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Phylogenetic Analysis of Hemagglutinin and Neuraminidase Genes of H9N2 Viruses Isolated from Migratory Ducks*

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Abstract. Genetic analysis indicated that the pandemic influenza strains derived from wild aquatic birds harbor viruses of 15 hemagglutinin (HA) and 9 neuraminidase (NA) antigenic subtypes. Surveillance studies have shown that H9N2 subtype viruses are worldwide in domestic poultry and could infect mammalian species, including humans. Here, we genetically analyzed the *HA* and *NA* genes of five H9N2 viruses isolated from the migratory ducks in Hokkaido, Japan, the flyway of migration from Siberia during 1997–2000. The results showed that *HA* and *NA* genes of these viruses belong to the same lineages, respectively. Compared with those of A/quail/Hong Kong/G1/97-like and A/duck/Hong Kong/Y280/97-like viruses, HA and NA of the migratory duck isolates had a close relationship with those of H9N2 viruses isolated from the chicken in Korea, indicating that the Korea H9N2 viruses might be derived from the migratory ducks. The *NA* genes of the five isolates were located in the same cluster as those of N2 viruses, which had caused a human pandemic in 1968, indicating that the *NA* genes of the previous pandemic strains are still circulating in waterfowl reservoirs. The present results further emphasize the importance of carrying out molecular epidemiological surveillance of H9N2 viruses in wild ducks to obtain more information for the future human influenza pandemics preparedness.

Key words: H9N2, HA and NA, influenza virus, migratory ducks, phylogenetic analysis

Introduction

Influenza A virus consists of 15 hemagglutinin (HA) and 9 neuraminidase (NA) subtypes. Aquatic birds, especially migratory ducks, appear to serve as a reservoir of all of the known subtypes of influenza A viruses [1,2]. Surveillance studies have shown that influenza viruses perpetuated in ducks nesting in Siberia could have contributed genes to emergence of the H5N1 virus in Hong Kong [3]. Antigenic and genetic analyses of influenza virus isolates from migratory ducks, domestic ducks, pigs, and humans indicated that the *HA* gene of A/Hong Kong/68 (H3N2) strain was introduced into the precedent human H2N2 Asian influenza virus by genetic reassortment in pigs through domestic ducks from an H3 influenza virus circulating in migratory ducks in southern China [4,5]. Pandemic influenza viruses arise by genetic reassortment between human and nonhuman viruses [2,6]. Thus, it is important to have information on influenza viruses circulating in avian species in the world.

H9N2 influenza virus has been isolated from domestic avian in many countries [7–9]. H9N2 avian influenza reported in Asian countries has caused great economic losses during recent years.

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Phylogenetic and antigenic analysis of Asian H9N2 isolates revealed that there are three lineages among *HA* genes of these H9N2 viruses [8]. One lineage represented by A/quail/Hong Kong/G1/97 (Qa/HK/G1/97), which consists of the H9N2 human viruses, Pakistan H9N2 chicken viruses, and some of the avian H9N2 viruses isolated in Hong Kong. Viruses in this lineage could have been the donor of the internal protein genes of the highly pathogenic H5N1 influenza virus that caused the Hong Kong influenza incident in 1997 [10]. Another lineage is composed mainly of the isolates from chickens in China, which are represented by A/duck/Hong Kong/Y280/97 (Dk/HK/Y280/97) [11,12]. The third lineage consisted of viruses isolated from chickens in Korea in 1996 [13]. These findings suggested that the H9N2 influenza viruses from Asia were not the result of spread of the same virus or a virus from a common origin in the region.

The migratory ducks congregate in Hokkaido of Japan on their flyway of migration from Siberia every year. In our laboratory, we collect the fecal samples from the migratory ducks in Hokkaido from 1995 to 2001. Five H9N2 subtype strains were isolated and identified during the past years. Here, to show the relationship between the H9N2 viruses isolated from migratory ducks and the H9N2 viruses isolated from the domestic chickens, which caused outbreaks in chicken populations, *HA* and *NA* genes of the migratory duck isolates were sequenced and analyzed.

