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FULL PAPER

Virological surveillance and phylogenetic analysis of the PB2 genes of influenza viruses isolated from wild water birds flying from their nesting lakes in Siberia to Hokkaido, Japan in autumn

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Abstract

Recent introduction of H5N1 highly pathogenic avian influenza virus (HPAIV) in wild birds from poultry in Eurasia signaled the possibility that this virus may perpetuate in nature. Surveillance of avian influenza especially in migratory birds, therefore, has been conducted to provide information on the viruses brought by them to Hokkaido, Japan, from their nesting lakes in Siberia in autumn. During 2008–2009, 62 influenza viruses of 21 different combinations of hemagglutinin (HA) and neuraminidase (NA) subtypes were isolated. Up to September 2010, no HPAIV has been found, indicating that H5N1 HPAIV has not perpetuated at least dominantly in the lakes where ducks nest in summer in Siberia. The PB2 genes of 54 influenza viruses out of 283 influenza viruses isolated in Hokkaido in 2000–2009 were phylogenetically analysed. None of the genes showed close relation to those of H5N1 HPAIVs that were detected in wild birds found dead in Eurasia on the way back to their northern territory in spring.

Keywords: *Avian influenza, migratory ducks, PB2 gene, surveillance*

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Introduction

Ecological studies have revealed that a vast influenza virus gene pool for avian and mamalian influenza exists in migratory ducks[10]. Each of the sixteen hemagglutinin (HA) and nine neuramidase (NA) subtypes of influenza A viruses are perpetuated among migratory ducks and their nesting lake water in nature[4,7,15,23]. Transmission of H5 or H7 influenza viruses to domestic birds and especially in chickens may result in the emergence of highly pathogenic avian influenza viruses (HPAIV)[14].

Since 2003, HPAIVs H5N1 have spread to 62 countries in Eurasia and Africa and seriously affected poultry in Asia. Over 400 million birds have died from the infection or been killed for control purposes. A HPAIV is generated when a non-pathogenic virus brought in by migratory birds from nesting lakes in the north is transmitted to chickens via domestic ducks, geese, quails, turkeys and acquires pathogenicity for chickens. During over-wintering, some migratory birds were conversely infected with HPAIV H5N1 from poultry and have been found dead at lakes in northern China, Mongolia, Japan, Russia, Europe and Africa in April to May on the way back to their nesting lakes in northern territories. It was found that each of the viruses isolated from these birds were genetically closely related to those of the isolates from poultry in China[2,10,16,17]. Thus HPAIV strains that are currently circulating in poultry have returned to migratory water birds and spread world wide[10].

Since it is of concern that this H5N1 virus may perpetuate in the lakes in Siberia where migratory ducks nest in summer, virological surveillance and phylogenetic analysis of influenza viruses have been carried out in autumn when these birds flew to Hokkaido, Japan in 2008–2009.

It is known that the PB2 protein is a component of the viral polymerase complex that plays an important role in virus replication[5,11,19], and is a determinant of host range and pathogenicity of influenza viruses[18,20]. Therefore, PB2 genes of influenza viruses isolated from migratory ducks have been phylogenetically analyzed in the present study.

Materials and Methods

Sample collection and virus isolation: A total of 1,626 fecal samples of wild water birds were collected in autumn in 2008–2009 from Lake Ohnuma, Wakkanai, and Ohno pond, Hokkaido University, Sapporo, Japan. The fecal samples collected were kept in chilled containers and transported to our laboratory. Virus isolation and subtyping were performed as previously described[9]. One virus of each of the HA and NA combinations was selected randomly by year of isolation for genetic analyses (Table 1).

