., Belshe, R.B., Frey, S.E., Tumpey, T.M. & García-Sastre, A.  
429 (2013) Glycosylations in the globular head of the hemagglutinin protein modulate the  
430 virulence and antigenic properties of the H1N1 influenza viruses. *Sci Transl Med*, 5:

431 187ra70.

432 32 Yamamoto, N., Sakoda, Y., Motoshima, M., Yoshino, F., Soda, K., Okamatsu, M. & Kida, H.

433 (2011) Characterization of a non-pathogenic H5N1 influenza virus isolated from a

434 migratory duck flying from Siberia in Hokkaido, Japan, in October 2009. *Virology*, 8: 65.

435 33 Chai N, Swem LR, Park S, Nakamura G, Chiang N, Estevez A, Fong R, Kamen L, Kho E,

436 Reichelt M, Lin Z, Chiu H, Skippington E, Modrusan Z, Stinson J, Xu M, Lupardus P,

437 Ciferri C, Tan MW. (2017) A broadly protective therapeutic antibody against influenza

438 B virus with two mechanisms of action. *Nat Commun* 8:14234.

439 34 Tan Y, Ng Q, Jia Q, Kwang J, He F. (2015) A novel humanized antibody neutralizes H5N1

440 influenza virus via two different mechanisms. *J Virol*. 89(7):3712-22.

441 35 Prabakaran, M., Ho, H.T., Prabhu, N., Velumani, S., Szyport, M., He, F., Chan, K.P., Chen,

442 L.M., Matsuoka, Y., Donis, R.O. & Kwang, J. (2009) Development of epitope-blocking

443 ELISA for universal detection of antibodies to human H5N1 influenza viruses. *PLoS*

444 *One*, 4: e4566.

445 36 Du, A., Daidoji, T., Koma, T., Ibrahim, M.S., Nakamura, S., De Silva, U.C., Ueda, M., Yang,

446 C.S., Yasunaga, T., Ikuta, K. & Nakaya, T. (2009) Detection of circulating Asian H5N1

447 viruses by a newly established monoclonal antibody. *Biochem Biophys Res Commun*,

448 378: 197-202.

449 37 Yi, H., Lee, M.S., Lee, J.Y., Lee, H.K. & Kang, C. (2015) Immunological characterization of

450 monoclonal antibodies used in rapid influenza diagnostic test for detection of the 2009

451 pandemic influenza A(H1N1)pdm09 infection. *J Microbiol*, 53: 166-75.



- 452 38 Hu, T., Song, J., Zhang, W., Zhao, H., Duan, B., Liu, Q., Zeng, W., Qiu, W., Chen, G., Zhang,  
453 Y., Fan, Q. & Zhang, F. (2015) Emergence of novel clade 2.3.4 influenza A (H5N1)  
454 virus subgroups in Yunnan Province, China. *Infect Genet Evol*, 33: 95-100.
- 455 39 Swayne, D.E. & Spackman, E. (2013) Current status and future needs in diagnostics and  
456 vaccines for high pathogenicity avian influenza. *Dev Biol (Basel)*, 135: 79-94.

457 **Figure legend**

458 Fig. 1. Positions of the amino acid substitutions selected by the MAbs based on the three-  
459 dimensional structure of the monomeric H5 HA. The positions of the amino acid changes  
460 observed in each escape mutant selected by each MAb against Kum/1-7 (H5N8) (Red) and  
461 Dk/Penn (H5N2) (Blue) are mapped onto the three-dimensional structure of the monomeric HA  
462 of A/Indonesia/5/2005 (H5N1) (PDB accession number: 4k62) (27). Each antigenic site (A to  
463 E) defined in H3 HA (9) is encircled. The numbering of the amino acid positions follows H3  
464 numbering (28).

