responses specific for Vac-2 antigen in the unvaccinated macaques were elevated on day 8 after infection with NL2586 alone (Fig. 6, left panels) and the levels on day 14 were higher than those on day 8. IgG responses specific for the Vac-2 in sera from the unvaccinated macaques infected with NL2586 and *S. pneumoniae* were comparable to those from the macaques infected with NL2586 alone (Fig. 6, middle panels). Recall IgG responses in the vaccinated macaques were observed on day 8 after the infection as the antibody responses on day 8 had rapidly increased to the similar level on day 14 (Fig. 6, right panels). Infection with *S. pneumoniae* did not affect IgG responses against H7N7 virus. This finding was also seen in a neutralization assay against NL2586 (Table 4): no difference in neutralization activities of sera between macaques infected with NL2586 alone and macaques infected with NL2586 and *S. pneumoniae* was observed.

Next, we examined IgG responses against *S. pneumoniae* (Fig. 7). Two of the three unvaccinated macaques (Nos 435 and 464) showed IgG specific responses against *S. pneumoniae* after inoculation with NL2586 and *S. pneumoniae*, and one macaque (No. 406) showed very weak IgG responses against *S. pneumo-*

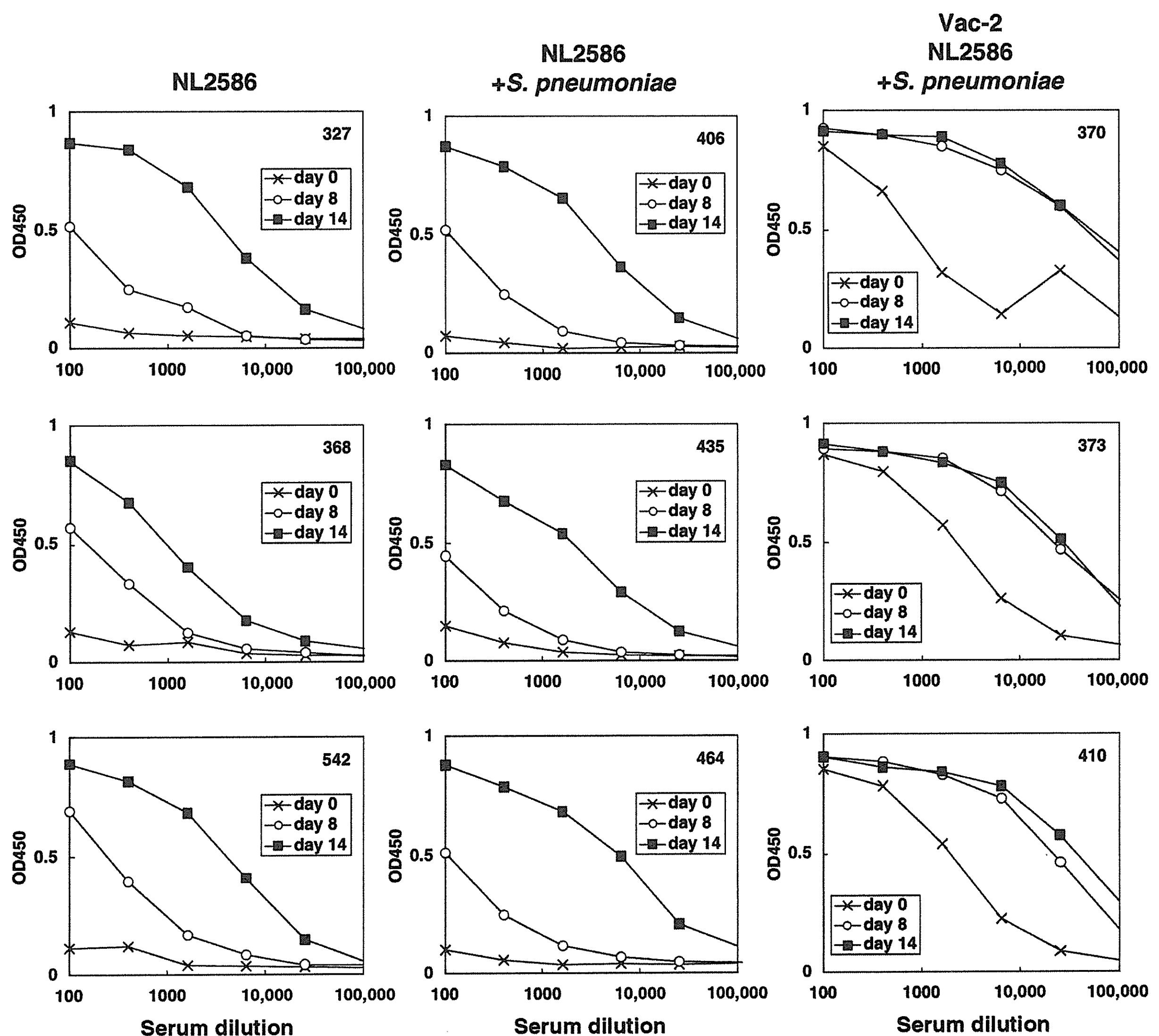

Fig. 6 Antibody responses specific for H7N7 vaccine antigens in cynomolgus macaques after challenge infection with NL2586. Sera were collected before (day 0) and after inoculation with NL2586 with or without *Streptococcus pneumoniae* (days 8 and 14). IgG antibodies specific for Vac-2 antigens in sera were analyzed at indicated dilutions as described in Fig. 2.

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Table 4 Neutralization activity of NL2586 with sera obtained after challenge with NL2586 and *Streptococcus pneumoniae*

Inoculation	Animal	50% neutralization titer (log 2)
NL2586	327	6.67
	368	5.50
	542	5.33
NL2586	406	5.17
+*S. pneumoniae*	435	4.00
	464	5.67
Vac-2	370	4.67
NL2586	373	5.83
+*S. pneumoniae*	410	6.23

Serum samples were collected 14 days after challenge infection with NL2586 with or without *S. pneumoniae*. The averages of 50% neutralization titers against NL2586 were 5.83, 4.95 and 5.58 in sera from macaques infected with NL2586 alone, macaques infected with NL2586 and *S. pneumoniae*, and macaques infected with NL2586 and *S. pneumoniae* after vaccination respectively. *P*-values with Student's *t*-test are >0.05 (NL2586 vs. NL2586 + *S. pneumoniae*, NL2586 vs. Vac-2 + NL286 + *S. pneumoniae*, and NL2586 + *S. pneumoniae* vs. Vac-2 + *S. pneumoniae*).

niae (Fig. 7, middle panels). A vaccinated macaque 370 showed higher increase in IgG specific for *S. pneumoniae* on day 8 than in that on day 14. This seems that immunological memory against *S. pneumoniae* has been present due to previous infection (Fig. 7, right panels). In two other vaccinated macaques (Nos 373 and 410), very weak or almost no IgG response was detected in sera. These findings suggest that viral replication may enhance IgG responses against *S. pneumoniae* in naïve animals, although IgG detected on day 14 was not sufficient to inhibit bacterial growth in the lungs (Table 2). Alternatively, as bacterial colonies in the vaccinated macaques were fewer than those in the unvaccinated macaques (Tables 1 and 2), IgG responses against *S. pneumoniae* might not be significant in the vaccinated macaques (Nos 373 and 410).

Discussion

Seasonal influenza virus infection occasionally induces secondary bacterial infection that results in substantial morbidity and mortality. Suppression of host immunity, including neutrophil function and macrophage-mediated microbial clearance [1, 4, 13, 24], and induction of inhibitory IL-10 [27, 28] are thought to be involved in the increased morbidity and mortality. It has not been determined whether mixed infection with HPAIV and bacteria causes more severe morbidity and higher mortality than infection with HPAIV alone, although co-infection with HPAIV and bacteria has not been detected in patients [5, 26]. Thus, in order to prevent severe morbidity and mortality in humans in future pandemics, it should be determined whether HPAIV infection with bacterial infection causes more severe morbidity than does HPAIV infection alone and whether regulation of HPAIV replication ameliorates bacterial pneumonia in experimental mixed infection using a macaque model as a preclinical study.

Some studies have shown that preceding influenza virus infection enhanced bacterial pneumonia with severe morbidity [6, 9, 11, 14]. Influenza viruses induce lung epithelial apoptosis via macrophage activation, resulting in the loss of defense capacity against bacteria in lung epithelia and the establishment of appropriate environment for bacterial growth [7]. Nonetheless, simultaneous infection with H7N7 HPAIV and *S. pneumoniae* in macaques did not induce severe pneumonia including diffuse alveolar damage at autopsy; only modest pneumonia immediately following lymphocyte infiltration was observed. Histology of the lungs of macaques simultaneously challenged with H7N7 HPAIV and *S. pneumoniae* indicated that the pneumonia seemed to be in a repairing phase with inflammatory cells infiltrating the alveoli being mainly lymphocytes with a few neutrophils, although pneumonia caused by *S. pneumoniae* occurred in addition to pneumonitis caused by H7N7 HPAIV, and pneumonia with neutrophils was not significant in lungs of macaques inoculated with NL2586 alone. As lung epithelial apoptosis via macrophage activation by influenza viruses seems to be responsible for severe pneumonia in co-infection, simultaneous infection but not preceding influenza viral infection might induce modest pneumonia. Alternatively, it is possible that pathogenicity of *S. pneumoniae* in macaques was low or *S. pneumoniae* growth might be regulated in some degree by host responses as *S. pneumoniae* might have been inoculated before desensitization of TLR by influenza virus infection in the simultaneous inoculation with HPAIV and *S. pneumonia* [4]. Further study should be required to reveal whether *S. pneumoniae* infection after HPAIV infection causes severe morbidity and mortality in a macaque model.