RNA extraction, RT-PCR, and nucleotide sequencing: RNA extraction and RT-PCR were conducted as previously described[13]. Partial-length PB2 genes were amplified using PB2 gene-specific primer set PB2–625F (5'-CAT GTA TGC TAC CAT CAA GGG-3'), and the universal primer Ba-PB2-2341R[6]. The PCR products were separated by 0.8% agarose gel electrophoresis and purified using the MiniElute™ Gel Extraction Kit (Qiagen, USA) as recommended by the manufacturer. The purified products were used as templates in sequencing reactions using a BigDye terminator cycle sequencing ready reaction kit and analyzed on a 3130 Genetic Analyzer (Applied Biosystems). DNA sequences were assembled and edited using the program Genetyx ATGC (2008 Genetyx Corp.). The accession numbers of PB2 genes sequenced in this study are available from DDBJ/EMBL/GenBank under accession numbers given in Table 2.

Phylogenetic analysis of the PB2 genes: Phylogenetic analysis was conducted using PB2 gene sequences of 36 representative strains from a total of 54 that were sequenced. Published sequences used in this study for phylogenetic comparison were obtained using BLAST homology searches from the influenza sequence database (http://www.ncbi.nlm.nih.gov/genomes/FLU/FLU.html). The PB2 gene tree was generated using the Neighbor Joining (NJ) bootstrap method (1,000 replicates) implemented in the Molecular Evolutionary Genetics Analysis program (MEGA, version 4.0)[22]. The evolutionary distances were calculated by the Maximum Composite Likelihood method[21].

Results

Influenza A viruses isolated from fecal samples of wild water birds flying from their nesting lakes in Siberia to Hokkaido, Japan in autumn

In the surveillance of avian influenza conducted in Hokkaido in autumn 2008–2009, 62 influenza viruses have been isolated from a total of 1,626 fecal samples. The HA (H1, H3-H7, H9-H12) and NA (N1-N3, N5-N9) subtypes of the isolates were identified. Twenty one HA and NA combinations were detected in the present study (Table 1). In the surveillance studies in 2000–2009 performed by our laboratory, no H5N1 HPAIV was isolated from wild water birds that flew from their nesting lakes in Siberia to Hokkaido, Japan in autumn[13].

Sequencing and phylogenetic analysis of the PB2 genes of influenza virus isolates from migratory birds

Randomly selected 54 isolates out of 283 avian influenza viruses isolated in the surveillance studies in 2000–2009 were sequenced of which 36 were phylogenetically analyzed. A phylogenetic tree was constructed on the basis of the partial nucleotide sequences of the PB2 genes (positions 1425–2192) of viruses isolated from wild water birds in Hokkaido in 2000 to 2009 (Fig. 1).

Table 1. Influenza viruses isolated from fecal samples of free-flying water birds 2008–2009

Subtypes of influenza viruses isolated in following years	
2008	2009
H3N2 (1)[a]	H1N3 (1)
H3N6 (3)	H1N5 (1)
H4N6 (11)	H4N6 (5)
H5N2 (1)	H5N1 (1)
H6N1 (4)	H5N2 (1)
H6N2 (1)	H6N1 (4)
H6N5 (1)	H6N8 (2)
H6N8 (1)	H11N9 (3)
H6N9 (1)	H12N5 (1)
H7N7 (1)	
H9N5 (1)	
H9N9 (1)	
H10N9 (2)	
H10N7 (11)	
H11N9 (2)	
H12N2 (1)	

[a]Number of isolates of subtypes were shown in parenthesis.

Phylogenetic tree of the PB2 genes was divided into American and Eurasian lineages. Duan *et al.*[3] showed that Eurasian lineage could be further divided into early and contemporary sublineages. Phylogenetic analysis of the PB2 genes of the isolates in the present study, belonged to the Eurasian lineage and were grouped (bootstrap values more than 85) into contemporary sublineages I and II. All the viruses analyzed in the present study belonged to either contemporary sublineage I or sublineage II. The majority of the PB2 genes under study clustered in different groups of sublineage I. They either clustered together or showed close relation to the PB2 genes of influenza viruses isolated from domestic and wild birds in China, Russia, Australia and Korea. It was noted that some of the strains, A/duck/Hokkaido/WZ76/2008

