PHYLOGENIC ANALYSIS OF THE M GENES OF INFLUENZA VIRUSES ISOLATED FROM SHOREBIRDS

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ABSTRACT

Tracheal and cloacal swabs were collected from a total of 195 juvenile shorebirds in the area in 2012 in Eastern Hokkaido, Japan. Viruses showing hemagglutination activity were successfully recovered from 28 bird samples. All viruses were identified as AIV by RT-PCR to detect viral M genes of influenza A virus, and were determined to be H4N7 subtype by RT-PCR for subtyping of AIV. Phylogenic analysis of the matrix (M) genes of 12 strains selected on the basis of their different places and date of surveillance. The M genes of 12 strains belonged to the Eurasian Lineages.

KEYWORDS: Shorebirds, Influenza virus, M gene, Phylogenetic analysis

INTRODUCTION

Avian influenza (AI) caused by influenza A virus of the *Orthomyxoviridae* and one of the important zoonotic diseases in the world. Influenza A viruses have been isolated from many species, including humans, pigs, horses, mink, felids, marine mammals and a wide range of domestic birds.
The influenza A virus genome consists of eight segments of negative-stranded RNA, which code for 11 proteins. Influenza viruses are classified on the basis of two of these proteins: the hemagglutinin (HA) and neuraminidase (NA) glycoproteins [1].

Sixteen HA subtypes and nine NA subtypes of influenza A viruses have been found in AI viruses (AIVs) which were isolated from wild aquatic birds. Thus, wild birds such as waterfowl and shorebirds were considered as natural reservoirs for influenza A viruses. AIVs are known to be transmitted by such wild birds in their migration periods. The viruses replicate in the epithelial cells forming crypts in the colon and are shed in fecal materials therefore transmission among birds is mainly by fecal-oral route [3, 5]. Influenza A viruses are in evolutionary stasis in their natural hosts [7] that do not show clinical signs [5].

MATERIALS AND METHODS

Sample collection
Tracheal and cloacal swabs were collected from a total of 195 juvenile shorebirds between August 31 and September 14 in 2012 around the Komuke and Furen Lakes. After collection, all the samples were kept in virus transport medium (VTM) at -80°C until use.

Virus isolation

Tracheal and cloacal swab samples were suspended into VTM together. Each of the suspension was centrifuged at 12,00xg for 10 min and the supernatant was inoculated into 2 eggs (9-11 days-old embryonated chicken eggs). The eggs were incubated at 37°C for 3 days. After 72 hours of incubation at 37°C, the inoculated eggs were chilled overnight at 4°C and then allontoic fluids were collected and tested for hemagglutination activity. Later, all hemagglutinating agents were identified by hemagglutination inhibition [2] and neuraminidase inhibition [6] tests using the panel of specific anti-sera against reference strains of influenza viruses [4].

RNA extraction, RT-PCR, and nucleotide sequencing

Total RNA was extracted from harvested allantoic fluid by using Isogen reagent according to manufacturer instructions. cDNA was synthesized using Uni 12 primer.

cDNA samples were used for the identification of AIV HA and NA subtyping using 15 sets of H primers (H1-H15) [9] and 9 sets of N primers (N1-N9) [10].

For the sequencing, cDNA was also synthesized using Uni 12 primer and full-length M gene was amplified using M gene-specific primer set (Bm-M-1 and Bm-M-1027R) [8].

The PCR products were separated by 1% agarose gel electrophoresis and purified using QIAquick Gel Extraction Kit according to the company`s protocol.

The purified products were used as templates in sequencing reactions using a BigDye terminator ver. 3.1 cycle sequencing kit with the same primers as used for amplification.

Phylogenetic analysis

M genes of selected isolates, BLAST homology searches were used to retrieve sequences for tree construction. Phylogenetic analysis was based on sequence of M genes (position 1-950bp) of influenza viruses. The Nucleotide sequences were analyzed using GENETYX ver. 10 software (GENETYX Corp., Japan) and compared with other available sequences using BLAST homology searches.

The nucleotide sequences were aligned by Clustal W [9].