Vaccination against H7N7 HPAIV decreased morbidity caused by H7N7 HPAIV and *S. pneumoniae* in the macaques. Bacterial growth in the lungs was decreased by vaccination against H7N7 HPAIV, although the reduction in bacterial colonies was not statistically significant. Similarly, mitigation of the pneumonia was histologically observed in the lungs of vaccinated macaques. It is likely that the aforementioned lung epithelial apoptosis caused by influenza viruses is somehow involved in the enhancement

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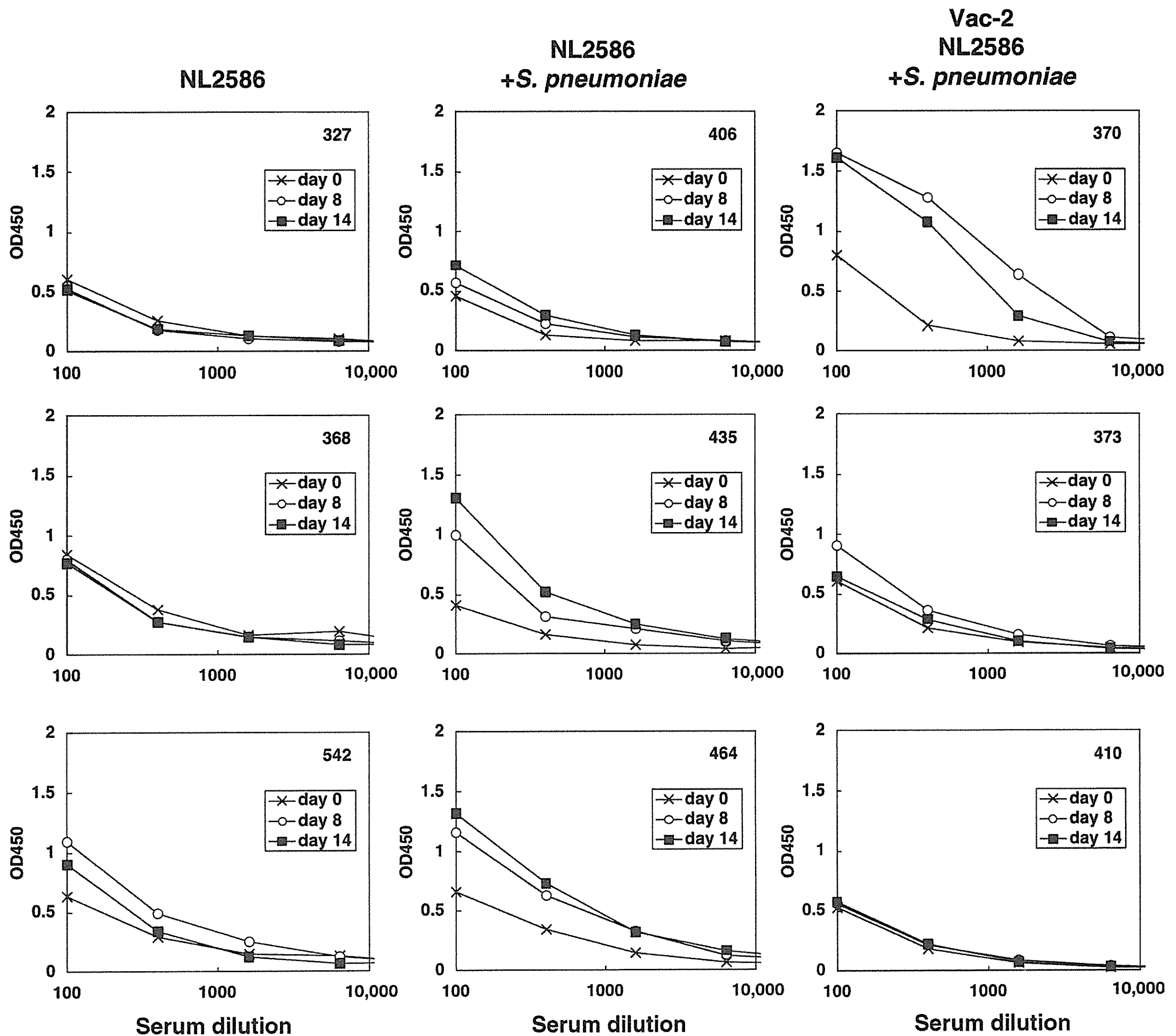

Fig. 7 Antibody responses specific for *Streptococcus pneumoniae* antigens in cynomolgus macaques after challenge infection with NL2586. Sera were collected before (day 0) and after inoculation of NL2586 with or without *S. pneumoniae* (days 8 and 14). IgG antibodies specific for *S. pneumonia* antigens in sera were analyzed at indicated dilutions as described in Fig. 2.

of bacterial pneumonia and that H7N7 HPAIV and *S. pneumoniae* interact with each other directly and indirectly in the development of pneumonia and pneumonitis.

In general, IL-10 expression is upregulated in influenza virus and *S. pneumoniae* infection of mice and IL-10 inhibits severe inflammatory responses in the lungs [12, 27, 28]. However, in the present study, *S. pneumoniae* infection suppressed IL-10 production, supporting histological findings and efficacy of antiviral vaccination. Furthermore, the reduction of IL-10 is assumed to finally induce cytokine storm after mixed infection with HPAIV and *S. pneumoniae* [3]. This assumption should be evidenced in near future.

Vaccination against HPAIV decreased the severity of pneumonia caused by bacterial superinfection, and prognosis of HPAIV-infected patients might be improved. Therefore, it might be crucial to prepare vaccines against future pandemic strains in order to prevent severe bacterial pneumonia as observed in the H1N1 pandemic in 1918 [10].

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Predicting the Antigenic Structure of the Pandemic (H1N1) 2009 Influenza Virus Hemagglutinin

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Abstract

The pandemic influenza virus (2009 H1N1) was recently introduced into the human population. The hemagglutinin (HA) gene of 2009 H1N1 is derived from "classical swine H1N1" virus, which likely shares a common ancestor with the human H1N1 virus that caused the pandemic in 1918, whose descendant viruses are still circulating in the human population with highly altered antigenicity of HA. However, information on the structural basis to compare the HA antigenicity among 2009 H1N1, the 1918 pandemic, and seasonal human H1N1 viruses has been lacking. By homology modeling of the HA structure, here we show that HAs of 2009 H1N1 and the 1918 pandemic virus share a significant number of amino acid residues in known antigenic sites, suggesting the existence of common epitopes for neutralizing antibodies cross-reactive to both HAs. It was noted that the early human H1N1 viruses isolated in the 1930s–1940s still harbored some of the original epitopes that are also found in 2009 H1N1. Interestingly, while 2009 H1N1 HA lacks the multiple *N*-glycosylations that have been found to be associated with an antigenic change of the human H1N1 virus during the early epidemic of this virus, 2009 H1N1 HA still retains unique three-codon motifs, some of which became *N*-glycosylation sites via a single nucleotide mutation in the human H1N1 virus. We thus hypothesize that the 2009 H1N1 HA antigenic sites involving the conserved amino acids will soon be targeted by antibody-mediated selection pressure in humans. Indeed, amino acid substitutions predicted here are occurring in the recent 2009 H1N1 variants. The present study suggests that antibodies elicited by natural infection with the 1918 pandemic or its early descendant viruses play a role in specific immunity against 2009 H1N1, and provides an insight into future likely antigenic changes in the evolutionary process of 2009 H1N1 in the human population.

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Introduction

In April 2009, pandemic (H1N1) 2009 influenza virus (2009 H1N1) was first found in patients with febrile respiratory illness in the United States and Mexico, and has spread rapidly across the world by human-to-human transmission. On the 11th of June 2009, the World Health Organization declared a global pandemic of 2009 H1N1 infection. H1N1 influenza virus caused a pandemic in 1918 (1918 H1N1) [1], and its descendant virus with highly altered antigenicity of the viral surface protein, hemagglutinin (HA) has been causing "seasonal flu" in humans.

The 2009 H1N1 resulted from genetic reassortment between the recently circulating swine H1 viruses in North America and the avian-like swine viruses in Europe [2]. Phylogenetic analysis showed that the HA gene of 2009 H1N1 was derived from the so-called "classical swine H1N1" virus, which likely shares a common ancestor with the recent human H1N1 virus [2]. Accordingly, it has been reported that the early strains of the classical swine H1N1 virus, which was first identified in North America in 1930, were antigenically similar to the prototype strain of 1918 H1N1, A/South Carolina/1/1918 (SC1918), detected from a few victims of the pandemic in 1918 [3,4]. Since antigenic changes occur more slowly in swine than in the human population [5], HA of the classical swine H1N1 virus was antigenically highly conserved until the late 1990s [4,6], raising the possibility that the recently emerged 2009 H1N1 may still retain an antigenic structure similar to that of SC1918 and the early isolates of its descendants.

In this study, we generated three-dimensional (3D) structures of the HA molecules of 1918 H1N1, its descendent, recent seasonal H1N1 viruses, and 2009 H1N1, and compared their antigenic structures to look for evidence for the existence of shared epitopes for neutralizing antibodies. Since the 2009 H1N1 HA antigenic sites will be targeted by antibody-mediated selection pressure in humans in the near future, we further discuss possible directions of antigenic changes in the evolutionary process of this pandemic virus.

