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FULL PAPER

An H5N1 highly pathogenic avian influenza virus that invaded Japan through waterfowl migration

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Abstract

In 2010, an H5N1 highly pathogenic avian influenza virus (HPAIV) was isolated from feces of apparently healthy ducks migrating southward in Hokkaido, the northernmost prefecture of Japan. The H5N1 HPAIVs were subsequently detected in domestic and wild birds at multiple sites corresponding to the flyway of the waterfowl having stopovers in the Japanese archipelago. The Hokkaido isolate was genetically nearly identical to H5N1 HPAIVs isolated from swans in the spring of 2009 and 2010 in Mongolia, but less pathogenic in experimentally infected ducks than the 2009 Mongolian isolate. These findings suggest that H5N1 HPAIVs with relatively mild pathogenicity might be selected and harbored in the waterfowl population during the 2009-2010 migration seasons. Our data provide "early warning" signals for preparedness against the unprecedented situation in which the waterfowl reservoirs serve as perpetual sources and disseminators of HPAIVs.

Key words: H5N1, Highly pathogenic avian influenza virus, natural host, waterfowl migration

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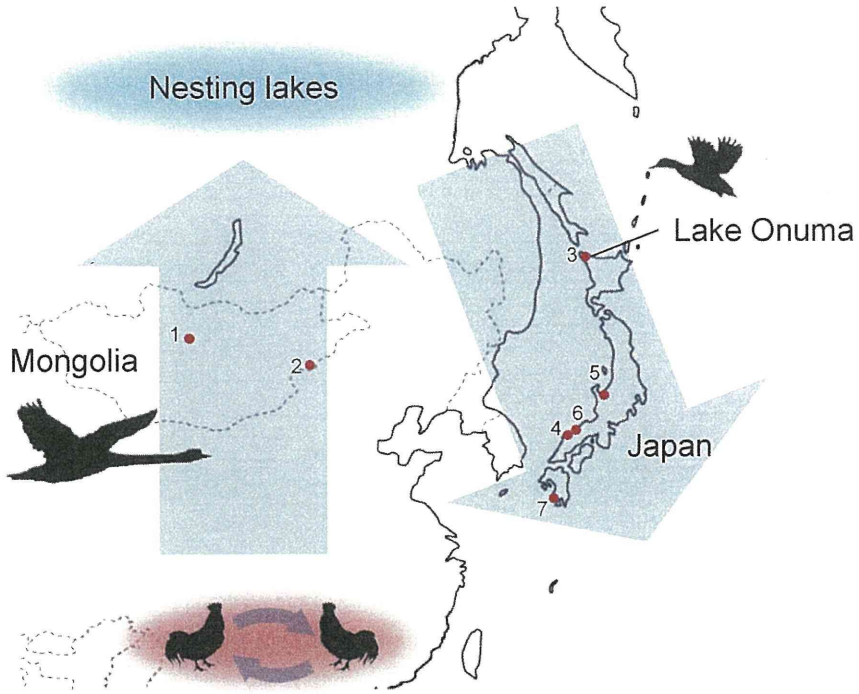
Introduction

Influenza A viruses are zoonotic pathogens that are widely distributed in birds and mammals, including humans. Wild aquatic birds, especially migratory ducks, are the natural reservoir host of influenza A viruses. Viruses of 16 hemagglutinin (HA; H1-H16) and 9 neuraminidase (NA; N1-N9) subtypes have been identified in the waterfowl reservoirs[4]. Influenza A viruses circulating in the reservoir are usually nonpathogenic and evolutionally stable[11,12]. It is known that low pathogenic viruses of the H5 or H7 subtype from wild aquatic birds may become highly pathogenic after circulating in domestic birds[1,29]. Since its first emergence in southern China in 1996, H5N1 highly pathogenic avian influenza viruses (HPAIVs) have been circulating in poultry for more than a decade and causing unprecedented outbreaks in wild birds and poultry in Asia, the Middle East and Africa[30]. These H5N1 HPAIVs occasionally infect humans and pose a significant pandemic threat[15,21].

It was generally believed that ducks could tolerate infection with influenza A viruses, including highly pathogenic viruses. However, in 2002, a large number of water birds, including ducks, geese, and other species, died because of H5N1 HPAIV infection in Hong Kong[25]. In 2005, approximately 6,000 aquatic birds were found dead with H5N1 HPAIV infection in Qinghai Lake, China[2,17], and this virus rapidly extended its geographical distribution to other continents in the following year[28]. Since 2005, H5N1 HPAIVs originating from southern China have been isolated almost annually from dead aquatic birds such as swans and geese on their migratory routes to the north in spring in Japan, Mongolia, and Russia[20,23,27,32].

Although aquatic birds have succumbed to infection with these viruses, some species of ducks such as mallards (*Anas platyrhynchos*) were shown to be resistant to H5N1 HPAIVs[3,9]. Because of this resistance to H5N1 HPAIV and their global migration patterns, wild mallards have been suspected to act as long-distance vectors and disseminators of H5N1 HPAIVs[9,13]. Nevertheless, isolation of H5N1 HPAIVs from wild aquatic birds in eastern Eurasia was mainly geographically linked to particular areas where the viruses have persisted in poultry[25] or restricted to the periods when the waterfowl were migrating to their northern territory in spring[20,23,27], suggesting that multiple strains of H5N1 HPAIVs were independently introduced into migratory birds from the virus pool in avian influenza-endemic areas (e.g., China), and not maintained in their populations over the years. Accordingly, H5N1 HPAIVs isolated from dead birds in Mongolia in 2009 and 2010 were phylogenetically distinct from those isolated in 2005 and 2006[23]. Moreover, during the active surveillance in Japan and Mongolia in the fall and winter months of 2005–2009, hundreds of nonpathogenic influenza A viruses of different subtypes were isolated from fecal samples of wild ducks[18,23,34], but no H5N1 HPAIVs could be detected when the birds migrated southward from their northern territory. Taken together, these previous data provided no definite evidence supporting the notion that H5N1 HPAIVs persisted over the year among the wild migratory bird population until 2009.

In October 2010, two H5N1 HPAIV strains, A/duck/Hokkaido/WZ83/2010 (H5N1) (WZ83) and A/duck/Hokkaido/WZ101/2010 (H5N1) (WZ101), were isolated from the fecal samples of migratory ducks collected at Lake Onuma in Wakkanai, Hokkaido, the northernmost stopover site of the birds in Japan. The viruses closely related to the Hokkaido strain were subsequently isolated from domestic and wild birds at multiple distinct sites in the Japanese archipelago where the migratory flyway of the waterfowl overlaps (Fig. 1)[32,35]. Here we show that the H5N1 HPAIV found in Hokkaido in October 2010 was almost identical to the H5N1 HPAIVs isolated from dead whooper swans (*Cygnus cygnus*) in May 2009 and 2010 in Mongolia[23], and that the pathogenicity of the Hokkaido strain in ducks, and even chickens,



No.	Prefecture	Date	Host
1	Arkhanghai	2009 May 23	whooper swan
2	Sukhbaatar	2010 May 10	whooper swan
3	Hokkaido	2010 Oct 14	duck
4	Shimane	2010 Nov 29	chicken
5	Toyama	2010 Dec 16	mute swan
6	Tottori	2010 Dec 4	tundra swan
7	Kagoshima	2010 Dec 19	hooded crane, white-naped crane

