FULL PAPER *Avian Pathology*

Surveillance of Avian Influenza Virus in Migratory Water Birds in Eastern Hokkaido, Japan

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ABSTRACT. The epidemiological information has obtained on avian influenza virus (AIV) in eastern Hokkaido, Japan, where AIV surveillance has not been performed. Cloacal or fecal samples obtained from migratory water birds were screened for AIV both by real-time reverse transcriptase polymerase chain reaction to detect the influenza A virus matrix (M) gene and by egg inoculation. Between 2007 and 2009, a total of 2,488 samples were collected from various avian species in Abashiri, Kushiro, Nemuro and Tokachi districts of eastern Hokkaido. AIVs were isolated from 18 of those samples (0.7%). No AIV was isolated from the 1,449 samples collected in Abashiri, Kushiro and Nemuro districts, although 6 were positive for the M gene by RRT-PCR. In contrast, 52 (5.0%) of the 1,039 samples collected from ducks in Tokachi district were M gene positive; AIVs were isolated from 18 of those samples (1.7%). The isolates included H3N5 (1 isolate), H3N6 (1), H3N8 (9), H4N2 (1), H4N6 (2), H6N5 (1), H6N8 (1), and H11N3 (2) subtypes. H3N5 and H11N3 subtypes have not been frequently isolated, and our study is the first to report H3N5 and the second to report H11N3 in Japan. Phylogenetic analysis revealed that the M genes of all isolates belonged to the Eurasian lineage.

KEY WORDS: avian influenza virus, eastern Hokkaido, surveillance, Tokachi district, water birds.

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Wild birds, predominantly waterfowl, are considered reservoirs for influenza A viruses. The 16 hemagglutinin and 9 neuraminidase antigenic subtypes of influenza A virus have been reported in wild birds, and avian influenza viruses (AIVs) are known to spread widely during the migration of wild birds [12, 29]. The interspecies transmission of whole AIV or AIV gene segments has been documented and may lead to the occurrence of new strains due to reassortment or adaptation to other species, resulting in rare but deadly outbreaks such as the 1918 Spanish flu epidemic [24].

Transport of highly pathogenic avian influenza (HPAI) viruses to different and distant geographic areas by wild birds was unknown until 2005–2006, when the HPAI H5N1 subtype viruses, which originated in Asia, were introduced into Russia, the Middle East, Europe, and Africa by infected migratory waterfowl [2, 3, 11, 12, 20, 28]. Information and understanding of the HPAI H5N1 virus in wild birds is limited, and it is unclear whether H5N1 viruses, like low pathogenic AI (LPAI) viruses, are perpetuated in these natural hosts.

Japan has experienced several outbreaks of the HPAI H5N1 subtype in poultry in recent years [23]. Migratory bird flyways from breeding areas in other locations to Japan are assumed to include the routes from the Kamchatka Peninsula to Hokkaido, Sakhalin Island to Hokkaido, the Korean Peninsula to Kyushu and others [4]. However, it should be noted that migratory routes are not always fixed [32]. However, there is no evidence that H5N1 viruses were introduced to poultry by migratory wild birds outside of Japan. In 2008, several dead or moribund whooper swans were found to be infected with the HPAI H5N1 virus in Tohoku district and Hokkaido. However, no infections with the virus were observed in poultry. Japan is part of the East Asia/Australia Shorebird Flyway, where not only eastern Asian and Australian countries but also Alaska is involved. This shorebird flyway also has an overlapping area with the Pacific and Mississippi Americas Flyways in Alaska [1, 11, 12]. Thus, water birds flying across those continents could distribute AIVs on a near-global scale. Our understanding of the global distribution of AIV and the significance of wild birds in the perpetuation of influenza A viruses remains limited, even though extensive surveillance has been conducted. Continued surveillance studies are necessary to increase our understanding of the ecology of AIV in natural hosts.

This study was performed to collect epidemiological information on AIV in eastern Hokkaido, the northernmost island in Japan. Hokkaido is an important area of Japan for AIV surveillance, because it is located within multiple wild bird flyways [1, 12]. Surveillance studies have been conducted in central and northern Hokkaido [10], but none so far have reported on samples from eastern Hokkaido, including Abashiri, Kushiro, Nemuro, and Tokachi districts. In this study, we report the first isolation of AIVs in Tokachi district, Hokkaido, Japan, and present a phylogenetic analysis of the isolates.