Results and Discussion

It is known that the H1 HA molecules have four distinct antigenic sites: Sa, Sb, Ca, and Cb [7,8,9,10] (Figure 1). As a result, these sites consist of the most variable amino acids in the HA molecule of the seasonal human H1N1 viruses that have been subjected to antibody-mediated immune pressure since its

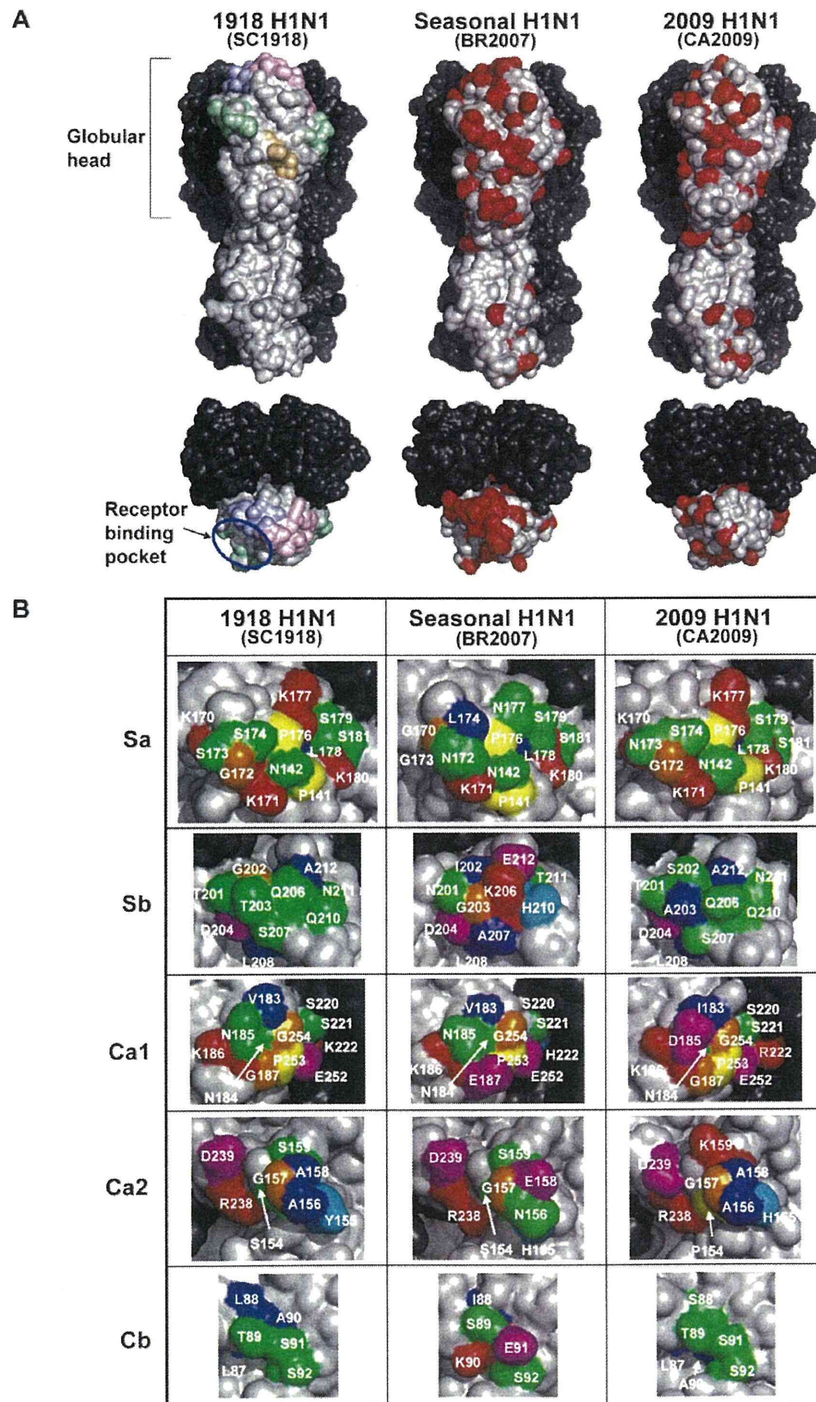

Figure 1. Comparison of the structures of antigenic sites on the HA molecules among 1918 H1N1 (SC1918), recent seasonal H1N1 (BR2007), and 2009 H1N1 (CA2009). Three-dimensional models of the H1 HA molecules of SC1918, BR2007, and CA2009 were constructed based on the HA crystal structures of A/South Carolina/1/18, A/Puerto Rico/8/34, and A/swine/Iowa/30, respectively (PDB codes: 1RUZ, 1RU7, and 1RUY, respectively). Models with solvent-accessible surface representation were generated by a molecular modeling method as described in the Methods section. Molecular surface of the HA trimers viewed on its side (upper) and top (lower) are shown (A). One monomer (center) is colored gray and the others are colored dark gray. The antigenic sites, Sa (light pink), Sb (light blue), Ca (pale green), and Cb (light orange) are indicated on the model of SC1918 HA. The spatial locations of amino acid residues that are distinct from those of SC1918 HA are shown in red on the models of BR2007 and CA2009 HAs. Each amino acid residue is mapped on the close-up views of each antigenic site of SC1918, BR2007, and CA2009 HAs (B). The Ca site is divided into subregions, Ca1 and Ca2. Amino acids are colored by the default ClustalX color scheme [29]: Trp, Leu, Val, Ile, Met, Phe, and Ala (blue); Lys and Arg (red); Thr, Ser, Asn, and Gln (green); Cys (pink); Asp and Glu (magenta); Gly (orange); His and Tyr (cyan); Pro (yellow).
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Table 1. Amino acid similarity in the HA antigenic sites among recent seasonal H1N1 (BR2007), 2009 H1N1 (CA2009), and 1918 H1N1 (SC1918).

Antigenic sites	No. amino acids involved	No. of amino acids identical to SC1918	
		BR2007	CA2009
Sa	13	8	12
Sb	12	4	10
Ca	19	13	13
Cb	6	2	5

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emergence in 1918 [3]. To investigate the structures of these antigenic sites of 2009 H1N1, 3D structures of the HA molecules of SC1918, the recent seasonal human H1N1 virus A/Brisbane/59/2007 (BR2007), and 2009 H1N1 A/California/04/2009 (CA2009) [2] were constructed by a homology modeling approach, and compared by mapping all the amino acid residues that were distinct from those of SC1918 HA (Figure 1 and Table S1). We found that most of these antigenic sites of BR2007 HA predominantly contained altered amino acid residues if compared with SC1918. By contrast, amino acid residues at these positions were relatively conserved in CA2009 HA. Notably, the Sa and Sb sites that contain many amino acids involved in neutralizing epitopes near the receptor binding pockets [8,10] remain almost intact in CA2009 HA (Table 1), suggesting that antibodies raised by natural infection with SC1918 or its antigenically related descendant viruses play a role in specific immunity against CA2009.

We then constructed 3D structures of the representative strains of seasonal H1 viruses that had been isolated since 1934, and tracked the amino acid substitutions on their HA molecules (Figure 2 and Figure S1). We confirmed that amino acid substitutions associated with the antigenic changes gradually accumulated on the globular head region of HA and were distributed over four distinct antigenic sites. However, it was noted that the early isolates represented by the A/Puerto Rico/8/1934 and A/Bellamy/1942 strains, but not the strains isolated after the

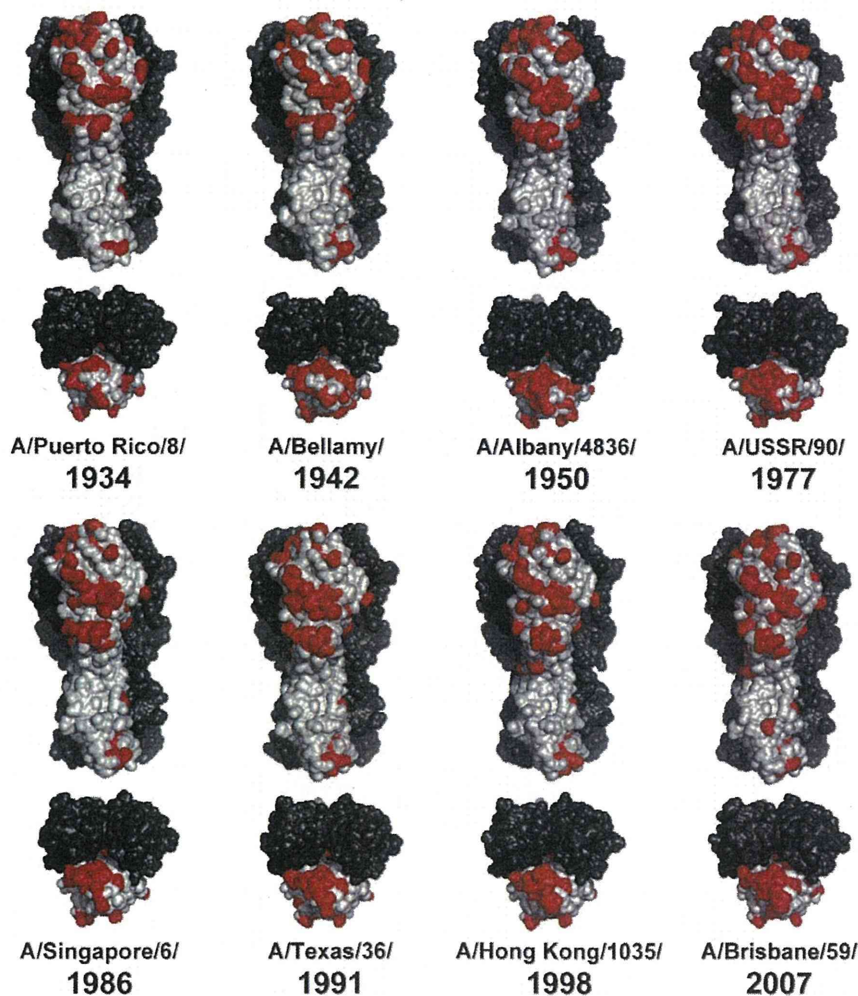

Figure 2. Amino acid substitutions associated with antigenic changes of seasonal human H1N1 virus HAs. All models were generated and shown by a molecular modeling method as described in the Methods section and the legend of Figure 1.
doi:10.1371/journal.pone.0008553.g002

1950s, still harbored unchanged amino acids forming potential neutralizing epitopes in the Sa and Sb sites (Figure 2). It seems likely that most of the amino acids on these antigenic sites were eventually substituted in the late 1940s (Figure S1).

