



## Interregional Transmission of the Internal Protein Genes of H2 Influenza Virus in Migratory Ducks from North America to Eurasia

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**Abstract.** H2 influenza virus caused a pandemic in 1957 and has the possibility to cause outbreaks in the future. To assess the evolutionary characteristics of H2 influenza viruses isolated from migratory ducks that congregate in Hokkaido, Japan, on their flyway of migration from Siberia in 2001, we investigated the phylogenetic relationships among these viruses and avian and human viruses described previously. Phylogenetic analysis showed that the PB2 gene of Dk/Hokkaido/107/01 (H2N3) and the PA gene of Dk/Hokkaido/95/01 (H2N2) belonged to the American lineage of avian virus and that the other genes of the isolates belonged to the Eurasian lineage. These results indicate that the internal protein genes might be transmitted from American to Eurasian avian host. Thus, it is further confirmed that interregional transmission of influenza viruses occurred between the North American and Eurasian birds. The fact that reassortants could be generated in the migratory ducks between North American and Eurasian avian virus lineage further stresses the importance of global surveillance among the migratory ducks.

**Key words:** H2 subtype, influenza virus, migratory duck, phylogenetic analysis

### Introduction

Influenza A virus can be divided into a number of subtypes based on the antigenicity of the surface glycoproteins, hemagglutinin (HA) (15 subtypes) and neuraminidase (NA) (nine subtypes) [1]. Each of the known subtypes of influenza viruses has been isolated from aquatic birds, especially migratory ducks [2,3]. Influenza viruses bearing three HA (H1 to H3) and two NA (N1 and N2) subtypes have caused widespread disease in human beings [4]. Genetic analyses have shown that pandemic influenza viruses arise by genetic reassortment between human and non-human viruses.

It has been proposed that pigs could be the “mixing vessel” or intermediate host for genetic reassortment between avian and human influenza viruses since they can be infected with both groups of viruses [5–7]. Genetic characterization of the viruses that caused the 1957 and 1968 pandemics revealed that they were reassortant viruses. The Singapore/57 (H2N2) strain derived the HA, NA, and PB1 genes and the Hong Kong/68 (H3N2) strain derived the HA and PB1 genes from circulating avian influenza viruses. The remaining genes were derived from previously circulating human influenza viruses [8,9]. It was thought that avian influenza viruses scarcely infected human beings, while the H5N1 Hong Kong incident in 1997 [10,11], H9N2 cases in Hong Kong and Guangdong province in 1999 [12,13], and the H7N7 case

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in Holland in 2003 [14] indicated that direct transmission of the virus from birds is also an important way of invading the human population.

H2 influenza viruses disappeared from the human population in 1968, although they have been isolated from domestic and wild birds during the recent years. Antigenic analysis indicated that the majority of H2N2 influenza viruses isolated from wild birds, including mallard ducks and gulls before 1991 are antigenically closely related to the early human H2N2 viruses, suggesting that the Singapore/57-like virus strains are continuously circulating in birds [15]. On the other hand, antigenically distinct H2N2 viruses were isolated from shorebirds in Delaware in 1993 and 1997 [16]. Since no H2 virus has been isolated from human beings during the past 35 years, young people should be very susceptible to H2 virus. These facts emphasize the importance of surveillance of H2 influenza viruses in their natural reservoir.

The migratory ducks congregate in Hokkaido, Japan on their flyway of migration from Siberia in fall. We have been collecting fecal samples from the birds in Hokkaido since 1996 and have isolated many strains of different subtypes of influenza virus [17]. However, only four strains of H2 virus were isolated in 2001. Here, to elucidate the evolutionary characteristics of these H2 influenza virus strains isolated from the ducks, we investigated the phylogenetic relationships among these viruses, avian and human influenza virus strains described previously.

## Materials and Methods

### *Viruses*

Four H2 (three H2N3 and one H2N2) influenza virus strains were isolated from the fecal samples of the migratory ducks in Hokkaido in 2001. Initial isolation of the virus was performed in 10-day-old embryonated chicken eggs (ECE). Subtype of the viruses was determined in standard haemagglutination inhibition and neuraminidase inhibition tests using specific antisera to the reference strains of influenza virus [18]. Allantoic fluids were harvested from ECE inoculated with the viruses and used as a stock for sequence analysis.