The M gene tree was generated using the Neighbor Joining (NJ) method and bootstrap analysis (1,000 replicates) implemented in the Molecular Evolutionary Genetics Analysis program (MEGA 5.1).

RESULTS

Isolation of influenza A viruses from tracheal and cloacal swabs of shorebirds

Tracheal and cloacal swabs were collected from a total of 195 juvenile shorebirds between August 31 and September 14 in 2012. From these, 28 influenza A isolated viruses were H4N7 subtype virus by RT-PCR (tab.1).

Table 1

Result summary of AIV subtyping by RT-PCR for the 28 isolates

Num. of sample	Sample ID	Species	Place	HA subtype	NA subtype
1	12EY0012	red-necked stint	Komuke lake	H4	N7
2	12EY0029	red-necked stint	Furen lake	H4	N7
3	12EY0044	red-necked stint	Furen lake	H4	N7
4	12EY0072	red-necked stint	Komuke lake	H4	N7
5	12EY0089	red-necked stint	Komuke lake	H4	N7
6	12EY0094	red-necked stint	Komuke lake	H4	N7
7	12EY0098	red-necked stint	Komuke lake	H4	N7
8	12EY0101	red-necked stint	Komuke lake	H4	N7
9	12EY0130	red-necked stint	Furen lake	H4	N7
10	12EY0131	red-necked stint	Furen lake	H4	N7
11	12EY0132	red-necked stint	Furen lake	H4	N7
12	12EY0133	red-necked stint	Furen lake	H4	N7
13	12EY0134	red-necked stint	Furen lake	H4	N7
14	12EY0135	red-necked stint	Furen lake	H4	N7

15	12EY0142	red-necked stint	Furen lake	H4	N7
16	12EY0144	red-necked stint	Furen lake	H4	N7
17	12EY0146	red-necked stint	Furen lake	H4	N7
18	12EY0154	red-necked stint	Furen lake	H4	N7
19	12EY0169	red-necked stint	Furen lake	H4	N7
20	12EY0170	red-necked stint	Furen lake	H4	N7
21	12EY0172	red-necked stint	Furen lake	H4	N7
22	12EY0173	red-necked stint	Furen lake	H4	N7
23	12EY0174	red-necked stint	Furen lake	H4	N7
24	12EY0178	red-necked stint	Furen lake	H4	N7
25	12EY0179	red-necked stint	Furen lake	H4	N7
26	12EY0180	red-necked stint	Furen lake	H4	N7
27	12EY0191	red-necked stint	Furen lake	H4	N7
28	12EY0193	red-necked stint	Furen lake	H4	N7