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MATERIALS AND METHODS

Sample collection: Cloacal swab or fecal samples were collected from wild birds in eastern Hokkaido, Japan, during 2007 through 2009 (Table 1). Cloacal samples were collected by licensed bird banders, who also identified bird species. Fresh fecal samples were collected near the birds' habitats. The samples were placed in sterile tubes containing a virus transport medium (VTM) (M4RT; Remel, Inc., Lenexa, KS, U.S.A.) and stored at –80ºC until use. Fecal samples were placed in plastic bags and stored at –80ºC.

RNA extraction and cDNA synthesis: Viral RNA was extracted from 50 μl of the original samples or allantoic fluids (AFs) using KingFisher Purification Systems (Thermo Fisher Scientific, Waltham, MA, U.S.A.) and a MagMAX-96 AI/ND Viral RNA Isolation Kit (Thermo) according to the manufacturer's instructions. The RNA was eluted with 50 μl of the elution buffer. First-strand cDNA was produced using 2.0 μl random hexamer primers (0.5 $\mu g/\mu l$), 1.0 μl 10 mM dNTP, 4.0 μl RNA, 6 μl DEPC-water, 4.0 μl 5× first-strand buffer, 2.0 μl 0.1 M DTT, 1 μl RNAse inhibitor, and 1.0 μl MMLV reverse transcriptase (Invitrogen) (total volume: 21 μl). The reaction was incubated at 25°C for 10 min, 37°C for 50 min, and 65°C for 10 min and placed on ice.

Real-time reverse transcription-polymerase chain reaction (RRT-PCR): RRT-PCR to detect the influenza A virus matrix (M) gene was carried out using a TaqMan probe assay as described in a previous report [19]. In brief, each reaction mixture included 12.5 μl of the 2× universal master mix (Applied Biosciences, Foster City, CA, U.S.A.), 1 μl of each primer (20 mM), 1 μl 5 μM FAM probe, 3 μl cDNA, and 6.5 μl DEPC water (total volume: 25 μl). Cycling conditions in an ABI PRISM Sequence Detection System 7900HT (Applied Biosciences) were as follows: Stage 1—95°C for 10 min, and Stage 2—40 cycles of 95°C for 15 sec and 60°C for 1 min. The primer sets, and the probe for partial M gene detection. The sequence of primers and probes used are as follows: forward primer 5'-aratgagtcttctraccgaggtcg-3', reverse primer 5'-tgcaaagacatcytcaagyytctg-3', and probe FAM-tcaggccccctcaaagccga-TAMRA. When samples gained the threshold cycle (Ct) value, they were tentatively regarded as M gene positive in this study. A positive control (A/Eq/Miami/1/63) was placed in the RRT-PCR, and the resulting Ct value was approximately 30.

Virus isolation: Virus isolation from the samples was carried out using 10-day-old embryonated chicken eggs. Prior to the inoculation into the eggs, the samples in VTM were centrifuged at 1,000 × *g* for 10 min at 4°C. The supernatants were supplemented with penicillin G (final concentration of 1,000 U/m*l*), streptomycin (1 mg/m*l*), gentamycin (100 μg/m*l*), and amphotericin B (10 μg/m*l*) and were kept at room temperature for 2 hr. A 20% fecal suspension in minimum essential medium (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) supplemented with antibiotics and antimycotics was prepared and kept at room temperature for 2 hr followed by centrifugation.

Then, 0.1 m*l* of the sample supernatant was inoculated into the allantoic cavity of each egg (2 eggs per sample). After 3 days of incubation at 37°C, the eggs were chilled overnight at 4°C. The AFs collected from the eggs (E1) were tested using a hemagglutination (HA) test according to the World Health Organization (WHO) manual [30]. HA-negative E1 samples were forwarded to the second egg inoculation, and the obtained AFs (E2) were tested for HA activity.

Samples that showed positive results for both the M gene in the original samples and HA in the E1 or E2 samples were determined to be AIV isolate. Samples that showed M gene positive results only were regarded as AIV suspect.

