Original article

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

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

Abstract

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

Keywords

antigenicity; H5N8; hemagglutinin; highly pathogenic avian influenza virus

Introduction

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

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

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

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

Materials and methods

Viruses and cells

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

calf serum and antibiotics.

MAbs

MAbs against Kum/1-7 (H5N8) designated as A32/2, A262/2, B3/2, and B157/1 were prepared as described by Kida et al. (22). Briefly, BALB/c mice (Japan SLC, Shizuoka, Japan) were immunized with formalin-inactivated Kum/1-7 (H5N8) virus and splenocytes were fused with Sp_2O-Ag14 myeloma cells (23). The hybridoma cells that secreted MAbs specific to the virus antigen were selected using an enzyme-linked immunosorbent assay (ELISA), as follows: 50 µl of the cell culture supernatant was added to each well of a 96-well plate coated with the virus antigens and the specific MAbs were detected by horseradish peroxidase-conjugated goat IgG to mouse IgG (MP Biomedicals, Santa Ana, CA, USA). The hybridoma cells were then cloned in 0.4% bacto-agar (Becton, Dickinson and Company, New Jersey, USA). The isotypes of the MAbs were determined using Mouse Monoclonal Antibody Isotyping Reagents (Sigma Aldrich, St Louis, MO, USA). Ascitic fluid of mice that contains each MAb were obtained and the aliquots were used for characterization of MAbs and antigenic analysis of H5 HPAIVs. Neutralizing MAbs that recognized the HA of A/duck/Pennsylvania/10218/1984 (Dk/Penn) (H5N2) (24) were also used in the present study.

Hyperimmune sera and single immunized sera

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

114 immunized intramuscularly with 500 µl 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 ($TCID_{50}$) 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**

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

Sequence analysis of virus genes

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

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.

Results

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

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

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

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

Antigenic analysis of H5 viruses with MAbs

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

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

Antigenic analysis of H5 viruses using antiserum

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

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

Discussion

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

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

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

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

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

Acknowledgements

We are grateful to Dr. Takehiko Saito of the National Institute of Animal Health, Japan for kindly providing A/chicken/Kumamoto/1-7/2014 (H5N8), A/chicken/Miyazaki/7/2014 (H5N8), A/chicken/Yamaguchi/7/2004 (H5N1), and A/chicken/Ibaraki/1/2005 (H5N2). We thank Dr. Luk S.M. Geraldine of the University of Hong Kong for providing A/peregrine falcon/Hong Kong/810/2009 (H5N1). We also thank Animal Health Institute, Taiwan for providing A/chicken/Taiwan/0502/2012 (H5N2). The present study was supported partly by the Japan Initiative for Global Research Network on Infectious Diseases (J-GRID) from Japan Agency for Medical Research and development, AMED. The present study was also partially supported by the Program for Leading Graduate Schools (F01) from Japan Society for promotion of Science (JSPS) and by JSPS KAKENHI Grant number JP26850178.

Disclosure

The authors have no conflicts of interest to declare.

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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).

Supplementary data

Fig. S1. Phylogenetic tree for the H5 HA genes of influenza viruses. The HA genes which used in this study were analyzed by the maximum-likelihood (ML) method along with that of reference strains using MEGA 6.0 software (http://www.megasoftware.net/). Horizontal distances are proportional to the minimum number of nucleotide differences required to join nodes and sequences. Digits at the nodes indicate the probability of confidence levels in a bootstrap analysis with 1000 replications. The viruses used in this study are indicated in bold. The 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
$TCID_{50}$	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[†]			
						Nucleotide		Amino acid	
	ELISA[‡]	HI	NT			Position	Change	Position[§]	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
						865	C→A	287	His→Asn
B157/1	5.6	<20	640	IgG1	mtB157/1	405	G→T	124	Lys→Asn

[†]Mutants were selected by each MAbs from Kum/1-7 (H5N8).

[‡]Titers are expressed in $\log_{10}$.

