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ORIGINAL ARTICLE

Characterization of avian influenza viruses isolated from domestic ducks in Vietnam in 2009 and 2010

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Abstract In the surveillance of avian influenza in Vietnam, 26 H9N2, 1 H3N2, 1 H3N8, 7 H4N6, 3 H11N3, and 1 H11N9 viruses were isolated from tracheal and cloacal swab samples of 300 domestic ducks in April 2009, and 1 H9N6 virus from 300 bird samples in March 2010. Out of the 27 H9 virus isolates, the hemagglutinins of 18 strains were genetically classified as belonging to the sublineage G1, and the other nine belonged to the Korean sublineage. Phylogenetic analysis revealed that one of the 27 H9 viruses was a reassortant in which the PB2 gene belonged to the Korean sublineage and the other seven genes belonged to the G1 sublineage. Three representative H9N2 viruses were intranasally inoculated into ducks, chickens, pigs, and mice. On the basis of experimental infection studies, it was found that each of the three viruses readily infected pigs and replicated in their upper respiratory tracts, and they infected chickens with slight replication. Viruses were recovered from the lungs of mice inoculated with two of the three isolates. The present results reveal that H9 avian influenza viruses are prevailing and genetic reassortment occurs among domestic ducks in Vietnam. It is recommended that careful surveillance of swine influenza with H9 viruses should be performed to prepare for pandemic influenza.

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Introduction

Avian influenza viruses of various subtypes are circulating in poultry in Asian countries [1, 15, 20, 30, 40]. In particular, H9N2 influenza virus is present in poultry in Eurasian countries [9–11, 25]. Since H9N2 viruses were isolated from quails in Hong Kong in 1988, they have become prevalent in live-bird markets and poultry farms in Asia [8, 34]. H9N2 virus infections have greatly affected not only the poultry industry but also public health [8, 40]. The hemagglutinin (HA) genes of Eurasian H9N2 viruses have been phylogenetically divided into G1, Y280, and Korean sublineages [10]. H9N2 viruses do not usually cause severe disease in poultry, but co-infection of H9N2 viruses with bacteria such as *Staphylococcus aureus*, *Haemophilus paragallinarum*, or attenuated coronavirus vaccine exacerbates the disease [12, 21]. H9N2 viruses were also isolated from domestic pigs in China [39] and Korea, and from humans with febrile respiratory illness in Hong Kong in 1998, 1999, 2003, 2008, and 2009 [4, 7, 23, 33, 43]. Thus, it is postulated that H9N2 virus may cause pandemic influenza in humans.

In our laboratory, avian influenza has been surveyed in Japan, Alaska, Siberia, Mongolia, and Australia since 1977