Fig. 1. The putative transmission dynamics of H5N1 HPAIV in eastern Eurasia in 2010. A parental H5N1 HPAIV circulating in domestic poultry in China was introduced into migratory birds (e.g., swans)[23], and carried by aquatic birds through Mongolia to nesting lakes, most likely in Siberia. The aquatic bird population might maintain the virus in their northern territory during the whole summer period, and then disseminated the virus on their southward migration. Arrows indicate the putative routes of transmission of H5N1 HPAIVs. Red dots represent the sites where the H5N1 viruses were isolated from domestic or wild birds in Japan and Mongolia in 2009 and 2010[23,32]. Information on the isolates (i.e., place, date, and host avian name) is shown in the lower table.

was lower than that of the 2009 Mongolian strain. These findings suggest that H5N1 HPAIVs with decreased virulence could be naturally selected. The putative situation in which H5N1 HPAIVs are maintained in the natural reservoir population may complicate strategies for the control of avian influenza and also damage the ecology of the wild birds, and possibly other wildlife.

Materials and Methods

Virus isolation and identification: Virus isolation from fecal samples was performed by using 10-day-old embryonated chicken eggs as previously described[10]. The subtypes of isolates were determined by hemagglutination inhibition and NA inhibition tests[10] as well as by sequencing of the HA and NA genes. The viruses

were passaged once in eggs before being used in the animal experiments. Viral RNA extraction, cDNA synthesis, PCR, and sequencing were carried out according to Simulundu *et al.*[24].

Phylogenetic analyses: The phylogenetic trees of each gene segment of H5N1 influenza A virus strains were constructed by the neighbor-joining method in the Molecular Evolutionary Genetics Analysis program (MEGA, version 4.1)[26]. The evolutionary distances were computed by using the Kimura 2-parameter method[14]. To support tree topology, 1,000 bootstrap replicates were performed. Nucleotide sequences for H5N1 influenza A virus genes were downloaded from the Influenza Virus Resource at the National Center for Biotechnology Information (NCBI). The GenBank/EMBL/DDBJ accession numbers for the sequences reported in this paper are AB612898-AB612913.

Experimental infection of chickens and ducks: Four-week-old domestic chickens (White Leghorn) and ducks (Cherry Valley, kindly provided by Takikawa Shinseien, Hokkaido, Japan) were infected intravenously or intranasally with each virus and monitored clinically over a period of 14 days. Birds exhibiting severe disease signs were euthanized by intravenous injection of pentobarbital and recorded as having died on the next day. To assess viral replication in ducks, 3 birds from each group were euthanized and brain, trachea, lung, kidney, liver, and colon tissues were aseptically collected at 3 days post inoculation (dpi). Viral titers in these tissues were determined by using eggs. Briefly, a 10% tissue homogenate was prepared with minimal essential medium. The tissue homogenates were clarified by centrifugation and ten-fold serially diluted with PBS followed by inoculation into 10-day-old embryonated chicken eggs. Viral titers were calculated as the $\log_{10}$ 50% egg infectious dose (EID_{50})/gram of tissue by the method of Reed and Muench[22]. Experimental infections were carried out in the biosafety level 3 facility at the Hokkaido University Research Center for Zoonosis Control, Japan, according to the guidelines of the institutional animal care and use committee of Hokkaido University.

Results

Isolation and identification of H5N1 HPAIVs from fecal samples of wild ducks

On October 14, 2010, 183 fecal samples of wild ducks were collected at Lake Onuma in Wakkanai, Hokkaido, the northernmost stopover site of the birds in Japan. There were approximately 3,000 ducks and 800 swans, most of which were migrating southward from their northern breeding territory. The waterfowl were apparently healthy, and no appreciable outbreak of highly pathogenic avian influenza was reported around the lake before or after the sampling date. Two strains, A/duck/Hokkaido/WZ83/2010 (H5N1) (WZ83) and A/duck/Hokkaido/WZ101/2010 (H5N1) (WZ101), were isolated from the fecal samples. Sequence analyses revealed that WZ83 and WZ101 were almost identical and that the HA of these viruses had multiple basic amino acid residues at the cleavage site (i.e., Arg-Glu-Arg-Arg-Arg-Lys-Arg), which is a characteristic signature of HPAIVs. Both viruses killed 10-day-old chicken embryos within 48 hours post inoculation. These data suggested that WZ83 and WZ101 were HPAIVs.

Phylogenetic analyses of H5N1 viruses isolated from wild ducks

Nucleotide sequences of all 8 gene segments of WZ83 and WZ101 were analyzed phylogenetically (Fig. 2). The viral surface glycoprotein (i.e., HA and NA) genes of WZ83 and WZ101 showed high similarity with those of A/whooper swan/Mongolia/6/2009 (H5N1) (MON09), a highly pathogenic virus strain isolated from a dead whooper swan in 2009 in Mongolia[23], A/grebe/Tyva/3/2009 (H5N1) isolated from a dead grebe at Lake Uvs nuur located at the Mongolia-Russia boundary[32], and A/great crested grebe/Qinghai/1/2009 (H5N1)[16] (Fig. 2A and B). The HA genes