### *RNA Extraction and RT-PCR*

Viral RNA was extracted from allantoic fluid by Trizol reagents (Gibco-BRL) and reverse transcription was done using oligonucleotide influenza universal primer Unil2: 5'-AGC AAA AGC AGG-3'. cDNA was amplified by polymerase chain reaction (PCR) as described by Shu [19]. The primers used for the H2 HA gene amplification were HAF (5'-GGC CAC CAG TCA ACA AAC TC-3') and HAR (5'-ACA TGG CCC AGA ACA AGA AG-3'), corresponding to the nucleotide positions 70–89 and 1629–1648, respectively. The primers used for other gene fragments were described previously [20].

### *Gene Sequence and Phylogenetic Analysis*

Polymerase chain reaction products were purified with the QIAquick PCR purification kit (Sapporo, Japan, Qiagen). The purified PCR products were then sequenced. Gene sequencing and phylogenetic analysis were performed as previously described [21].

### *Nucleotide Sequence Accession Numbers*

The nucleotide sequences determined in this study are available in the GenBank under accession numbers AY422014–AY422042.

## Results

We isolated 55 influenza virus strains from the fecal samples of the migratory ducks in Hokkaido between 1996 and 2001. Of these, only one H2N2 strain (Dk/Hokkaido/95/01) and three H2N3 strains (Dk/Hokkaido/17/01, Dk/Hokkaido/86/01, and Dk/Hokkaido/107/01) were isolated in 2001. The phylogenetic relationships among the HA1 fragments of the four isolates and those of the other H2 viruses from the database were analyzed based on the nucleotide sequences (positions 1–1050) (Fig. 1a). The phylogenetic tree confirms that the avian H2 influenza viruses divide into two distinct geographical lineages: American and Eurasian. All the H2 virus strains isolated from the ducks in Hokkaido formed a unique cluster and belonged to the Eurasian lineage, which also