AIV subtyping: The AF samples (E1 or E2) of the AIV isolates were subjected to RT-PCR for HA and NA subtyping using 15 sets of H primers (H1–H15) [9] and 9 sets of N primers (N1–N9) [18]. PCR was performed using 1 μl cDNA, 1 μl of each primer (20 μM), 1.6 μl dNTP (all 2.5 mM), 2 μl 10× Taq Buffer, 13.3 μl DEPC water, and 0.1 μl Takara Ex Taq (Takara, Shiga, Japan). The PCR conditions were as follows: 94ºC for 5 min; 40 cycles of denaturation at 94°C for 30 sec, annealing at 50–60°C for 30 sec (depending on the primer set), and extension at 72°C for 40 sec; and final extension at 72°C for 10 min. The annealing temperatures for the primer sets varied and were 50°C for H1, H5, H6, H7, H8, H9, H10, H12, and H13; 55°C for H2, H3, H4, H11, H14, and H15; and 60°C for all N-primer sets.

AIV subtyping was also performed using hemagglutination inhibition (HI) and neuraminidase inhibition (NI) tests according to the WHO Manual [30]. The reference antisera against influenza viruses and the reference viruses for the HI and NI tests were provided by Dr. H. Kida, Hokkaido University, Japan.

Nucleotide sequencing and phylogenetic analysis: Total RNA extracted from AF containing the virus was transcribed into cDNA using the Uni12 primer (5'-agcraaagcagg-3') and SuperScript III Reverse Transcriptase (Invitrogen) at 42°C for 60 min followed by 70°C for 10 min. The cDNA samples were used as template for PCR to amplify a full length of the M gene using the primer sets described by Hoffmann *et al.* [6]. The PCR products obtained were separated by 1% agarose gel electrophoresis and purified using a QIAquick PCR Purification Kit (Qiagen, Hilden, Germany). The purified products were used as a template for sequencing reactions using a BigDye terminator ver. 3.1 cycle sequencing kit (Applied Biosystems) and the WHO M gene primers M30F, M264R2 [31], and M-1023R [30]. Nucleotide sequencing was performed with an ABI PRISM 310 Genetic Analyzer (Applied Biosystems). The obtained sequences (nucleotide positions 1–783) were analyzed by GENETYX ver. 9.0 software (GENETYX Corp., Tokyo, Japan) and compared with other available sequences using BLAST homology searches. Evolution distances were calculated using the aligned sequences and the Tamura-Nie model [26, 27]. Phylogenetic trees were constructed using the Neighbor Joining (NJ) method supported by 1,000 bootstrap replicates [21] and Mega 4.0 software [25].

RESULTS

Sample collection: Samples collected during this study are summarized in Table 1. Between February 2007 and May 2008, 1,449 samples were collected from a variety of water bird species at lakefronts and seashores in Abashiri, Kushiro, and Nemuro districts of eastern Hokkaido. During February through December 2007, 449 samples that mainly consisted of shorebirds were collected in multiple locations. The remaining 1,000 samples were collected during May 2008 at the Notsuke Peninsula and Lake Saroma after dead or moribund whooper swans were found to be infected with the H5N1 virus. These samples were collected with the intent of HPAI surveillance. The examined species were biased toward wigeons.

In Tokachi district, 1,039 duck fecal samples were collected along a riverside. Among those, 720 were collected between October and December 2008, and 319 were collected between January and February 2009.

Virus isolation: Of the 1,449 samples collected in eastern Hokkaido excluding Tokachi district, no AIV was isolated (Table 2). RRT-PCR results showed that 6 samples (4 collected in 2007 and 2 collected in 2008) were M gene positive, with Ct values ranging from 36.99 to 41.58. However, no virus was detected in the eggs even after the passage E2; therefore, the 6 samples were regarded as AIV suspect (0.4% prevalence).

In contrast, 52 of the 1,039 samples (5.0%) collected in Tokachi district were M gene positive. Among those, 18 were recovered as AIV isolates (1.7%) and 34 were determined to be AIV suspect (3.3%). All of the 18 AIV isolates were from the 720 samples collected between October and December 2008 (2.5%). In addition to the AIV isolates, 32 samples (4.4%) were regarded as AIV suspect in the October–December samples (Table 2).

Among the 18 AIV isolates, 16 were M gene positive by RRT-PCR from the original samples, and Ct values ranged from 29.18 to 39.56. The remaining 2 samples, KI0040 and KI0184, were negative in the first RRT-PCR but were positive in the second RRT-PCR of E1 samples (Table 3).

Ct values of the 34 AIV suspect samples in the first RRT-PCR were between 30.49 and 42.36; 9 samples had values below 35.00, and 6 samples had values over 40.00. All of the samples except 1 (KI0066) was M gene negative in the second RRT-PCR of E1 samples. Although KI0066 was positive in both the first and second RRT-PCR, no virus was isolated from eggs inoculated with the samples (E1 and E2). The E2 sample was further forwarded through another passage into eggs (E3), but virus was not isolated (Table 4).