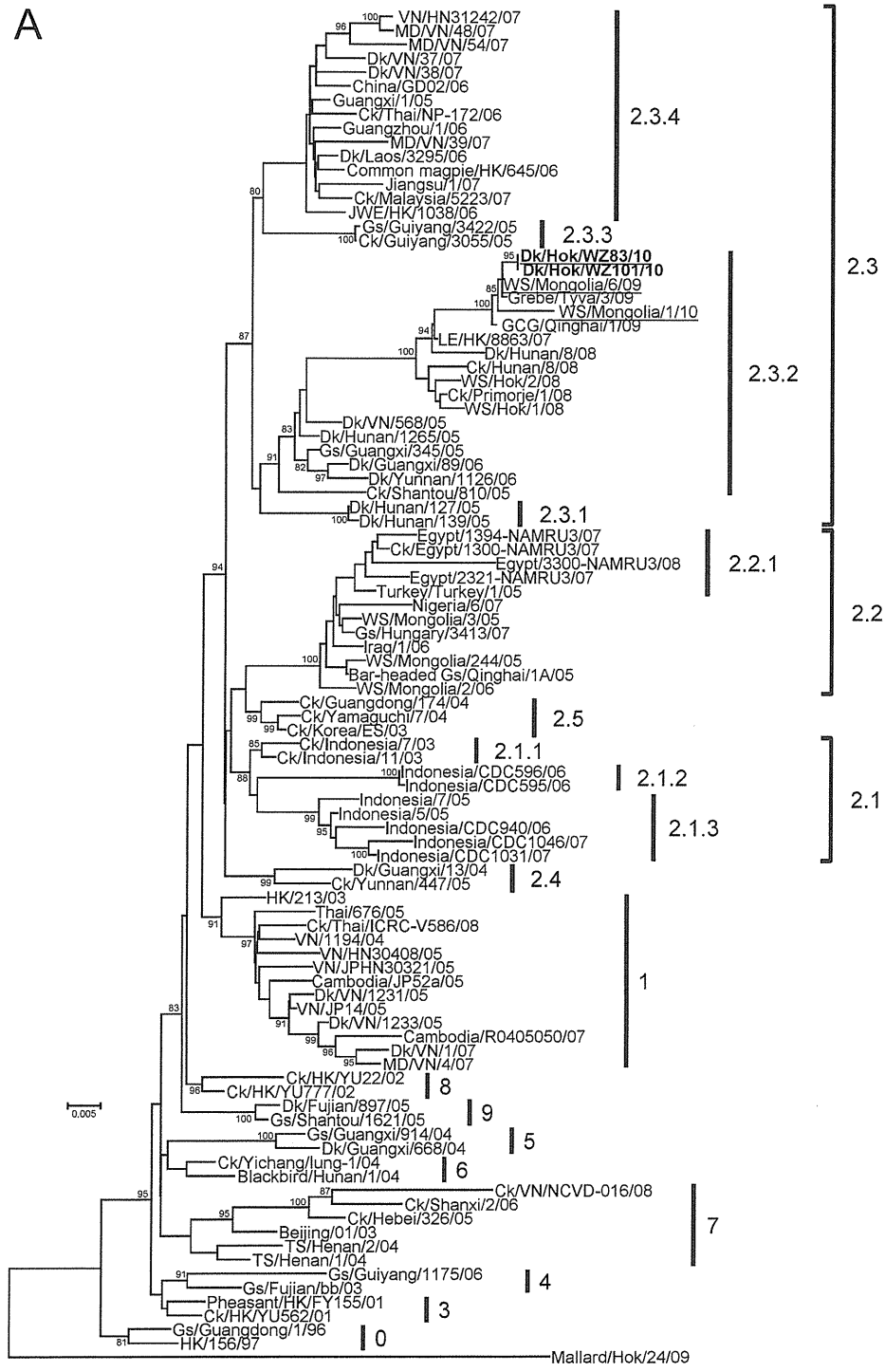
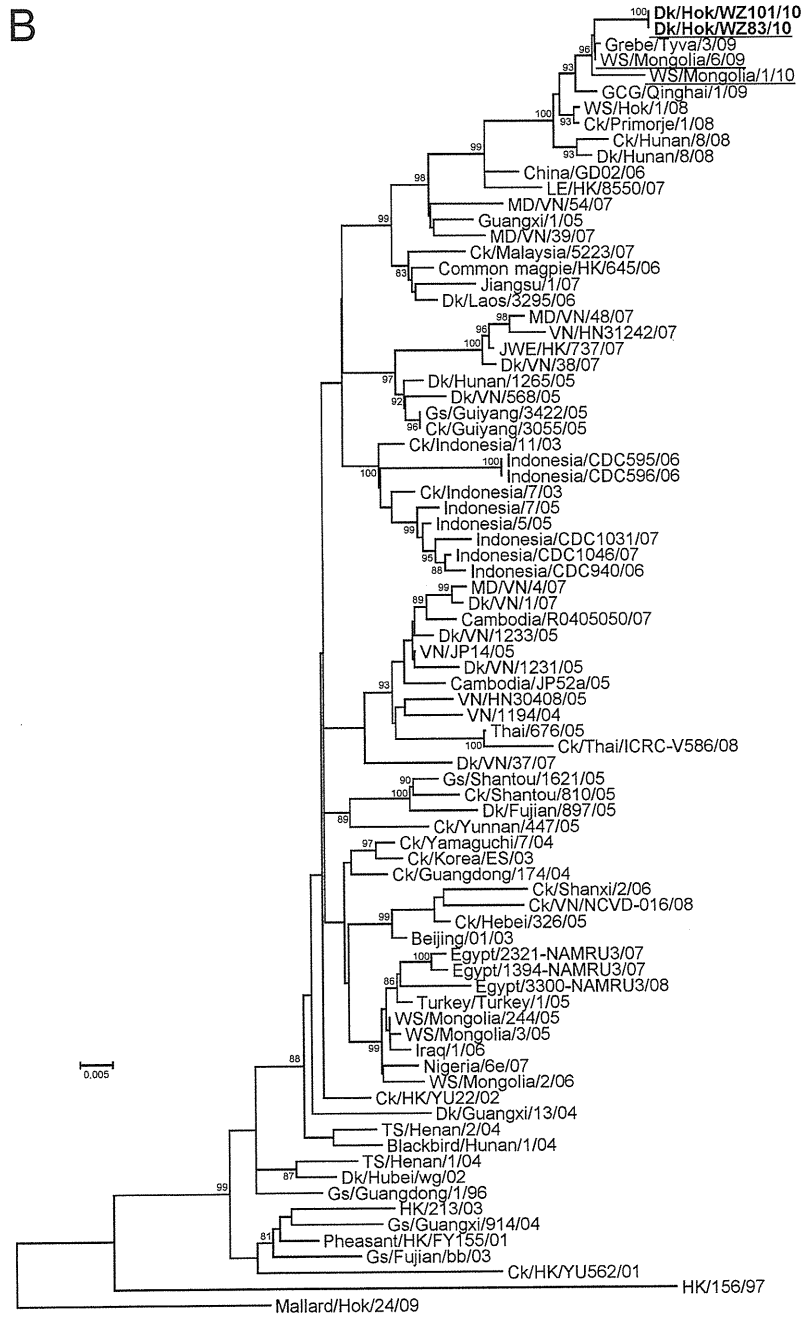
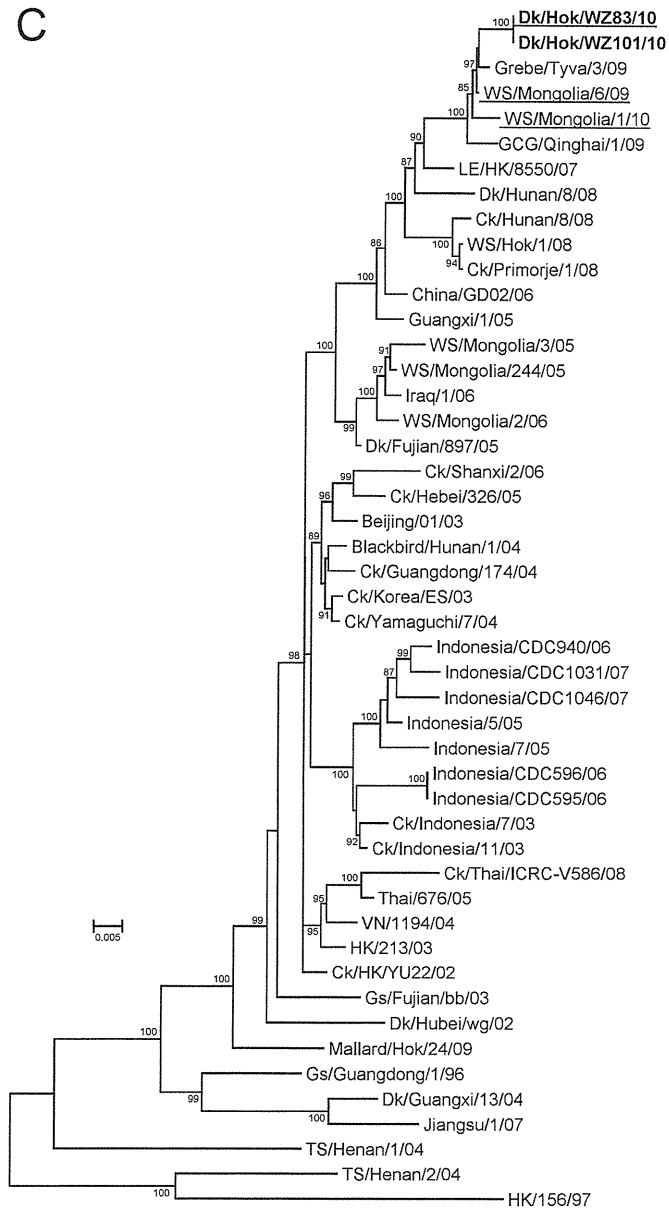