Materials and Methods

Viruses

Five H9N2 influenza virus strains were isolated from fecal samples of migratory ducks which were collected in October 1997–2000 at Lake Ohnuma in Wakkanai, Hokkaido, Japan. Initial isolation of the virus was performed in 10-day-old embryonated chicken eggs (ECE). Subtype identification of the viruses were determined in standard haemagglutination inhibition and neuraminidase inhibition tests using specific antisera to the reference strains of influenza viruses [14]. Allantoic fluids were harvested from ECE-passaged virus and used as a stock for sequence analysis. The HA and NA sequence of other H9N2 virus used in the study were from the GenBank database.

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 Uni12: 5′-AGC AAA AGC AGG-3′. cDNA was amplified by PCR as described by Shu [15]. The primers used for HA amplication were HAF (5′-GGC CAC CAG TCA ACA AAC TC-3′) and HAR (5′-ACA TGG CCC AGA ACA AGA AG-3′), corresponding to positions 70–89 and 1629–1648, respectively. The primers used for NA amplication were NAF (5′-GCA ATT GGC TCT GTT TCT CT-3′) and NAR (5′-CTT TGG TCT TCC TCT TAT CA-3′), corresponding to positions 25–44 and 1277–1296, respectively.

Gene Sequence

PCR products were purified with the QIAquick PCR purification kit (Qiagen). The purified PCR products were then sequenced using the CEQ Dye Terminal Cycle Sequence (DTCS) Quick Start Kit and a CEQ 2000 Beckman Coulter sequencer (Beckman Coulter). Assembly of sequences, translation of nucleotide sequence into protein sequence, and initial multiple sequence alignments were performed with the Clustal V method in MegAlign software version 1.03 (SNASTar Inc., Madison, WI).

Phylogenetic Analysis

Phylogenetic analysis was carried out by analyzing the data obtained here with those of other sequences of influenza viruses from the GenBank database by using PHYLIP software package (http://www.ddbj.nig.ac.jp/E-mail/clustalw-e.html). The tree was drawn using TREEVIE (version 1.40, Roderic D. M. Page, 1997).

Nucleotide Sequence Accession Numbers

The nucleotide sequences determined in this study are available in the GenBank under accession number AY330332–AY330341.

Results

Amino Acids Sequence at the Cleavage Site of the HA of the H9N2 Isolates

In this study, 1472 bp nucleotides of *HA* gene of the five H9N2 viruses were sequenced. The amino acid sequences (39–528) of the HA of the five isolates were deduced from the nucleotide sequence. Amino acids sequence at the cleavage site of HA of the isolates possessed a -P-A-A-S-D-R/G-L- motif. While those of mainland China chicken H9N2 isolates, the representative strain Qa/HK/G1/97, human H9N2, and Pakistan chicken H9N2 strains had -P-A-R-S-S-R/G-L- motif (Table 1). The Korea H9N2 isolates possessed -P-A-A-S-Y(V)-R/G-L- motif. That of representative virus A/turkey/Wisconsin/66(Ty/WI/66) had -P-A-V-S-S-R/G-L- motifs. Thus, two amino acid differences at the cleavage site exist between those of migratory duck isolates and those of China and Pakistan H9N2 isolates, while only one amino acid difference was between those of migratory duck strains and those of the Korea isolates. The pathogenicity of H5 and H7 influenza viruses is related to the multiple basic amino acids at the site of cleavage to HA1 and HA2 [16,17]. It is not clear that the amino acids sequence at the cleavage site of HA of H9 subtype viruses is related to the pathogenicity.