Subtypes of the AIV isolates in the October samples included H3N5 (1 isolate), H3N6 (1), H3N8 (9), H4N2 (1), H4N6 (2), H6N5 (1), and H6N8 (1). Among these subtypes, H3N8 was the most prevalent. The other 2 AIV isolates obtained from the December samples were identified as subtype H11N3 (Table 3). The H3N5 subtype was the first isolated in Japan, and the H11N3 subtype was the first isolated

Table 1. Cloacal and fecal samples collected from water birds in eastern Hokkaido

Location (district)	Sampling period	No. of samples	Origin species (number)
Abashiri, Kushiro, Nemuro[a)]	Feb-Dec/2007	449[c)]	Grey-rumped Sandpiper (127), Rufous-necked Stint (95), Slaty-backed Gull (95), Pintail (2), Black-tailed Gull (26), Dunlin (21), Green-winged Teal (11), Mallard (10), Others (62)
Abashiri, Nemuro	May/2008	1,000[c,d)]	Wigeon (831), Wigeon or Common Teal (49), Grey-tailed Tattler (42), Others (78)
Tokachi[b)]	Oct-Dec/2008	720[d)]	Duck (720)
	Jan-Feb/2009	319[d)]	Duck (319)

a) Latitude and longitude coordinates for the location are 42°59' to 44°16'N, 143°29' to 145°35'E. b) Location of sample collection: 42°59'N, 143°11'E. c) Cloacal sample. d) Fecal sample.

Table 2. AIV screening by RRT-PCR and egg inoculation[a)]

Location (district)	Sampling period	AIV isolates	AIV suspects	AIV isolates + suspects
Abashiri, Kushiro, Nemuro	Feb-Dec/2007	0/449 (0.0%)	4/449 (0.9%)	4/449 (0.9%)
Abashiri, Nemuro	May/2008	0/1,000 (0.0%)	2/1,000 (0.2%)	2/1,000 (0.2%)
Tokachi	Oct-Dec/2008	18/720 (2.5%)[b)]	32/720 (4.4%)	50/720 (6.9%)
	Jan-Feb/2009	0/319 (0.0%)	2/319 (0.6%)	2/319 (0.6%)
	Total	18/2,488 (0.7%)	40/2,488 (1.6%)	58/2,488 (2.3%)

a) Original samples were tested by both RRT-PCR for the M gene and egg inoculation followed by the HA test; samples with positive results for both the M gene and HA test were determined as AIV isolates, and samples that were M gene positive only were regarded as AIV suspect. b) Among the 18 AIV isolates, 16 were from October samples (16/414; 3.9%), and 2 were from December samples (2/209; 1.0%).

Table 3. Comparison between Ct values and HA titers of the AIV isolates in Tokachi district

Sample ID	Date at collection	Ct value		HA titer		AIV subtype determined
		Original	E1	E1	E2	
KI0040	Oct 08	N[a)]	28.44	<2	128	H3N5
KI0041	Oct 08	35.35	33.61	<2	128	H3N8
KI0067	Oct 08	31.59	18.58	128	NT[b)]	H3N8
KI0068	Oct 08	35.65	21.37	512	NT	H3N6
KI0095	Oct 08	29.95	19.34	64	NT	H3N8
KI0102	Oct 08	38.40	19.39	128	NT	H3N8
KI0129	Oct 08	34.22	19.12	256	NT	H3N8
KI0135	Oct 08	29.63	18.14	64	NT	H6N5
KI0148	Oct 08	31.54	20.07	128	NT	H4N2
KI0150	Oct 08	33.23	18.94	128	NT	H3N8
KI0162	Oct 08	33.22	19.11	128	NT	H3N8
KI0180	Oct 08	36.13	18.58	64	NT	H3N8
KI0184	Oct 08	N	19.51	128	NT	H4N6
KI0185	Oct 08	31.31	23.99	2	64	H4N6
KI0195	Oct 08	29.18	20.95	64	NT	H6N8
KI0236	Oct 08	39.56	20.95	128	NT	H3N8
KI0647	Dec 08	33.58	31.72	128	NT	H11N3
KI0665	Dec 08	32.08	30.63	256	NT	H11N3

a) Ct value was not obtained and was regarded as M gene negative. b) Not tested.