[§]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	-	**A/chicken/Kumamoto/1-7/2014 (H5N8)**	40,960	2,560	640	640	<20	<20
	2.3.4.4	-	**A/duck/Vietnam/HU1-1151/2014 (H5N6)**	40,960	1,280	1,280	640	<20	<20
	2.3.4.4	Group C	**A/chicken/Miyazaki/7/2014 (H5N8)**	10,240	2,560	1,280	640	<20	<20
	2.3.4		**A/peregrine falcon/Hong Kong/810/2009 (H5N1)**	<20	640	640	<20	1,280	<20
	0		**A/Hong Kong/156/1997 (H5N1)**	640	1,280	640	<20	<20	1,280
	1.1		**A/muscovy duck/Vietnam/OIE-559/2011 (H5N1)**	<20	2,560	640	<20	640	<20
	2.2		**A/whooper swan/Mongolia/3/2005 (H5N1)**	<20	1,280	320	<20	<20	40
	2.3.2.1		**A/whooper swan/Hokkaido/1/2008 (H5N1)**	<20	1,280	320	<20	<20	<20
	2.5		**A/chicken/Yamaguchi/7/2004 (H5N1)**	640	2,560	1,280	80	<20	1,280
	-		**A/tern/South Africa/1961 (H5N3)**	640	1,280	<20	<20	1,280	1,280
	-		A/mallard/Hokkaido/24/2009 (H5N1)	640	640	<20	<20	1,280	1,280
North American	-		**A/chicken/Taiwan/0502/2012 (H5N2)**	<20	1,280	<20	<20	1,280	1,280
	-		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	288
Eurasian	2.3.4.4	-	**A/chicken/Kumamoto/1-7/2014 (H5N8)**	CDLNGV		PKSSWPN		KKNDAYP		VEYGHC	
	2.3.4.4	-	**A/duck/Vietnam/HU1-1151/2014 (H5N6)**	·····		·····T·		·····		·····	
	2.3.4.4	Group A	**A/environment/Kagoshima/KU-ngr-H/2014 (H5N8)**	·····		·····		·····		·····	
	2.3.4.4	Group B	**A/crane/Kagoshima/KU1/2014 (H5N8)**	·····		·····		·····		M····	
	2.3.4.4	Group B	**A/crane/Kagoshima/KU13/2014 (H5N8)**	·····		·R····		·····		M····	
	2.3.4.4	Group C	**A/crane/Kagoshima/KU41/2014 (H5N8)**	·····		·····		·····		·····	
	2.3.4.4	Group C	**A/mallard duck/Kagoshima/KU116/2015 (H5N8)**	·····		·····		·····		·····	
	2.3.4.4	Group C	**A/chicken/Miyazaki/7/2014 (H5N8)**	·····		·····		·····		·····	
	2.3.4		**A/peregrine falcon/Hong Kong/810/2009 (H5N1)**	·····		·····D		···NT··		·G··N·	
	0		**A/Hong Kong/156/1997 (H5N1)**	·····		·····S·		···S···		L···N·	
	1.1		**A/muscovy duck/Vietnam/OIE-559/2011 (H5N1)**	···D·I		·····S		···ST··		L···N·	
	2.2		**A/whooper swan/Mongolia/3/2005 (H5N1)**	···D··		·····SD		··DN···		L···N·	
	2.3.2.1		**A/whooper swan/Hokkaido/1/2008 (H5N1)**	·····		··D··SD		··DN···		V···N·	
	2.5		**A/chicken/Yamaguchi/7/2004 (H5N1)**	···D··		·····SD		···S···		L···N·	
	-		**A/tern/South Africa/1961 (H5N3)**	·S····		·R···S·		E··N···		L···N·	
	-		A/mallard/Hokkaido/24/2009 (H5N1)	·S····		·R···S·		···N···		L···N·	
North	-		**A/chicken/Taiwan/0502/2012 (H5N2)**	·S·K··		·R···S·		···NV··		LS·SN·	
American	-		A/chicken/Ibaraki/1/2005 (H5N2)	·S·K··		·R···S·		··DNV··		LD··N·	
			A/duck/Pennsylvania/10218/1984 (H5N2)	·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	-	**A/chicken/Kumamoto/1-7/2014 (H5N8)**	640	320	20	80	20	<20
	2.3.4.4	-	**A/duck/Vietnam/HU1-1151/2014 (H5N6)**	640	320	20	40	20	<20
	2.3.4.4	Group A	**A/environment/Kagoshima/KU-ngr-H/2014 (H5N8)**	640	640	<20	80	40	<20
	2.3.4.4	Group B	**A/crane/Kagoshima/KU1/2014 (H5N8)**	640	160	<20	80	20	<20
	2.3.4.4	Group B	**A/crane/Kagoshima/KU13/2014 (H5N8)**	640	160	<20	80	20	<20
	2.3.4.4	Group C	**A/crane/Kagoshima/KU41/2014 (H5N8)**	160	160	<20	40	<20	<20
	2.3.4.4	Group C	**A/mallard duck/Kagoshima/KU116/2015 (H5N8)**	160	160	<20	40	<20	<20
	2.3.4.4	Group C	**A/chicken/Miyazaki/7/2014 (H5N8)**	320	160	20	40	20	<20
	2.3.4		**A/peregrine falcon/Hong Kong/810/2009 (H5N1)**	20	2,560	20	80	<20	<20
	2.3.2.1		**A/whooper swan/Hokkaido/1/2008 (H5N1)**	80	40	640	640	40	<20
	2.5		**A/chicken/Yamaguchi/7/2004 (H5N1)**	80	80	320	5,120	320	320
	-		A/mallard/Hokkaido/24/2009 (H5N1)	2,560	40	80	1,280	1,280	1,280
North American	-		A/chicken/Ibaraki/1/2005 (H5N2)	<20	<20	20	1,280	320	20,480