Fig. 2. Phylogenetic trees of influenza A viruses of the H5N1 subtype. Analyses are based on 1,322, 1,305, and 2,239 bp of HA (A), NA (B), and PB2 (C) genes, respectively. Horizontal distances are proportional to the minimum number of nucleotide differences required to join nodes and sequences. Numbers next to branches indicate neighbor-joining bootstrap values of ≥80%. The isolates from migratory ducks in Hokkaido are shown in bold and the viruses used in pathogenic analyses are underlined. Abbreviations: HK (Hong Kong), Hok (Hokkaido), Thai (Thailand), VN (Vietnam), Ck (chicken), Dk (duck), GCG (great crested grebe), Gs (goose), JWE (Japanese white-eye), LE (little egret), MD (muscovy duck), TS (tree sparrow), and WS (whooper swan).



of WZ83, WZ101, and MON09 belonged to clade 2.3.2, which includes viruses isolated from Chinese poultry during 2005–2008. This clade also includes an H5N1 HPAIV strain isolated from a whooper swan in Hokkaido in 2008. The internal protein gene phylogenies also showed a close relation between the Hokkaido strains and MON09 (as a representative, the phylogeny of PB2 genes is shown in Fig. 2C). For each segment, WZ83 and MON09 shared 98.9%-99.8% nucleotide sequence identity. Nineteen amino acid differences between WZ83 and MON09 were identified in several viral proteins (1 each in HA, M1, and M2; 2 each in PB2, PA, and NP; 5 each in PB1 and NA).



Pathogenicity of WZ83 in chickens

To assess the pathogenicity of the isolate, we inoculated WZ83 into chickens and determined its intravenous pathogenicity index according to the manual of the World Organisation for Animal Health (OIE)[31]. All chickens infected intravenously with WZ83 died within 4 dpi, giving an index of 2.76 that met the OIE criteria for HPAIVs[31]. However, this value was lower than those of the recent H5N1 HPAIVs isolated from wild birds such as MON09, whose index was 2.97[23], a finding that was also in agreement with the longer survival time of WZ83-infected chickens (Fig. 3A). We then compared the pathogenic potentials of WZ83 and MON09 in chickens by inoculating the viruses through the intranasal route to mimic the natural route of infection. Chickens were infected intranasally with a $10^{6.0}$ EID_{50} of WZ83 or MON09, and observed for clinical symptoms. All chickens infected with WZ83 or MON09 died, showing typical clinical signs of highly pathogenic avian influenza such

as cyanosis and edema of the head region and legs. Interestingly, similarly to intravenous infection, a remarkable difference between WZ83 and MON09 was seen in the survival periods of the infected chickens (Fig. 3B).

Pathogenicity of WZ83 in ducks

Finally, we tested the pathogenicities of WZ83 and MON09 in ducks. Another Mongolian strain isolated in 2010, A/whooper swan/Mongolia/1/2010 (H5N1) (MON10), belonging to clade 2.3.2 (Fig. 2A)[23], was also tested. Eleven or eight ducks in each group were infected intranasally with WZ83, MON09, or MON10 ($10^{8.0}$ EID_{50}/bird). At 3 dpi, 3 infected ducks in each group were euthanized to determine virus titers in various organs (Table). WZ83, MON09, and MON10 were detected in the tissue samples from trachea, lung, kidney, and colon of all the euthanized ducks examined, indicating systemic infection of these birds. However, the titers of WZ83 in the brain and colon tissues were either undetectable or lower than those of MON09 and MON10. A more prominent difference among these viruses was found in their virulence for ducks. Five of the eight ducks infected with MON09 died at 4–8 dpi, showing severe clinical symptoms such as complete inactivity, rotational torticollis, and tremors. Even the surviving ducks manifested severe depression and anorexia. MON10 also caused decreased locomotor activity and appetite and 1 of the 5 ducks showed mild torticollis, but none of the ducks died. By contrast, all ducks infected with WZ83 were nearly asymptomatic throughout the observation period, although some of them showed slight hypoactivity on 3–5 dpi.

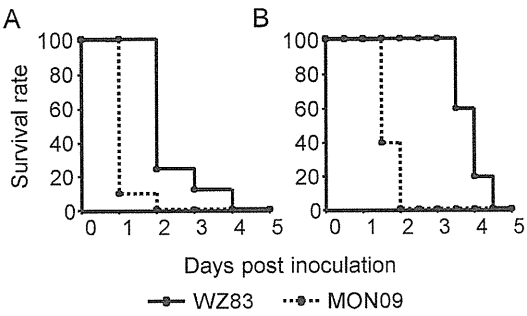


Fig. 3. Difference in disease progression in H5N1 HPAIV-infected chickens. For intravenous infection, 8 chickens of each group were infected with $10^{7.0}$ EID_{50} of the viruses and observed for clinical symptoms every 24 hours (A). Five chickens of each group were infected intranasally with $10^{6.0}$ EID_{50} of the viruses and observed for clinical symptoms every 12 hours (B).

Discussion

In the eastern Eurasian region, some species of wild migratory birds such as ducks, geese, and swans nest and breed at the lakes in their northern territory close to the Arctic Circle during summer, migrate southward in autumn, and return to the northern nesting lakes in spring[33,35]. Considering the migratory flyway of the ducks in this region, the genetic similarity among the isolates of Japan, Mongolia, and Russia suggests that the H5N1 HPAIV experienced a north-south round trip in eastern Eurasia during 2009–2010 (Fig. 1). This also implies that the

Table. Different virulence among the H5N1 HPAIV strains in ducks

Virus[a]	Lethality (dead/total)	Virus titers in organs (log EID_{50}/g)[b]				
		Brain	Trachea	Lung	Kidney	Colon
WZ83	0/8	-[c], 1.7, 2.5	4.7, 5.0, 3.5	6.5, 6.7, 8.3	5.3, 6.5, 6.7	2.5, 4.7, 2.3
MON10	0/5	4.3, 4.5, 5.7	3.5, 6.3, 4.5	5.0, 5.3, 6.7	5.3, 5.3, 5.5	5.5, 5.3, 5.5
MON09[d]	5/8	4.3, 7.3, 7.3	5.7, 6.8, 8.5	6.5, 6.8, 7.8	6.0, 7.5, 8.3	4.8, 5.8, 7.6

a) WZ83; A/duck/Hokkaido/WZ83/2010 (H5N1), MON10; A/whooper swan/1/2010 (H5N1), MON09; A/whooper swan/6/2009 (H5N1).
b) Virus titers of 3 ducks are shown.
c) < 1.5 log EID_{50}/g.
d) Data are cited partially from a previous study[23].

aquatic bird population might have harbored H5N1 HPAIVs in their northern territory during the whole spring-summer period. Because Lake Onuma may serve as the northernmost stopover site for migratory birds in Japan during their southward migration, it is highly likely that the H5N1 HPAIV was introduced into Japan by these migratory birds in the fall of 2010. Indeed, following the detection of the H5N1 HPAIV in Hokkaido, the viruses closely related to the Hokkaido strain were subsequently isolated from chickens, swans, cranes, and ducks sporadically at multiple distinct sites in the Japanese archipelago[32], suggesting that this virus rapidly spread longitudinally along the migratory flyway of the waterfowl (Fig. 1).

