[14, 22, 27, 31, 36, 41]. The isolates were antigenically and phylogenetically analyzed and assessed for pathogenicity in birds and mammals by experimental infection [22, 27, 36, 41]. In the present study, a surveillance of avian influenza was carried out in Vietnam in domestic ducks and wild birds in 2009 and 2010, and the isolates were antigenically and phylogenetically analyzed and their pathogenicity in birds and mammals was assessed.

Materials and methods

Viruses

A/duck/Hong Kong/Y280/1997 (H9N2), A/chicken/Hong Kong/G9/1997 (H9N2), and A/duck/Hong Kong/W213/1998 (H9N2) of the Y280 sublineage and A/quail/Hong Kong/G1/1997 (H9N2) of the G1 sublineage were provided by Dr. K. F. Shortridge, the University of Hong Kong, China. A/turkey/Wisconsin/1/1966 (H9N2) of the North American lineage was provided by Dr. R. G. Webster, St. Jude Children's Research Hospital, United States of America. A/duck/Hokkaido/49/1998 (H9N2) and A/duck/Hokkaido/13/2000 (H9N2) of Korean sublineage were isolated from ducks under surveillance in our laboratory [27, 31]. Viruses isolated from domestic ducks in Vietnam in 2009 and 2010 were grown in 10-day-old embryonated chicken eggs, and infectious allantoic fluids were stored at −80°C until use.

Virus isolation and phylogenetic analysis

One hundred tracheal and cloacal swab samples that were viral gene positive from 600 domestic ducks and 207 wild birds (night heron, *Nycticorax nycticorax*; grey heron, *Ardea cinerea*; purple heron, *Ardea purpurea*; chinese pond heron, *Ardeola bacchus*; chinese egret, *Egretta eulophotes*; little egret, *Egretta garzetta*; intermediate egret, *Egretta intermedia*; cormorant, *Phalacrocorax carbo*; little cormorant, *Microcarbo niger*; Japanese bush warbler, *Cettia diphone*; black-browed reed warbler, *Acrocephalus bistrigiceps*; olive bulbul, *Iole virescens*; black capped kingfisher, *Halcyon pileata*; collared kingfisher, *Halcyon chloris*; racket tailed treepie, *Crypsirina temia*; oriental magpie robin, *Copsychus saularis*; tiger shrike, *Lanius tigrinus*; yellow bittern, *Ixobrychus sinensis*; indian cuckoo, *Cuculus micropterus*; common koel, *Eudynamys scolopacea*; and black collared starling, *Sturnus nigricollis*) in April 2009 and March 2010 in southern Vietnam were inoculated into the allantoic cavities of 10-day-old embryonated chicken eggs. Viral RNA was detected by the reverse transcription loop-mediated isothermal amplification (RT-LAMP) method described previously [42] as a screening test for virus isolation. Viral RNAs were extracted from the allantoic fluids of chicken embryos infected with viruses by TRIzol LS Reagent (Invitrogen, CA, USA) and reverse-transcribed using the Uni12 primer [13] and M-MLV reverse transcriptase (Invitrogen). Polymerase chain reaction for amplification of the viral genes was performed using a PTC-200 thermal cycler (Bio-Rad, CA, USA). Direct sequencing of the viral genes was performed using an autosequencer CEQ 2000XL (Beckman Coulter, CA, USA). For phylogenetic analysis, sequence data for these genes together with those from public database were analyzed by the neighbor-joining method [35] using MEGA 5.0 software (http://www.megasoftware.net/). Accession numbers of the gene sequences of the isolates in the present study are as follows: AB545593, AB545594, AB639351-AB639356 (OIE-2313), AB621343, AB639024-AB639030 (OIE-2326), AB545591, AB545592, AB571519-AB571524 (OIE-2327), AB571525-AB571532 (OIE-2328), AB638754-AB638761 (OIE-2390), AB638722-AB638729 (OIE-2576), AB638746-AB638753 (OIE-2581), AB638730-AB638737 (OIE-2582), AB571533-AB571539, AB572587 (OIE-2583), AB638738-AB638745 (OIE-2584), AB638603-AB638610 (OIE-2587), AB638320-AB638327 (OIE-2592), AB638312-AB638319 (OIE-2593), and AB636530-AB636537 (OIE-2595). Subtypes of influenza virus isolates were identified by hemagglutination-inhibition (HI) and neuraminidase-inhibition tests using chicken antisera to the reference strains of influenza viruses [17].

Animals

Four-week-old Chelly Valley ducks were purchased from Takikawa Shinseien (Hokkaido, Japan). Four-week-old Boris brown chickens were purchased from Hokuren Co. (Hokkaido, Japan). Three-week-old crossbred (Landrace × Duroc × Yorkshire) specific-pathogen-free pigs were purchased from Yamanaka Chikusan (Hokkaido, Japan). Four-week-old female BALB/c mice were purchased from Japan SLC, Inc. (Shizuoka, Japan). All procedures were performed according to the animal experiment guidelines of Graduate school of Veterinary Medicine, Hokkaido University.

Experimental infection

A/duck/Vietnam/OIE-2327/2009 (Dk/VN/OIE-2327/2009), A/duck/Vietnam/OIE-2328/2009 (Dk/VN/OIE-2328/2009), and A/duck/Vietnam/OIE-2583/2009 (Dk/VN/OIE-2583/2009) were inoculated intranasally into three ducks (100 µl/duck), six chickens (100 µl/chicken), two pigs (1 ml/pig), and ten mice (30 µl/mouse) at a 50% egg infectious dose (EID_{50}) of $10^{5.8}$ EID_{50}, $10^{5.8}$ EID_{50}, $10^{6.8}$ EID_{50}, and $10^{5.0}$ EID_{50}, respectively.

After the inoculation of each influenza virus into three ducks, laryngopharyngeal and cloacal swabs were collected in minimal essential medium (MEM; Nissui, Tokyo, Japan) with antibiotics (penicillin G potassium, streptomycin sulfate, gentamicin sulfate, and nystatin) daily from 1 to 7 days post-infection (d.p.i.). All ducks were clinically observed for 14 days after inoculation with influenza viruses.

After the inoculation of each influenza virus into six chickens, three chickens were sacrificed at 3 d.p.i., and the brain, trachea, lung, and colon were collected and homogenized to make 10% (w/v) suspensions in MEM. The remaining three chickens were clinically observed for 14 days after inoculation.

After the inoculation of each influenza virus into two pigs, nasal swabs from these pigs were collected in MEM from 1 to 7 d.p.i. daily, and two pigs were clinically observed for 14 days after inoculation.

After the inoculation of each influenza virus into ten mice, five mice were sacrificed at 3 d.p.i., and the lungs were collected and homogenized to make 10% (w/v) suspensions in MEM. The other five mice were clinically observed and their body weight was monitored for 14 days after inoculation.

Virus titers in the supernatants of the swabs and the tissue homogenates were determined in 10-day-old embryonated chicken eggs and expressed as the EID_{50}/ml and g of tissue, respectively. Antibody responses to the inoculated viruses in ducks, chickens, and pigs at 14 d.p.i. were examined by HI test or enzyme-linked immunosorbent assay (ELISA) [18].

Results

Isolation of influenza viruses from domestic ducks and wild birds

In the present study, surveillance of avian influenza was carried out in Vinh Loi district, Bac Lieu town, and Hoa Binh district in Vietnam in April 2009 and March 2010. Twelve strains (1 H3N2, 1 H3N8, 6 H4N6, 2 H9N2, 1 H11N3, and 1 H11N9) were isolated from 34 RT-LAMP-positive tracheal and cloacal swab samples from 240 domestic ducks in Vinh Loi district. Nine strains (7 H9N2 and 2 H11N3) were isolated from 38 RT-LAMP-positive swab samples from 160 domestic ducks in Bac Lieu town. Nineteen strains (1 H4N6, 17 H9N2, and 1 H9N6) were isolated from 28 RT-LAMP-positive swab samples of 200 domestic ducks in Hoa Binh district (Table 1). All of the viruses were isolated from domestic ducks in households, live-bird markets, and slaughterhouses in Vinh Loi district, Bac Lieu town, and Hoa Binh district in Vietnam (Fig. 1). No virus was isolated from 207 wild-bird samples in April 2009 and March 2010.

Genetic characterization of viruses isolated from domestic ducks in southern Vietnam

The sequence data of the HA genes of 27 H9 isolates, including reference strains of three different sublineages, were phylogenetically analyzed by the neighbor-joining method (Fig. 2). All of the H9 HA genes were classified as belonging to the Eurasian lineage, and the HA genes of 19 and 8 isolates were grouped into the G1 and Korean sublineage, respectively.

The partial nucleotide sequence of each gene segment of the isolates was analyzed phylogenetically (Fig. 2). Gene constellations of the H9N2 virus isolates were divided into three patterns. H9N2 viruses belonging to the G1 sublineage were isolated from domestic ducks in households A and E. On the other hand, H9N2 viruses belonging to the Korean sublineage were isolated in live-bird market G. Furthermore, one of these H9N2 viruses of the G1 sublineage of the HA gene was isolated in live-bird market G, and this virus also possessed a PB2 gene of the Korean sublineage. Representative isolates of these three patterns are Dk/VN/OIE-2583/2009, Dk/VN/OIE-2327/2009, and Dk/VN/OIE-2328/2009, respectively.