It is well-documented that antigenic changes of HA occasionally result in the acquisition of carbohydrate side chains on the HA molecule [8,11]. Since the carbohydrate side chains in the vicinity of antigenic sites mask the neutralizing epitopes on the HA surface, amino acid substitutions associated with acquisition of carbohydrate chains are believed to efficiently generate antigenic variants. Accordingly, recent seasonal H1N1 viruses have acquired 4–5 *N*-glycosylation sites (Asn-Xaa-Ser/Thr, where Xaa is any amino acid except Pro) in the globular head region of HA [12,13], whereas SC1918 HA had only one site, at Asn 104 (Figure 3).

Interestingly, CA2009 also has a single potential *N*-glycosylation site at the same position in the globular head region of HA (Figure 3), despite the fact that the classical swine H1N1 virus emerged in the early 1900s and was circulating in the pig population until recently. This prompted us to estimate the potential of 2009 H1N1 to acquire additional *N*-glycosylation sites on its HA, which may be related to its future evolutionary process in the human population. We previously defined a three-codon motif that becomes an *N*-glycosylation site with a single-nucleotide mutation as "*Cand1*", and suggested that the presence of the *Cand1* sites in the HA sequence is one of the key factors for human influenza A viruses to rapidly acquire *N*-glycosylation sites during the early epidmic in the human population [13]. We compared the number of the *Cand1* sites in the HA globular head region between SC1918 and CA2009 (Figure 3 and Table S1). We found that CA2009 HA possessed three *Cand1* sites on the antigenic sites Sa and Ca, all of which were also present at the same position in SC1918 HA (positions of the first Asn residue, 177, 179, and 184). Of these, the *Cand1* sites with positions at 177 and 179 had actually become potential *N*-glycosylation sites in human H1N1 viruses, although these two sites did not exist concurrently [12]. It is noted that these two *Cand1* sites are still present on the surface of CA2009 HA, suggesting the likelihood of additional *N*-glycosylation at these sites during future antigenic changes of 2009 H1N1 HA.

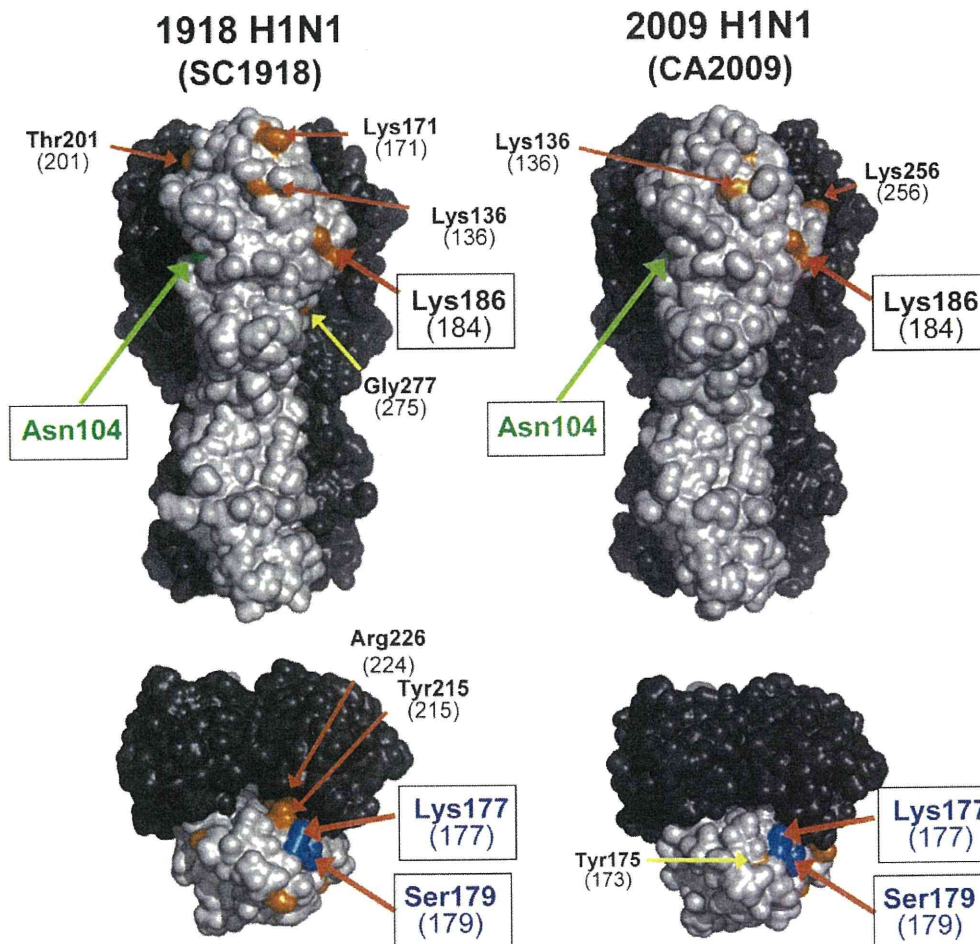

Figure 3. Comparison of the *N*-glycosylation potential of HA between SC1918 and CA2009. Residues shown in green represent Asn at the actually existing *N*-glycosylation sites. Residues shown in orange or blue represent the amino acids in *Cand1* sites that require a nucleotide substitution to produce *N*-glycosylation sites. Residues shown in blue represent the amino acids that were actually substituted, resulting in the acquisition of *N*-glycosylation sites during the antigenic evolution of human H1N1 viruses. Numbers in parentheses show the positions of Asn residues that may be linked to carbohydrate chains, if respective *Cand1* sites mutate to have *N*-glycosylation sites. All models were generated as described in the Methods section and the legend of Figure 1.
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In this paper, we employed 3D structures constructed by a homology modeling method to map amino acid residues on the antigenic sites of HA. When compared to the presentation of simple primary sequences, the 3D presentation has following advantages: (a) There are several amino acid residues that are buried beneath the surface of the HA molecule, even if they are included in the antigenic sites described by the primary amino acid sequences. Since such amino acid residues do not directly contribute to the interaction with antibodies, the surface structures of antigenic sites that are accessible for antibodies can be compared more precisely in the presentation by 3D models than by the primary amino acid sequence. (b) An epitope likely consists of multiple amino acid residues belonging to different antigenic regions presented by the primary amino acid sequence. Such conformational epitopes can be illustrated only by the 3D presentation. (c) One of the purposes of this study is to provide a structural basis to confirm antigenic similarity between the 1918 H1N1 and the pandemic 2009 H1N1 viruses. For this purpose, we employed a homology modeling method rather than simply mapping on the existing crystal structure (e.g. 1918 H1N1 HA), since this method is generally used to generate a 3D structure of a protein molecule if there is no available crystal structure of the target protein [14]. Thus, we believe that this method produces more likely HA structure models of the viruses whose HA crystal structure are not available (e.g. CA2009). In fact, our homology modeling approach suggests that several amino acid residues were occasionally buried beneath or exposed to the surface of HA molecule, depending on the substitutions found in the viruses examined (Figure 1B and Figure S1). The homology modeling approach might enable us to analyze such dynamics of antigenic changes at molecular levels.

Our analysis indicated that 2009 H1N1 had undergone less significant antigenic changes of HA in the pig population than human H1N1 virus since their emergence in the early 1900s. The Centers for Disease Control and Prevention reported that vaccination with recent (2005–2009) human H1N1 viruses was unlikely to provide protection against 2009 H1N1 [15]; however, cross-reactive antibodies were detected in 33% of people aged 60 and over. Another report showed that appreciable neutralizing antibodies against CA2009 were present in the sera collected from individuals born before 1918 [16]. Our 3D models provide a protein-structural basis supporting these observations, and further suggest that infection with the 1918 H1N1 or early human H1N1 viruses (viruses present before the 1940s), but not with antigenically divergent human H1N1 viruses circulating after the 1950s, elicited cross-neutralizing antibodies to 2009 H1N1.

This virus will soon be subjected to complex immunological selection pressure by the antibody response that will be induced in the human population by vaccination and/or natural infection with homologous viruses, and pre-existing immunity cross-reactive to the early descendants of 1918 H1N1. In the present study, we showed that the antigenic structure of 2009 H1N1 HA might still be similar, at least in part, to that of the 1918 H1N1 HA. We speculate that the 2009 H1N1 HA antigenic sites involving the conserved amino acids will soon be targeted by neutralizing antibodies in humans. Thus, it is of interest to monitor whether these antigenic sites of 2009 H1N1 will undergo similar patterns of amino acid substitutions to those seen in seasonal H1N1 viruses during its epidemic period (Figure 4). Interestingly, we found that some of the recent variants of the 2009 H1N1 virus (as of November 3, 2009) have indeed undergone substitutions identical to those predicted in Figure 4. Although the present study still needs to be supported by experimental data, our approach may provide new perspectives on collective immunity against 2009 H1N1 and an insight into future antigenic changes of this new human pandemic influenza virus.