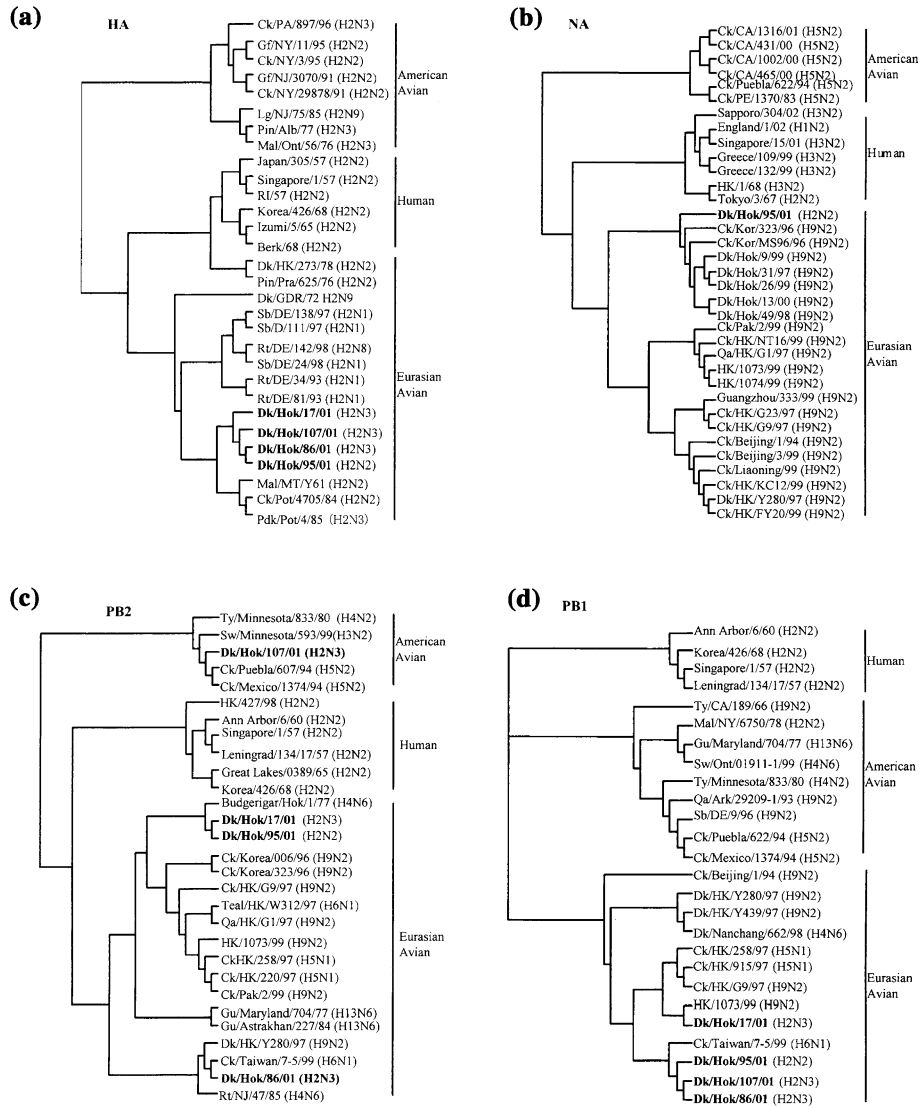


Fig. 1. Continued.

contained viruses isolated from shorebirds in Delaware. Phylogenetic analysis of the N2 NA gene shows that the N2 NA gene of Dk/Hokkaido/95/01 was closely related to those of the H9N2 viruses isolated from chickens in Korea and belonged to the lineage different from those of human H2N2 and H3N2 viruses (Fig. 1b).

The genes encoding the internal protein of the H2 influenza viruses were also phylogenetically analyzed. Like the surface glycoprotein genes, the internal protein genes could be divided into three lineages: the American avian, Eurasian avian and

human virus lineages. Phylogenetic analysis of the PB2 genes shows that the gene of Dk/Hokkaido/107/01 belonged to the American lineage and had a sister relationship with those of H5N2 viruses which caused outbreaks in America and Mexico in 1994 (Fig. 1c). The PB2 genes of Dk/Hokkaido/95/01 and Dk/Hokkaido/17/01 had a very close relationship and fell into the same cluster, while that of Dk/Hokkaido/86/01 belonged to another cluster and had a close relationship with that of the H9N2 virus representative strain, Dk/Hong Kong/Y280/97, which is prevalent in chickens in China.