Table 1. Amino acids at the cleavage site and the receptor-binding sites of hemagglutinin

Viruses	Amino Acid Sequence at the Cleavage Site	Amino Acid Residue*		
		183	190	226
Dk/Hok/31/97	PAASDR/GL	H	E	Q
Dk/Hok/49/98	PAASDR/GL	H	E	Q
Dk/Hok/9/99	PAASDR/GL	H	E	Q
Dk/Hok/26/99	PAASDR/GL	H	E	Q
Dk/Hok/13/00	PAASDR/GL	H	E	Q
Ty/CA/189/66	PAVSDR/GL	H	E	Q
Ty/WI/66	PAVSDR/GL	H	E	Q
Dk/HK/Y439/97	PAASNR/GL	H	E	Q
Ck/Kor/006/96	PAASVR/GL	H	E	Q
Ck/Kor/323/96	PAASYR/GL	H	E	Q
Ck/BJ/1/94	PARSSR/GL	N	V	Q
Ck/BJ/1/95	PARSSR/GL	N	A	Q
Ck/HB/1/96	PARSSR/GL	N	A	Q
Ck/BJ/2/97	PARSSR/GL	N	A	L
Ck/GD/97	PARSSR/GL	N	T	Q
Ck/HN/98	PARSSR/GL	N	V	Q
Ck/BJ/3/99	PARSSR/GL	N	A	Q
Ck/LN/99	PARSSR/GL	N	V	L
Ck/HK/KC12/99	PARSSR/GL	N	A	L
Dk/HK/Y280/97	PARSSR/GL	N	T	L
Ck/HK/G9/97	PARSSR/GL	N	A	L
Qa/HK/Gl/97	PARSSR/GL	H	E	L
Ck/Par/2/99	PARSSR/GL	H	A	Q
HK/1073/99	PARSSR/GL	H	E	L
Qa/HK/A17/99	PARSSR/GL	H	E	L
Ck/HK/NT16/99	PARSSR/GL	H	E	L
Pg/HK/FY6/99	PARSSR/GL	H	E	L
Pa/Narina/92A/98	PARSSR/GL	H	E	M

*Represents the number according to H3 HA. Amino acids at positions H-183, E-190, and Q-226 in the receptor-binding site were considered as conserved at these positions in the avian virus consensus sequence. Abbreviations are listed in Fig. 1. Viruses in bold were sequenced in this study.

Relevant Amino Acids in the Receptor Binding Site of the HA of the H9N2 Viruses

Amino acids in the receptor binding sites of the HAs are associated with differences in receptor binding specificity [18]. Table 1 shows the amino acids at positions 183, 190, and 226 (numbering according to H3 HA), which are responsible for receptor binding specificity in the receptor binding sites of the HA. All of the five isolates had H, E, and Q at positions 183, 190, and 226 which are considered to be conserved sequence in the avian virus consensus sequence. The H9N2 viruses isolated from chickens in Korea also had the H, E, and Q at these positions. However, H9N2 viruses isolated in mainland China possessed N, A (V), and L at the same positions. Qa/HK/G1/97-like viruses had H, E, and L at these positions.

Phylogenetic Analysis of HA and NA Genes

The phylogenetic relationships among the *HA* genes of the five isolates and those of other H9N2 viruses from the database were analyzed based on the nucleotide sequence (position 114–1585). Figure 1 illustrates that there are two distinct lineages: North America avian and Eurasian avian lineages. The Eurasian avian lineage consists of three sublineages. All of the tested migratory duck strains together with the Korea chicken H9N2 viruses belonged to one sublineage, which was represented by Dk/HK/Y439/97. The results revealed that these viruses may have the same progenitor. All the mainland China H9N2 viruses belong to one sublineage, which was represented by the representative strain Dk/HK/Y280/97. The human H9N2 virus, Pakistan chicken H9N2