Methods

Sequence Data of HA Genes

Nucleotide sequences for HA genes of SC1918 (AF117241), BR2007 (CY030230), CA2009 (FJ966082), A/Puerto Rico/8/1934/Mount Sinai (AF389118), A/Bellamy/1942 (CY009276), A/Albany/4836/1950 (CY021701), A/USSR/90/1977 (DQ508897), A/Singapore/6/1986 (CY020477), A/Texas/36/1991 (AY289927), and A/Hong Kong/1035/1998 (AF386777) [2,3,17,18,19,20] were obtained from Influenza Virus Resource at the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/genomes/FLU/FLU.html).

Molecular Modeling

MODELLER 9v6 [21] was used for homology modeling of HA structures. After one hundred models of the HA trimer were generated, the model was chosen by a combination of the MODELLER objective function value and the discrete optimized protein energy (DOPE) statistical potential score [22]. After addition of hydrogen atoms, the model was refined by energy minimization (EM) with the minimization protocols in the Discovery Studio 2.1 software package (Accelrys, San Diego, CA) using a CHARMm force field. Steepest descent followed by conjugate gradient minimizations was carried out until the root mean square (rms) gradient was less than or equal to 0.01 kcal/mol/Å. The generalized Born implicit solvent model [23,24] was used to model the effects of solvation. The HA model was finally evaluated by using PROCHECK [25], WHATCHECK [26], and VERIFY-3D [27]. All figures are shown as a solvent-accessible surface representation prepared by PyMOL (DeLano Scientific LLC) [28]. All HA structures constructed by a homology modeling method are available in Supplementary Files S1, S2, S3, S4, S5, S6, S7, and S8.

Sequence Data Analyses for *N*-Glycosylation Sites

Custom-made programs were developed with the Ruby language and used for investigating the numbers of potential *N*-glycosylation sites and candidate codons (*Cand1*) in HA sequences. The programs are available upon request.

Supporting Information

Table S1
Found at: doi:10.1371/journal.pone.0008553.s001 (0.04 MB PDF)

Figure S1 Amino acid substitutions of seasonal human H1N1 virus HAs shown in close-up views of each antigenic site. The strains used in this analysis are corresponding to those shown in Figure 2. Amino acids are colored according to the scheme in the legend of Figure 1B.
Found at: doi:10.1371/journal.pone.0008553.s002 (1.02 MB PDF)

File S1 PDB file of the homology model of H1 HA (A/California/04/2009) after energy minimizations.
Found at: doi:10.1371/journal.pone.0008553.s003 (0.20 MB ZIP)

File S2 PDB file of the homology model of H1 HA (A/Bellamy/1942) after energy minimizations.
Found at: doi:10.1371/journal.pone.0008553.s004 (0.20 MB ZIP)

File S3 PDB file of the homology model of H1 HA (A/Albany/4836/1950) after energy minimizations.

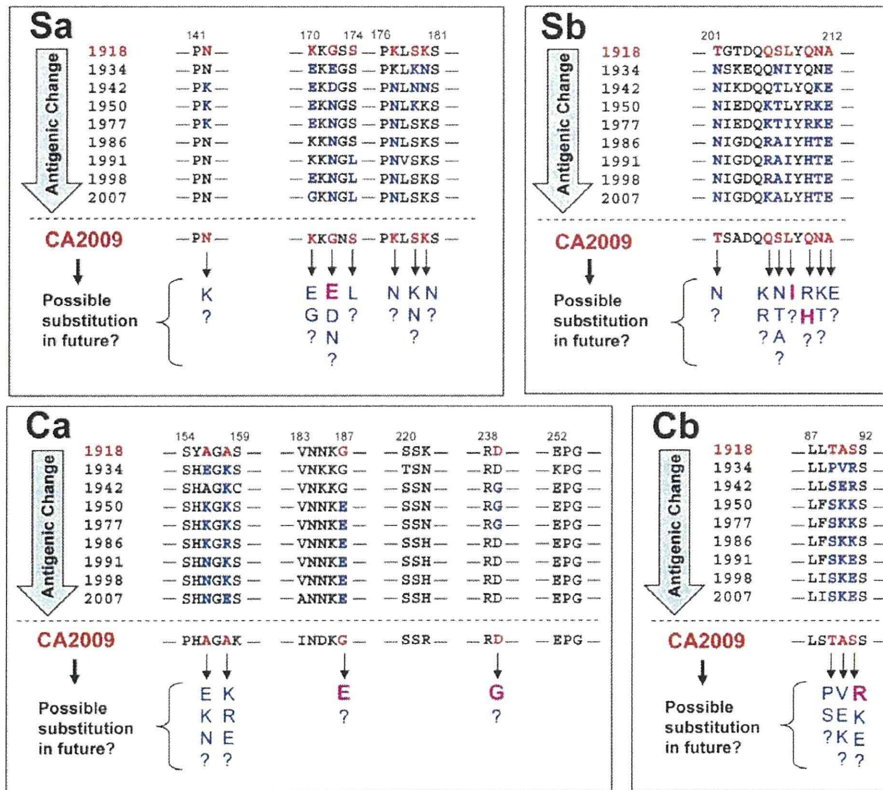

Figure 4. Prediction of the future amino acid substitutions on the antigenic sites of 2009 H1N1 HA. Amino acid sequences of HA antigenic sites of human H1N1 viruses are shown. Sequence data are corresponding to those of virus strains shown in Figures 1 and 2. Amino acid residues shared between 1918 H1N1 (SC1918) and 2009 H1N1 (CA2009) are shown in red, and those that have been substituted since 1934 are shown in blue. Amino acid residues indicated by arrows represent the predicted substitutions which might be associated with antigenic changes of 2009 H1N1 in the near future. The amino acid substitutions which have already been found in the recent variants of the 2009 H1N1 virus (as of November 3, 2009) are shown in bold pink letters.
doi:10.1371/journal.pone.0008553.g004

Found at: doi:10.1371/journal.pone.0008553.s005 (0.20 MB ZIP)

File S4 PDB file of the homology model of H1 HA (A/USSR/90/1977) after energy minimizations.
Found at: doi:10.1371/journal.pone.0008553.s006 (0.20 MB ZIP)

File S5 PDB file of the homology model of H1 HA (A/Singapore/6/1986) after energy minimizations.
Found at: doi:10.1371/journal.pone.0008553.s007 (0.20 MB ZIP)

File S6 PDB file of the homology model of H1 HA (A/Texas/36/1991) after energy minimizations.
Found at: doi:10.1371/journal.pone.0008553.s008 (0.20 MB ZIP)

File S7 PDB file of the homology model of H1 HA (A/Hong Kong/1035/1998) after energy minimizations.
Found at: doi:10.1371/journal.pone.0008553.s009 (0.20 MB ZIP)

File S8 PDB file of the homology model of H1 HA (A/Brisbane/59/2007) after energy minimizations.
Found at: doi:10.1371/journal.pone.0008553.s010 (0.20 MB ZIP)

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Author Contributions

Conceived and designed the experiments: MI KI HK AT. Analyzed the data: MI RY DT. Wrote the paper: MI AT.

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RESEARCH **Open Access**

Characterization of a non-pathogenic H5N1 influenza virus isolated from a migratory duck flying from Siberia in Hokkaido, Japan, in October 2009

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Abstract

Background: Infection with H5N1 highly pathogenic avian influenza viruses (HPAIVs) of domestic poultry and wild birds has spread to more than 60 countries in Eurasia and Africa. It is concerned that HPAIVs may be perpetuated in the lakes in Siberia where migratory water birds nest in summer. To monitor whether HPAIVs circulate in migratory water birds, intensive surveillance of avian influenza has been performed in Mongolia and Japan in autumn each year. Until 2008, there had not been any H5N1 viruses isolated from migratory water birds that flew from their nesting lakes in Siberia. In autumn 2009, A/mallard/Hokkaido/24/09 (H5N1) (Mal/Hok/24/09) was isolated from a fecal sample of a mallard (*Anas platyrhynchos*) that flew from Siberia to Hokkaido, Japan. The isolate was assessed for pathogenicity in chickens, domestic ducks, and quails and analyzed antigenically and phylogenetically.

Results: No clinical signs were observed in chickens inoculated intravenously with Mal/Hok/24/09 (H5N1). There was no viral replication in chickens inoculated intranasally with the isolate. None of the domestic ducks and quails inoculated intranasally with the isolate showed any clinical signs. There were no multiple basic amino acid residues at the cleavage site of the hemagglutinin (HA) of the isolate. Each gene of Mal/Hok/24/09 (H5N1) is phylogenetically closely related to that of influenza viruses isolated from migratory water birds that flew from their nesting lakes in autumn. Additionally, the antigenicity of the HA of the isolate was similar to that of the viruses isolated from migratory water birds in Hokkaido that flew from their northern territory in autumn and different from those of HPAIVs isolated from birds found dead in China, Mongolia, and Japan on the way back to their northern territory in spring.

Conclusion: Mal/Hok/24/09 (H5N1) is a non-pathogenic avian influenza virus for chickens, domestic ducks, and quails, and is antigenically and genetically distinct from the H5N1 HPAIVs prevailing in birds in Eurasia and Africa. H5 viruses with the HA gene of HPAIV had not been isolated from migratory water birds in the surveillance until 2009, indicating that H5N1 HPAIVs had not become dominant in their nesting lakes in Siberia until 2009.