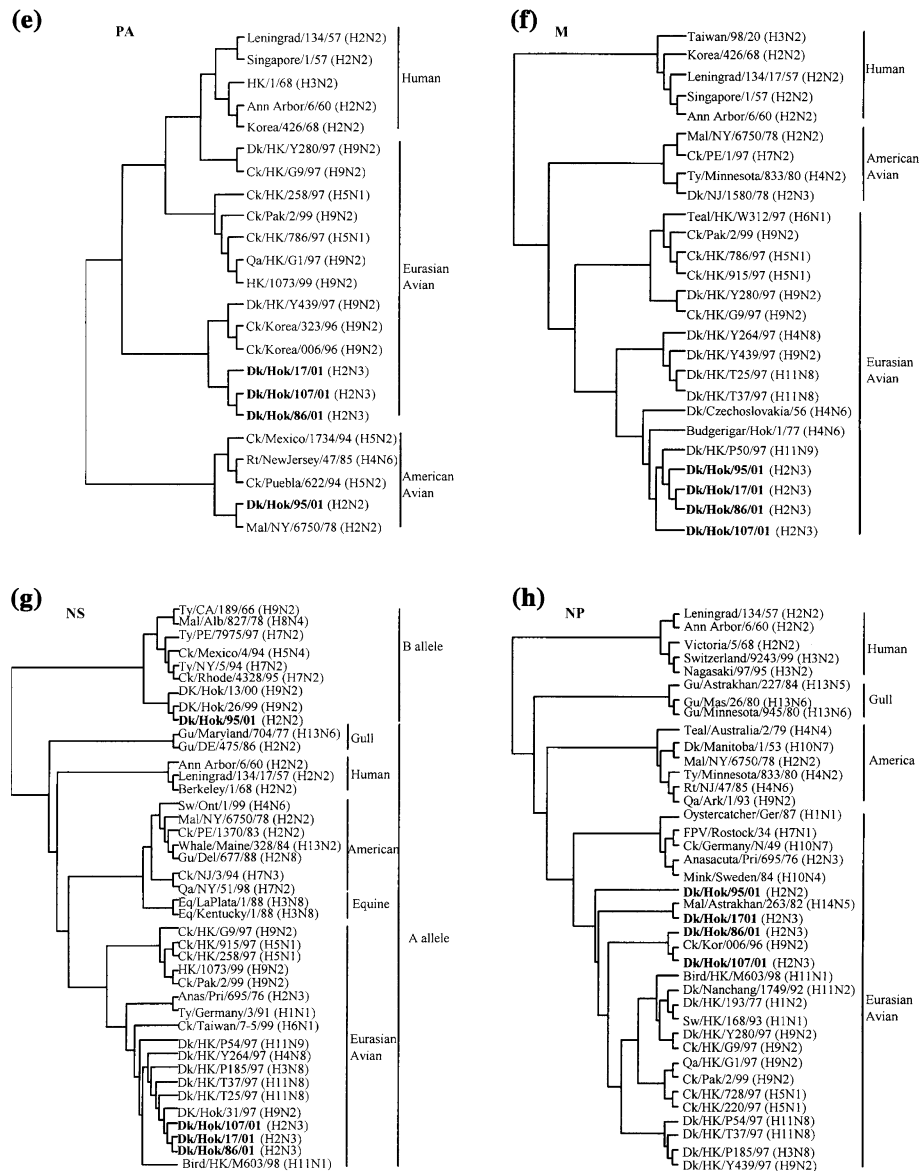


Fig. 1. Phylogenetic trees for the eight gene segments of influenza viruses. Nucleotides 1–1050 (1050 bp) of HA (A), 110–1267 (1158 bp) of NA (B), 1593–2157 (565 bp) of PB2 (C), 1168–1733 (566 bp) of PB1 (D), 720–1221 (502 bp) of PA (E), 55–759 (705 bp) of M (F), 25–693 (669 bp) of NS (G), and 1097–1478 (382 bp) of NP (H) were used for the phylogenetic analyses. Virus strains sequenced in the present study are in bold. Dk, duck; Pdk, Peking duck; Pin, pintail duck; Qa, quail; Ck, chicken; Gf, guinea fowl; Rt, ruddy turnstone; Mal, mallard; Gu, gull; Sb, shorebird; Sw, swine; Kor, Korea; Ark, Arkansas; CA, California; PE, Pennsylvania; Pak, Pakistan; DE, Delaware; NJ, New Jersey; NY, New York; Pot, Potsdam; Alb, Alberta; Pra, Praimoric; Ont, Ontario; HK, Hong Kong; Hok, Hokkaido.

The PB2 genes of human isolates also belonged to a different lineage from those of the viruses isolated from the migratory ducks.

Like the PB2 gene of Dk/Hokkaido/107/01, the PA gene of Dk/Hokkaido/95/01 also belonged to

the American avian virus lineage and had a close relationship with those of the H5N2 viruses that caused outbreaks in chickens in America and Mexico (Fig. 1e). The present results reveal that the PA gene of Dk/Hokkaido/95/01 may be

derived from viruses belonging to the American lineage. The PA genes of Dk/Hokkaido/17/01, Dk/Hokkaido/107/01, and Dk/Hokkaido/86/01 formed a cluster, which had a sib-relation with those of the H9N2 viruses isolated from chickens in Korea in 1996. The PB1 gene of Dk/Hokkaido/17/01 had a close relationship with that of H9N2 virus isolated from human beings in 1999, while those of the other three virus strains tested formed another cluster (Fig. 1d).