Table 2. Influenza virus strains analyzed in this study

Virus strain[a]	Subtype	Accession number	Virus strain	Subtype	Accession number
A/duck/Hokkaido/379/00	H4N6	AB478622	A/duck/Hokkaido/277/06	H6N2	AB478604
A/duck/Hokkaido/69/00	H5N3	AB300036	A/duck/Hokkaido/W162/06	H6N5	AB478618
A/duck/Hokkaido/18//00	H10N4	AB282876	A/duck/Hokkaido/W299/06	H9N2	AB478621
A/duck/Hokkaido/1169/01	H1N1	AB478607	A/duck/Hokkaido/W95/06	H10N8	AB569460
A/duck/Hokkaido/95/01	H2N2	AY422042	A/duck/Hokkaido/W73/07	H1N1	AB478614
A/duck/Hokkaido/17/01	H2N3	AY422040	A/duck/Hokkaido/W282/07	H4N6	AB478623
A/duck/Hokkaido/86/01	H2N3	AY422041	A/duck/Hokkaido/167/07	H5N3	AB378679
A/duck/Hokkaido/1005/01	H3N6	AB478606	A/duck/Hokkaido/201/07	H5N3	AB378687
A/duck/Hokkaido/56/01	H3N8	AB478611	A/duck/Hokkaido/69/07	H8N4	AB569464
A/duck/Hokkaido/1058/01	H4N5	AB569458	A/duck/Hokkaido/75/08	H3N6	AB569452
A/duck/Hokkaido/1019/01	H4N6	AB569457	A/duck/Hokkaido/W79/08	H4N6	AB569462
A/duck/Hokkaido/24/02	H11N9	AB478596	A/duck/Hokkaido/69/08	H4N6	AB569448
A/duck/Hokkaido/83/04	H1N1	AB478598	A/duck/Hokkaido/WZ21/08	H5N2	AB569454
A/duck/Hokkaido/18/04	H3N8	AB478595	A/duck/Hokkaido/W67/08	H6N1	AB569588
A/duck/Hokkaido/143/04	H4N2	AB569459	A/duck/Hokkaido/WZ76/08	H6N2	AB569453
A/duck/Hokkaido/W5/04	H4N6	AB569461	A/duck/Hokkaido/W112/08	H6N5	AB569466
A/duck/Hokkaido/193/04	H5N3	AB299377	A/duck/Hokkaido/W54/08	H6N8	AB569449
A/duck/Hokkaido/257/04	H6N1	AB478601	A/duck/Hokkaido/W76/08	H6N9	AB569465
A/duck/Hokkaido/W109/04	H6N2	AB478616	A/duck/Hokkaido/229/08	H7N7	AB569456
A/duck/Hokkaido/W12/04	H6N2	AB478609	A/duck/Hokkaido/238/08	H9N2	AB569467
A/duck/Hokkaido/W59/04	H8N4	AB478612	A/duck/Hokkaido/131/08	H10N7	AB569451
A/duck/Hokkaido/89/04	H10N5	AB478599	A/duck/Hokkaido/WZ16/08	H10N9	AB569463
A/duck/Hokkaido/W259/05	H2N5	AB478620	A/duck/Hokkaido/W45/08	H11N9	AB569455
A/duck/Hokkaido/12/05	H3N2	AB478594	A/ws/Hokkaido/OIE110/08	H12N2	AB569450
A/duck/Hokkaido/W70/05	H3N8	AB478613	A/duck/Hokkaido/WZ75/09	H5N2	AB569468
A/duck/Hokkaido/W268/05	H6N1	AB478603			
A/duck/Hokkaido/260/05	H8N4	AB478602			
A/duck/Hokkaido/279/06	H4N6	AB478605			
A/duck/Hokkaido/W206/06	H6N1	AB478619			

[a]Name of the virus with corresponding accession number of PB2 genes sequenced in this study.

