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Citation	Japanese Journal of Veterinary Research, 59(2&3), 89-100
Issue Date	2011-08
DOI	10.14943/jjvr.59.2-3.89
Doc URL	<a href="http://hdl.handle.net/2115/47149">http://hdl.handle.net/2115/47149</a>
Type	bulletin (article)
File Information	JJVR59-2&3_3.pdf



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

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Received for publication, June 20, 2011; accepted, July 13, 2011

## 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

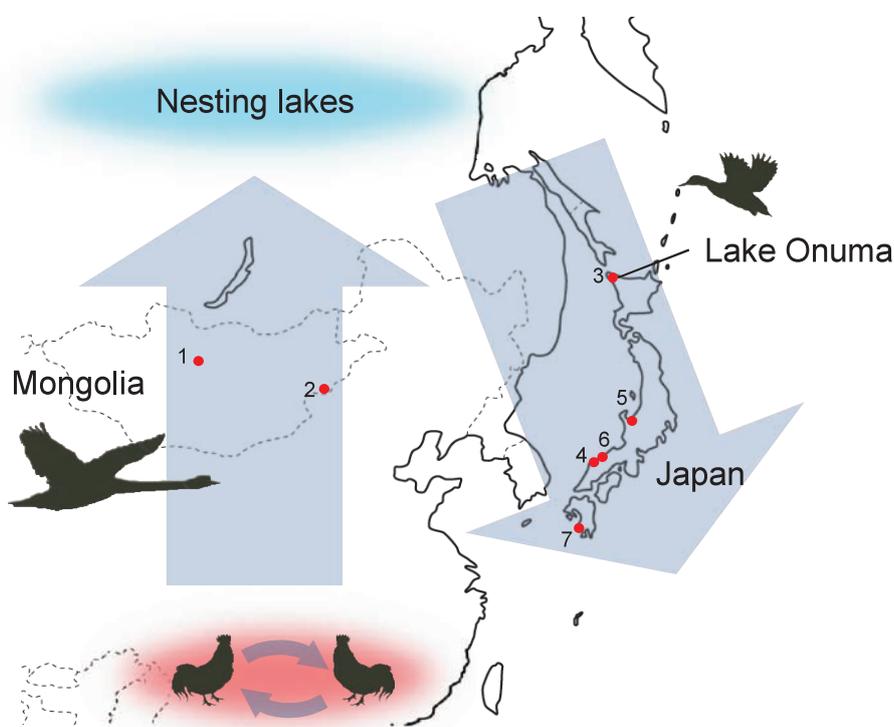
Influenza A viruses are zoonotic pathogens that are widely distributed in birds and mammals, including humans. Wild aquatic birds, especially migratory ducks, are the natural reservoir host of influenza A viruses. Viruses of 16 hemagglutinin (HA; H1-H16) and 9 neuraminidase (NA; N1-N9) subtypes have been identified in the waterfowl reservoirs<sup>4</sup>. Influenza A viruses circulating in the reservoir are usually nonpathogenic and evolutionally stable<sup>11,12</sup>. 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<sup>1,29</sup>. 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<sup>30</sup>. These H5N1 HPAIVs occasionally infect humans and pose a significant pandemic threat<sup>15,21</sup>.

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<sup>25</sup>. In 2005, approximately 6,000 aquatic birds were found dead with H5N1 HPAIV infection in Qinghai Lake, China<sup>2,17</sup>, and this virus rapidly extended its geographical distribution to other continents in the following year<sup>28</sup>. 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<sup>20,23,27,32</sup>.

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<sup>3,9</sup>. 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<sup>9,13</sup>. 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<sup>25</sup> or restricted to the periods when the waterfowl were migrating to their northern territory in spring<sup>20,23,27</sup>, 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<sup>23</sup>. 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<sup>18,23,34</sup>, 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)<sup>32,35</sup>. 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<sup>23</sup>, and that the pathogenicity of the Hokkaido strain in ducks, and even chickens,



No.	Prefecture	Date	Host
1	Arkhangai	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)<sup>23)</sup>, 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<sup>23,32)</sup>. 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<sup>10)</sup>. The subtypes of isolates were determined by hemagglutination inhibition and NA inhibition tests<sup>10)</sup> 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.*<sup>24)</sup>.

*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)<sup>26)</sup>. The evolutionary distances were computed by using the Kimura 2-parameter method<sup>14)</sup>. 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<sub>50</sub>)/gram of tissue by the method of Reed and Muench<sup>22)</sup>. 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.