**465 Supplementary data**

466 Fig. S1. Phylogenetic tree for the H5 HA genes of influenza viruses. The HA genes which used  
467 in this study were analyzed by the maximum-likelihood (ML) method along with that of reference  
468 strains using MEGA 6.0 software (<http://www.megasoftware.net/>). Horizontal distances are  
469 proportional to the minimum number of nucleotide differences required to join nodes and  
470 sequences. Digits at the nodes indicate the probability of confidence levels in a bootstrap  
471 analysis with 1000 replications. The viruses used in this study are indicated in bold. The  
472 viruses used for MAbs production were underlined.

### List of the abbreviations

Abbreviation	Definition
CA	State of California
Dk/Penn (H5N2)	A/duck/Pennsylvania/10218/1984 (H5N2)
ELISA	enzyme-linked immunosorbent assay
FCA	Freund's complete adjuvant
FITC	fluorescein isothiocyanate
HA	hemagglutinin
HI	hemagglutination-inhibition
HPAIVs	highly pathogenic avian influenza viruses
IFA	immunofluorescent antibody assay
IgG	Immunoglobulin G
Kum/1-7 (H5N8)	A/chicken/Kumamoto/1-7/2014 (H5N8)
MAB	monoclonal antibody
MDCK cells	Madin-Darby canine kidney cells
MEM	minimal essential medium
NT	neutralization
PBS	phosphate-buffered saline
Pf/HK (H5N1)	A/peregrine falcon/Hong Kong/810/2009 (H5N1)
RNA	ribonucleic acid
TCID <sub>50</sub>	the 50% tissue culture infectious dose
USA	the United States of America

**Table 1.** Biological properties of neutralizing MAbs recognizing HA molecule of Kum/1-7 (H5N8).

MAb	Antibody titer			Isotype	Escape mutant	Substitution of escape mutant <sup>†</sup>			
	ELISA <sup>‡</sup>	HI	NT			Nucleotide		Amino acid	
						Position	Change	Position <sup>§</sup>	Change
A32/2	7.2	80	40,960	IgG1	mtA32/2	514	G→A	160	Ala→Thr
A262/2	7.4	<20	2,560	IgG1	mtA262/2	183	G→A	50	Gly→Arg
B3/2	6.8	20	640	IgG1	mtB3/2-D47N-H287N	175	G→A	47	Asp→Asn
B157/1	5.6	<20	640	IgG1	mtB157/1	865	C→A	287	His→Asn
						405	G→T	124	Lys→Asn

<sup>†</sup>Mutants were selected by each MAbs from Kum/1-7 (H5N8).

<sup>‡</sup>Titers are expressed in log<sub>10</sub>.

<sup>§</sup>Amino acid position is based on Wilson et al. (28).

**Table 2.** Reactivity of MAbs with each escape mutant by IFA.

MAb	Kum/1-7	Escape mutant from Kum/1-7					
		mtA32/2	mtA262/2	mtB3/2-D47N-H287N	mtB157/1	mtB3/2-D47N	mtB3/2-H287N
A32/2	+	-	+	+	+	+	+
A262/2	+	+	-	+	+	+	+
B3/2	+	+	+	-	+	+	+
B157/1	+	+	+	+	-	+	+