[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

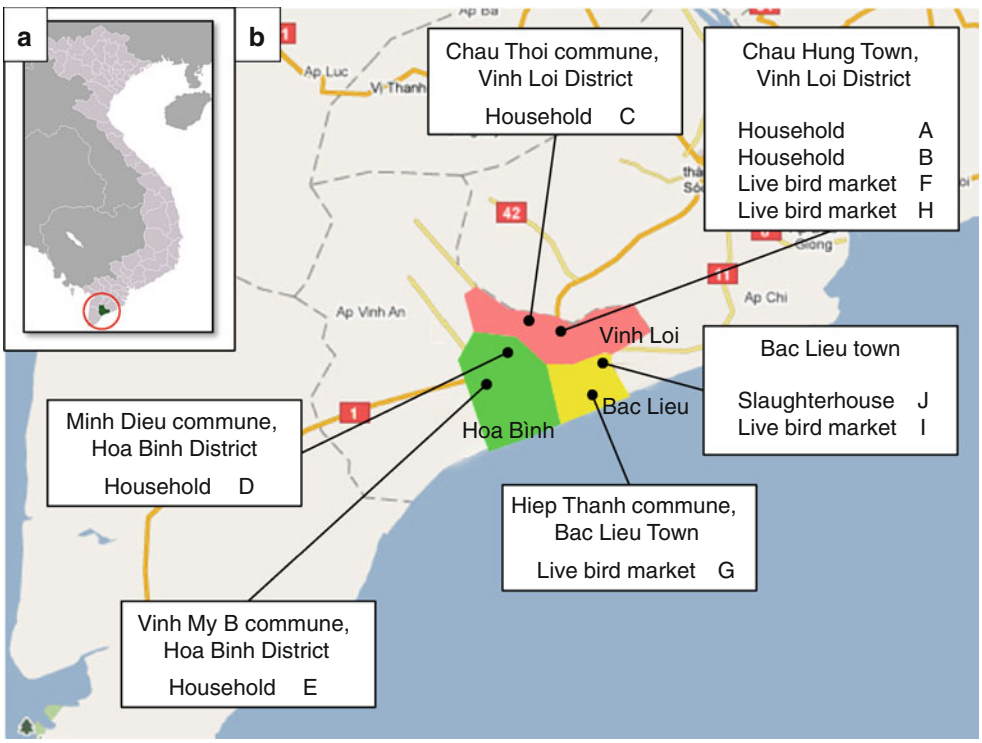
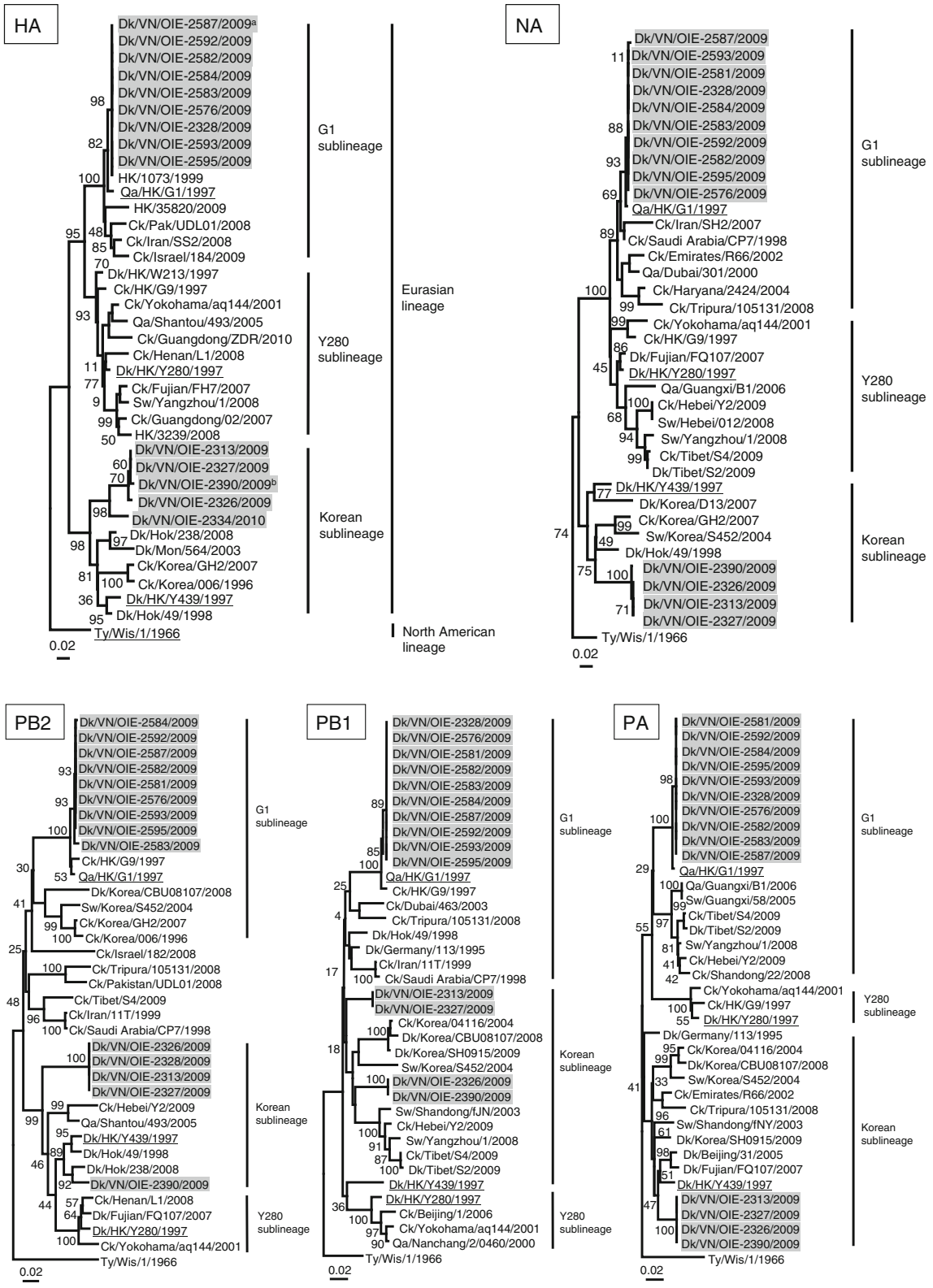


Fig. 1 Sampling points in southern Vietnam in the present study. Location of Bac Lieu province in Vietnam (**a**). Magnification of the circle in Fig. 1a and sampling points in Vinh Loi district, Bac Lieu town, and Hoa Binh district in Bac Lieu province (**b**). Avian influenza viruses were isolated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