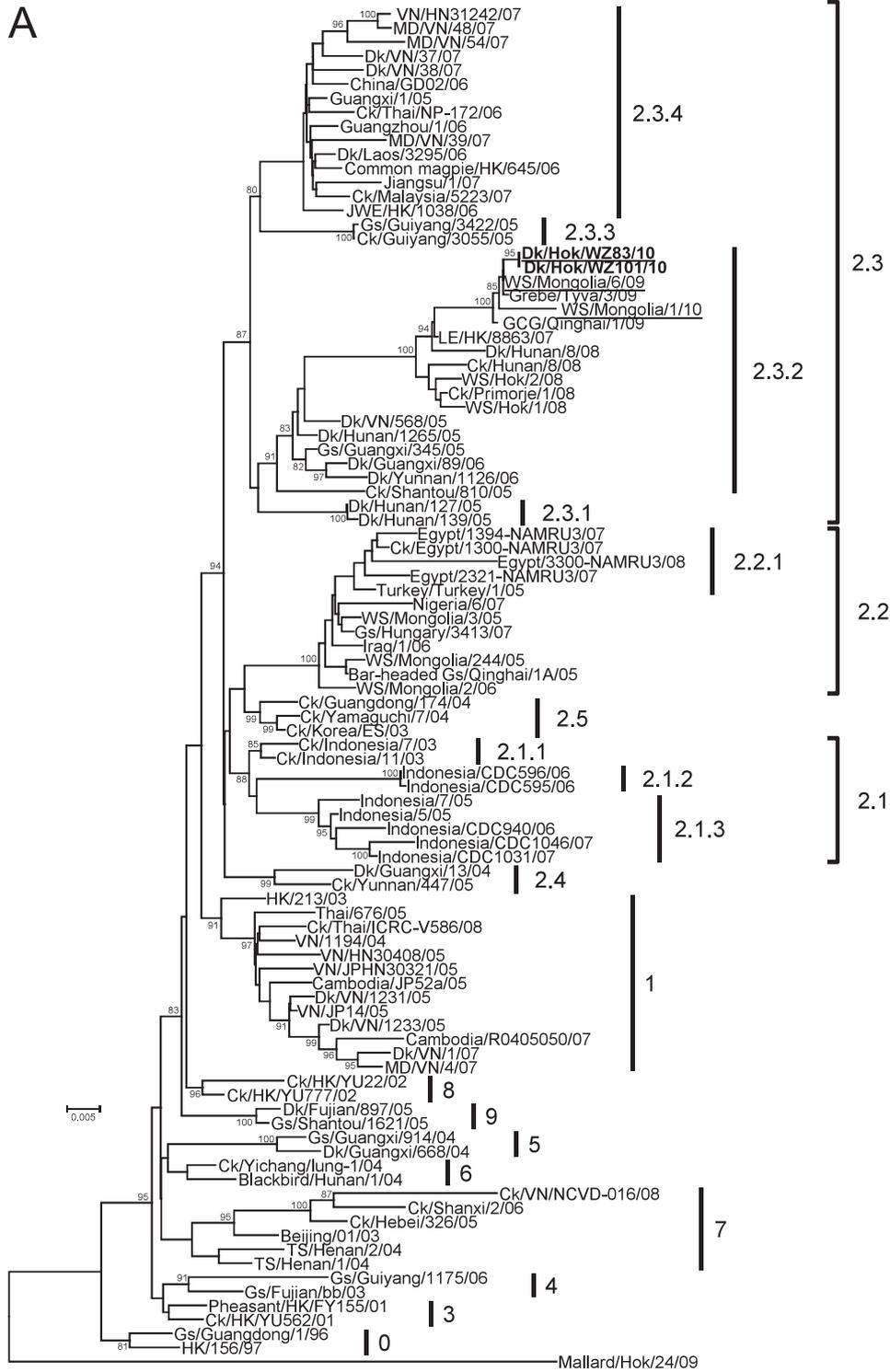
## Results

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

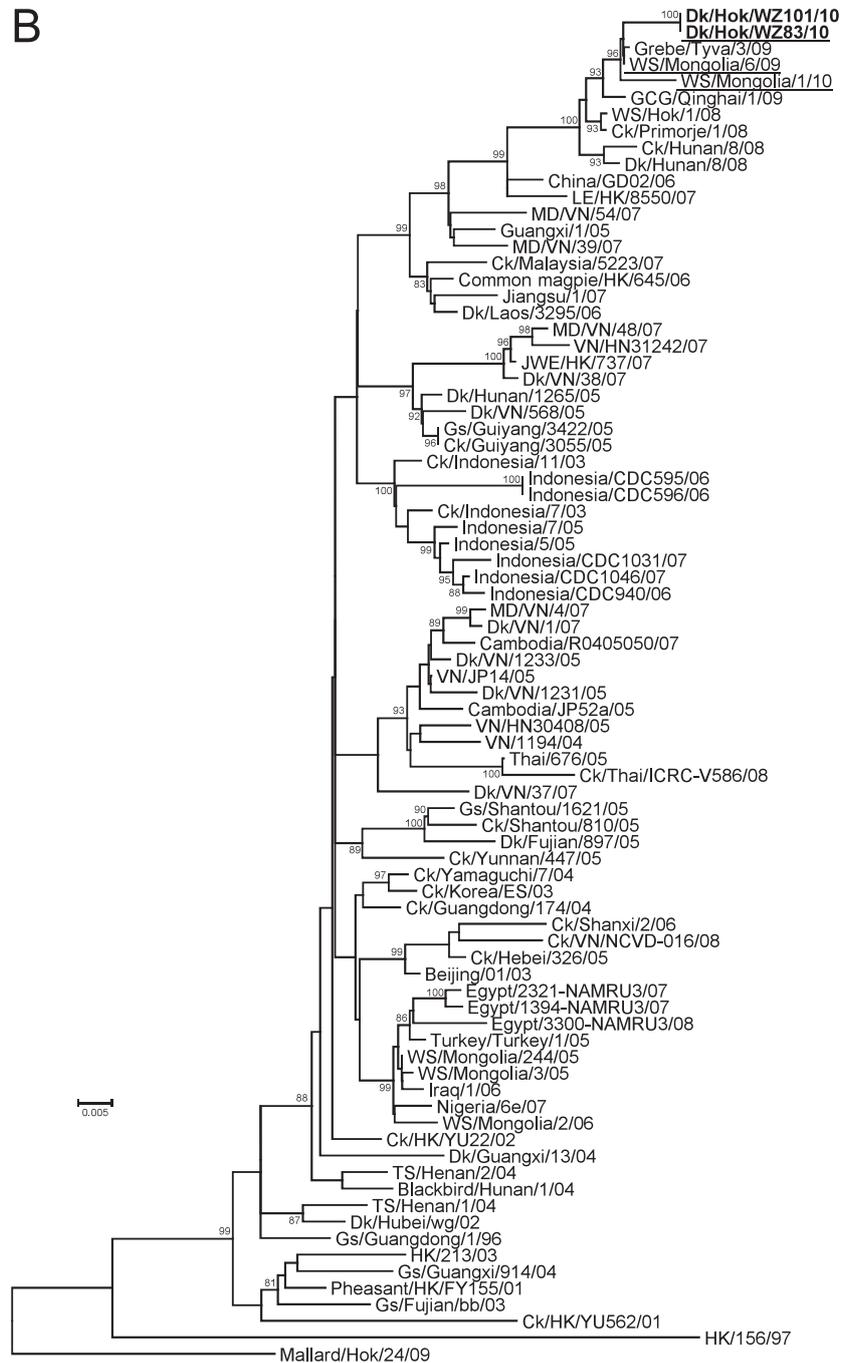
On October 14, 2010, 183 fecal samples of wild ducks were collected at Lake Onuma in Wakkanai, Hokkaido, the northernmost stopover site of the birds in Japan. There were approximately 3,000 ducks and 800 swans, most of which were migrating southward from their northern breeding territory. The waterfowl 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<sup>23)</sup>, A/grebe/Tyva/3/2009 (H5N1) isolated from a dead grebe at Lake Uvs nuur located at the Mongolia-Russia boundary<sup>32)</sup>, and A/great crested grebe/Qinghai/1/2009 (H5N1)<sup>16)</sup> (Fig. 2A and B). The HA genes