Table 1 Viruses isolated from domestic ducks in southern Vietnam in 2009 and 2010

Place of sampling	Subtypes of isolates	ID number of samples
Household		
A	H4N6	OIE-2454, OIE-2455
	H9N2	OIE-2448
B	H4N6	OIE-2470, OIE-2471
C	H4N6	OIE-2480, OIE-2481
D	H4N6	OIE-2577
	H9N2	OIE-2574, OIE-2575, OIE-2576
E	H9N2	OIE-2580, OIE-2581, OIE-2582, OIE-2583, OIE-2584, OIE-2585, OIE-2586, OIE-2587, OIE-2590, OIE-2591, OIE-2592, OIE-2593, OIE-2594, OIE-2595
Live-bird market		
F	H3N8	OIE-2403
G	H9N2	OIE-2322, OIE-2323, OIE-2325, OIE-2326, OIE-2327, OIE-2328
	H11N3	OIE-2329, OIE-2336
H	H3N2	OIE-2382
	H9N2	OIE-2390
	H11N3	OIE-2391
	H11N9	OIE-2386
I	H9N6	OIE-2334[a]
Slaughter house		
J	H9N2	OIE-2313

[a] This virus was isolated in 2010

Antigenic analysis of the HAs of H9 influenza viruses

H9 influenza viruses isolated from domestic ducks in Vietnam were analyzed by the HI test (Table 2). All of the H9 isolates tested reacted with antisera against the H9 viruses of the Korean and G1 sublineages. However, the isolates of the Korean sublineage showed low cross-reactivity to antisera against the H9 viruses of the G1 sublineage, and all isolates in this study showed moderate and low cross-reactivity to antisera against the H9 viruses of the Y280 sublineage and North American lineage, respectively. This suggests that the antigenicity of the H9 isolates of the Korean sublineage is different from that of viruses of the G1 sublineage. It was also found that reactivity patterns of H9 isolates belonging to the G1 and Korean sublineage in the present study were the same as those of the reference strains.

Susceptibility of ducks, chickens, pigs, and mice to infection with H9N2 isolates

H9N2 isolates were inoculated intranasally into ducks, chickens, pigs, and mice. Clinical signs were not observed during 14 days in any of the ducks. Viruses were recovered from laryngopharyngeal swabs from duck #1 inoculated with DK/VN/OIE-2327/2009 at 3 and 4 d.p.i. Viruses were also recovered from a laryngopharyngeal swab from duck #5 inoculated with Dk/VN/OIE-2328/2009 at 3 d.p.i. In the experimental infection with Dk/VN/OIE-2583/2009, viruses were recovered from a cloacal swab from duck #7 at 5 d.p.i. and laryngopharyngeal swabs from duck #8 at 1 and 3 d.p.i. Antibodies to H9 HA were detected from the sera of all ducks at 14 d.p.i (Table 3).

Fig. 2 Phylogenetic trees for the eight gene segments of H9 influenza viruses. Nucleotides 70-417 (347 bp) of HA, 67-468 (402 bp) of NA, 1,318-1,902 (585 bp) of PB2, 1,135-1,610 (476 bp) of PB1, 756-1,167 (412 bp) of PA, 1,139-1,434 (296 bp) of NP, 55-893 (839 bp) of M, and 25-790 (766 bp) of NS were used for phylogenetic analysis. Horizontal distances are proportional to the minimum number of nucleotide differences required to join nodes and sequences. Numbers at the nodes indicate confidence levels in bootstrap analysis with 1,000 replications. Viruses isolated in this study are highlighted in gray. Representative viruses in each sublineage are underlined. Abbreviations: Ck, chicken; Dk, duck; Qa, quail; Ty, turkey; HK, Hong Kong; Hok, Hokkaido; Pak, Pakistan; and Wis, Wisconsin. **a** The nucleotide sequences of the HA genes of 11 isolates were the same as that of Dk/VN/OIE-2587/2009. The ID numbers of these 10 isolates are OIE-2448, OIE-2574, OIE-2575, OIE-2580, OIE-2581, OIE-2585, OIE-2586, OIE-2590, OIE-2591, and OIE-2594. **b** The nucleotide sequences of the HA genes of two isolates were the same as that of Dk/VN/OIE-2390/2009. The ID numbers of these two isolates are OIE-2322 and OIE-2323

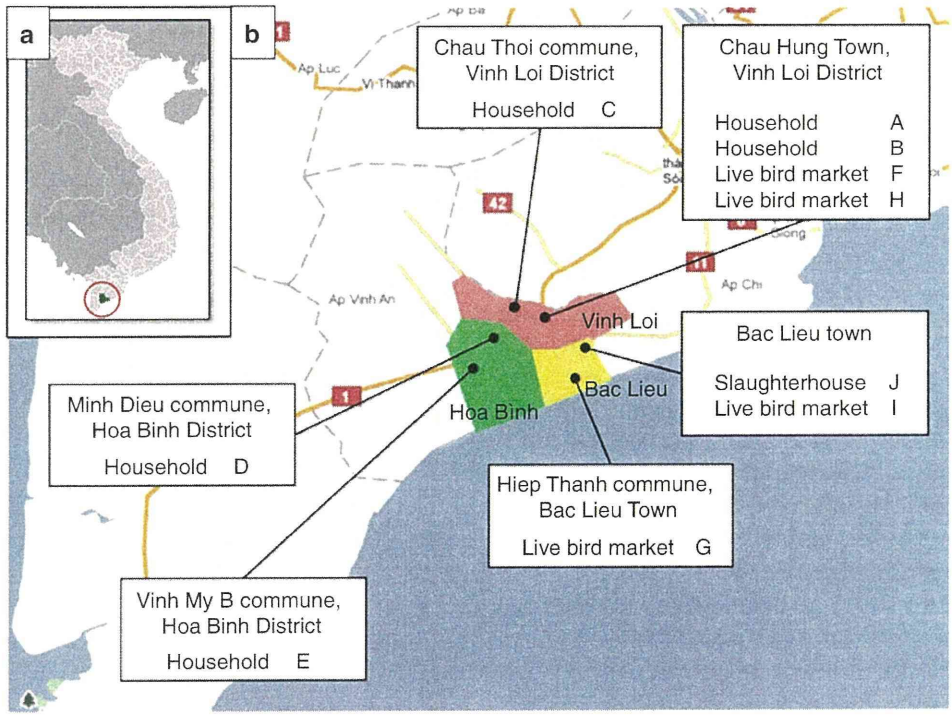
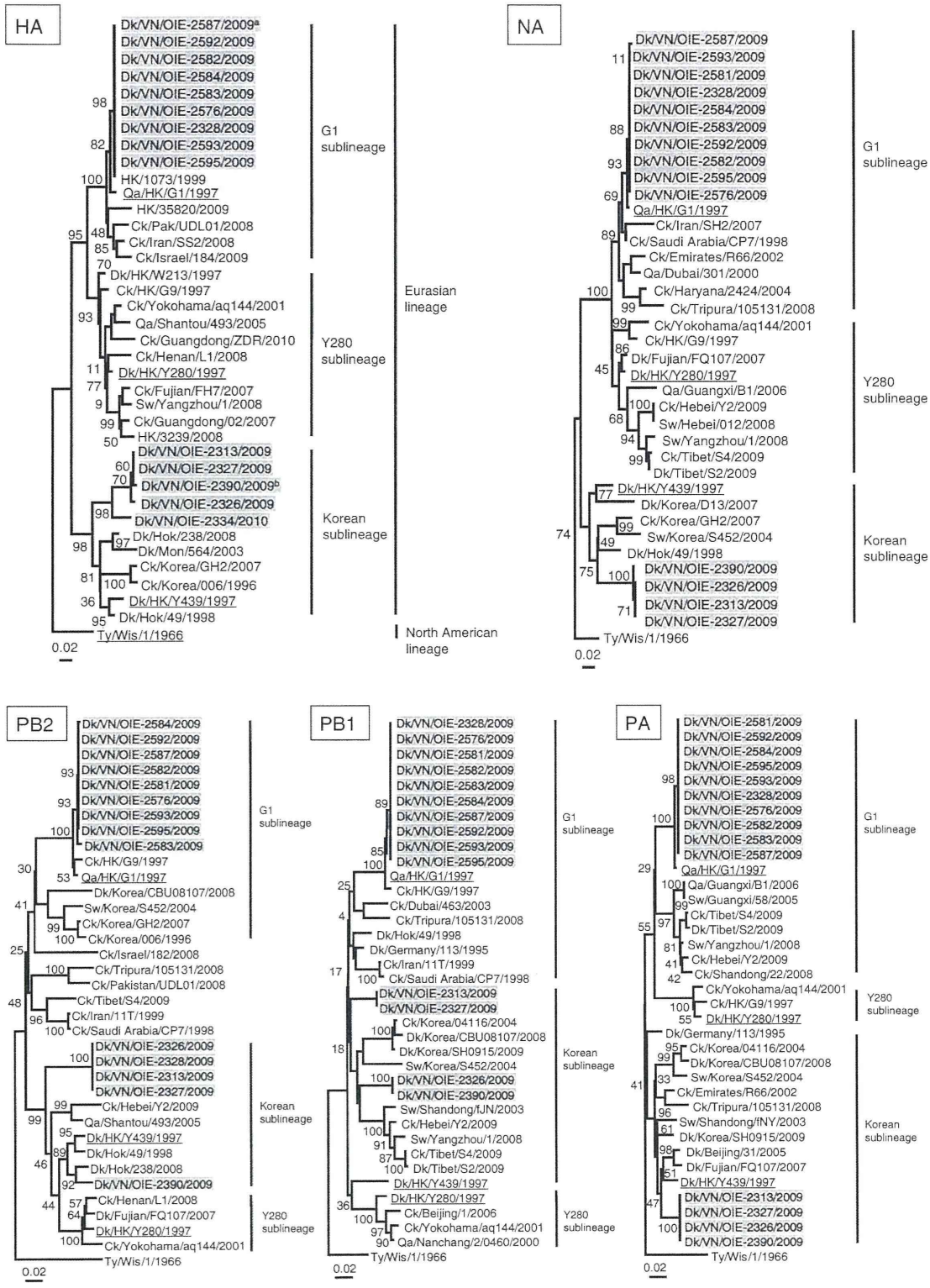