It has been proved that the NS gene of influenza virus falls into two lineages called A and B alleles [22,23]. Phylogenetic analysis of the NS genes of the four H2 virus strains showed that Dk/Hokkaido/95/01 (H2N2) was located in the B allele which was found exclusively among avian virus strains (Fig. 1g). The other three H2N3 virus strains belonged to A allele which contained avian virus strains and all mammalian isolates tested so far.

The M and NP genes of the four strains had a close relationship with each other and also belonged to the Eurasian avian virus lineage (Fig. 1f,h).

The phylogenetic types of the gene segments of these viruses tested in the study are summarized in Table 1.

## Discussion

H2 influenza viruses were first isolated in 1957, circulating in human beings until 1968. The first avian H2 influenza virus was isolated in 1972 and has continued to circulate in wild and domestic birds [15]. Although no H2 virus was isolated from pig, direct transmission of influenza virus from bird to human has occurred occasionally

[10,11,14]. We also reported that influenza viruses perpetuated in ducks nesting in Siberia should have contributed genes in the emergence of the highly virulent H5N1 virus isolated from chicken and human in Hong Kong in 1997 [17].

Phylogenetic analysis of avian influenza viruses demonstrated geographically distinct lineages, the North American and Eurasian ones. However, previous studies showed that the H2 HA genes of the Eurasian lineage were transmitted from Eurasian to American birds; for example, the genes of some isolates in Delaware in 1988, 1993, 1997 and 1998 belonged to the Eurasian lineage [15,16]. No report has shown that the internal protein genes of viruses isolated in European area belonged to the North American lineage. Here, we found that the PB2 gene of Dk/Hokkaido/107/01 and PA gene of Dk/Hokkaido/95/01 belonged to the American lineage, indicating that these genes also could be transmitted from American to Eurasian birds. Thus, the present results further confirm that interregional transmission of the internal protein genes of influenza virus occurred between the North American and Eurasian migratory ducks.

Most migratory ducks follow North–South migration routes that are separated for each hemisphere. How did the reassortant form between the North American and Eurasian lineage viruses? Some of the birds maybe migrate across the Atlantic after the North–South migration. Curry-Lindahl [24] reported that the Canadian terns and gulls crossing the Atlantic arrived in Europe after the European birds had already migrated to Africa. We previously reported that influenza viruses were kept in lake water after the migration and preserved in frozen water in winter [25]. It was likely that the birds were infected with viruses from the water and that reassortment

Table 1. Genotyping of H2 subtype influenza viruses isolated from migratory ducks in Hokkaido in 2001

Virus	Gene segment							
	PB2	PB1	PA	HA	NP	NA	M	NS
Dk/Hok/17/01	–	–	–	–	–	N	–	A
Dk/Hok/86/01	–	–	–	–	–	N	–	A
Dk/Hok/95/01	–	–	+	–	–	–	–	B
Dk/Hok/107/01	+	–	–	–	–	N	–	A

The symbols + and – indicate that the gene segments belong to North American and Eurasian lineage, respectively. A and B represent the gene segments belonging to A and B allele, respectively. N means not done here.

occurred between the North American and Eurasian lineage viruses.

The present results also indicate that each gene of the four H2 viruses tested belonged to a lineage different from that of human H2 viruses. Previous studies indicated that most H2N2 viruses in wild and domestic birds were antigenically closely related to those circulating in human population between 1957 and 1968 [15]. Now we are preparing a panel of monoclonal antibodies against the H2 virus isolated from migratory ducks in Hokkaido to provide information about antigenic relationship between the recent duck isolates and the previous H2 virus strains.

Although a small number of H2 viruses were isolated from birds, the present findings reveal that H2 influenza viruses are still circulating in their reservoir. Since no H2 virus was isolated from human for the past 35 years, it is possible that H2 virus could cause human pandemic again. Vaccine prepared from avirulent influenza virus isolated from duck was potent enough to protect mice from lethal challenge with pathogenic virus [26]. Therefore, intensive surveillance studies of aquatic birds to get information on the future pandemic influenza virus strains and for vaccine preparation is strongly recommended.

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