HA
Dk/VN/OIE-2587/2009a
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-2390/2009b
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
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Dk/Tibet/S2/2009
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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
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Ck/Korea/GH2/2007
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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
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Dk/Hok/238/2008
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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
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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
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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
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0.02
G1 sublineage
Y280 sublineage
Korean sublineage

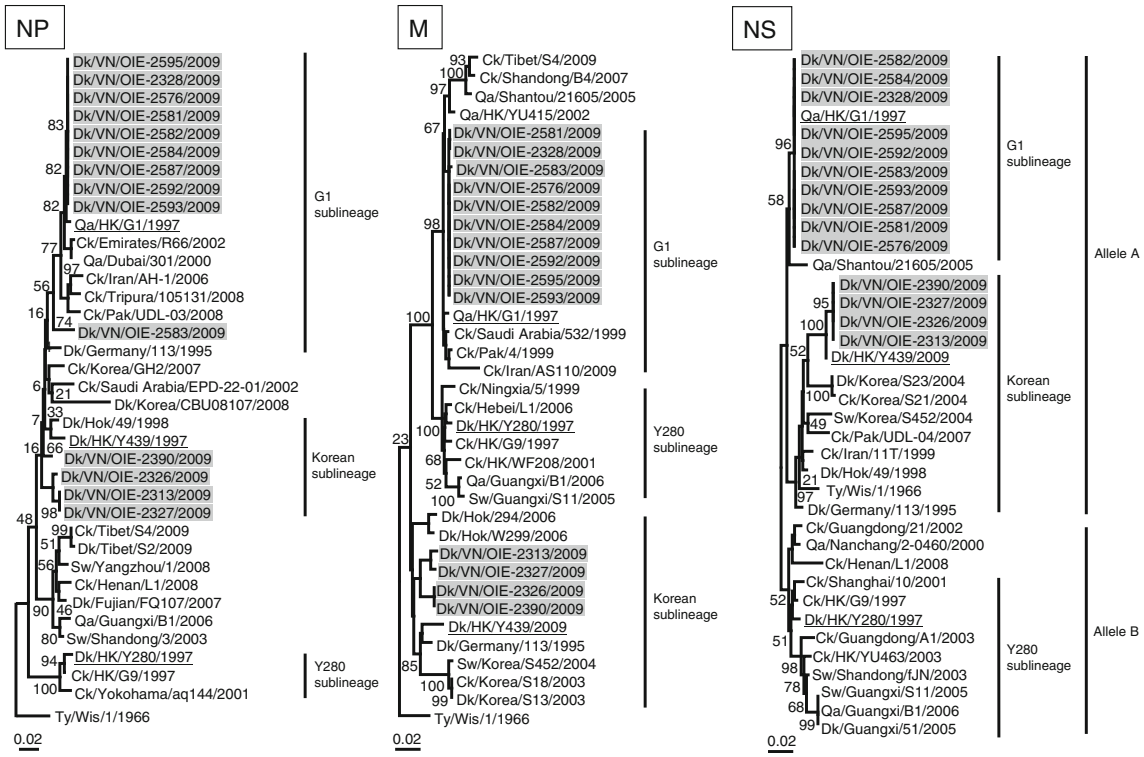

Fig. 2 continued

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.

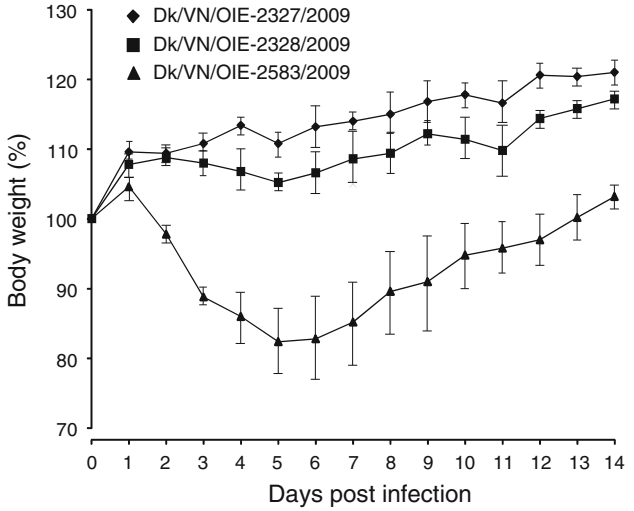

Fig. 3 Changes in body weight of mice inoculated with influenza viruses. Five mice were inoculated intranasally with Dk/VN/OIE-2327/2009, Dk/VN/OIE-2328/2009, and Dk/VN/OIE-2583/2009. The body weight of the mice was monitored for 14 days after inoculation with each influenza virus. Data are shown as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

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

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