Fig. 1 Sampling points in southern Vietnam in the present study. Location of Bac Lieu province in Vietnam (**a**). Magnification of the circle in Fig. 1a and sampling points in Vinh Loi district, Bac Lieu town, and Hoa Binh district in Bac Lieu province (**b**). Avian influenza viruses were isolated from domestic ducks in households A-E, live-bird markets F-I, and slaughterhouse J in Vinh Loi district, Bac Lieu town, and Hoa Binh district in Vietnam


HA
Dk/VN/OIE-2587/2009[a]
Dk/VN/OIE-2592/2009
Dk/VN/OIE-2582/2009
Dk/VN/OIE-2584/2009
Dk/VN/OIE-2583/2009
Dk/VN/OIE-2576/2009
Dk/VN/OIE-2328/2009
Dk/VN/OIE-2593/2009
Dk/VN/OIE-2595/2009
HK/1073/1999
Qa/HK/G1/1997
HK/35820/2009
Ck/Pak/UDL01/2008
Ck/Iran/SS2/2008
Ck/Israel/184/2009
Dk/HK/W213/1997
Ck/HK/G9/1997
Ck/Yokohama/aq144/2001
Qa/Shantou/493/2005
Ck/Guangdong/ZDR/2010
Ck/Henan/L1/2008
Dk/HK/Y280/1997
Ck/Fujian/FH7/2007
Sw/Yangzhou/1/2008
Ck/Guangdong/02/2007
HK/3239/2008
Dk/VN/OIE-2313/2009
Dk/VN/OIE-2327/2009
Dk/VN/OIE-2390/2009[b]
Dk/VN/OIE-2326/2009
Dk/VN/OIE-2334/2010
Dk/Hok/238/2008
Dk/Mon/564/2003
Ck/Korea/GH2/2007
Ck/Korea/006/1996
Dk/HK/Y439/1997
Dk/Hok/49/1998
Ty/Wis/1/1966
0.02
G1 sublineage
Y280 sublineage
Korean sublineage
Eurasian lineage
North American lineage
NA
Dk/VN/OIE-2587/2009
Dk/VN/OIE-2593/2009
Dk/VN/OIE-2581/2009
Dk/VN/OIE-2328/2009
Dk/VN/OIE-2584/2009
Dk/VN/OIE-2583/2009
Dk/VN/OIE-2592/2009
Dk/VN/OIE-2582/2009
Dk/VN/OIE-2595/2009
Dk/VN/OIE-2576/2009
Qa/HK/G1/1997
Ck/Iran/SH2/2007
Ck/Saudi Arabia/CP7/1998
Ck/Emirates/R66/2002
Qa/Dubai/301/2000
Ck/Haryana/2424/2004
Ck/Tripura/105131/2008
Ck/Yokohama/aq144/2001
Ck/HK/G9/1997
Dk/Fujian/FQ107/2007
Dk/HK/Y280/1997
Qa/Guangxi/B1/2006
Ck/Hebei/Y2/2009
Sw/Hebei/012/2008
Sw/Yangzhou/1/2008
Ck/Tibet/S4/2009
Dk/Tibet/S2/2009
Dk/HK/Y439/1997
Dk/Korea/D13/2007
Ck/Korea/GH2/2007
Sw/Korea/S452/2004
Dk/Hok/49/1998
Dk/VN/OIE-2390/2009
Dk/VN/OIE-2326/2009
Dk/VN/OIE-2313/2009
Dk/VN/OIE-2327/2009
Ty/Wis/1/1966
0.02
G1 sublineage
Y280 sublineage
Korean sublineage
PB2
Dk/VN/OIE-2584/2009
Dk/VN/OIE-2592/2009
Dk/VN/OIE-2587/2009
Dk/VN/OIE-2582/2009
Dk/VN/OIE-2581/2009
Dk/VN/OIE-2576/2009
Dk/VN/OIE-2593/2009
Dk/VN/OIE-2595/2009
Dk/VN/OIE-2583/2009
Ck/HK/G9/1997
Qa/HK/G1/1997
Dk/Korea/CBU08107/2008
Sw/Korea/S452/2004
Ck/Korea/GH2/2007
Ck/Korea/006/1996
Ck/Israel/182/2008
Ck/Tripura/105131/2008
Ck/Pakistan/UDL01/2008
Ck/Tibet/S4/2009
Ck/Iran/11T/1999
Ck/Saudi Arabia/CP7/1998
Dk/VN/OIE-2326/2009
Dk/VN/OIE-2328/2009
Dk/VN/OIE-2313/2009
Dk/VN/OIE-2327/2009
Ck/Hebei/Y2/2009
Qa/Shantou/493/2005
Dk/HK/Y439/1997
Dk/Hok/49/1998
Dk/Hok/238/2008
Dk/VN/OIE-2390/2009
Ck/Henan/L1/2008
Dk/Fujian/FQ107/2007
Dk/HK/Y280/1997
Ck/Yokohama/aq144/2001
Ty/Wis/1/1966
0.02
G1 sublineage
Korean sublineage
Y280 sublineage
PB1
Dk/VN/OIE-2328/2009
Dk/VN/OIE-2576/2009
Dk/VN/OIE-2581/2009
Dk/VN/OIE-2582/2009
Dk/VN/OIE-2583/2009
Dk/VN/OIE-2584/2009
Dk/VN/OIE-2587/2009
Dk/VN/OIE-2592/2009
Dk/VN/OIE-2593/2009
Dk/VN/OIE-2595/2009
Qa/HK/G1/1997
Ck/HK/G9/1997
Ck/Dubai/463/2003
Ck/Tripura/105131/2008
Dk/Hok/49/1998
Dk/Germany/113/1995
Ck/Iran/11T/1999
Ck/Saudi Arabia/CP7/1998
Dk/VN/OIE-2313/2009
Dk/VN/OIE-2327/2009
Ck/Korea/04116/2004
Dk/Korea/CBU08107/2008
Dk/Korea/SH0915/2009
Sw/Korea/S452/2004
Dk/VN/OIE-2326/2009
Dk/VN/OIE-2390/2009
Sw/Shandong/fJN/2003
Ck/Hebei/Y2/2009
Sw/Yangzhou/1/2008
Ck/Tibet/S4/2009
Dk/Tibet/S2/2009
Dk/HK/Y439/1997
Dk/HK/Y280/1997
Ck/Beijing/1/2006
Ck/Yokohama/aq144/2001
Qa/Nanchang/2/0460/2000
Ty/Wis/1/1966
0.02
G1 sublineage
Korean sublineage
Y280 sublineage
PA
Dk/VN/OIE-2581/2009
Dk/VN/OIE-2592/2009
Dk/VN/OIE-2584/2009
Dk/VN/OIE-2595/2009
Dk/VN/OIE-2593/2009
Dk/VN/OIE-2328/2009
Dk/VN/OIE-2576/2009
Dk/VN/OIE-2582/2009
Dk/VN/OIE-2583/2009
Dk/VN/OIE-2587/2009
Qa/HK/G1/1997
Qa/Guangxi/B1/2006
Sw/Guangxi/58/2005
Ck/Tibet/S4/2009
Dk/Tibet/S2/2009
Sw/Yangzhou/1/2008
Ck/Hebei/Y2/2009
Ck/Shandong/22/2008
Ck/Yokohama/aq144/2001
Ck/HK/G9/1997
Dk/HK/Y280/1997
Dk/Germany/113/1995
Ck/Korea/04116/2004
Dk/Korea/CBU08107/2008
Sw/Korea/S452/2004
Ck/Emirates/R66/2002
Ck/Tripura/105131/2008
Sw/Shandong/fNY/2003
Dk/Korea/SH0915/2009
Dk/Beijing/31/2005
Dk/Fujian/FQ107/2007
Dk/HK/Y439/1997
Dk/VN/OIE-2313/2009
Dk/VN/OIE-2327/2009
Dk/VN/OIE-2326/2009
Dk/VN/OIE-2390/2009
Ty/Wis/1/1966
0.02
G1 sublineage
Y280 sublineage
Korean sublineage

