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Antigenic and genetic analyses of H5 influenza viruses isolated from ducks in Asia

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Abstract

The hemagglutinin (HA) of six H5 influenza virus strains isolated from ducks in Japan and China in 1976 to 1996 were analyzed antigenically and genetically. Antigenic analysis using a panel of monoclonal antibodies revealed that the HA of H5 influenza viruses isolated from ducks are antigenically closely related to each other. Phylogenetic analysis indicates that the isolates from ducks in Hokkaido were derived from an ancestor common with the highly pathogenic isolates from chickens and humans in Hong Kong in 1997.

Key words: Duck, Hemagglutinin, H5, Influenza virus

Introduction

Influenza A viruses infect humans, pigs, horses, seals, whales, and variety of domestic and wild birds. Each of the known subtypes of the hemagglutinin (HA, H1-H15) and the neuraminidase (NA, N1-N9) of influenza A viruses has been isolated from aquatic birds⁹⁾. In general, influenza A virus isolates do not cause symptomatic infections in birds. However, a few influenza viruses of H5 and H7 subtypes become highly virulent, causing outbreaks of severe systemic disease of high morbidity and mortality³⁾

Nonpathogenic H5 avian influenza viruses acquire pathogenicity most consistently by molecular changes at the cleavage activation site of the HA¹⁵⁾. Highly pathogenic H5 avian influenza viruses possess multiple basic amino acid inserts at the cleavage site that is recognized by

ubiquitous intracellular proteases^{10,31)}, allowing the virus to spread systemically in birds with high mortality. In contrast, nonpathogenic viruses possess only one or two basic amino acids at the cleavage site of the HA and are not susceptible to these proteases^{2,3)}.

Influenza A viruses of avian origin have been implicated in outbreaks of influenza in mammals, such as seals^{8,33,34)}, pigs²⁶⁾, minks²¹⁾, and horses⁶⁾ as well as in domestic poultries^{1,7)}. Since each pandemic of human influenza first appeared in China³²⁾, the possibility has been raised that southern China is an influenza epicenter²⁹⁾. New human pandemic strains are believed to arise by genetic reassortment between human and non-human viruses^{5,22,36)}. It has been shown that H2N2 Asian and H3N2 Hong Kong pandemic influenza viruses are genetic reassortants between strains of human and avian

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origin^{12,27,35}). Antigenic and genetic analyses of H3 influenza viruses isolated from wild ducks captured on the Asian Pacific flyway in Japan, domestic ducks and pigs in southern China have shown that the HA of these viruses were closely related to those of the earliest human H3 viruses^{18,19,38}. These findings indicated that HA gene of the human pandemic strain, A/Hong Kong/68 (H3N2) was derived from such an avian virus¹⁷. Therefore, epidemiological surveillance of avian influenza would provide information on the next epidemics for other animal species, including human pandemics.

In May 1997, H5N1 influenza A virus was isolated from a 3-year-old child in Hong Kong with fatal respiratory illness¹¹. Preceding this incident, an outbreak of avian influenza virus (H5N1) was reported in Hong Kong. Nucleotide sequence analysis of all gene segments revealed that the human influenza H5N1 isolate was genetically closely related to highly pathogenic H5N1 avian influenza viruses isolated in Hong Kong⁴. The evidence indicates that H5 viruses with an avian HA gene may have the potential to adapt to humans and to cause an influenza outbreak. The extent of antigenic and genetic diversity among the HA of Asian duck H5 influenza viruses, therefore, should be informed.

In the present study, we analyzed the HA of the H5 influenza viruses isolated from ducks in Asia antigenically and genetically. The results indicate that the HA genes of H5 influenza viruses of ducks are conserved in nature.

Materials and Methods

Viruses

The influenza viruses used in the present study are from the repository in the Department of Disease Control, Graduate School of Veterinary Medicine, Hokkaido University; A/duck/ Miyagi/54/76 (Dk/Mi/54/76) (H5N3), A/duck/ Hong Kong/342/78 (Dk/HK/342/78) (H5N2), A/duck/Hong Kong/698/79 (Dk/HK/

698/79) (H5N3) A/duck/ Hong Kong/ 820/80 (Dk/HK/820/80) (H5N3), A/duck/ Hokkaido/4/96 (Dk/Hok/4/96) (H5N3), A/duck/ Hokkaido/ 67/96 (Dk/Hok/67/96) (H5N4), A/HongKong/156/97 (HK/156/97) (H5N1). These viruses were grown in 10-day-old embryonated hens' eggs at 35°C for 2 days.

Monoclonal antibodies (MAbs)

MAbs against A/chicken/Pennsylvania/ 1370/83 (H5N2)¹⁴ and A/chicken/ Pennsylvania/ 1/83 (H5N2)¹³ were kindly provided by R. G. Webster, St. Jude Children's Research Hospital.

Serological assays

Hemagglutination-inhibition (HI) tests were performed in microtiter plates with ascitic fluids²⁹.