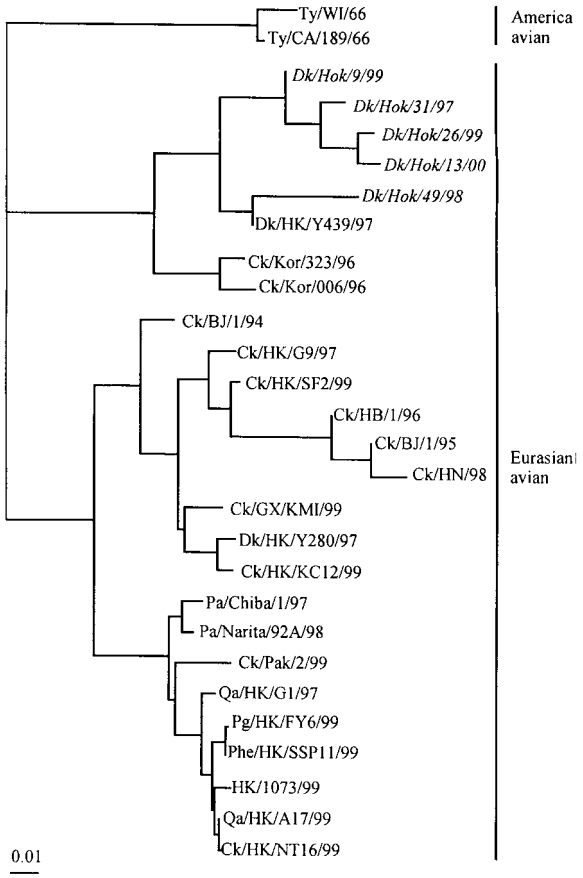

Fig. 1. Phylogenetic tree for the H9 *HA* gene of influenza A viruses. Nucleotides 114–1585 (1,472 bp) of HA were used for the phylogenetic analyses. Horizontal distances are proportional to the minimum number of nucleotide differences required to join nodes and sequences. Virus strains isolated and sequenced in the present study are in italic. *Abbreviations*: WI, Wisconsin; CA, California; Hok, Hokkaido; Kor, Korea; Pak, Pakistan; HB, Hebei; HN, Henan; GX, Guangxi; BJ, Beijing; HK, Hong Kong; Ty, Turkey; Qa, quail; Dk, duck; Sw, swine; Pg, pigeon; Pa, parakeet; Phe, pheasant; Ck, chicken.

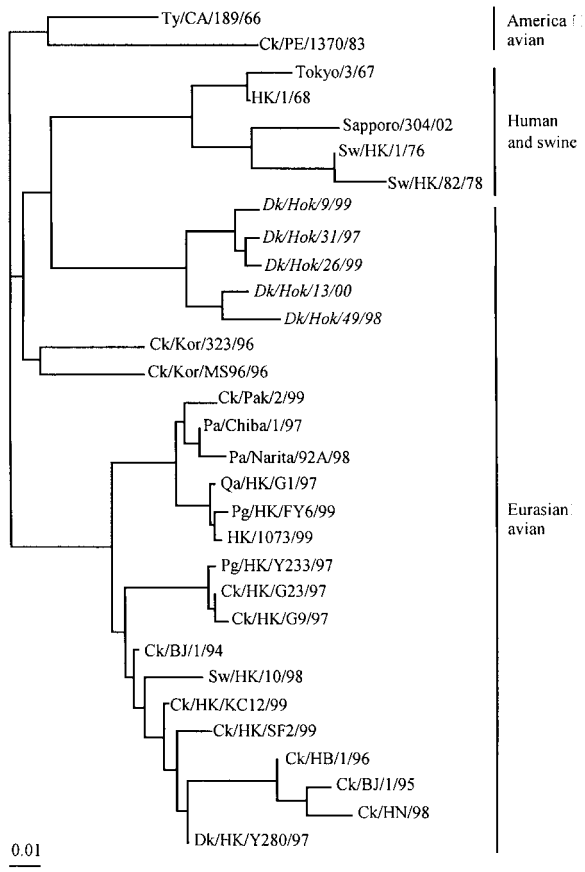

Fig. 2. Phylogenetic tree for the N2 *NA* gene of influenza A viruses. Nucleotides 58–1255 (1,167 bp) of NA were used for the phylogenetic analyses. Virus strains isolated and sequenced in the present study are in italic. Abbreviations are listed in Fig. 1.

virus, and some of the Hong Kong H9N2 viruses formed one group, which was represented by the prototype strain Qa/HK/G1/97, that was presumed to be the donor of the internal protein genes of the highly pathogenic H5N1 influenza virus in Hong Kong in 1997.