Background

Influenza viruses widely distribute in birds and mammals including humans. Viruses of each of the known hemagglutinin (HA) and neuraminidase (NA) subtypes (H1-H16 and N1-N9, respectively) have been isolated from migratory water birds. Ducks are orally infected with influenza viruses by waterborne transmission at their nesting lakes in Siberia, Alaska, and Canada close to the Arctic Circle during their breeding season, in summer [1]. These viruses replicate in the columnar epithelial cells forming crypts in the colon, and are excreted in feces [2]. The viruses are preserved in frozen lake water in winter after the ducks leave for migration to the south [3]. Nesting lakes for migratory ducks, thus, serve as influenza virus gene pools in nature.

Since late 2003, H5N1 highly pathogenic avian influenza viruses (HPAIVs) have seriously affected poultry in

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Eurasia and Africa. Non-pathogenic avian influenza viruses (NPAIVs) circulating in waterfowl transmit to terrestrial birds such as quails and turkeys through domestic water birds such as ducks and geese in live bird markets. Then HPAIVs are generated during multiple transmission of low pathogenic H5 or H7 viruses in chicken population [1]. After 2005, H5N1 HPAIVs have been isolated from dead migratory water birds in China, Mongolia, Russia, and Japan on the way back to their nesting lakes in Siberia in spring [4-8]. It is a serious concern that HPAIVs may be perpetuated in the lakes where migratory water birds nest in summer, and that those migratory water birds may then bring HPAIVs to the south in autumn.

Since Japan and Mongolia are located on the flyways of migratory water birds that flew from their nesting lakes in Siberia to the south [1,9-11], intensive surveillance of avian influenza has been performed in autumn in Hokkaido, Japan, and Mongolia every year since 1996. The subtypes and the numbers of isolates in the surveillance in autumn between 1996 and 2009 have been reported [6,11-13]. A total of 634 viruses including 17 H5 viruses were isolated from fecal samples of migratory water birds in the surveillance (Tables 1 and 2). Until 2008, H5N1 virus had not been isolated from those of migratory water birds. In autumn 2009, an H5N1 virus, A/mallard/Hokkaido/24/09 (H5N1) (Mal/Hok/24/09), was isolated from the fecal sample of a mallard (*Anas platyrhynchos*) in Hokkaido, Japan. Pathogenicity of the isolate for chickens, domestic ducks, and quails was assessed by experimental infection studies, and the isolate was phylogenetically and antigenically analyzed.

Materials and methods

Isolation and identification of viruses

A total of 711 fecal samples were collected from migratory water birds at lakeside of Ono Pond on the campus of Hokkaido University, Sapporo and Lake Ohnuma in Wakkanai, Hokkaido, Japan, between September and November 2009. Each sample was mixed with Minimum Essential Medium (Nissui) containing antibiotics and inoculated into the allantoic cavities of ten-day-old chicken embryos. The subtypes of influenza viruses were identified by hemagglutination inhibition (HI) and neuraminidase inhibition (NI) tests with antisera to the reference influenza virus strains [14].

Sequencing and phylogenetic analysis

Viral RNA was extracted from the allantoic fluid of chicken embryos infected with the isolates by TRIzol LS Reagent (Invitrogen) and reverse-transcribed with the Uni12 primer [15] and SuperScript Reverse Transcriptase III (Invitrogen) or M-MLV Reverse Transcriptase (Invitrogen). The full-length of each gene segment was amplified by polymerase chain reaction with gene-specific primer sets [15]. Direct sequencing of each gene segment was performed using an auto-sequencer CEQ 2000XL (Beckman Coulter) or 3500 Genetic Analyzer (Applied Biosystems).

The nucleotide sequences were phylogenically analyzed based on those of the H5 HA and N1 NA genes of influenza viruses by the neighbor-joining method [3,16]. Sequence data of the viral genes were compared with those from GenBank/EMBL/DDBJ.

Experimental infection of chickens, domestic ducks, and quails with Mal/Hok/24/09 (H5N1)

To determine the intravenous pathogenicity index (IVPI), 0.2 ml of the 1:10 dilution of infectious allantoic fluid of embryonated eggs was inoculated intravenously into ten seven-week-old chickens (White Leghorn). The score for IVPI was calculated according to the manual of World Organisation for Animal Health (OIE) [17].

To assess the intranasal pathogenicity for poultry, Mal/Hok/24/09 (H5N1) of $10^{6.0}$ 50% egg infectious dose (EID_{50}) was inoculated intranasally into eight four-week-old chickens (Boris Brown), domestic ducks (Chelly Valley), and quails (Japanese Quail). Four of eight birds were euthanized three days post-inoculation (dpi), and the trachea and cloaca swabs, brain, trachea, lungs, kidneys, and colon were collected aseptically for virus recovery. The birds were observed daily for disease signs for 14 days after inoculation. Sera were collected from them on the day of inoculation and 14 dpi to test for antibodies against H5N1 virus. The swabs and tissue homogenates were inoculated into ten-day-old chicken embryos and the infectivity titers of virus were calculated and expressed as the EID_{50} per milliliter of swab or gram of tissue samples. Sera were examined for the presence of antibodies against H5N1 virus by enzyme-linked immunosorbent assay (ELISA) [18]. The purified A/duck/Hokkaido/Vac-1/04 (H5N1) generated from H5N2 and H7N1 viruses isolated from migratory water birds by genetic reassortment in embryonated chicken eggs [19] was used as antigen for ELISA. Each of the birds was housed in a self-contained isolator unit (Tokiwa Kagaku) at a BSL-3 facility at the Graduate School of Veterinary Medicine, Hokkaido University, Japan.

Antigenic analysis

The antigenic properties of H5 viruses, A/duck/Hokkaido/WZ21/08 (H5N2), A/duck/Hokkaido/WZ75/09 (H5N2), Mal/Hok/24/09 (H5N1), A/whooper swan/Hokkaido/1/08 (H5N1), and A/peregrine falcon/Hong Kong/810/09 (H5N1), were determined by the fluorescent antibody method with monoclonal antibodies (MAbs) against H5 HA produced previously [20]. MDCK cells infected with H5 influenza viruses were fixed with cold 100% acetone for eight hours post-inoculation. The reactivity

Table 1 Influenza viruses isolated from fecal samples of migratory water birds in autumn between 1996 and 2009

Locations	Subtypes of influenza viruses isolated in following years													
	1996	1997	1998	1999	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009
Sapporo, Japan	NP[a]	NP	NP	NP	NP	H1N1 (9) H3N6 (1) H4N5 (1) H4N6 (1)	H3N8 (3) H5N3 (1) H11N9 (3)	H3N8 (11) H6N8 (2) H7N1 (18) H8N4 (1)	H1N1 (1) H3N8 (1) H4N2 (7) H5N3 (3) H6N1 (5) H6N8 (2) H10N5 (7)	H3N2 (1) H6N2 (4) H8N4 (2)	H3N8 (5) H4N6 (1) H6N2 (1) H9N2 (1) H11N9 (3)	H3N8 (2) H4N6 (2) H5N3 (2) H8N4 (2) H12N5 (1)	H3N2 (1) H3N6 (3) H4N6 (9) H7N7 (1) H9N5 (1) H10N7 (11)	H1N3 (1) H1N5 (1) H4N6 (5) H5N1 (1) H6N1 (4) H6N8 (2) H11N9 (3) H12N5 (1)
Wakkanai, Japan	H1N1 (1)[b] H3N8 (1) H5N3 (3) H6N1 (1)	H1N1 (1) H6N1 (2) H9N2 (1) H11N9 (1)	H6N2 (1) H9N2 (1)	H2N2 (1) H3N8 (2) H6N2 (4) H9N2 (2)	H4N6 (1) H5N3 (2) H6N2 (2) H8N4 (1) H9N2 (1) H10N4 (12)	H2N2 (1) H2N3 (4) H3N8 (6) H6N2 (4) H12N5 (2)	NP	NP	H4N6 (6) H6N2 (12) H6N8 (2) H7N7 (13) H8N4 (1) H10N6 (1) H11N9 (1) H12N5 (1)	H2N5 (1) H3N8 (3) H6N1 (1) H6N2 (3)	H3N6 (2) H3N8 (1) H4N9 (3) H6N1 (4) H6N5 (1) H9N2 (1) H10N8 (1) H11N9 (11) H13N6 (2)	H1N1 (1) H3N8 (1) H4N6 (2) H8N4 (1) H10N2 (1) H10N7 (1)	H4N6 (2) H5N2 (1) H6N1 (4) H6N2 (1) H6N5 (1) H6N8 (1) H6N9 (1) H9N9 (1) H10N9 (2) H11N9 (2)	H5N2 (1)
Mongolia	NP	NP	NP	NP	NP	H1N1 (1) H3N2 (1) H3N6 (3) H3N8 (11) H4N2 (1) H4N6 (12) H5N2 (1) H5N3 (2) H7N1 (1) H10N3 (4)	H1N1 (3) H3N6 (20) H3N8 (55) H4N6 (12) H4N7 (1) H4N8 (1) H7N1 (1) H7N7 (9) H8N4 (5) H10N7 (1) H12N5 (1)	H1N1 (1) H2N3 (1) H3N6 (6) H3N8 (28) H4N2 (1) H4N6 (25) H9N2 (1) H10N5 (5)	NP	H3N2 (1) H3N6 (2) H3N8 (10) H4N6 (6) H8N4 (1) H10N3 (11) H10N7 (1)	H2N2 (1) H3N8 (8) H4N6 (9)	H3N8 (14) H4N3 (1) H7N6 (1) H7N7 (4)	H3N6 (3) H3N8 (23) H4N6 (8) H4N8 (3) H7N9 (3)	H1N8 (1) H3N8 (2) H4N6 (3) H8N4 (3)

Surveillance data were referred from Okazaki *et al.* [11], Manzoor *et al.* [12], Sakoda *et al.* [6], and Asmah *et al.*[13].