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)	64	8	640
mtA32/2	8	16	320

Underlines indicate homologous titer.

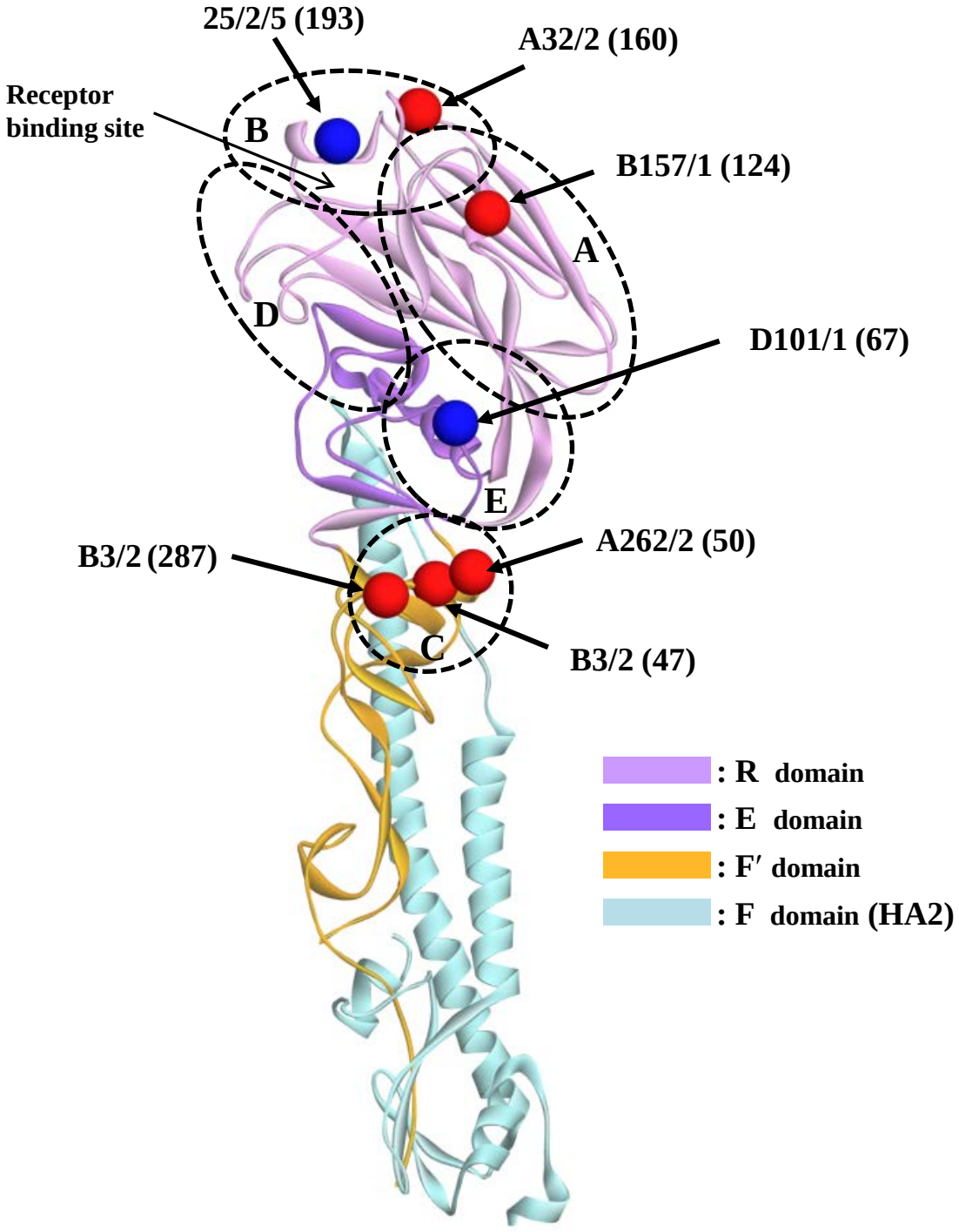