1167 bp nucleotides of *NA* gene of the five H9N2 viruses were sequenced. Phylogenetic analysis of *NA* genes revealed that there are three distinct lineages: North American avian, human and swine, and Eurasian avian lineages (Fig. 2). *NA* genes of the five isolates belong to the same lineage. Notably, viruses of this lineage had a sister relationship with those of the human N2 virus strains, which had caused human pandemic in 1968. The results revealed that *NA N2* genes that had caused previous pandemics are still maintained in the migratory ducks.

Discussion

Only five H9N2 subtype viruses were isolated from fecal samples of migratory ducks congregated on their flyway of migration in Hokkaido during 1997–2000. No H9N2 viruses were isolated from the same place in 1996 and 2001 in our laboratory. Surveillance studies showed that H9N2 influenza viruses were only found in apparently healthy wild ducks in Alberta, Canada, from 1976 to 1990 and in Asia, Hong Kong from 1975 to 1985 [19–21]. In China,

the H9N2 subtype of influenza virus is the most prevalent in the chicken population since 1994. The H9N2 subtype was the second-most commonly isolated virus from birds in live poultry markets during the H5N1 outbreak in Hong Kong in 1997 [22]. In 1996, H9N2 subtype influenza was also reported in Korea, which caused outbreak at several broiler breeder flocks [13].

Previous studies have shown that all HAs of influenza viruses isolated from avian species possess H, E, and Q at amino acid position 183, 190, and 226, respectively, at the receptor binding sites [23]. HAs of H9N2 viruses isolated in Korea and North America maintained the specificity, while most of the HAs of H9N2 belonged to the Qa/HK/G1/97 and Dk/HK/Y280/97 lineage had different types. In this study, we also analyzed the connecting peptide of the HAs of the five migratory duck isolates. All of them possessed a motif of -P-A-A-S-D-R/G-L-, which was identical to those of H9N2 viruses isolated in Ireland, A/avian/Ireland/PV46B/93 and A/pheasant/Ireland/PV18/97. The pathogenicity of H5 and H7 influenza viruses is associated with the presence of multiple basic amino acids at the cleavage site of HA [16], yet no motif standards have been established to evaluate the high or low pathogenic H9 subtype viruses. Infection experiments revealed that only slight depression and decreased feed consumption could be seen 4 days postinoculation by intravenous inoculation of 6-week-old chickens with Dk/Hok/31/97 and no mortalities were observed (data not shown).

HAs of the Eurasian H9 viruses are divided into three different groups, whose representative strains are Qa/HK/G1/97, Ck/HK/Y280/97, and Dk/HK/Y439/97 [8]. It was found in the present study that the HAs of the five strains isolated from migratory ducks in Hokkaido since 1997–2000 belonged to one lineage as well as those of H9N2 Korea strains. The results indicated that *HA* genes of the migratory duck strains had genetically close relationships with those of the Korea H9N2 strains. Figure 1 also showed that the HAs of the migratory ducks isolates were distinct from those of viruses that belong to Qa/HK/G1/97 or Dk/HK/Y280/97 lineage. The *NA* genes of the H9N2 virus isolated from migratory ducks showed the same phylogeny as the *HA* genes. These findings indicate that the major surface glycoprotein genes of the H9N2 viruses maintained in chickens in Korea may be derived from the migratory ducks. Genetic characterization of the viruses that caused the 1957 and 1968 pandemics revealed that the H2N2 strains derived the *HA*, *NA*, and *PB1* genes and the H3N2 strain derived the *HA* and *PB1* genes from circulating avian influenza A viruses, while the remaining genes were derived from previously circulating human influenza A viruses. Remarkably, the present results showed that the N2 *NA* genes of the 1968 pandemic virus strains have a close relationship with those of the H9N2 viruses isolated from the migratory ducks. These results suggested that the *NA* genes of the previous pandemic strains are still circulating in waterfowl reservoirs.

The overall conclusion from the present study is that *HA* and *NA* genes of the H9N2 viruses isolated from the migratory ducks in Hokkaido may be the precursor for the Korea H9N2 viruses, not for the mainland China or Pakistan chicken H9N2 influenza viruses. N2 *NA* of these viruses has a close relationship with those of previous pandemic strains indicating that the *NA* genes of the pandemic strains are still preserved in aquatic birds. The present study also emphasizes the need to continue carrying out influenza virus surveillance studies in waterfowl to have more information for the prognostication and control of the future pandemic influenza.