(H6N2), A/duck/Hokkaido/W76/2008 (H6N9) and A/duck/Hokkaido/69/2008 (H4N6) characterized in this study were closely related to strains A/mallard/Korea/gH170/2007 (H7N7) and A/magpie/Korea/YJDI74/2007 (H7N7) isolated from domestic birds in Korea. Some viruses that fell in sublineage I were phylogenetically closely related to an isolate obtained from pintails in Alaska, virus strain A/northernpintail/Alaska/44204–108/06 (H3N1). Novel reassortant H5N1 HPAIV, A/chicken/Laos/P0130/2007 (H5N1) isolated from Laos[1)] also belonged to this sublineage but was most closely related to a virus isolated in migratory birds in Korea, virus strain A/shorebird/Korea/S6/2006 (H1N2). The Eurasian sublineage II consisted of only one group (Fig 1). The H5N1

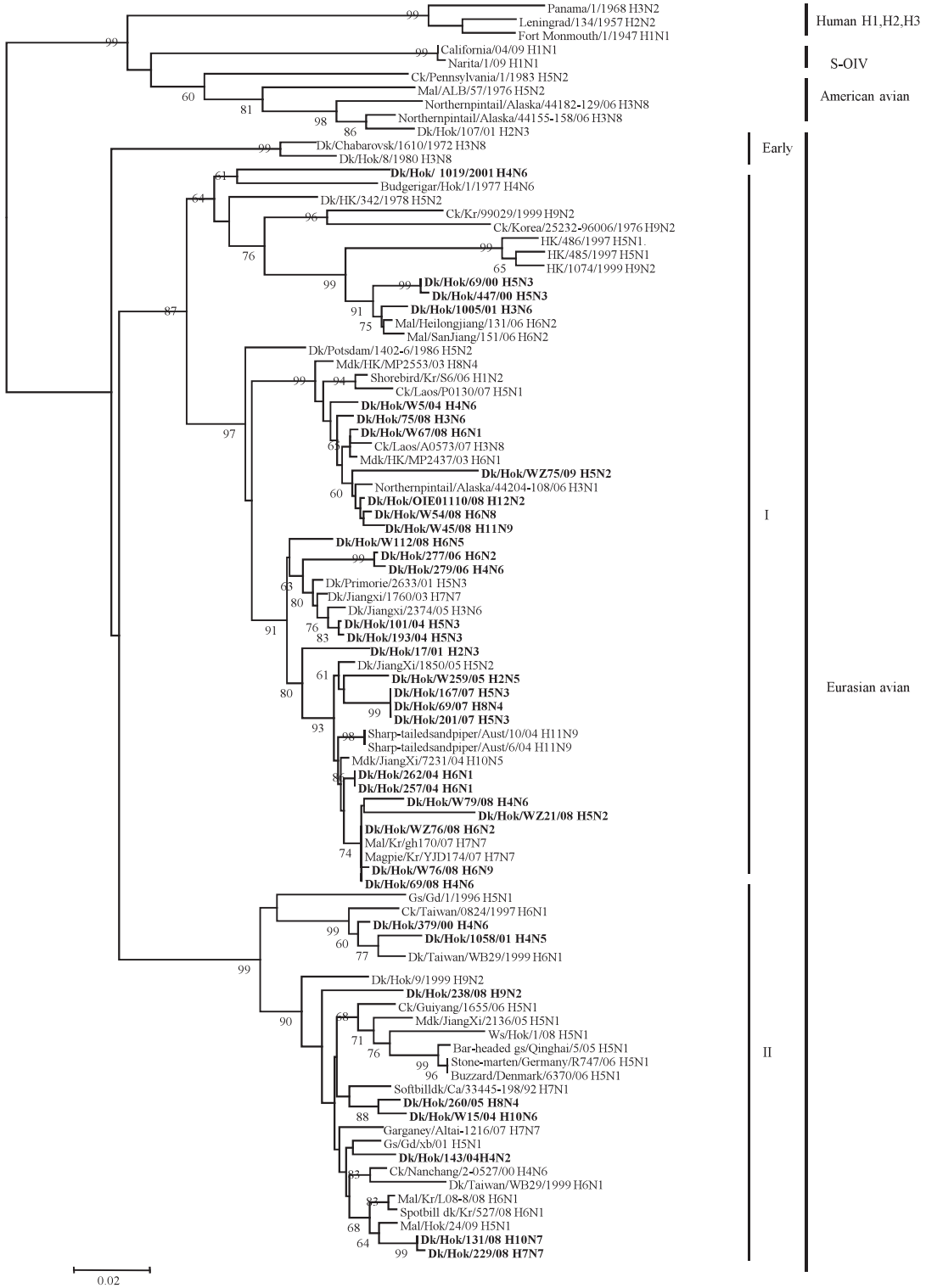

Fig. 1. Phylogenetic tree of influenza A virus PB2 genes. The phylogenetic tree was constructed using neighbour joining (NJ) method (1,000 replicates). For construction of this tree, 36 representative strains from a total of 54 that were sequenced. PB2 gene sequences each comprising 767 nucleotides (positions 1425–2192) were analyzed. This figure showing complete phylogram of avian influenza virus lineages with overall lineage of these isolates were of Eurasian avian and divided further into 2 distinct contemporary sublineages I and II. Bootstrap values below 60 are not shown. The strains sequenced in this study are indicated in bold.

HPAIV isolated from wild birds in China, Europe, and Japan belonged to this sublineage but none of the isolates tested in this study are closely related to these H5N1 HPAIVs.

Discussion

Rapid world wide spread of HPAIV to 62 countries in Eurasia and Africa with H5N1 viruses isolated from water birds found dead in Mongolia on the way back to their nesting lakes in Siberia in spring 2005, 2006, 2009 and 2010 raises concern that they may perpetuate in the northern nesting lakes in Siberia in summer. Since it was found that these H5N1 HPAIVs were genetically closely related to those influenza viruses isolated from birds in China, Iraq, Croatia, Nigeria, Korea and Japan, intensive surveillance of avian influenza in migratory water birds needs to be continued. In 2008–2009 avian influenza surveillance, we isolated 62 influenza viruses from