[a] Surveillance did not be performed.

[b] Number of isolates of each antigenic subtype is shown in parenthesis.

Table 2 H5 viruses isolated from migratory water birds in the surveillance in autumn between 1996 and 2009

Years	Locations	Names	Subtypes
1996	Wakkanai, Japan	Swan/Hokkaido/4/96	H5N3
		Swan/Hokkaido/51/96	H5N3
		Swan/Hokkaido/67/96	H5N3
2000	Wakkanai, Japan	Dk/Hokkaido/447/00	H5N3
		Dk/Hokkaido/69/00	H5N3
2001	Mongolia	Dk/Mongolia/54/01	H5N2
		Dk/Mongolia/500/01	H5N3
		Dk/Mongolia/596/01	H5N3
2002	Sapporo, Japan	Dk/Hokkaido/84/02	H5N3
2004	Sapporo, Japan	Dk/Hokkaido/101/04	H5N3
		Dk/Hokkaido/193/04	H5N3
		Dk/Hokkaido/299/04	H5N3
2007	Sapporo, Japan	Dk/Hokkaido/167/07	H5N3
		Dk/Hokkaido/201/07	H5N3
2008	Wakkanai, Japan	Dk/Hokkaido/WZ21/08	H5N2
2009	Wakkanai, Japan	Dk/Hokkaido/W75/09	H5N2
	Sapporo, Japan	Mal/Hokkaido/24/09	H5N1

Abbreviations: Dk (Duck), Mal (Mallard).

patterns of the MAbs to H5 viruses were investigated by the immunofluorescent method with a FITC-conjugated goat IgG to mouse IgG (ICN Biomedicals). Fluorescence was visualized with the Axiovert 200 (Carl Zeiss).

Results

Isolation of influenza A viruses from fecal samples of migratory water birds

In 2009, a total of 19 viruses were isolated from 711 fecal samples of migratory water birds. Those were 1 H1N3, 1 H1N5, 5 H4N6, 1 H5N1, 1 H5N2, 4 H6N1, 2 H6N8, 3 H11N9, and 1 H12N5 viruses. In our previous surveillance until 2008, H5N1 virus had not been isolated (Table 1) [6,11-13]. In autumn 2009, Mal/Hok/24/09 (H5N1) was isolated from a fecal sample of a mallard in Hokkaido, Japan.

Pathogenicity of Mal/Hok/24/09 (H5N1) in chickens, domestic ducks, and quails

The pathogenicity of Mal/Hok/24/09 (H5N1) was evaluated by IVPI test using chickens. None of the ten birds intravenously inoculated with Mal/Hok/24/09 (H5N1) showed clinical signs during ten days of observation (IVPI = 0.00). None of the chickens, domestic ducks, and quails intranasally inoculated with $10^{6.0}$ EID_{50} of Mal/Hok/24/09 (H5N1) showed clinical signs during 14 days of observation (Table 3). The virus was not recovered from the tracheal and cloacal swabs and tissues of chickens intranasally inoculated with Mal/Hok/24/09 (H5N1) on three dpi, and there were no antibodies to H5N1 virus detected by ELISA on 14 dpi (Table 3), indicating that chickens were not infected with the isolate. Although virus was not recovered from the swabs and tissues of domestic ducks inoculated with the virus on three dpi, antibodies against H5N1 virus were detected in the sera of the birds, indicating that domestic ducks were infected with the isolate. Viruses of $10^{3.3}$ and $10^{3.6}$ EID_{50}/ml were recovered from tracheal swabs of two of four quails inoculated with the virus on three dpi, respectively. Antibodies against H5N1 virus were detected in the sera of the birds on 14 dpi. These findings indicate that quails are susceptible to infection with the isolate.

Table 3 Virus recovery from birds experimentally inoculated with A/mallard/Hokkaido/24/09 (H5N1)

Birds	No. of Birds	Days[a] p.i.	Clinical signs	Virus recovery[d]							Antibody[e] response
				Swabs (log EID_{50}/ml)		Tissues (log EID_{50}/g)					
				Trachea	Cloaca	Brain	Trachea	Lungs	Kidneys	Colon	
Chickens	1 - 4	3	-[b]	<	<	<	<	<	<	<	NT
	5 - 8	14	-	NT[c]	NT	NT	NT	NT	NT	NT	< 40[f]
Domestic ducks	1 - 4	3	-	<	<	<	<	<	<	<	NT
	5 - 8	14	-	NT	NT	NT	NT	NT	NT	NT	1,600[f]
Quails	1	3	-	3.3	<	<	<	<	<	<	NT
	2	3	-	3.6	<	<	<	<	<	<	NT
	3 - 4	3	-	<	<	<	<	<	<	<	NT
	5 - 8	14	-	NT	NT	NT	NT	NT	NT	NT	800[f]

[a] All birds were sacrificed.
[b] -: Birds did not show any clinical signs during observation days.
[c] NT: Not tested.
[d] Digit: Virus titers. <: Virus titer was less than 0.8 log EID_{50}/ml swab or 1.5 log EID_{50}/g tissue.
[e] ELISA titers on 14 dpi.
[f] ELISA titers of three birds were equal.

Genetic analysis of Mal/Hok/24/09 (H5N1)

Each gene of Mal/Hok/24/09 (H5N1) was phylogenetically analyzed. The HA and NA genes of Mal/Hok/24/09 (H5N1) were classified into the Eurasian lineage, and were different from HA and NA genes of H5N1 HPAIVs, respectively (Figure 1). In addition, the other six genes of Mal/Hok/24/09 (H5N1) were not closely related to those of HPAIVs, but related to those of NPAIVs isolated from migratory water birds (data not shown). The eight segments of Mal/Hok/24/09 (H5N1) were analyzed by the Basic Local Alignment Search Tool (BLAST) available from the DDBJ/EMBL/GenBank (Table 4). It was found that all genes of Mal/Hok/24/09 (H5N1) were derived from those of the viruses circulating in water birds in nature. M gene of the virus was classified into North American lineage, and the other genes were classified into Eurasian lineage (Table 4), indicating that genetic reassortment occurs between the viruses whose genes classified into North American and Eurasian lineages. The amino acid sequence of the HA cleavage site of Mal/Hok/24/09 (H5N1) was RETR/GLF, and insertion or substitution of multiple basic amino acids found in the HAs of HPAIVs [21] was not observed.

Antigenic analysis of the HA of Mal/Hok/24/09 (H5N1)

The HA of Mal/Hok/24/09 (H5N1) was antigenically analyzed using a panel of MAbs recognizing six different epitopes on the HA of A/duck/Pennsylvania/10218/84 (H5N2) [20]. Each of the MAb bound to the antigen of Mal/Hok/24/09 (H5N1) as well as those of the other non-pathogenic H5 viruses, and few MAbs bound to the antigen of H5N1 HPAIVs recently isolated in Mongolia, Japan, and Hong Kong (Table 5), indicating that the HA of Mal/Hok/24/09 (H5N1) is antigenically closely related to the H5 HA of the viruses circulating in migratory water bird.

Discussion

Efforts to monitor avian influenza in migratory water birds have increased worldwide in recent years due to concern that migratory water birds may disseminate HPAIVs. Intensive surveillance of avian influenza has been conducted every autumn in Hokkaido, Japan, and