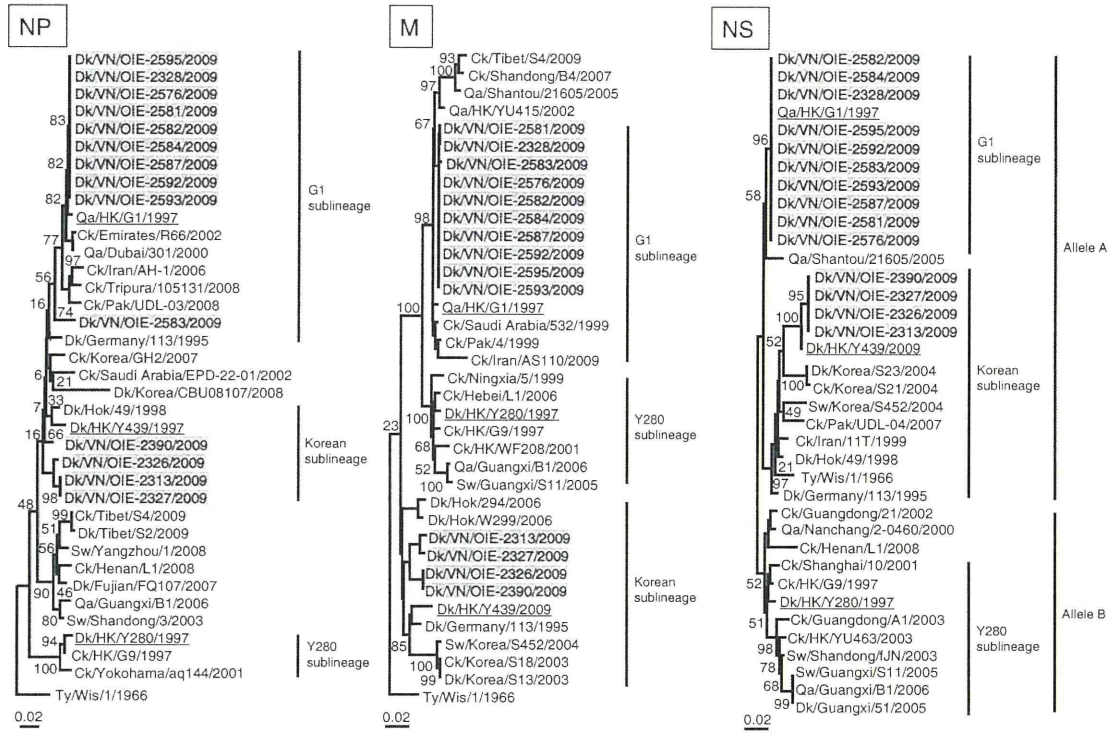


Fig. 2 continued

Clinical signs were not observed during the experiments in any of the chickens. Virus was not recovered from the brains, tracheas, lungs, or colons of chickens inoculated with the three H9N2 viruses at 3 d.p.i., while anti-H9 HA antibodies were detected in the sera of the chickens on 14 d.p.i. (data not shown), indicating that virus replication had occurred at a low level.

Clinical signs were not observed during the experiment in any of the pigs. Viruses were recovered from the nasal swabs of pigs inoculated with each of the three H9N2 isolates, and anti-H9 HA antibodies were detected in the sera of pigs at 14 d.p.i (Table 4). Antibodies were not detected in the sera of pig #1 inoculated with Dk/VN/OIE-2327/2009, indicating that the pig was not infected with influenza viruses.

Body weight fell in mice inoculated with Dk/VN/OIE-2583/2009, with 15-20% loss from 4 to 8 d.p.i (Fig. 3). Viruses were recovered from the lungs of mice inoculated with Dk/VN/OIE-2328/2009 and Dk/VN/OIE-2583/2009 at 3 d.p.i. (Table 5), and anti-H9 HA antibodies were detected in the sera of all mice at 14 d.p.i (Table 6).

Discussion

Recently, H9N2 viruses of the G1, Y280, and Korean sublineages have been isolated from wild birds and poultry worldwide [2, 3, 8, 29, 40]. H9N2 viruses have been isolated from pigs and humans in China [4, 39] and Korea, suggesting that the H9N2 virus is a candidate to cause pandemic influenza in humans. Live-bird markets provide an ideal environment for genetic reassortment and interspecies transmission of influenza viruses [24, 26, 28, 38]. In Asia, H9N2 influenza viruses had been isolated only from feral ducks until 1988 [37], but since then, H9N2 viruses have been isolated from domestic ducks and chickens [5]. H9N3 viruses belonging to the Korean sublineage have been isolated from domestic ducks in Vietnam [30]. In the present study, it was found that H9 viruses belonging to the Korean and G1 sublineages are circulating in domestic ducks in Vietnam, and one of these H9N2 viruses, belonging to the G1 sublineage but possessing the PB2 gene of the Korean sublineage, was isolated from domestic ducks. Thus, genetic reassortment has occurred between viruses of the G1 and Korean sublineages in the poultry population in Vietnam.

In this study, H9N2 viruses did not replicate well in chickens and ducks. It has been reported that H9N2 viruses isolated from ducks replicate slightly in chickens [34], suggesting that the similar results in this study were due to the low susceptibility of chickens to H9N2 viruses. It has also been reported that H9N2 viruses isolated from ducks replicate in only some of the organs in ducks, and viruses of low titer are recovered from tracheal and cloacal swabs [11, 32]. The present results were similar to those of the previous reports. In this animal experiment, we collected

Table 2 Cross-reactivity between antisera and H9 viruses by HI test[a]

Lineage	Sublineage	Virus	Antiserum to					
			Korean		G1	Y280		North American
			Dk/Hok/49/1998	Dk/Hok/13/2000	Qa/HK/G1/1997	Dk/HK/Y280/1997	Ck/HK/G9/1997	Ty/Wis/1/1966
Eurasian	Korean	Dk/Hok/49/1998	2,560	2,560	80	320	320	640
		Dk/Hok/13/2000	5,120	2,560	40	320	320	320
		Dk/VN/OIE-2313/2009	640	2,560	640	640	320	320
		Dk/VN/OIE-2322/2009	1,280	1,280	320	320	320	160
		Dk/VN/OIE-2323/2009	640	1,280	640	640	320	320
		Dk/VN/OIE-2325/2009	640	1,280	640	640	160	320
		Dk/VN/OIE-2326/2009	1,280	2,560	640	320	320	160
		Dk/VN/OIE-2327/2009	640	1,280	320	640	320	320
		Dk/VN/OIE-2390/2009	1,280	1,280	320	640	320	320
		Dk/VN/OIE-2334/2010	5,120	5,120	320	640	320	640
	G1	Qa/HK/G1/1997	1,280	1,280	5,120	1,280	640	320
		Dk/VN/OIE-2328/2009	1,280	1,280	10,240	5,120	1,280	320
		Dk/VN/OIE-2448/2009	1,280	1,280	5,120	1,280	1,280	640
		Dk/VN/OIE-2574/2009	1,280	2,560	2,560	5,120	1,280	160
		Dk/VN/OIE-2575/2009	1,280	2,560	5,120	2,560	1,280	320
		Dk/VN/OIE-2576/2009	640	1,280	5,120	2,560	1,280	160
		Dk/VN/OIE-2580/2009	1,280	2,560	2,560	2,560	640	320
		Dk/VN/OIE-2581/2009	1,280	1,280	5,120	2,560	640	320
		Dk/VN/OIE-2582/2009	640	1,280	2,560	1,280	1,280	160
		Dk/VN/OIE-2583/2009	1,280	2,560	5,120	2,560	1,280	320
		Dk/VN/OIE-2584/2009	1,280	640	1,280	1,280	1,280	160
		Dk/VN/OIE-2585/2009	2,560	1,280	2,560	1,280	640	160
		Dk/VN/OIE-2586/2009	1,280	1,280	2,560	2,560	1,280	160
		Dk/VN/OIE-2587/2009	1,280	2,560	5,120	5,120	640	160
		Dk/VN/OIE-2590/2009	2,560	2,560	1,280	1,280	320	160
		Dk/VN/OIE-2591/2009	1,280	2,560	1,280	1,280	640	320
		Dk/VN/OIE-2592/2009	640	1,280	5,120	1,280	640	320
		Dk/VN/OIE-2593/2009	640	640	2,560	1,280	320	160
		Dk/VN/OIE-2594/2009	1,280	1,280	2,560	5,120	1,280	160
		Dk/VN/OIE-2595/2009	1,280	1,280	2,560	2,560	1,280	320
	Y280	Dk/HK/Y280/1997	2,560	5,120	5,120	20,480	20,480	40
		Ck/HK/G9/1997	1,280	2,560	2,560	10,240	40,960	320
		Dk/HK/W213/1998	1,280	2,560	2,560	20,480	40,960	80
North American		Ty/Wis/1/1966	320	320	<20	20	80	640

[a] Homologous reactions are underlined

laryngopharyngeal swabs because it was hard to collect tracheal swabs daily from the ducks inoculated with H9N2 viruses in the safety cabinet. Furthermore, the strain of ducks used in experimental infection (Chelly Valley) may not be identical to that of domestic ducks in Vietnam. These factors might affect the titer of recovered virus.