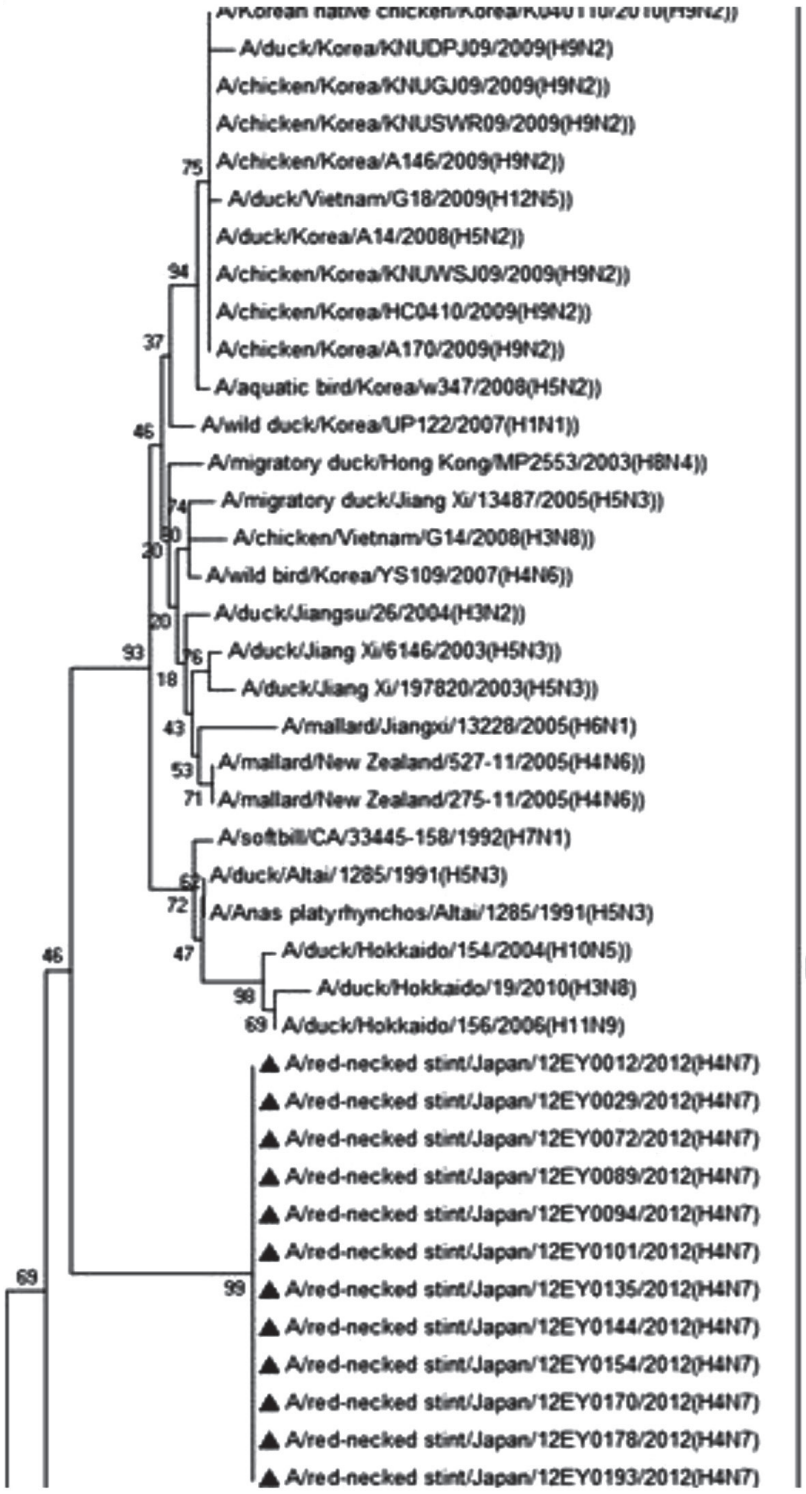
Phylogenic analysis of M genes of influenza virus isolates

For sequencing, we selected twelve samples, which derived from different places and date of surveillance.