Table 4. Comparison between Ct values and HA titers of the AIV suspects in the Tokachi samples

Number of samples	Ct values		HA titers	
	Original	E1	E1	E2
1 (KI0066)[a)]	33.59	40.5	<2	<2
8[b)]	30.49–34.80	N[c)]	<2	<2
19	35.44–39.94	N	<2	<2
6	40.11–42.36	N	<2	<2

a) Sample ID is in parentheses; for this sample, additional egg passage of the E2 AF was performed, but HA activity was not detected in the AF (E3). b) Number does not include KI0066. c) Ct value was not obtained and was regarded as M gene negative.

in Hokkaido.

Phylogenetic analysis of the AIV isolates: The M gene sequences of the 18 isolates and others from GenBank were analyzed phylogenetically, and all 18 isolates were found to belong to the Eurasian lineage, which can be grouped into sublineages I and II (Fig. 1).

In Eurasian sublineage I, 7 of 9 H3N8 viruses and 1 each of H4N2, H6N5, and H6N8 viruses were included. The remaining H3N8 viruses (8KI0095 and 8KI0150) were grouped into Eurasian sublineage II, although all H3N8 viruses were isolated from samples collected on the same day at the same location. Other isolates, including the H3N5 and H11N3 subtypes, were also grouped into Eurasian sublineage II. The H11N3 viruses were phylogenetically different from other H11N3 viruses isolated in New Zealand, China, and Sweden and were most closely related to A/duck/Hokkaido/201/07 (H5N3).

No isolates were grouped into the North American lineage (Fig. 1).

DISCUSSION

AIV surveillance studies have been conducted in multiple locations in Japan, including the San-in district (western part of Japan) [5, 13–16, 22], Tohoku district (northeastern part of Japan) [7, 8], and Hokkaido (northernmost prefecture) [10]. In the previous Hokkaido study, fecal samples from free-flying water birds were collected during 2000–2007 at 2 distinct locations in central Hokkaido (Sapporo) and northern Hokkaido (Wakkanai). The prevalence of AIVs isolated from these 2 locations was high, 6.4 and 5.4%, respectively [10]. In the current study, we conducted AIV surveillance in multiple locations in eastern Hokkaido, in areas where no such studies had been conducted previously (Table 1).

A total of 18 AIVs were isolated from the 720 duck fecal samples collected along the riverside in Tokachi district during October through December 2008 (2.5% prevalence). Among these, 16 originated from the October samples, and 2 originated from the December samples. No AIV was isolated from the samples collected between January and February 2009 (Table 2). In the prior studies, the prevalence of AIV varies by year or by sampling location [5, 7, 10]. Our studies are continuing to accumulate epidemiological information on AIV in eastern Hokkaido.

No AIV was isolated from samples collected in eastern Hokkaido, excluding Tokachi district (Table 2). Sampling in 2007 focused on shorebirds rather than ducks; prior to 2007, studies of shorebirds had been limited. However, because bird species, times, and locations varied during sampling, interpretation of the results was difficult. In 2008, several dead or moribund whooper swans infected with lethal H5N1 viruses were found in Abashiri and Nemuro districts in May. The negative results of the 2008 samples collected