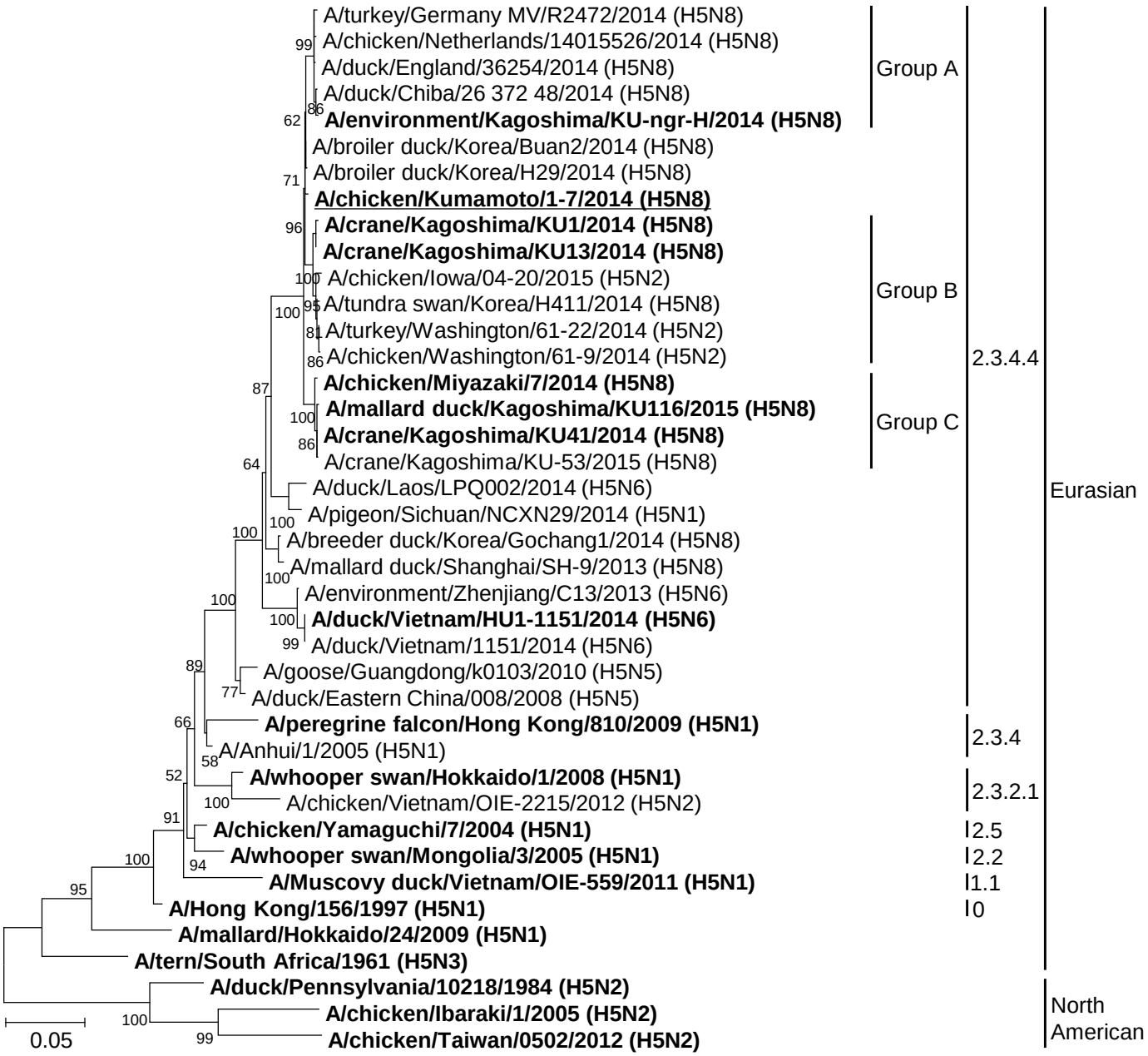


Fig. 1 Ohkawara et al.

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



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