In mice, H9N2 viruses replicate in the lungs, and body weight losses are observed [16, 28]. In this experiment, viruses replicated efficiently in the lungs of mice inoculated with Dk/VN/OIE-2328/2009 and Dk/VN/OIE-2583/2009. Body weight losses were observed in the mice inoculated with Dk/VN/OIE-2583/2009 and not in those with Dk/VN/OIE-2328/2009, indicating that Dk/VN/OIE-2583/2009 replicated more efficiently than Dk/VN/OIE-2328/2009 at the early stage of infection in mice. The genetic analysis suggested that the PB2 genes may be responsible for the higher replication rate in mice, since the sublineages of the PB2 gene are different in these two

Table 3 Virus titers of the laryngopharyngeal and cloacal swabs and antibody responses of ducks inoculated with H9N2 viruses[a]

Virus	Animal no.	Swab	Virus titer on the following d.p.i.[b] (log EID_{50}/ml)								Serum antibody titer[c]
			0	1	2	3	4	5	6	7	
Dk/VN/ OIE-2327/2009	#1	Laryngopharyngeal	–	–	–	0.7	1.5	–	–	–	80
		Cloacal	–	–	–	–	–	–	–	–	
	#2	Laryngopharyngeal	–	–	–	–	–	–	–	–	160
		Cloacal	–	–	–	–	–	–	–	–	
	#3	Laryngopharyngeal	–	–	–	–	–	–	–	–	160
		Cloacal	–	–	–	–	–	–	–	–	
Dk/VN/ OIE-2328/2009	#4	Laryngopharyngeal	–	–	–	–	–	–	–	–	80
		Cloacal	–	–	–	–	–	–	–	–	
	#5	Laryngopharyngeal	–	–	–	0.8	–	–	–	–	160
		Cloacal	–	–	–	–	–	–	–	–	
	#6	Laryngopharyngeal	–	–	–	–	–	–	–	–	80
		Cloacal	–	–	–	–	–	–	–	–	
Dk/VN/ OIE-2583/2009	#7	Laryngopharyngeal	–	–	–	–	–	–	–	–	80
		Cloacal	–	–	–	–	–	0.7	–	–	
	#8	Laryngopharyngeal	–	1.7	–	1.3	–	–	–	–	40
		Cloacal	–	–	–	–	–	–	–	–	
	#9	Laryngopharyngeal	–	–	–	–	–	–	–	–	80
		Cloacal	–	–	–	–	–	–	–	–	

[a] Laryngopharyngeal and cloacal swabs of three inoculated ducks were collected daily from 1 to 7 d.p.i

[b] Bar (–) indicate that virus was not detected

[c] HI antibody titers to the inoculated viruses at 14 d.p.i

Table 4 Virus isolation from nasal swabs of pigs inoculated with H9N2 viruses[a]

Virus	Animal no.	Virus titer on the following d.p.i. (log EID_{50}/ml)								Serum antibody titer
		0	1	2	3	4	5	6	7	
Dk/VN/OIE-2327/2009	#1	-	-	-	-	-	-	-	-	-
	#2	-	-	2.7	1.5	2.5	2.7	1.5	-	640
Dk/VN/OIE-2328/2009	#3	-	-	3.8	-	5.3	4.3	2.7	1.7	320
	#4	-	3.5	3.5	1.8	4.3	4.5	3.5	3.0	640
Dk/VN/OIE-2583/2009	#5	-	5.0	3.5	2.8	4.3	3.8	2.8	1.5	320
	#6	-	4.3	3.8	3.7	4.8	3.8	1.8	-	320

[a] Nasal swabs of two inoculated pigs were collected daily from 1 to 7 d.p.i., -:< 0.5. Antibody responses to the inoculated viruses in pigs at 14 d.p.i. were examined by ELISA

viruses. Further study is needed to clarify the pathogenicity of H9N2 viruses in mice.

Experimental infection studies revealed that pigs are highly susceptible to infection with avian influenza viruses of each of the known HA subtypes, and genetic reassortment can take place in pigs [19]. Thus, pigs have been suggested to serve as intermediate hosts to generate genetic reassortants [19]. Three H9N2 viruses were recovered from swabs from pigs in this experiment, and the results were similar to those of previous reports [6, 19]. Especially, viruses were recovered efficiently from nasal swabs from pigs inoculated with Dk/VN/OIE-2328/2009 and Dk/VN/OIE-2583/2009, which belong to the G1 sublineage and replicate efficiently in mice. In addition, H9N2 viruses isolated from humans in Hong Kong were genetically classified as belonging to the G1 sublineage [4, 7, 23, 33, 43], suggesting that H9N2 viruses belonging to the G1 sublineage have the potential to replicate efficiently in mammals. The findings indicate that H9N2 virus is one of the candidates for pandemic influenza in humans. Surveillance of influenza in wild birds, domestic birds, and pigs is important in order to prepare for pandemic influenza in humans.

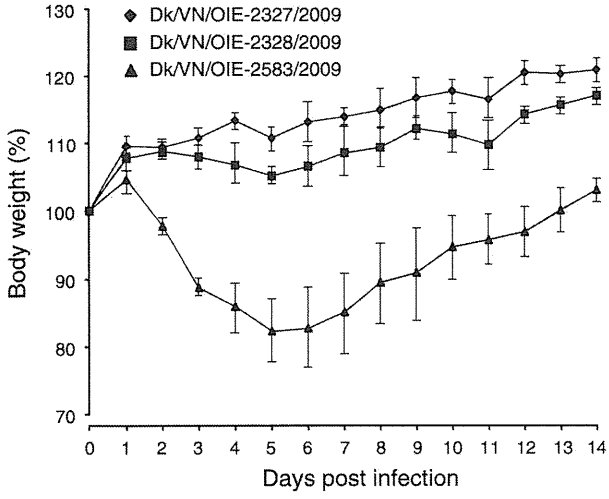


Fig. 3 Changes in body weight of mice inoculated with influenza viruses. Five mice were inoculated intranasally with Dk/VN/OIE-2327/2009, Dk/VN/OIE-2328/2009, and Dk/VN/OIE-2583/2009. The body weight of the mice was monitored for 14 days after inoculation with each influenza virus. Data are shown as averages of body weight changes in each group with the corresponding standard deviation

Table 5 Virus titers of the lungs of mice inoculated with H9N2 viruses

Virus	Animal no.	Virus titer[a] (log EID_{50}/g)
Dk/VN/OIE-2327/2009	#1	–
	#2	–
	#3	–
	#4	–
	#5	–
Dk/VN/OIE-2328/2009	#6	5.3
	#7	5.3
	#8	5.3
	#9	5.0
	#10	5.7
Dk/VN/OIE-2583/2009	#11	5.5
	#12	5.7
	#13	5.8
	#14	5.5
	#15	5.3

[a] Five mice were sacrificed at 3 d.p.i., and the lungs were collected for virus titration. –: virus was not detected

Acknowledgments We thank Dr. Kennedy F. Shortridge for providing H9N2 influenza viruses. We are also grateful for the support of the Programme on Surveillance of Wild Birds and Domestic Animals along Migratory Flyways under the OIE/JTF Project for Strengthening HPAI Control in Asia. This work was supported by J-GRID; the Japan Initiative for Global Research Network on Infectious Diseases of Ministry of Education, Culture, Sports, Science and Technology of Japan. This work was also supported by Japan Science and Technology Agency Basic Research Programs. We are grateful for the support of the Global Center of Excellence (GCOE) Program of Hokkaido University.