Since 2005, H5N1 HPAIVs isolated from wild aquatic birds have been shown to be highly virulent, even to ducks[2,3,17,23]. Consistently, our data indicated that MON09 caused systemic and lethal infection in experimentally infected ducks, as well as infected chickens (Table). By contrast, the pathogenicity of WZ83 in ducks, and even chickens, was notably lower than that of MON09. Interestingly, MON10 did not kill experimentally infected ducks, although mild clinical symptoms were observed. In general, because a highly lethal virus kills the hosts before it can fully exploit opportunities for transmission to new hosts, less virulent mutants of the virus tend to increase over time in the host population[5,6]. Thus, it is likely that H5N1 HPAIV variants with decreased pathogenicity for ducks were naturally selected and harbored among the wild aquatic bird populations in eastern Eurasia during 2009–2010. Importantly, WZ83 still retained high pathogenicity in chickens and thus asymptomatically infected ducks may serve as a perpetual source of the viruses.

Another concern lies in the possibility that H5N1 HPAIVs could be preserved in the frozen water of the nesting lakes during winter as hypothesized by previous studies[8]. According to this hypothesis, it is conceivable that H5N1 HPAIVs may be disseminated again by wild birds moving from their northern nesting lakes to the south in every fall migration season in eastern Eurasia. Although the American continent has not recorded outbreaks of avian influenza caused by Eurasian H5N1 HPAIVs, there is a potential risk of virus introduction from Asia, because some ethological studies of northern pintails (*Anas acuta*) revealed that North American birds cross into Siberia and share the nesting lakes with pintails from Eurasia[19,35].

At the moment, the harmful effect on wildlife under the unprecedented eco-epidemiological situation in which wild waterfowl maintain H5N1 HPAIVs in their natural ecosystems is unclear. However, considering that the Hokkaido 2010 strain seems to be low pathogenic for ducks but still highly lethal for other species of birds, particularly for terrestrial birds, it is reasonable to envision that such strains can potentially negatively affect the ecology of wild birds, and possibly other wildlife. As a matter of fact, this virus killed a number of hooded cranes (*Grus monacha*) and white-naped cranes (*Grus vipio*)[32], both of which are in the vulnerable category of threatened species of the International Union for Conservation of Nature[7], in southern Japan. Because numerous wild bird flocks that have diverse migratory routes come together at the same stopover sites in Japan[35], it is also reasonable to assume that many species of wild birds may be affected by H5N1 HPAIV infection in the near future. We further speculate that some mammalian hosts sharing the same habitats with some waterfowl could be infected with H5N1 HPAIVs, as recently demonstrated through epidemiological surveys of these viruses in wild pikas in China[36].

Although the earliest detection of H5N1 HPAIV in migratory ducks at their northernmost stopover site in Japan in October 2010 provided an alarm to the poultry industry in Japan, the subsequent southward migration of aquatic birds resulted in the spread of H5N1 HPAIVs to farmed poultry and wild birds throughout the

Japanese archipelago. Our data underscore the need for continued global monitoring of H5N1 HPAIVs and provide "early warning" signals for preparedness against the unprecedented situation in which the natural reservoirs maintain HPAIVs consistently, as is the case with nonpathogenic influenza A viruses.

Acknowledgments

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ORIGINAL ARTICLE

Improvement of the H5N1 influenza virus vaccine strain to decrease the pathogenicity in chicken embryos

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Abstract The avian influenza vaccine strain A/duck/Hokkaido/Vac-1/2004 (H5N1) (Vac-1) was found to be pathogenic in chicken embryos (CEs). In order to decrease the pathogenicity of Vac-1 in CEs, a series of reassortant viruses was generated between Vac-1 and A/Puerto Rico/8/1934 (H1N1) (PR8), and their pathogenicity and growth potential were compared in CEs. The results indicated that either the PB1 or PA protein was responsible for the pathogenicity of Vac-1 in CEs. The HA titers of the allantoic fluids of CEs inoculated with the recombinant H5N1 viruses, of which pathogenicity was lower than that of the recombinant Vac-1 prepared by reverse genetics in CEs, were equivalent to those of CEs inoculated with the recombinant Vac-1. One of the reassortant viruses, rg-PR8-PA/Vac-1 (H5N1), in which the PA gene was replaced with the corresponding gene of PR8, yielded allantoic fluids with the same HA titer as that of Vac-1, indicating that this reassortant should be a good candidate as an improved vaccine strain.

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Introduction

Outbreaks of highly pathogenic avian influenza (HPAI) caused by H5N1 viruses have spread to 62 countries in Eurasia and Africa from Southeast Asia since 1996, and well over 400 million birds have died or been culled [27, 28]. This has greatly affected not only the poultry industry but also public health. H5N1 HPAI viruses infected 18 humans, and 6 died in Hong Kong in 1997 [2]. Since 2003, there have been 498 human cases of H5N1 virus infection, with 294 deaths in 15 countries as of May 6, 2010 [27]. "Stamping-out" is the basic measure for the control of HPAI. Vaccination may be an additional option when the disease spreads widely [18]. Inactivated H5 and H7 avian influenza virus vaccines have been prepared and evaluated by several research groups [9, 12, 20, 24, 25]. It is, therefore, important to assess the antigenicity, pathogenicity, and growth potential of the vaccine strains in chicken embryos (CEs).

CEs are currently used as the host in which influenza viruses can grow in sufficient amounts for vaccine production. It is well known that the surface glycoproteins hemagglutinin (HA) and neuraminidase (NA) are responsible for extensive replication of influenza viruses in embryonated chicken eggs [1, 4, 7, 14, 15]. Amino acid substitutions in the vicinity of the receptor-binding site of HA are responsible both for growth potential in CEs and antigenicity [1]. After several passages in embryonated chicken eggs, influenza A and B viruses with amino acid substitutions in the vicinity of the receptor-binding pocket on the HA molecule show high growth potential [11, 15, 16]. The NA contributes to enhancement of virus yield in embryonated chicken eggs [7]. Amino acid substitutions in the HA and/or NA, however, lead not only to extensive growth but also to antigenic variation of the virus [15]. Internal proteins, such as PB2 and NP, which are components of the ribonucleoprotein (RNP)

complex, may also contribute to the replication of influenza viruses in embryonated chicken eggs [16]. The pathogenicity of influenza viruses in CEs also affects the yield of the virus suspension, because embryo death makes it difficult to harvest infectious allantoic fluids due to postmortem change.