fecal samples collected from migratory ducks that flew from their northern nesting lakes to Hokkaido, Japan in autumn. Influenza viruses of different subtypes have been isolated from these wild water birds. Twenty one combinations of the HA and NA subtypes of influenza viruses were detected. No H5N1 HPAIV was found during the surveillance period, indicating that the H5N1 HPAIV has not been perpetuated, at least dominantly in wild water birds that nest in northern territory in summer. The present findings are in agreement with previous study[13] showing that the H5N1 HPAIV has not persisted yet in wild water birds that nest in Siberia in summer.

The phylogenetic analyses in the present study revealed that none of the PB2 gene sequences of influenza viruses tested were closely related to HPAIV and none belonged to the American lineage. However, previous studies conducted in our laboratory found some internal protein genes (PB2, PA, and M) of influenza viruses isolated from migratory birds in Hokkaido which phylogenetically clustered with those of influenza viruses of the American lineage[12,13], indicating that interregional transmission of influenza virus genes do occur between the American and Eurasian gene pools among viruses obtained in Hokkaido. The grouping together of the PB2 gene of an influenza virus isolated from a pintail *(Anas acuta)* in Alaska with those of some viruses examined presently testifies to this phenomenon. The pintail (*Anas acuta*) species has been implicated in the inter-hemispheric transmission of influenza viruses between the American and Eurasian gene pools[8].

In conclusion, intensive surveillance of avian influenza conducted in Hokkaido in autumn in 2008–2009, has demonstrated that no HPAIVs were isolated from wild water birds flying from their nesting lakes in Siberia, indicating that the HPAIV has not yet persisted in their nesting lakes where they nest in summer. However, there is no guarantee that the absence of H5N1 HPAIV in wild water birds that come to Hokkaido is a permanent status. Therefore, the present study highlights the need for continued surveillance of avian influenza viruses in wild and domestic birds for the prevention and control of influenza.

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