**Table 3.** NT titer of MAb against H5 viruses.

Lineage	Clade	Subgroup	Virus	Monoclonal antibody prepared from					
				Kum/1-7				Dk/Penn	
				A32/2	A262/2	B3/2	B157/1	D101/1	25/2/5
Eurasian	2.3.4.4	-	<b>A/chicken/Kumamoto/1-7/2014 (H5N8)</b>	40,960	2,560	640	640	<20	<20
	2.3.4.4	-	<b>A/duck/Vietnam/HU1-1151/2014 (H5N6)</b>	40,960	1,280	1,280	640	<20	<20
	2.3.4.4	Group C	<b>A/chicken/Miyazaki/7/2014 (H5N8)</b>	10,240	2,560	1,280	640	<20	<20
	2.3.4		<b>A/peregrine falcon/Hong Kong/810/2009 (H5N1)</b>	<20	640	640	<20	1,280	<20
	0		<b>A/Hong Kong/156/1997 (H5N1)</b>	640	1,280	640	<20	<20	1,280
	1.1		<b>A/muscovy duck/Vietnam/OIE-559/2011 (H5N1)</b>	<20	2,560	640	<20	640	<20
	2.2		<b>A/whooper swan/Mongolia/3/2005 (H5N1)</b>	<20	1,280	320	<20	<20	40
	2.3.2.1		<b>A/whooper swan/Hokkaido/1/2008 (H5N1)</b>	<20	1,280	320	<20	<20	<20
	2.5		<b>A/chicken/Yamaguchi/7/2004 (H5N1)</b>	640	2,560	1,280	80	<20	1,280
	-		<b>A/tern/South Africa/1961 (H5N3)</b>	640	1,280	<20	<20	1,280	1,280
	-		A/mallard/Hokkaido/24/2009 (H5N1)	640	640	<20	<20	1,280	1,280
North	-		<b>A/chicken/Taiwan/0502/2012 (H5N2)</b>	<20	1,280	<20	<20	1,280	1,280
American	-		A/chicken/Ibaraki/1/2005 (H5N2)	<20	1,280	<20	<20	<20	<20

HPAIVs are shown in bold.

**Table 4.** Comparison of amino acid sequence of H5 strains used in the present study.

Lineage	Clade	Subgroup	Virus	Amino acid sequence of the HA						
				46	51	123	129	156	162	283
Eurasian	2.3.4.4	-	<b>A/chicken/Kumamoto/1-7/2014 (H5N8)</b>	CD <u>DL</u> NGV		PK <u>SS</u> WPN		KKND <u>A</u> YP		VEYGH <u>C</u>
	2.3.4.4	-	<b>A/duck/Vietnam/HU1-1151/2014 (H5N6)</b>	•••••		•••••T•		•••••		•••••
	2.3.4.4	Group A	<b>A/environment/Kagoshima/KU-ngr-H/2014 (H5N8)</b>	•••••		•••••		•••••		•••••
	2.3.4.4	Group B	<b>A/crane/Kagoshima/KU1/2014 (H5N8)</b>	•••••		•••••		•••••		M•••••
	2.3.4.4	Group B	<b>A/crane/Kagoshima/KU13/2014 (H5N8)</b>	•••••		•R•••••		•••••		M•••••
	2.3.4.4	Group C	<b>A/crane/Kagoshima/KU41/2014 (H5N8)</b>	•••••		•••••		•••••		•••••
	2.3.4.4	Group C	<b>A/mallard duck/Kagoshima/KU116/2015 (H5N8)</b>	•••••		•••••		•••••		•••••
	2.3.4.4	Group C	<b>A/chicken/Miyazaki/7/2014 (H5N8)</b>	•••••		•••••		•••••		•••••
	2.3.4		<b>A/peregrine falcon/Hong Kong/810/2009 (H5N1)</b>	•••••		•••••D		•••N <u>T</u> ••		•G•••N•
	0		<b>A/Hong Kong/156/1997 (H5N1)</b>	•••••		•••••S•		•••S•••		L••••N•
	1.1		<b>A/muscovy duck/Vietnam/OIE-559/2011 (H5N1)</b>	•••D•I		•••••S		•••S <u>T</u> ••		L••••N•
	2.2		<b>A/whooper swan/Mongolia/3/2005 (H5N1)</b>	•••D••		•••••SD		••DN•••		L••••N•
	2.3.2.1		<b>A/whooper swan/Hokkaido/1/2008 (H5N1)</b>	•••••		••D••SD		••DN•••		V••••N•
	2.5		<b>A/chicken/Yamaguchi/7/2004 (H5N1)</b>	•••D••		•••••SD		•••S•••		L••••N•
	-		<b>A/tern/South Africa/1961 (H5N3)</b>	•S•••••		•R••••S•		E••N•••		L••••N•
	-		<b>A/mallard/Hokkaido/24/2009 (H5N1)</b>	•S•••••		•R••••S•		•••N•••		L••••N•
North	-		<b>A/chicken/Taiwan/0502/2012 (H5N2)</b>	•S•K••		•R••••S•		•••NV••		LS•SN•
American	-		<b>A/chicken/Ibaraki/1/2005 (H5N2)</b>	•S•K••		•R••••S•		••DNV••		LD•••N•
	-		<b>A/duck/Pennsylvania/10218/1984 (H5N2)</b>	•S•K••		•R••••S•		•••N•••		L••••N•

HPAIVs are shown in bold.