In a previous study, a non-pathogenic H5N1 reassortant influenza virus, A/duck/Hokkaido/Vac-1/2004 (H5N1) (Vac-1), was generated between A/duck/Mongolia/54/2001 (H5N2) and A/duck/Mongolia/47/2001 (H7N1), both of which were isolated from migratory ducks in Asia [9]. Phylogenetic analysis of the H5 HA genes revealed that A/duck/Mongolia/54/2001 (H5N2) belonged to the Eurasian lineage [22]. In addition, antigenic analysis using a panel of monoclonal antibodies to the H5 HA proteins and antiserum to Vac-1 indicated that the HAs of HPAI viruses currently circulating in Asia were antigenically closely related to that of Vac-1 [22]. The inactivated vaccine prepared from Vac-1 was confirmed to be potent in a previous study [9]; however, the CEs in the eggs inoculated with Vac-1 died between 48 and 72 hours post-inoculation. Ideally, vaccine strains should be less pathogenic in chicken embryos for vaccine manufacture, although Vac-1 was defined as a nonpathogenic virus strain in 6-week-old chickens [9].

In the present study, it was revealed that the PB1 and PA proteins of Vac-1 influenza virus are responsible for pathogenicity in CEs. One of the reassortant viruses, rg-PR8-PA/Vac-1 (H5N1), in which PA gene was replaced with the corresponding gene of PR8, yielded allantoic fluids with the same HA titer as that of Vac-1, indicating that this reassortant should be a good candidate as a vaccine strain.

Materials and methods

Viruses

A/duck/Hokkaido/Vac-1/2004 (H5N1) was selected from reassortants between A/duck/Mongolia/54/2001 (H5N2) and A/duck/Mongolia/47/2001 (H7N1), both of which were isolated from fecal samples from migratory ducks in Asia [9, 23]. The NA and NS gene segments of Vac-1 are derived from A/duck/Mongolia/47/2001 (H7N1), and the other 6 segments from A/duck/Mongolia/54/2001 (H5N2) [23]. Nucleotide and amino acid sequences of the eight genes of Vac-1 (H5N1) were submitted to the DNA Data Bank of Japan under accession numbers AB253760 (PB2), AB253761 (PB1), AB257726 (PA), AB263192 (HA), AB263193 (NP), AB263194 (NA), AB263195 (M), and AB263196 (NS) [9]. A/Puerto Rico/8/1934 (H1N1) was provided by St. Jude Children's Research Hospital, USA. PR8 and Vac-1 were propagated in 10-day-old embryonated chicken eggs at 35°C for 48 hours, and the allantoic fluids were then harvested.

Generation of recombinant viruses

A series of plasmids carrying the eight gene segments of PR8 was provided by Drs. E. Hoffmann and R. Webby, St. Jude Children's Research Hospital. The universal primer set for influenza A viruses was used for RT-PCR [6]. Each of the PCR products of the eight gene segments of Vac-1 was cloned into pCR 2.1 TOPO vector (Invitrogen). Eight segments of Vac-1 were cloned into a dual-promoter plasmid, pHW2000 [5]. The plasmids carrying either the PB1 or PA gene of PR8 or Vac-1 were used to construct chimeric gene segments. To exchange the PB1 genes between PR8 and Vac-1, the plasmids carrying the PB1 gene of either PR8 or Vac-1 were digested with *Mfe*I (Takara Bio; cleavage site, nucleotide position 719 in the PB1 gene) and *Bam*HI (Takara Bio; cleavage site, nucleotide position 1997 in PB1 gene), and both of their reading frames were divided into three fragments. The fragments were cloned, and the resulting 6 plasmids were designated as pHW-PB1/P719V (nucleotide position 1 to 719 of PR8, and the other region of Vac-1), pHW-PB1/V719P (nucleotide position 1 to 719 of Vac-1, and the other region of PR8), pHW-PB1/V719P1997V (nucleotide position 720 to 1997 of PR8, and the other regions of Vac-1), pHW-PB1/P719V1997P (nucleotide position 720 to 1997 of Vac-1, and the other regions of PR/8), pHW-PB1/V1997P (nucleotide position 1 to 1997 of Vac-1, and the other region of PR8), and pHW-PB1/P1997V (nucleotide position 1 to 1997 of PR8, and the other region of Vac-1). To exchange the PA gene between PR8 and Vac-1, plasmids carrying the PA gene of either PR8 or Vac-1 were digested with *Csp*45I (Toyobo; cleavage site, nucleotide position 706 in the PA gene) and *Sph*I (Takara Bio; cleavage site, nucleotide position 1240 in the PA gene), and both of their reading frames were divided into three fragments. The fragments were cloned, and the resulting 6 plasmids were designated as pHW-PA/P706V (nucleotide position 1 to 706 of PR8, the other region of Vac-1), pHW-PA/V706P (nucleotide position 1 to 706 of Vac-1, and the other region of PR8), pHW-PA/V706P1240V (nucleotide position 707 to 1240 of PR8, and the other regions of Vac-1), pHW-PA/P706V1240P (nucleotide position 707 to 1240 of Vac-1, and the other regions of PR8), pHW-PA/V1240P (nucleotide position 1 to 1240 of Vac-1, and the other region of PR8), and pHW-PA/P1240V (nucleotide position 1 to 1240 of PR8, and the other region of Vac-1).

Recombinant viruses were generated by reverse genetic methods according to Hoffman et al. [5]. Briefly, 293T cells and Madin-Darby canine kidney (MDCK) cells were cocultured in 35-mm dishes and transfected with 1 μg of each of the eight plasmids and 16 μl of TransIT-293T (Promega) in a total volume of 1 ml of OPTI-MEM (Gibco). After 30 hours, 1 ml of OPTI-MEM with 5 μg/ml

Table 1 MDTs of chicken embryos inoculated with wild-type and recombinant influenza viruses

Virus	Gene segment [a]								MDT ± SD (h) [b]
	PB2	PB1	PA	HA	NP	NA	M	NS	
wt-PR8									95.0 ± 9.8
wt-Vac-1									64.0 ± 11.9
rg-PR8									86.2 ± 13.2
rg-Vac-1									55.2 ± 5.9
rg-Vac-PB2-PB1-PA/PR8									※62.3 ± 8.6
rg-PR8-PB2-PB1-PA/Vac-1									※※79.1 ± 12.9
rg-Vac-1-HA-NA/PR8									88.0 ± 10.1
rg-PR8-HA-NA/Vac-1									62.2 ± 14.3
rg-Vac-1-NP-M-NS/PR8									※68.4 ± 11.4
rg-PR8-NP-M-NS/Vac-1									60.4 ± 9.6
rg-Vac-1-PB2/PR8									82.2 ± 3.9
rg-PR8-PB2/Vac-1									※※72.0 ± 15.5
rg-Vac-1-PB1/PR8									※53.3 ± 5.7
rg-PR8-PB1/Vac-1									※※88.0 ± 18.7
rg-Vac-1-PA/PR8									※69.6 ± 9.3
rg-PR8-PA/Vac-1									※※90.2 ± 18.1

[a] White; derived from A/Puerto Rico/8/1934 (H1N1), Black; derived from A/duck/Hokkaido/Vac-1/2004 (H5N1)

[b] Values significantly different from that of rg-PR8 at the 5% level are indicated by a single asterisk, and values significantly different from that of rg-Vac-1 at the 5% level are indicated by double asterisks

acetyltrypsin was added, and the cells were incubated at 35°C for 48 hours. One hundred μl of the supernatant was inoculated into 10-day-old embryonated chicken eggs, which were then incubated at 35°C for 48 hours.