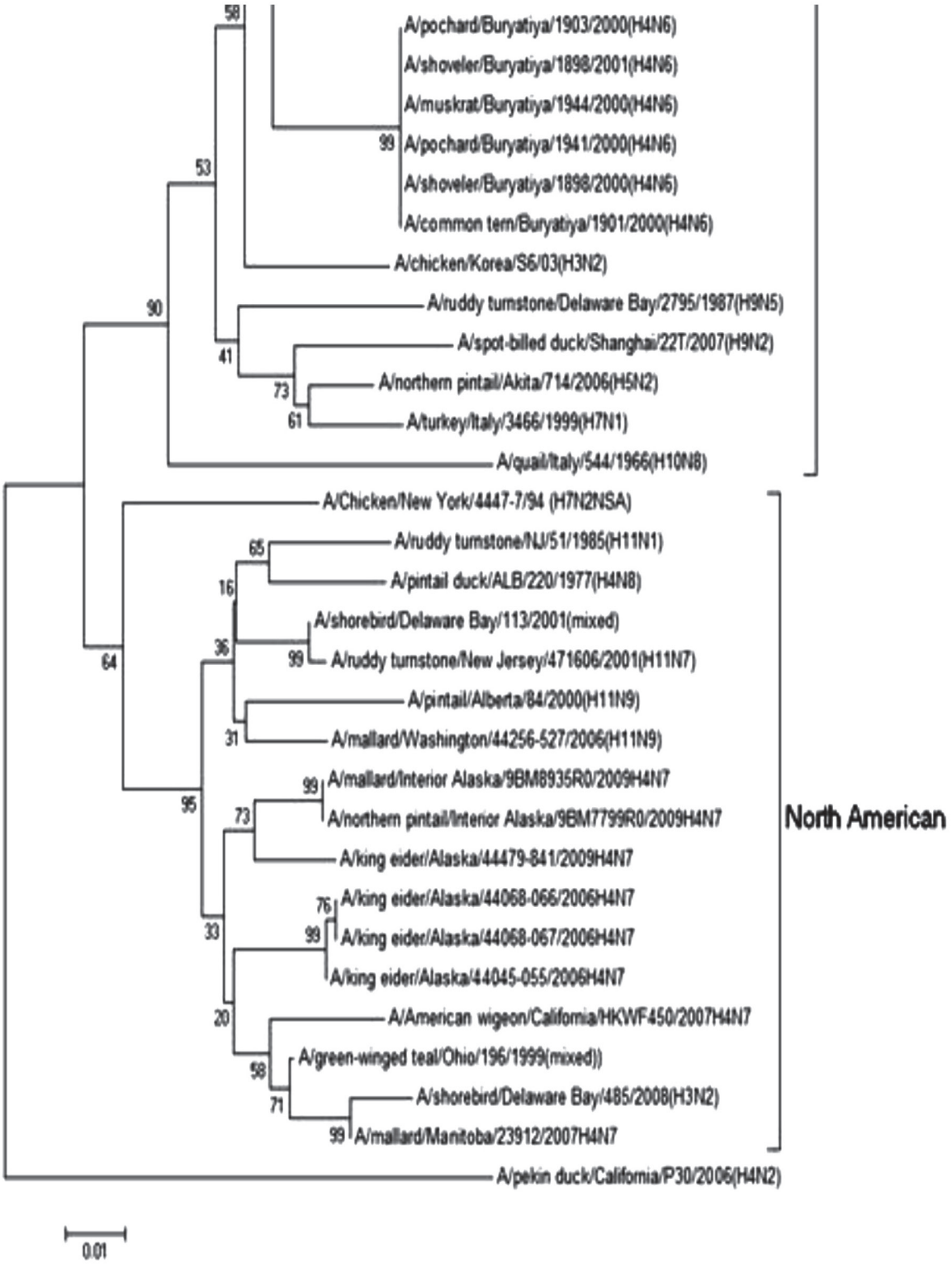
Full-length nucleotide sequences of the M gene were analyzed for all 12 isolates, and it was found that those isolates are 100% homologous in the M gene.

The BLAST homology searching showed that the M gene of all 12 isolates was close to A/duck/Jiangsu/26/2004(H3N2) in both nucleotide sequence homology (97.38%) and amino acid (99.6%). Furthermore, phylogenic analysis showed that M genes from 12 isolates belonged to the Eurasian lineage. The result and phylogenic analysis were shown in Figure 1.

Fig.1. Phylogenetic tree of influenza A virus M genes. The phylogenetic tree was constructed using Neighbor Joining (NJ) method (1,000 replicates) and Mega 5.1 software. The strains isolated in this study are highlighted by triangles.



Contd.,



DISCUSSION

We isolated 28 strains of the H4N7 subtype virus from red-necked stints in August of 2012. This is the first isolation of the H4N7 subtype virus in Japan. The M gene of all the 12 isolates analyzed was close to A/duck/Jiangsu/26/2004(H3N2) in both nucleotide sequence homology (97.38%) and amino acid (99.6%).

The result indicated that M genes of H4N7 isolates are phylogenetically grouped with Eurasian lineage viruses.

All of strains of the H4N7 subtype virus were isolated from red-necked stints. Red-necked stints are migratory birds. Many migratory birds are known to perform regular long-distance migrations [1], thereby potentially distributing LPAI viruses between countries or even

continents. Birds breeding in one geographic region often follow similar migratory flyways. Japan is included in the East Asian-Australian flyway, which covers part of eastern Siberia, western Alaska, southern to eastern Asia and Australia. Therefore, it is possible that virus-infected birds can transmit their pathogens to other populations that subsequently may bring the viruses to new areas.

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