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References

1. Rohm C., Zhou N., Suss J. and Mackenzie J. Webster R.G., Virology *217*, 508–516, 1996.
2. Webster R.G., Bean W.J., Gorman O.T., Chambers T.M., and Kawaoka Y., Microbiol Rev *56*, 152–179, 1992.
3. Okazaki K., Takada A., Ito T., Imai M., Takakuwa H., Hatta M., Ozaki H., Tanizaki T., Nagano T., Ninomiya A., Demenev V.A., Tyaptirganov M.M., Karatayeva T.D., Yamnikova S.S., Lvov D.K., and Kida H., Arch Virol *145*, 885–893, 2000.
4. Kida H., Shortridge K.F., and Webster R.G., Virology *162*, 160–166, 1998.
5. Yasuda J., Shortridge K.F., Shimizu Y., and Kida H., J Gen Virol *72*, 2007–2010, 1991.
6. Scholtissek C., Eur J Epidemiol *10*, 455–458, 1994.
7. Alexander D.J., Vet Microbiol *74*, 3–13, 2000.

8. Guan Y., Shortridge K.F., Krauss S., Chin P.S., Dyrting K.C., Ellis T.M., Webster R.G., and Peiris M., J Virol *74*, 9372–9380, 2000.
9. Guo Y.J., Krauss S., Senne D.A., Mo I.P., Lo K.S., Xiong X.P., Norwood M., Shortridge K.F., Webster R.G., and Guan Y., Virology *267*, 279–288, 2000.
10. Guan Y., Shortridge K.F., Krauss S., and Webster R.G., Proc Natl Acad Sci USA *96*, 9363–9367, 1999.
11. Liu H.Q., Liu X.F., Cheng J.A., Peng D.X., Jia L.J., and Huang Y., Avian Dis *47*, 116–127, 2003.
12. Li X., Yu K.Z., Tian G.B., Tang X.Y., Deng G.H., Wang X.R., and Meng Q.W., Chin J Prev Vet Med *24*(4), 249–251, 2002.
13. Lee C.W., Song C.S., Lee Y.J., Mo I.P., Garcia M., Suarez D.L., and Kirn S.J., Avian Dis *44*, 527–535, 2000.
14. Kendal A.P., Pereira M.S., and Skehel J.J., *Concepts and Procedures for Laboratory-based Influenza Surveillance*. U.S. Department of Health and Human Services, Centers for Disease Control and Prevention, Atlanta, GA, 1982.
15. Shu L.L., Lin Y.P., Wright S.M., Shortridge K.F., and Webster R.G., Virology *202*, 825–833, 1994.
16. Kawaoka Y. and Webster R.G., Proc Natl Acad Sci USA *85*, 324–328, 1988.
17. Matrosovich M., Zhou N., Kawaoka Y., and Webster R.G., J Virol *73*, 1146–1155, 1999.
18. Weis W., Brown J.H., Cusack S., Paulson J.C., Skehel J.J., and Wiley D.C., Nature *333*, 426–431, 1988.
19. Sharp G.B., Kawaoka Y., Wright S.M., Turner B., Hinshaw V., and Webster R.G., Epidemiol Infect *110*, 161–176, 1993.
20. Sharp G.B., Kawaoka Y., Jones D.J., Bean W.J., Pryor S.P., Hinshaw V., and Webster R.G., J Virol *71*(8), 6128–6135, 1997.
21. Shortridge K.F., Semin Respir Infect *7*, 11–25, 1992.
22. Shortridge K.F., Vaccine *17*, S26–S29, 1999.
23. Nobusawa E., Aoyama T., Kato H., Suzuki Y., Tateno Y., and Nakajima K., Virology *182*, 475–485, 1991.
24. Cox N.J. and Subbarao K., Annu Rev Med *51*, 407–421, 2000.