Mean death time of CEs inoculated with recombinant influenza viruses

Wild-type or recombinant viruses generated by the reverse genetic method were inoculated into the allantoic cavities of ten 9-day-old embryonated chicken eggs at 100 times the 50% egg infectious dose (EID_{50}). All eggs were incubated at 35°C and observed for embryo death every eight hours until 120 hours post-inoculation. The mean death time (MDT) of the CEs was calculated as the mean hours until the death of all ten inoculated CEs. Differences in pathogenicity between rg-PR8 or rg-Vac-1 and the recombinant viruses were evaluated statistically using Student's t-test at the 5% level.

Comparison of the HA titer of recombinant viruses in embryonated chicken eggs

Each recombinant virus was inoculated into the allantoic cavities of six 9-day-old embryonated chicken eggs at a dosage of 100 EID_{50} and incubated at 35°C for 72 hours. Allantoic fluids of the eggs were collected every 6 or 12 hours starting at 24 hours post-inoculation. Dead CEs were not sampled further. An HA test was performed to assess the HA titer of each of the collected allantoic fluids. The HA titers of allantoic fluids of CEs at each time point were evaluated statistically using the Welch t-test at the 5% level [26].

Results

MDTs of CEs inoculated with reassortant influenza viruses

The MDT of CEs inoculated with rg-PR8 and rg-Vac-1 generated by reverse genetic methods was 86.2 and 55.2 hours, respectively (Table 1). Although the MDTs of these recombinant viruses were not equal to those of the respective wild-type viruses, significant differences were not found between the MDTs of wild-type and recombinant viruses. The MDT of either the wild-type or reassortant of Vac-1 was approximately 30 hours shorter than that of PR8. The MDT of CEs inoculated with rg-Vac-1-PB2-PB1-PA/PR8 (H1N1), which had PB2, PB1, and PA gene

Table 2 MDTs of chicken embryos inoculated with influenza viruses with a chimeric gene segment

Virus	Gene segment [a, b]				MDT ± SD (h) [c]
	PB2	PB1	PA	Other genes	
rg-PR8					86.2 ± 13.2
rg-Vac-1					55.2 ± 11.9
	1 240 665 758				
rg-PB1/P719V					69.0 ± 20.0
rg-PB1/V719P					77.6 ± 23.0
rg-PB1/V719P1997V					※※82.4 ± 17.4
rg-PB1/P719V1997P					※68.0 ± 16.2
rg-PB1/V1997P					58.6 ± 17.3
rg-PB1/P1997V					81.4 ± 21.3
			1 235 413 716		
rg-PA/P706V					※※86.0 ± 20.0
rg-PA/V706P					※53.0 ± 10.4
rg-PA/V706P1240V					※※82.4 ± 21.0
rg-PA/P706V1240P					85.0 ± 23.9
rg-PA/V1240P					69.6 ± 20.3
rg-PA/P1240V					※67.2 ± 14.2

[a] White; derived from A/Puerto Rico/8/1934 (H1N1), Black; derived from A/duck/Hokkaido/Vac-1/2004 (H5N1)

[b] Chimeric parts are described in Materials and methods

[c] Values significantly different from that of rg-PR8 at the 5% level are indicated by single asterisks, and values significantly different from that of rg-Vac-1 at the 5% level are indicated by double asterisks

segments from Vac-1 and the others from PR8, was 62.3 hours. The MDT of CEs inoculated with rg-PR8-PB2-PB1-PA/Vac-1 (H5N1), which had PB2, PB1, and PA gene segments from PR8 and the others from Vac-1, was 79.1 hours. The pathogenicity of PR8 in CEs increased upon recombination of these three gene segments from Vac-1, and conversely, that of Vac-1 decreased upon recombination of these gene segments from PR8. The MDTs of CEs inoculated with rg-Vac-1-HA-NA/PR8 (H5N1) and rg-PR8-HA-NA/Vac-1 (H1N1) were not significantly different from those inoculated with rg-PR8 and rg-Vac-1, respectively. It is thus postulated that the virus glycoproteins of Vac-1 are not responsible for pathogenicity in CEs. The MDT of CEs inoculated with rg-Vac-1-NP-M-NS/PR8 (H1N1), which had NP, M, and NS gene segments from Vac-1 and the others from PR8, was 68.4 hours. Although an aggravation of the pathogenicity of recombinants in CEs upon the introduction of the gene segments from Vac-1 was observed, no significant difference was found in the pathogenicity in CEs between rg-Vac-1 and rg-PR8-NP-M-NS/Vac-1. These results indicate that, although it is possible that some of these three genes should be responsible for pathogenicity, it is unlikely that they are responsible for the pathogenicity of PR8 or Vac-1 in CEs.

The present results of the MDT test indicate that the viral polymerase proteins of Vac-1 are responsible for pathogenicity in CEs. To identify the virus protein(s) responsible for the pathogenicity in CEs, 6 clones of single gene reassortant viruses were prepared, and the MDT of each recombinant virus was determined. The MDTs of CEs inoculated with recombinant viruses possessing 7 gene segments from Vac-1 and one from PR8 were significantly different from those of CEs inoculated with rg-Vac-1. In addition, for three recombinant PR8 viruses possessing 7 gene segments from PR8 and one from Vac-1, the MDTs of CEs inoculated with each of the recombinant viruses except rg-Vac-1-PB2/PR8 (H1N1) were significantly different from those of CEs inoculated with rg-PR8. Recombinant PR8 viruses with either the PB1 or PA gene of Vac-1 were more pathogenic in CEs than rg-PR8, and recombinant Vac-1 viruses with either of the genes of PR8 were less pathogenic in CEs than rg-Vac-1. These results indicate that the PB1 and PA proteins of Vac-1 are responsible for the pathogenicity in CEs.

Identification of amino acid region(s) responsible for pathogenicity in CEs

Chimeric genes of the PB1 and PA gene segments of either PR8 or Vac-1 were constructed to identify the amino acid residue(s) of the encoded proteins responsible for the pathogenicity in CEs on the basis of the MDTs (Table 2).

A mutant PR8 virus with the pHW-PB1/P719V1997P gene was more pathogenic in CEs than rg-PR8. On the other hand, a mutant Vac-1 with the pHW-PB1/V719P1997V gene showed lower pathogenicity in CEs than rg-Vac-1. Sequence analysis showed that the nucleotide region from 720 to 1997 in the PB1 gene encodes amino acids 240 to 665 of the PB1 protein, indicating that this region of the PB1 protein is responsible for the pathogenicity of Vac-1 in CEs. The MDT of CEs inoculated with rg-PA/P706V (H5N1), which had a chimeric PA gene from nucleotide position 706 to 1240, was 86.0 hours. The MDT of CEs inoculated with rg-PA/V706P (H1N1), which was also chimeric at the same residues, was 53.0 hours. Sequence analysis showed that the nucleotide region from 1 to 707 in the PA gene encoding amino acids 1 to 235 in the PA protein is responsible for the pathogenicity of Vac-1 in CEs.