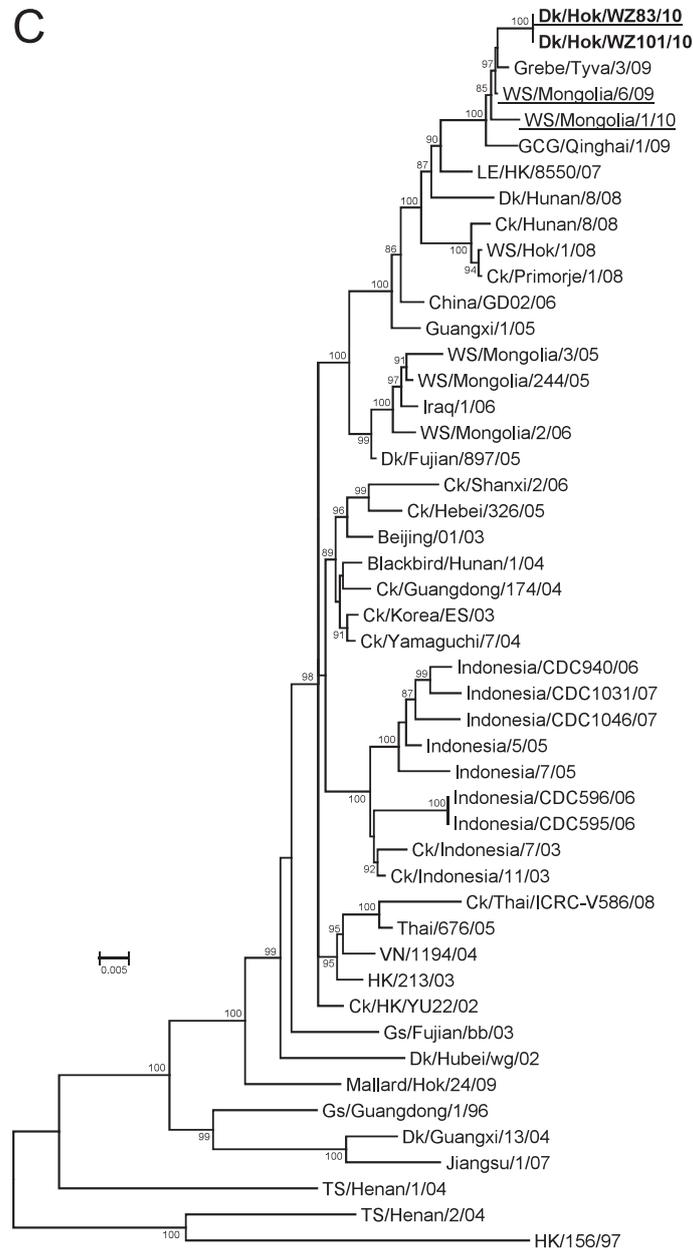


**Fig. 2. Phylogenetic trees of influenza A viruses of the H5N1 subtype.** Analyses are based on 1,322, 1,305, and 2,239 bp of HA (A), NA (B), and PB2 (C) genes, respectively. Horizontal distances are proportional to the minimum number of nucleotide differences required to join nodes and sequences. Numbers next to branches indicate neighbor-joining bootstrap values of  $\geq 80\%$ . 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)<sup>31</sup>. All chickens infected intravenously with WZ83 died within 4 dpi, giving an index of 2.76 that met the OIE criteria for HPAIVs<sup>31</sup>. However, this value was lower than those of the recent H5N1 HPAIVs isolated from wild birds such as MON09, whose index was 2.97<sup>23</sup>, a

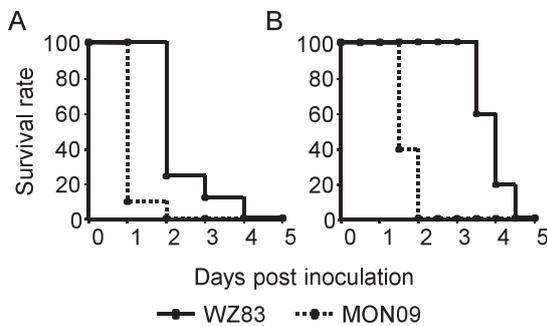
finding that was also in agreement with the longer survival time of WZ83-infected chickens (Fig. 3A). We then compared the pathogenic potentials of WZ83 and MON09 in chickens by inoculating the viruses through the intranasal route to mimic the natural route of infection. Chickens were infected intranasally with a  $10^{6.0}$  EID<sub>50</sub> 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)<sup>23)</sup>, was also tested. Eleven or eight ducks in each group were infected intranasally with WZ83, MON09, or MON10 ( $10^{8.0}$  EID<sub>50</sub>/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<sub>50</sub> 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<sub>50</sub> 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<sup>33,35)</sup>. 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 <sup>a)</sup>	Lethality (dead/total)	Virus titers in organs (log EID <sub>50</sub> /g) <sup>b)</sup>				
		Brain	Trachea	Lung	Kidney	Colon
WZ83	0/8	<sup>c)</sup> , 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 <sup>d)</sup>	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<sub>50</sub>/g.

d) Data are cited partially from a previous study<sup>23)</sup>.

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<sup>32)</sup>, 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<sup>2,3,17,23)</sup>. 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<sup>5,6)</sup>. 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 asymptotically 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<sup>8)</sup>. 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<sup>19,35)</sup>.

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*)<sup>32)</sup>, both of which are in the vulnerable category of threatened species of the International Union for Conservation of Nature<sup>7)</sup>, in southern Japan. Because numerous wild bird flocks that have diverse migratory routes come together at the same stopover sites in Japan<sup>35)</sup>, 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<sup>36)</sup>.

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

We thank Hiroko Miyamoto, Ayaka Yokoyama, Hiromi Yoshida, Mayumi Endo, and Aiko Ohnuma for their excellent technical assistance. We also thank Kim Barrymore for editing the manuscript. This work was supported by World Organisation for Animal Health (OIE), the Japan Initiative for Global Research Network on Infectious Diseases (J-GRID) and the Global COE Program from the Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan, Japan Science and Technology Agency Basic Research Programs, and a Grant-in-Aid for JSPS Fellows.

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