Table 6 Serum antibody responses of mice inoculated with H9N2 viruses

Virus	Animal no.	Serum antibody titer[a]
Dk/VN/OIE-2327/2009	#16	40
	#17	40
	#18	80
	#19	40
	#20	40
Dk/VN/OIE-2328/2009	#21	20
	#22	80
	#23	20
	#24	40
	#25	40
Dk/VN/OIE-2583/2009	#26	40
	#27	40
	#28	40
	#29	80
	#30	80

[a] HI antibody titer to the inoculated viruses at 14 d.p.i

References

1. Abbas MA, Spackman E, Swayne DE, Ahmed Z, Sarmento L, Siddique N, Naeem K, Hameed A, Rehmani S (2010) Sequence and phylogenetic analysis of H7N3 avian influenza viruses isolated from poultry in Pakistan 1995–2004. Virol J 7:137
2. Alexander DJ (2000) A review of avian influenza in different bird species. Vet Microbiol 74:3–13
3. Banet-Noach C, Perk S, Simanov L, Grebenyuk N, Rozenblut E, Pokamunski S, Pirak M, Tendler Y, Panshin A (2007) H9N2 influenza viruses from Israeli poultry: a five-year outbreak. Avian Dis 51:290–296
4. Butt KM, Smith GJ, Chen H, Zhang LJ, Leung YH, Xu KM, Lim W, Webster RG, Yuen KY, Peiris JS, Guan Y (2005) Human infection with an avian H9N2 influenza A virus in Hong Kong in 2003. J Clin Microbiol 43:5760–5767
5. Cameron KR, Gregory V, Banks J, Brown IH, Alexander DJ, Hay AJ, Lin YP (2000) H9N2 subtype influenza A viruses in poultry in Pakistan are closely related to the H9N2 viruses responsible for human infection in Hong Kong. Virology 278:36–41
6. Choi YK, Ozaki H, Webby RJ, Webster RG, Peiris JS, Poon L, Butt C, Leung YHC, Guan Y (2004) Continuing evolution of H9N2 influenza viruses in Southeastern China. J Virol 78:8609–8614
7. Fusaro A, Monne I, Salviato A, Valastro V, Schivo A, Amarin NM, Gonzalez C, Ismail MM, Al-Ankari AR, Al-Blowi MH, Khan OA, Maken Ali AS, Hedayati A, Garcia Garcia J, Ziay GM, Shoushtari A, Al Qahtani KN, Capua I, Holmes EC, Cattoli G (2011) Phylogeography and evolutionary history of reassortant H9N2 viruses with potential human health implications. J Virol 85:8413–8421
8. Ge FF, Zhou JP, Liu J, Wang J, Zhang WY, Sheng LP, Xu F, Ju HB, Sun QY, Liu PH (2009) Genetic evolution of H9 subtype influenza viruses from live poultry markets in Shanghai, China. J Clin Microbiol 47:3294–3300

9. Guan Y, Shortridge KF, Krauss S, Webster RG (1999) Molecular characterization of H9N2 influenza viruses: were they the donors of the "internal" genes of H5N1 viruses in Hong Kong? Proc Natl Acad Sci USA 96:9363–9367
10. Guan Y, Shortridge KF, Krauss S, Chin PS, Dyrting KC, Ellis TM, Webster RG, Peiris M (2000) H9N2 influenza viruses possessing H5N1-like internal genomes continue to circulate in poultry in southeastern China. J Virol 74:9372–9380
11. Guo YJ, Krauss S, Senne DA, Mo IP, Lo KS, Xiong XP, Norwood M, Shortridge KF, Webster RG, Guan Y (2000) Characterization of the pathogenicity of members of the newly established H9N2 influenza virus lineages in Asia. Virology 267:279–288
12. Haghighat-Jahromi M, Asasi K, Nili H, Dadras H, Shooshtari AH (2008) Coinfection of avian influenza virus (H9N2 subtype) with infectious bronchitis live vaccine. Arch Virol 153:651–655
13. Hoffmann E, Stech J, Guan Y, Webster RG, Perez DR (2001) Universal primer set for the full-length amplification of all influenza A viruses. Arch Virol 146:2275–2289
14. Ito T, Okazaki K, Kawaoka Y, Takada A, Webster RG, Kida H (1995) Perpetuation of influenza A viruses in Alaskan waterfowl reservoirs. Arch Virol 140:1163–1172
15. Jeong OM, Kim YJ, Choi JG, Kang HM, Kim MC, Kwon JH, Lee YJ (2010) Genetic characterization of H1 avian influenza viruses isolated from migratory birds and domestic ducks in Korea. Virus Genes 42:55–63
16. Bi J, Deng G, Dong J, Kong F, Li X, Xu Q, Zhang M, Zhao L, Qiao J (2010) Phylogenetic and molecular characterization of H9N2 influenza isolates from chickens in Northern China from 2007–2009. PLoS One 5. pii: e13063
17. Kida H, Yanagawa R (1979) Isolation and characterization of influenza a viruses from wild free-flying ducks in Hokkaido, Japan. Zentralbl Bakteriol Orig A 244:135–143
18. Kida H, Brown LE, Webster RG (1982) Biological activity of monoclonal antibodies to operationally defined antigenic regions on the hemagglutinin molecule of A/Seal/Massachusetts/1/80 (H7N7) influenza virus. Virology 122:38–47
19. Kida H, Ito T, Yasuda J, Shimizu Y, Itakura C, Shortridge KF, Kawaoka Y, Webster RG (1994) Potential for transmission of avian influenza viruses to pigs. J Gen Virol 75(Pt 9):2183–2188
20. Kim HR, Lee YJ, Lee KK, Oem JK, Kim SH, Lee MH, Lee OS, Park CK (2010) Genetic relatedness of H6 subtype avian influenza viruses isolated from wild birds and domestic ducks in Korea and their pathogenicity in animals. J Gen Virol 91:208–219
21. Kishida N, Sakoda Y, Eto M, Sunaga Y, Kida H (2004) Co-infection of Staphylococcus aureus or Haemophilus paragallinarum exacerbates H9N2 influenza A virus infection in chickens. Arch Virol 149:2095–2104
22. Kishida N, Sakoda Y, Shiromoto M, Bai GR, Isoda N, Takada A, Laver G, Kida H (2008) H2N5 influenza virus isolates from terns in Australia: genetic reassortants between those of the Eurasian and American lineages. Virus Genes 37:16–21
23. Lin YP, Shaw M, Gregory V, Cameron K, Lim W, Klimov A, Subbarao K, Guan Y, Krauss S, Shortridge K, Webster R, Cox N, Hay A (2000) Avian-to-human transmission of H9N2 subtype influenza A viruses: relationship between H9N2 and H5N1 human isolates. Proc Natl Acad Sci USA 97:9654–9658
24. Liu H, Liu X, Cheng J, Peng D, Jia L, Huang Y (2003) Phylogenetic analysis of the hemagglutinin genes of twenty-six avian influenza viruses of subtype H9N2 isolated from chickens in China during 1996–2001. Avian Dis 47:116–127
25. Liu J, Okazaki K, Ozaki H, Sakoda Y, Wu Q, Chen F, Kida H (2003) H9N2 influenza viruses prevalent in poultry in China are phylogenetically distinct from A/quail/Hong Kong/G1/97 presumed to be the donor of the internal protein genes of the H5N1 Hong Kong/97 virus. Avian Pathol 32:551–560
26. Liu M, He S, Walker D, Zhou N, Perez DR, Mo B, Li F, Huang X, Webster RG, Webby RJ (2003) The influenza virus gene pool in a poultry market in south central China. Virology 305:267–275
27. Manzoor R, Sakoda Y, Mweene A, Tsuda Y, Kishida N, Bai GR, Kameyama K, Isoda N, Soda K, Naito M, Kida H (2008) Phylogenic analysis of the M genes of influenza viruses isolated from free-flying water birds from their Northern Territory to Hokkaido, Japan. Virus Genes 37:144–152
28. Moon HJ, Song MS, Cruz DJ, Park KJ, Pascua PN, Lee JH, Baek YH, Choi DH, Choi YK, Kim CJ (2010) Active reassortment of H9 influenza viruses between wild birds and live-poultry markets in Korea. Arch Virol 155:229–241
29. Nagarajan S, Rajukumar K, Tosh C, Ramaswamy V, Purohit K, Saxena G, Behera P, Pattnaik B, Pradhan HK, Dubey SC (2009) Isolation and pathotyping of H9N2 avian influenza viruses in Indian poultry. Vet Microbiol 133:154–163
30. Nguyen DC, Uyeki TM, Jadhao S, Maines T, Shaw M, Matsuoka Y, Smith C, Rowe T, Lu X, Hall H, Xu X, Balish A, Klimov A, Tumpey TM, Swayne DE, Huynh LP, Nghiem HK, Nguyen HH, Hoang LT, Cox NJ, Katz JM (2005) Isolation and characterization of avian influenza viruses, including highly pathogenic H5N1, from poultry in live bird markets in Hanoi, Vietnam, in 2001. J Virol 79:4201–4212
31. Okazaki K, Takada A, Ito T, Imai M, Takakuwa H, Hatta M, Ozaki H, Tanizaki T, Nagano T, Ninomiya A, Demenev VA, Tyaptirganov MM, Karatayeva TD, Yamnikova SS, Lvov DK, Kida H (2000) Precursor genes of future pandemic influenza viruses are perpetuated in ducks nesting in Siberia. Arch Virol 145:885–893
32. Park KJ, Kwon HI, Song MS, Pascua PN, Baek YH, Lee JH, Jang HL, Lim JY, Mo IP, Moon HJ, Kim CJ, Choi YK (2011) Rapid evolution of low-pathogenic H9N2 avian influenza viruses following poultry vaccination programmes. J Gen Virol 92:36–50
33. Peiris M, Yuen KY, Leung CW, Chan KH, Ip PL, Lai RW, Orr WK, Shortridge KF (1999) Human infection with influenza H9N2. Lancet 354:916–917
34. Perez DR, Lim W, Seiler JP, Yi G, Peiris M, Shortridge KF, Webster RG (2003) Role of quail in the interspecies transmission of H9 influenza A viruses: molecular changes on HA that correspond to adaptation from ducks to chickens. J Virol 77:3148–3156
35. Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4:406–425
36. Sakoda Y, Sugar S, Batchluun D, Erdene-Ochir TO, Okamatsu M, Isoda N, Soda K, Takakuwa H, Tsuda Y, Yamamoto N, Kishida N, Matsuno K, Nakayama E, Kajihara M, Yokoyama A, Takada A, Sodnomdarjaa R, Kida H (2010) Characterization of H5N1 highly pathogenic avian influenza virus strains isolated from migratory waterfowl in Mongolia on the way back from the southern Asia to their northern territory. Virology 406:88–94
37. Shortridge KF (1992) Pandemic influenza: a zoonosis? Semin Respir Infect 7:11–25
38. Sun Y, Pu J, Jiang Z, Guan T, Xia Y, Xu Q, Liu L, Ma B, Tian F, Brown EG, Liu J (2010) Genotypic evolution and antigenic drift of H9N2 influenza viruses in China from 1994 to 2008. Vet Microbiol 146:215–225
39. Xu C, Fan W, Wei R, Zhao H (2004) Isolation and identification of swine influenza recombinant A/Swine/Shandong/1/2003(H9N2) virus. Microbes Infect 6:919–925
40. Xu KM, Smith GJ, Bahl J, Duan L, Tai H, Vijaykrishna D, Wang J, Zhang JX, Li KS, Fan XH, Webster RG, Chen H, Peiris JS, Guan Y (2007) The genesis and evolution of H9N2 influenza