Underlined amino acids are the positions that substitution(s) are observed in each escape mutant: mtB3/2-D47N-H287N, mtA262/2 (G50R), mtB157/1 (K124N) and mtA32/2 (A160T).

Double underline indicates the amino acid substitution of alanine 160 to threonine which consist of glycosilation site.



**Table 5.** HI titer of polyclonal antibodies against H5 influenza viruses.

Lineage	Clade	Subgroup	Virus	Antiserum against					
				Ck/Kum/ 1-7/14	Pf/HK/ 810/09	Ws/Hok/ 1/08	Ck/Yam/ 7/04	Mal/Hok/ 24/09	Ck/Ibr/ 1/05
Eurasian	2.3.4.4	-	<b>A/chicken/Kumamoto/1-7/2014 (H5N8)</b>	<u>640</u>	320	20	80	20	<20
	2.3.4.4	-	<b>A/duck/Vietnam/HU1-1151/2014 (H5N6)</b>	640	320	20	40	20	<20
	2.3.4.4	Group A	<b>A/environment/Kagoshima/KU-ngr-H/2014 (H5N8)</b>	640	640	<20	80	40	<20
	2.3.4.4	Group B	<b>A/crane/Kagoshima/KU1/2014 (H5N8)</b>	640	160	<20	80	20	<20
	2.3.4.4	Group B	<b>A/crane/Kagoshima/KU13/2014 (H5N8)</b>	640	160	<20	80	20	<20
	2.3.4.4	Group C	<b>A/crane/Kagoshima/KU41/2014 (H5N8)</b>	160	160	<20	40	<20	<20
	2.3.4.4	Group C	<b>A/mallard duck/Kagoshima/KU116/2015 (H5N8)</b>	160	160	<20	40	<20	<20
	2.3.4.4	Group C	<b>A/chicken/Miyazaki/7/2014 (H5N8)</b>	320	160	20	40	20	<20
	2.3.4		<b>A/peregrine falcon/Hong Kong/810/2009 (H5N1)</b>	20	<u>2,560</u>	20	80	<20	<20
	2.3.2.1		<b>A/whooper swan/Hokkaido/1/2008 (H5N1)</b>	80	40	<u>640</u>	640	40	<20
	2.5		<b>A/chicken/Yamaguchi/7/2004 (H5N1)</b>	80	80	320	<u>5,120</u>	320	320
	-		A/mallard/Hokkaido/24/2009 (H5N1)	2,560	40	80	1,280	<u>1,280</u>	1,280
	North American	-		A/chicken/Ibaraki/1/2005 (H5N2)	<20	<20	20	1,280	320

HPAIVs are shown in bold.

Underlines indicate homologous titer.

Ck: chicken, Pf: peregrine falcon, Ws: whooper swan, Mal: mallard, Kum: Kumamoto, HK: Hong Kong, Hok: Hokkaido, Yam: Yamaguchi, Ibr: Ibaraki

**Table 6.** HI titer of single immunized serum against Kum/1-7 (H5N8) and mtA32/2 .

Virus	Antiserum against		
	Single immunized		Hyperimmune
	Kum/1-7	mtA32/2	Kum/1-7
A/chicken/Kumamoto/1-7/2014 (H5N8)	<u>64</u>	8	<u>640</u>
mtA32/2	8	<u>16</u>	320

Underlines indicate homologous titer.