Nucleotide sequencing

Influenza virus RNA was extracted from infectious allantonic fluid with RNeasy Mini Kit (QIAGEN). Full-length cDNA was synthesized from the viral RNA template by using Moloney murine leukemia virus reverse transcriptase (Gibco) and random hexamer primers. Polymerase chain reaction (PCR) direct sequencing of the HA gene was carried out using an autosequencer (Applied Biosystems Inc., CA). Primers were designed on the basis of the published nucleotide sequences of influenza virus HA genes as follows: forward primer (H5-515) 5'-CRTACCCAACAATAAAGAGG-3' and reverse primer (H5-1201R) 5'-GTGTTTCAATTTGTYAATGAT-3'.

Phylogenetic analysis of the HA genes

Nucleotide sequences of the HA genes (positions 559-1056) of influenza virus isolates were analyzed and compared with those from Genbank, by unweighted-pair group method with arithmetic means (UPGMA)²³, using a computer software, GENETYX-MAC version 9.0.

Results

Antigenic comparison of the HA of H5 influenza

viruses

The H5 influenza viruses of duck origin were analyzed by HI tests with a panel of MAbs to the HA molecule of A/chicken/Pennsylvania/1370/83 (H5N2) and A/chicken/Pennsylvania/1/83 (H5N2) strains (Table 1).

Six influenza virus strains isolated from ducks in the period of 1976 to 1996 gave identical

reactivity patterns and reacted with majority of antibodies. The results indicate that the antigenicity of the HA of H5 influenza viruses was highly conserved in duck population for 20 years. *Amino acid sequence at the cleavage site of the HA of the isolates*

Amino acid sequence at the cleavage site of the HA was deduced from the nucleotide sequence of the corresponding gene of each of the six

Table 1. Reactivity of H5 influenza viruses with a panel of MAbs

Viruses	MAbs to the HA of														
	A/chicken/Pennsylvania/1370/83											A/chicken/Pennsylvania/1/83			
	CP24	CP25	CP28	CP31	CP34	CP52	CP55	CP58	CP62	CP79	CP91	176/26	264/1	406/7	777/1
Dk/Mi/54/76 (H5N3)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Dk/HK/342/78 (H5N2)	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+
Dk/HK/698/79 (H5N3)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Dk/HK/820/80 (H5N3)	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+
Dk/Hok/4/96 (H5N3)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Dk/Hok/67/69 (H5N4)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
HK/156/97 (H5N1)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

Note. HI tests were performed with MAbs to A/chicken/Pennsylvania/1370/83(H5N2)¹⁴ and A/chicken/Pennsylvania/1/83(H5N2)¹³. + indicates antibody titers of 1:100–51,200; - indicates < 1:100.

Table 2. HA cleavage site sequence of the H5 influenza viruses

Viruses ^a	HA cleavage site sequence ^b	Pathotype
Dk/Mi/54/76 (H5N3)	321PQ---- <u>R</u> <u>E</u> <u>T</u> <u>R</u> /G	avirulent
Dk/HK/342/78 (H5N2)	PQ---- <u>R</u> <u>E</u> <u>T</u> <u>R</u> /G	avirulent
Dk/HK/698/79 (H5N3)	PQ---- <u>R</u> <u>E</u> <u>T</u> <u>R</u> /G	avirulent
Dk/HK/820/80 (H5N3)	PQ---- <u>R</u> <u>E</u> <u>T</u> <u>R</u> /G	avirulent
Dk/Hok/4/96 (H5N3)	PQ---- <u>R</u> <u>E</u> <u>T</u> <u>R</u> /G	avirulent
Dk/Hok/67/96 (H5N4)	PQ---- <u>R</u> <u>E</u> <u>T</u> <u>R</u> /G	avirulent
Turkey/Ireland/113/83 (H5N8)	PQ-- <u>R</u> <u>K</u> <u>R</u> <u>K</u> <u>K</u> <u>R</u> /G	virulent
Turkey/England/50-92 (H5N1)	PQ-- <u>R</u> <u>K</u> <u>R</u> <u>K</u> <u>T</u> <u>R</u> /G	virulent
Chicken/Hong Kong/220/97 (H5N1)	PQ <u>R</u> <u>E</u> <u>R</u> <u>R</u> <u>R</u> <u>K</u> <u>K</u> <u>R</u> /G	virulent
Hong Kong/156/97 (H5N1)	PQ <u>R</u> <u>E</u> <u>R</u> <u>R</u> <u>R</u> <u>K</u> <u>K</u> <u>R</u> /G	virulent

^a Viruses analyzed in the present study are bolded.

^b Dashes are included to align the sequences; basic amino acids are underlined.

isolates from ducks in Japan and China in 1976 to 1996. As shown in Table 2, all isolates had RETR sequence at the HA cleavage site that is typical amino acid sequence found in the HA of avirulent viruses.