viruses in poultry from southern China, 2000 to 2005. J Virol 81:10389–10401
41. Yamamoto N, Sakoda Y, Motoshima M, Yoshino F, Soda K, Okamatsu M, Kida H (2011) Characterization of a non-pathogenic H5N1 influenza virus isolated from a migratory duck flying from Siberia in Hokkaido, Japan, in October 2009. Virol J 8:65
42. Yoshida H, Sakoda Y, Endo M, Motoshima M, Yoshino F, Yamamoto N, Okamatsu M, Soejima T, Senba S, Kanda H, Kida H (2011) Evaluation of the reverse transcription loop-mediated isothermal amplification (RT-LAMP) as a screening method for the detection of influenza viruses in the fecal materials of water birds. J Vet Med Sci 73:753–758
43. Yu H, Zhou YJ, Li GX, Ma JH, Yan LP, Wang B, Yang FR, Huang M, Tong GZ (2011) Genetic diversity of H9N2 influenza viruses from pigs in China: a potential threat to human health? Vet Microbiol 149:254–261

Japanese Journal of Veterinary Research 59(2&3): 89-100, 2011

FULL PAPER

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

Masahiro Kajihara[1)], Keita Matsuno[1)], Edgar Simulundu[1)], Mieko Muramatsu[1)], Osamu Noyori[1)], Rashid Manzoor[1)], Eri Nakayama[1)], Manabu Igarashi[2)], Daisuke Tomabechi[1)], Reiko Yoshida[1)], Masatoshi Okamatsu[3)], Yoshihiro Sakoda[3)], Kimihito Ito[2)], Hiroshi Kida[3, 4, 5, 6)] and Ayato Takada[1, 7, *)]

[1)]Department of Global Epidemiology, Hokkaido University Research Center for Zoonosis Control, Sapporo 001-0020, Japan

[2)]Department of Bioinformatics, Hokkaido University Research Center for Zoonosis Control, Sapporo 001-0020, Japan

[3)]Laboratory of Microbiology, Department of Disease Control, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo 060-0818, Japan

[4)]SORST, Japan Science and Technology Agency Basic Research Programs (JST), 4-1-8 Honcho Kawaguchi, Saitama 332-0012, Japan

[5)]OIE Reference Laboratory for Animal Influenza, Sapporo 060-0818, Japan

[6)]Hokkaido University Research Center for Zoonosis Control, Sapporo 001-0020, Japan

[7)]School of Veterinary Medicine, the University of Zambia, P. O. Box 32379, Lusaka, Zambia

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

*Corresponding author: Prof. Ayato Takada, Department of Global Epidemiology, Hokkaido University Research Center for Zoonosis Control, Kita 20 Nishi 10, Kita-ku, Sapporo 001-0020, Japan
Phone: +81-11-706-9502. Fax: +81-11-706-7310. E-mail: atakada@czc.hokudai.ac.jp