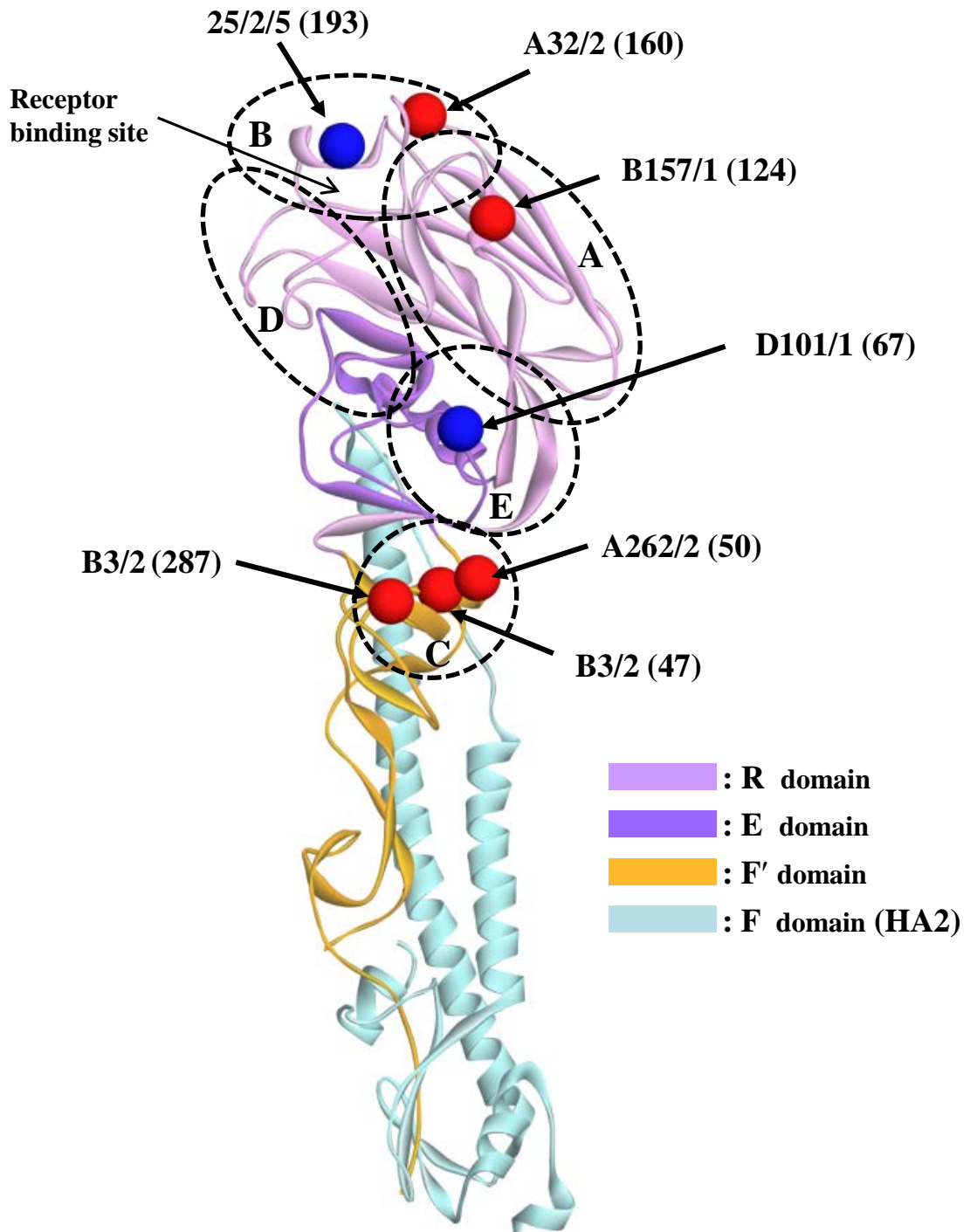


Fig. 1 Ohkawara et al.

Fig. S1  
Phylogenetic tree for the  
H5 HA genes of influenza  
viruses.