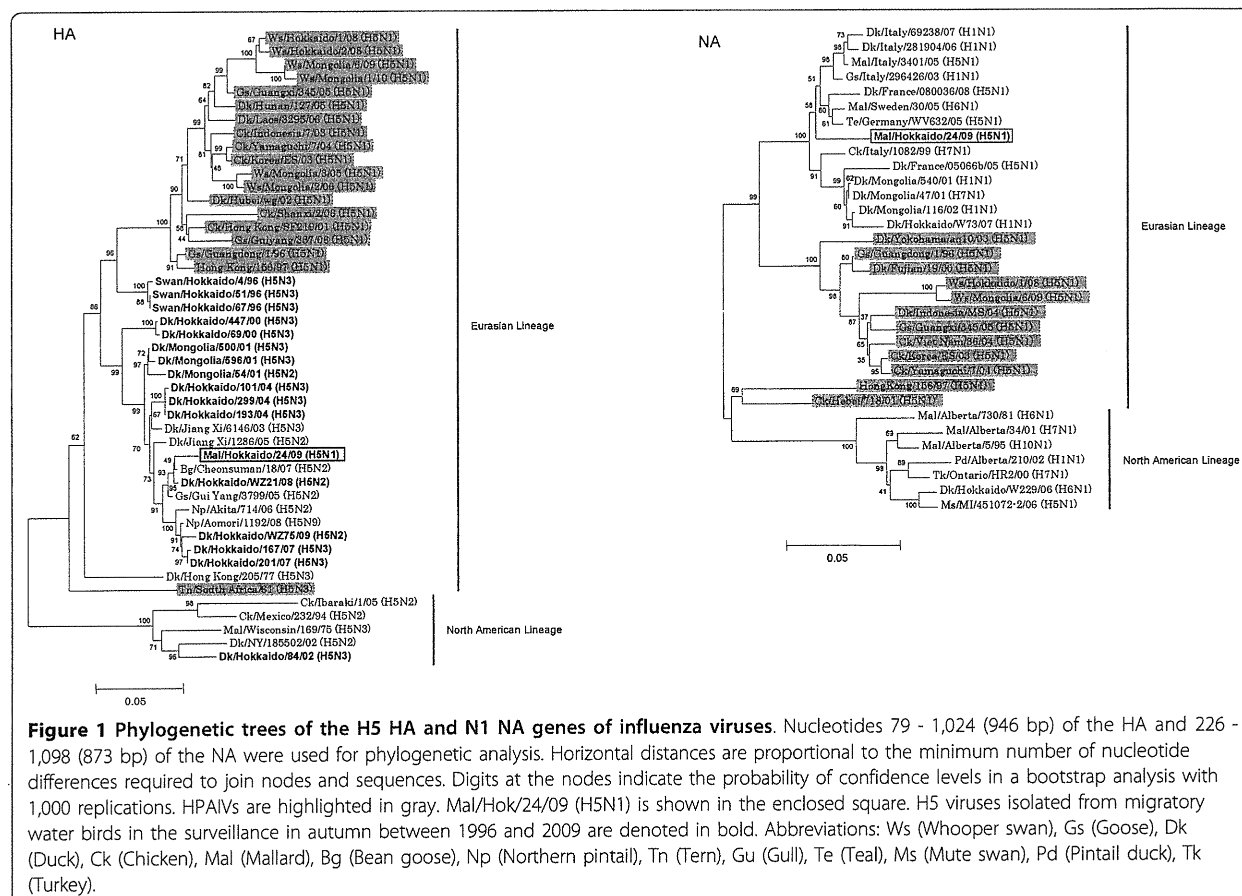

Figure 1 Phylogenetic trees of the H5 HA and N1 NA genes of influenza viruses. Nucleotides 79 - 1,024 (946 bp) of the HA and 226 - 1,098 (873 bp) of the NA were used for phylogenetic analysis. Horizontal distances are proportional to the minimum number of nucleotide differences required to join nodes and sequences. Digits at the nodes indicate the probability of confidence levels in a bootstrap analysis with 1,000 replications. HPAIVs are highlighted in gray. Mal/Hok/24/09 (H5N1) is shown in the enclosed square. H5 viruses isolated from migratory water birds in the surveillance in autumn between 1996 and 2009 are denoted in bold. Abbreviations: Ws (Whooper swan), Gs (Goose), Dk (Duck), Ck (Chicken), Mal (Mallard), Bg (Bean goose), Np (Northern pintail), Tn (Tern), Gu (Gull), Te (Teal), Ms (Mute swan), Pd (Pintail duck), Tk (Turkey).

Table 4 Characterization of the genes of A/mallard/Hokkaido/24/09 (H5N1)

Gene segments[a]	Region of examined nucleotides	Viruses with highest homology: Name[b]	Viruses with highest homology: Accession numbers	Homologies (%)	Lineages
PB2	14-2293	Sbd/Korea/619/08 (H6N2)	GQ414790	98	Eurasian
PB1	9-2269	Sbd/Korea/540/08 (H6N1)	GQ414822	98	Eurasian
PA	1-2200	Dk/Shiga/8/04 (H4N6)	AB304146	98	Eurasian
HA	79-1726	Bg/Cheonsuman/18/07 (H5N2)	FJ767718	98	Eurasian
NP	31-1527	Mal/SanJiang/151/06 (H6N2)	EF592496	99	Eurasian
NA	1-1422	Gs/Italy/296426/03 (H1N1)	FJ432780	97	Eurasian
M	1-983	Mal/Minnesota/153/98 (H9N2)	GU051519	98	North American
NS	1-838	Gu/Astrakhan/1846/98 (H13N6)	GU052231	98	Eurasian

[a] GenBank accession number of each gene of Mal/Hokkaido/24/09 (H5N1): PB2 [AB530989], PB1 [AB530990], PA [AB530991], HA [AB530992], NP [AB530993], NA [AB530994], M [AB530995], and NS [AB530996].
[b] Abbreviations: Sbd (Spot-billed duck), Dk (Duck), Bg (Bean goose), Mal (Mallard), Gs (Goose), Gu (Gull).

Mongolia. As shown in Table 1, H5N1 virus had not been isolated from migratory water birds in the surveillance until 2008. In autumn 2009, Mal/Hok/24/09 (H5N1) was isolated from a fecal sample of a mallard that flew from the northern territory in Siberia to Hokkaido, Japan. In the present study, the H5N1 isolate was examined for pathogenicity in chickens, domestic ducks, and quails. Based on the results of IVPI test, the isolate was designated a NPAIV. Chickens were not susceptible to infection with Mal/Hok/24/09 (H5N1) (Table 3). Domestic ducks and quails were infected with the isolate but did not show clinical signs. These findings indicate

Table 5 Reactivity of H5 viruses with MAbs against HA of A/duck/Pennsylvania/10218/84 (H5N2)

	Viruses[a]	Clades	D101/1 (88[b])	A310/39 (145)	64/1 (157)	B9/5 (168)	B59/5 (169)	25/2 (205)
			Monoclonal antibodies					
NPAIVs	Dk/Pennsylvania/10218/84 (H5N2)	-[c]	+	+	+	+	+	+
	Swan/Hokkaido/4/96 (H5N3)	-	+	+	+	+	+	+
	Swan/Hokkaido/51/96 (H5N3)	-	+	+	+	+	+	+
	Swan/Hokkaido/67/96 (H5N3)	-	+	+	+	+	+	+
	Dk/Hokkaido/447/00 (H5N3)	-	+	+	+	+	+	+
	Dk/Hokkaido/69/00 (H5N3)	-	+	+	+	+	+	+
	Dk/Mongolia/54/01 (H5N2)	-	+	+	+	+	+	+
	Dk/Mongolia/500/01 (H5N3)	-	+	+	+	+	+	+
	Dk/Mongolia/596/01 (H5N3)	-	+	+	+	+	+	+
	Dk/Hokkaido/84/02 (H5N3)	-	+	+	+	+	+	+
	Dk/Hokkaido/101/04 (H5N3)	-	+	+	+	+	+	+
	Dk/Hokkaido/193/04 (H5N3)	-	+	+	+	+	+	+
	Dk/Hokkaido/299/04 (H5N3)	-	+	+	+	+	+	+
	Dk/Hokkaido/167/07 (H5N3)	-	+	+	+	+	+	+
	Dk/Hokkaido/201/07 (H5N3)	-	+	+	+	+	+	+
	Dk/Hokkaido/WZ21/08 (H5N2)	-	+	+	+	+	+	+
	Dk/Hokkaido/WZ75/09 (H5N2)	-	+	+	+	+	+	+
	Mal/Hokkaido/24/09 (H5N1)	-	+	+	+	+	+	+
HPAIVs	Ws/Mongolia/3/05 (H5N1)	2.2	+	-	+	+	-	+
	Ws/Hokkaido/1/08 (H5N1)	2.3.2	+	-	-	-	-	-
	Pf/Hong Kong/810/09 (H5N1)	2.3.4	-	-	-	-	-	-

The results, except Dk/Hokkaido/WZ21/08 (H5N2), Dk/Hokkaido/WZ75/09 (H5N2), Mal/Hokkaido/24/09 (H5N1), Ws/Hokkaido/1/08 (H5N1), and Pf/Hong Kong/810/09 (H5N1), were referred from previous report [20].
[a] Abbreviations: Dk (Duck), Mal (Mallard), Ws (Whooper swan), Pf (Peregrine falcon).
[b] Location of amino acid substitutions in antigenic variants selected in the presence of respective monoclonal antibodies [20].
[c] Dashes (-) indicate classical HA gene which is not classified into the clades 0 - 9.

that the isolate is non-pathogenic in chickens, domestic ducks, and quails. Phylogenetic analyses demonstrated that Mal/Hok/24/09 (H5N1) was distinguished from H5N1 HPAIVs that are prevailing in birds in Eurasia and Africa. Antigenic comparisons of the HAs of H5 viruses indicated that the antigenicity of the HA of Mal/Hok/24/09 (H5N1) is closely related with the H5 NPAIVs circulating in nature (Table 5).

After 1996, H5N1 HPAIVs with both HA and NA genes of A/goose/Guangdong/1/96 (H5N1) have spread to Eurasia and Africa [22]. After 2005, H5N1 HPAIVs were isolated from dead migratory water birds in China, Mongolia, Russia, and Japan in spring [4-8], suggesting that the birds were infected with HPAIVs in the south during the spring and died on the way back to the northern territories. In the surveillance studies of avian influenza in autumn since 1996, H5 viruses with the HA gene of A/goose/Guangdong/1/96 (H5N1) had not been isolated from migratory water birds that flew from Siberia to Japan and Mongolia (Figure 1) indicating that H5N1 HPAIVs had not become dominant in their nesting lakes in Siberia until 2009.

On 14th October, 2010, H5N1 HPAIVs were isolated from migratory water birds that flew from Siberia to Japan (under publication). Then, H5N1 HPAIVs have been isolated from migratory water birds and poultry in other places in Japan.

For the control of HPAIV infection in birds and mammals, early detection of the viruses and stamping out to contain the viruses in the domestic poultry are essential.

Conclusion

In autumn 2009, Mal/Hok/24/09 (H5N1) was isolated from a fecal sample of a mallard. Mal/Hok/24/09 (H5N1) is a NPAIV for chickens, domestic ducks, and quails, and is antigenically and genetically distinct from H5N1 HPAIVs that are prevailing in birds in Eurasia and Africa. Phylogenetic analysis of the HA genes revealed that H5 viruses with the HA gene of HPAIV had not been isolated from migratory water birds in the surveillance until 2009. These findings indicate that H5N1 HPAIVs had not become dominant in their nesting lakes in Siberia until 2009.

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Authors' contributions

NY carried out the animal experiments and the antigenic and phylogenetic analyses, and drafted the manuscript. MM and FY collected the fecal samples and carried out the viral isolation and identification of subtypes. MO carried out the IVPI test. KS participated in the antigenic analysis. YS and HK participated in coordination of the study. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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