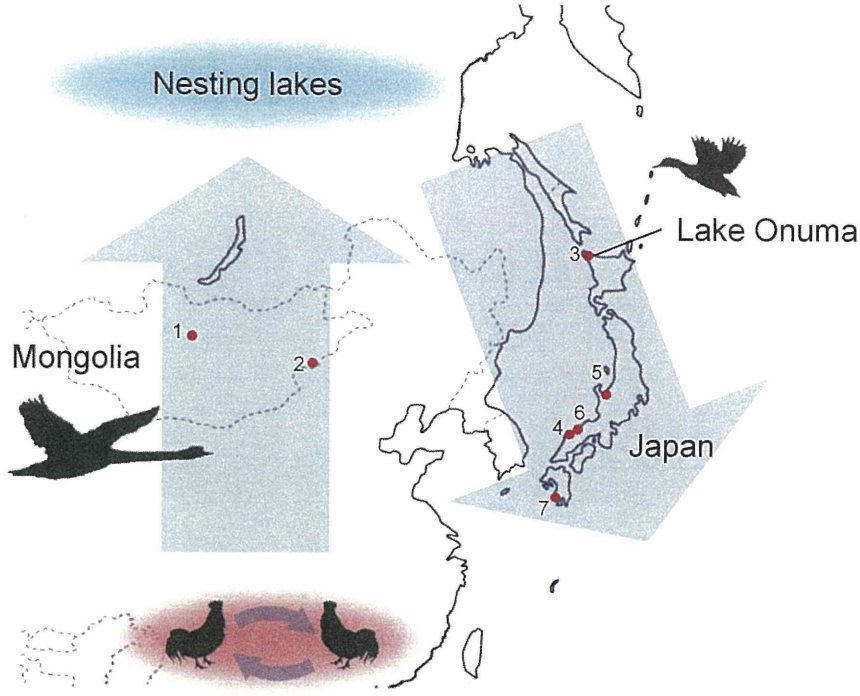
Introduction

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

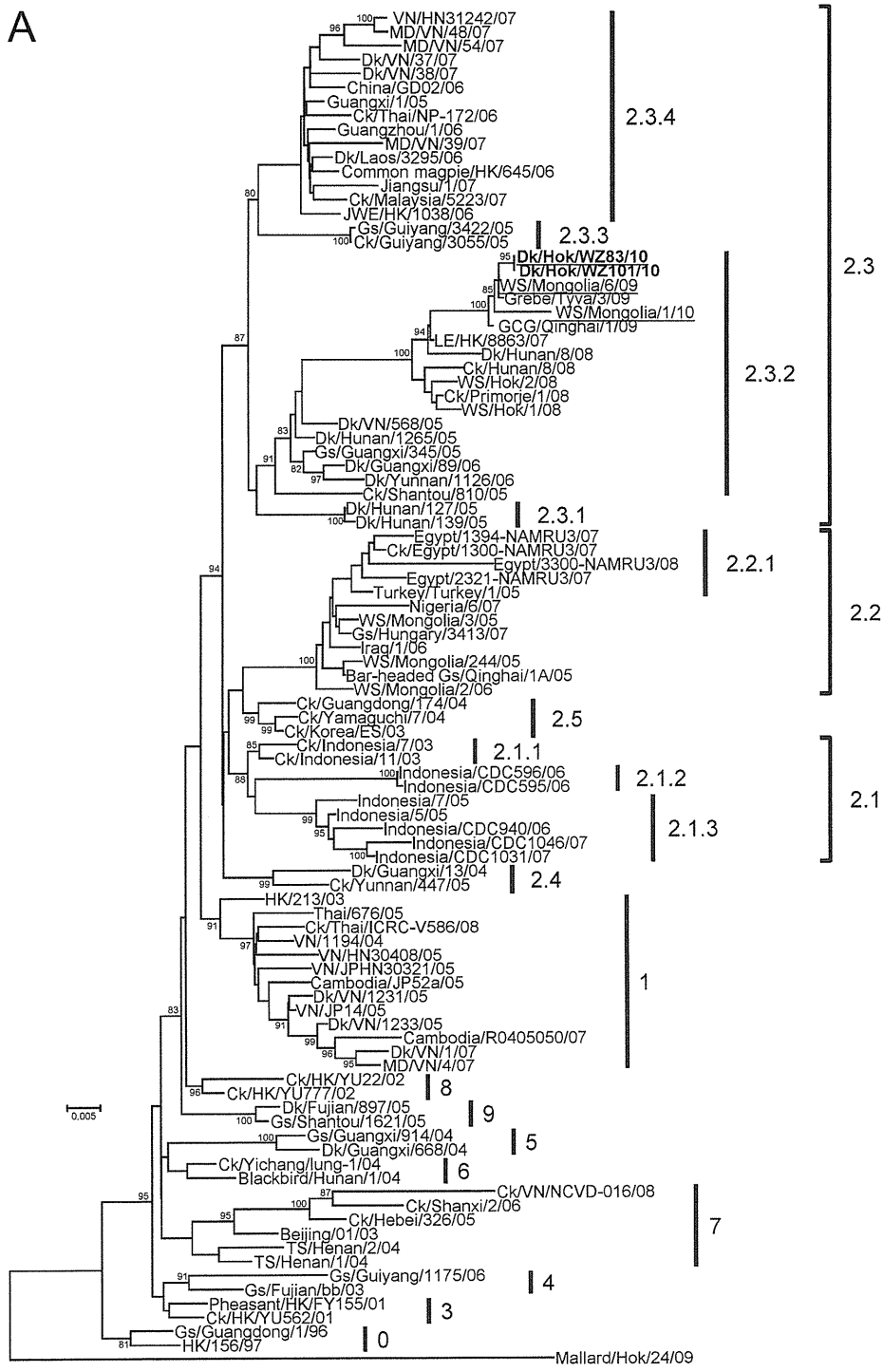
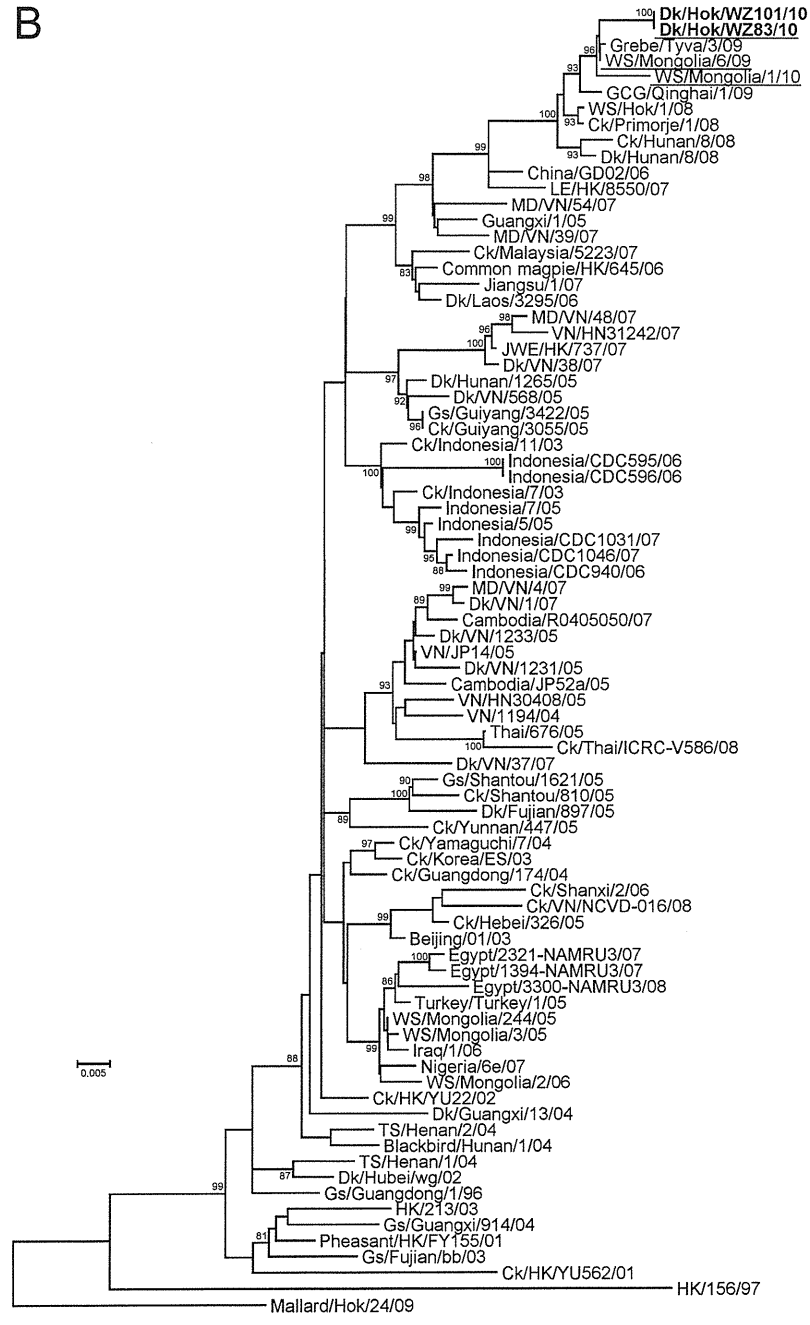
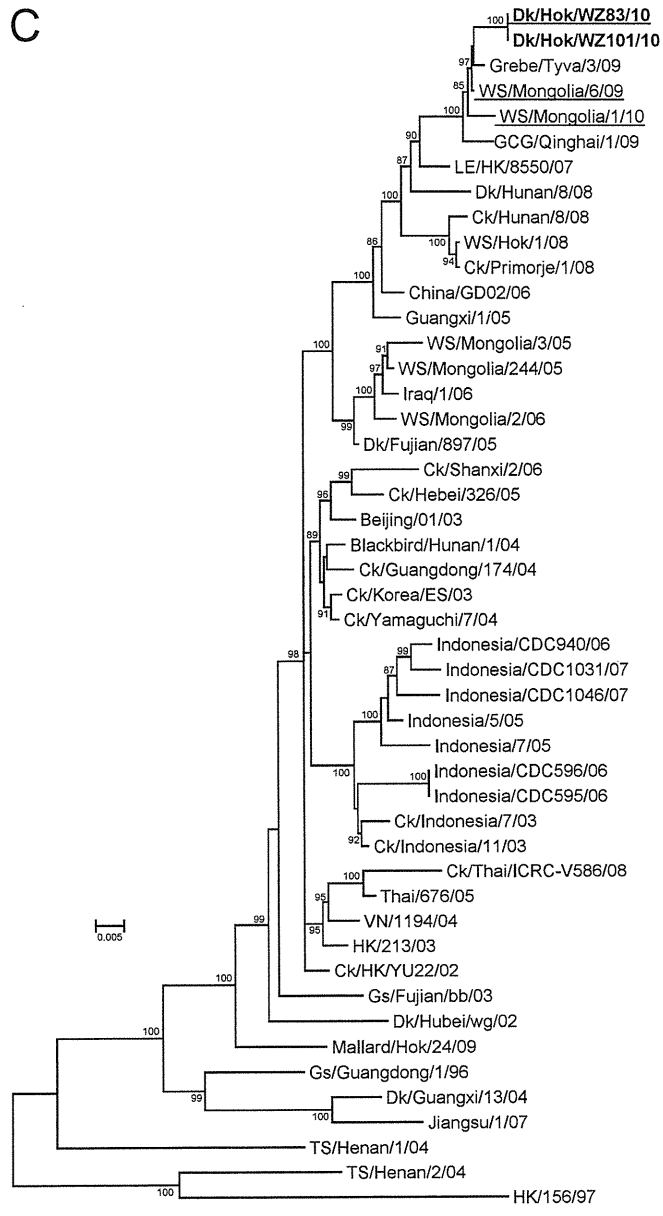


Fig. 2. Phylogenetic trees of influenza A viruses of the H5N1 subtype. Analyses are based on 1,322, 1,305, and 2,239 bp of HA (A), NA (B), and PB2 (C) genes, respectively. Horizontal distances are proportional to the minimum number of nucleotide differences required to join nodes and sequences. Numbers 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.

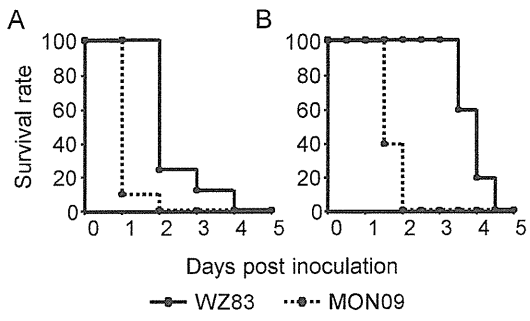


Fig. 3. Difference in disease progression in H5N1 HPAIV-infected chickens. For intravenous infection, 8 chickens of each group were infected with $10^{7.0}$ EID_{50} of the viruses and observed for clinical symptoms every 24 hours (A). Five chickens of each group were infected intranasally with $10^{6.0}$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