Comparison of HA titers of recombinant viruses in embryonated chicken eggs

The HA titers of recombinant viruses in embryonated chicken eggs were compared. Each of the recombinant H5N1 viruses that showed low pathogenicity in CEs was inoculated into 9-day-old embryonated chicken eggs. The infectious allantoic fluids were collected every 6 or 12 hours starting at 24 hours post-inoculation. Maximum HA titers of the allantoic fluids of embryonated chicken eggs inoculated with rg-Vac-1 and those inoculated with the other recombinant viruses were between 128 and 512 (Fig. 1). HA titers of rg-PR8-PB1/Vac-1 (H5N1) at 48 and 72 hours post-inoculation were significantly lower than those of rg-Vac-1. On the other hand, HA titers of rg-PR8-PA/Vac-1 (H5N1) were not lower than those of rg-Vac-1 at any of the time points. These results indicate that, for the four H5N1 recombinant viruses generated from Vac-1, which are less pathogenic in CEs than Vac-1, the HA titer of rg-PR8-PA/Vac-1 (H5N1) in CEs stayed at a high level similar to that of Vac-1 for 72 hours after inoculation.

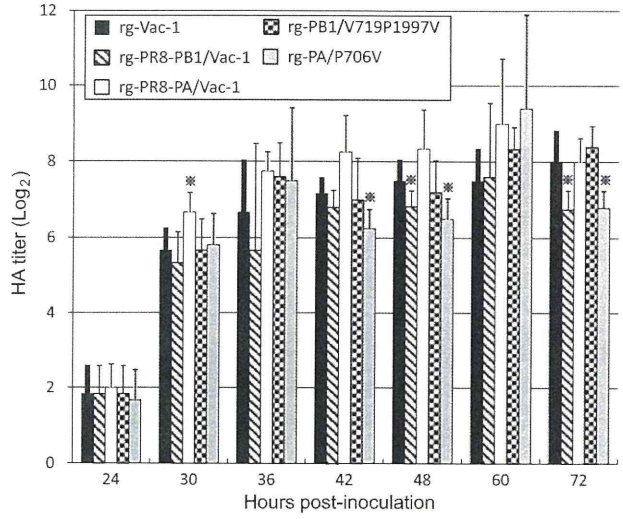

Fig. 1 Virus growth in chicken embryos. Each of six recombinant virus strains generated by reverse genetics was inoculated into six 9-day-old embryonated chicken eggs and incubated at 35°C for 72 hours. The allantoic fluids of the eggs inoculated with each virus were collected every 6 or 12 hours, starting at 24 hours post-inoculation, and samples were titrated by HA test. The mean (bar) and standard deviation (line) of the HA titer at each time point are shown. The differences in HA titer at each time point were evaluated statistically using the Welch t-test. An asterisk indicates a significant difference between the HA titers of the allantoic fluids of embryonated chicken eggs inoculated with rg-Vac-1 and those of each recombinant virus at the 5% level

Discussion

An influenza virus strain, A/duck/Hokkaido/Vac-1/2004 (H5N1), was generated by genetic reassortment between non-pathogenic H5N2 and H7N1 isolates from migratory ducks in order to prepare an inactivated avian influenza vaccine [9, 21, 23]. In the previous study, it was shown that the NA and NS gene segments of Vac-1 are derived from A/duck/Mongolia/47/2001 (H7N1), and the other segments from A/duck/Mongolia/54/2001 (H5N2) [23]. Although the pathogenicity of Vac-1 in CEs was not high (MDT = 64.0 hours), it should ideally be less pathogenic in CEs. In the present study, the contribution of the PB1 and PA proteins of the vaccine strains to pathogenicity in CEs was demonstrated. HA titers of the allantoic fluids of CEs inoculated with four of five recombinant viruses that were less pathogenic than Vac-1 were equal to that of rg-Vac-1 in CEs. The other recombinant virus, rg-PR8-PA/Vac-1 (H5N1), grew more efficiently than rg-Vac-1. Therefore, the present data provide information concerning how to establish a good vaccine strain with high growth potential and low pathogenicity in CEs.

Between PR8 and Vac-1, 10 amino acid differences were found from positions 240 to 666 in the PB1 protein, and seven amino acid differences were found from positions 1 to 235 in the PA protein (Table 3). In the present study, recombinant viruses with 10 and 7 amino acid substitutions in the PB1 and PA proteins, respectively, did not show significant differences in the MDT of CEs (data not shown). It has been shown that the virus polymerase proteins of avian influenza viruses are responsible for pathogenesis in different host animals [8, 13]. In the case of acquisition of pathogenicity in chickens by serial intracerebral passages, amino acid substitutions were identified not only in the HA but also in the internal proteins, including the PB1 and PA proteins [13]. The PB1 and PA proteins of H5N1 HPAI viruses were responsible for

Table 3 Amino acid differences in PB1 and PA proteins between PR8 and Vac-1

Virus	Amino acid at each position in the virus proteins																
	PB1 (from 240 to 665)										PA (from 1 to 235)						
	325	375	383	398	473	563	577	640	645	654	20	28	55	57	65	100	213
A/Puerto Rico/8/1934 (H1N1)	M	S	D	E	L	I	I	M	M	N	T	L	N	Q	L	A	K
A/duck/Hokkaido/Vac-1/2004 (H5N1)	I	N	E	D	V	R	L	V	V	S	A	P	D	R	S	V	R

lethality in mallard ducks, although the mechanism by which these two proteins play roles in pathogenicity has not been clarified [8]. It has been reported that strong interferon beta antagonism due to the accumulation of the virus NS1 proteins in the cytoplasm of infected cells is associated with high pathogenicity of the virus in avian hosts, including embryonated chicken eggs [10]. In the present study, it was considered that the virus pathogenicity in CEs may be associated with viral polymerase activity. However, we were not able to provide conclusive evidence relating to viral polymerase activity using a minigene luciferase assay and pathogenicity in CEs (data not shown). The association between the viral polymerase and pathogenicity in CEs needs to be clarified in a further study.

The crystal structure and function of the amino terminus of the PA protein have been analyzed [3, 17, 19, 29]. It has been shown that amino acid residues 1 to 209 in the PA protein contain the site of endonuclease activity [3]. The PB2 protein binds the 5' cap of host pre-messenger RNA that has been cleaved by the virus endonuclease, and the viral polymerase synthesizes viral messenger RNA. It has also been reported that amino acid residues 163 to 178 of the PA protein are directly or indirectly involved in complementary RNA promoter binding, suggesting a novel function for the PA protein in modulating promoter binding [17]. The sites responsible for these activities are located in the region of amino acids 1 to 235 in the PA protein.

In order to account for the defect in the avian influenza vaccine strain, the virus proteins responsible for pathogenicity in CEs were identified. Rg-PR8-PA/Vac-1 (H5N1), which shows lower pathogenicity in CEs than rg-Vac-1 and gives a high HA titer in CEs that is similar to that of Vac-1, was obtained as a new vaccine strain by using reverse genetics. The present data indicate that replacement of PB1 and PA genes of vaccine strains with those of the PR8 strain decreases their pathogenicity in CEs. This procedure could be applied for the establishment of influenza vaccine strains.

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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