Phylogenetic analysis of the HA genes

Fig. 1 shows an evolutionary tree constructed on the basis of partial sequences of the HA genes of the isolates from ducks together with those of other viruses from Genbank data base. The HA genes of H5 influenza A viruses diverge into two major lineages, Eurasian and North American²⁵). The HA gene of the H5 influenza virus isolates were clustered in the Eurasian influenza virus group. Miyagi and Hong Kong isolates (Dk/Mi/54/76, Dk/HK/

342/78, Dk/HK/698/79, Dk/HK/820/80) formed one distinct Eurasian sublineage, indicating that these are derived from a common ancestor, most likely a virus circulating among duck population. These isolates are most closely related to two pathogenic isolates from Ireland. Interestingly, the latest Hokkaido isolates (Dk/Hok/4/96, Dk/Hok/67/96) share a common ancestor with the highly pathogenic H5N1 influenza viruses (HK/156/97, Hong Kong/481/97, Hong Kong/482/97, Hong Kong/483/97, Chicken/Hong Kong/220/97, Chicken/Hong Kong/258/97, Chicken/Hong Kong/728/97, Chicken/Hong Kong/915/97) isolated from humans and chickens in Hong Kong in 1997.

Discussion

The present results of antigenic analysis of H5 influenza virus isolates from duck in Asia using a panel of MAbs has confirmed that the HA of duck influenza viruses are antigenically highly conserved in their reservoir hosts¹⁹). The reason why the HAs of influenza virus are antigenically conserved in duck population has been explained as follows: Laboratory studies have shown that the antibody response of ducks to avian influenza virus infection is weak and shortlived and ducks appear to be readily reinfected with the same virus²⁰). Even if they produce neutralizing antibodies, the serum antibodies may not be effective at inhibiting viral replication in the intestinal tract, the site where these viruses replicate in ducks^{30,37}).

It has been shown that the pathogenicity of avian influenza virus is associated with the presence of multiple basic amino acid at the cleavage site of the HA glycoprotein¹⁵). The present results showed all the HA of duck influenza viruses tested were nonpathogenic type, possessing only two monobasic amino acid residues at the cleavage site (321PQRETR/G327). Previous studies indicated that acquisition of the pathogenicity of avirulent virus strains

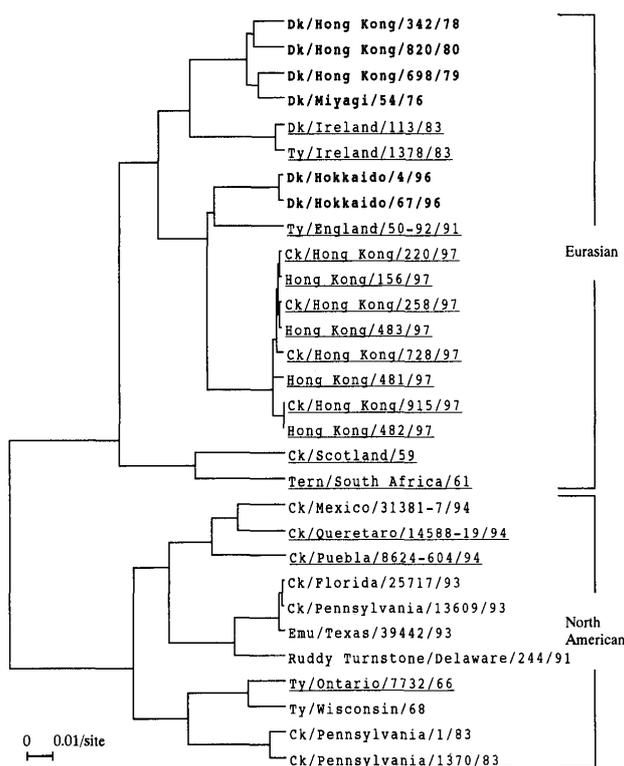


Fig. 1. Phylogenetic tree of the HA genes of H5 influenza viruses on the basis of their 500-base sequences. Horizontal distances are proportional to the minimum number of nucleotide differences required to join nodes and sequences. The tree was branched into Eurasian and North American lineages. Viruses analyzed in the present study are bolded. Virulent viruses are underlined.

was correlated with mutation at the cleavage site in the HA such as increase of the number of basic amino acid residues at the HA cleavage site or loss of carbohydrate attachment site at 11Asn in the vicinity of this site^{16,24}.

The transmission of avian H5N1 influenza virus from chickens to humans in Hong Kong in 1997 emphasized the need to be informed what subtypes and variants are dominant in aquatic birds as reservoir hosts. In the present study, phylogenetic analysis indicates that the isolates from ducks in Hokkaido (Dk/Hok/4/96, Dk/Hok/67/96) were derived from an ancestor common with the highly pathogenic isolates from chickens and humans in Hong Kong. Kida et al.¹⁸ proposed the mechanism of the emergence of new pandemic strains that the route of introduction of the HA gene is migratory ducks→domestic ducks→pigs→humans. The present results indicate that whatever the route of transmission of the new HA gene to humans is through pigs or directly from chickens, the gene is originated from influenza viruses circulating in duck population. To prepare for the emergence of next pandemic influenza, it is important to expand surveillance study of avian influenza in Siberia and Asia.

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