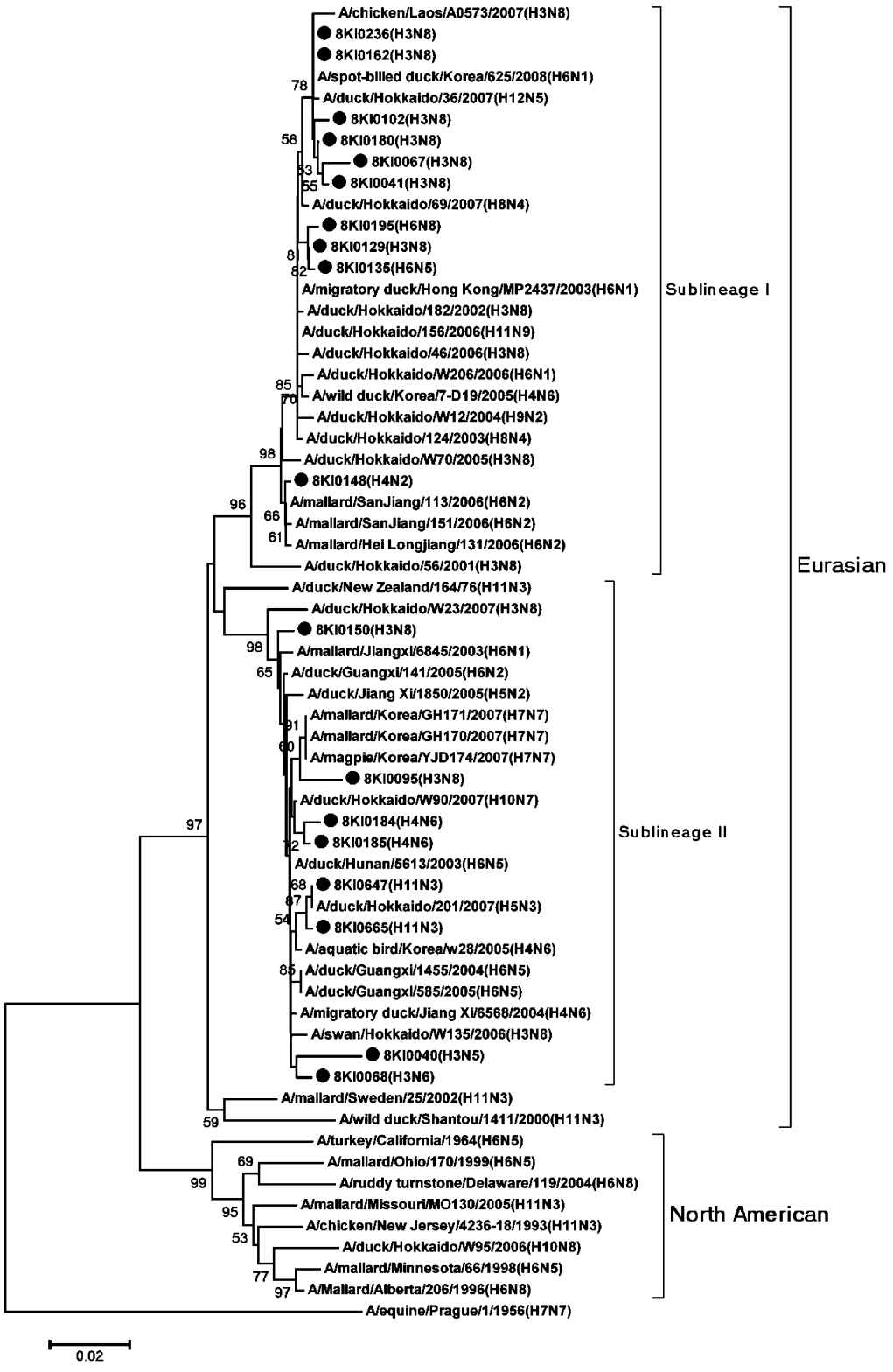

Fig. 1. Phylogenetic tree of AIV M genes. The phylogenetic tree was constructed using the Neighbor Joining (NJ) method (1,000 replicates) and Mega 4.0 software. The analysis was based on nucleotides 1–783 of the M gene. Numbers at each branch point indicate bootstrap values ≥50% in the bootstrap interior branch test. The strains isolated in this study are marked with closed circles.

in the middle of May suggested that widespread transmission of the H5N1 virus from the infected whooper swans to other bird species at the concerned spots was unlikely.

Predominant HA subtypes obtained in this study were H3, H4, and H6, corresponding to results reported in previous studies [10, 12]. Subtype combinations included H3N5 (1 isolate), H3N6 (1), H3N8 (9), H4N2 (1), H4N6 (2), H6N5 (1), H6N8 (1), and H11N3 (2); the most prevalent subtype was H3N8 (Table 3). In northern and central Hokkaido from 2000 to 2007, migratory water birds flying mainly via the Sakhalin Island route were investigated by other researchers [4, 10]. Eastern Hokkaido migratory water birds are assumed to fly from their breeding areas mainly via the Kamchatka Peninsula route. Combinations of the HA and NA subtypes, except for the 2 (H3N5 and H11N3) reported here, were coincident with the subtypes reported in the prior studies on Hokkaido, whereas only the H4N6, H6N5, and H6N8 subtypes isolated in this study were reported in San-in and Tohoku districts from 2001 to 2008 [5, 7, 8]. Interestingly, no H3N8 viruses were isolated in San-in and Tohoku districts during that time; only 2 H3N8 viruses have been isolated in San-in district, in 1981 and 1982 [15], although they have been isolated in Hokkaido more frequently and in surveillance studies by other researchers [10]. These results possibly suggest that some migratory wild birds flying to the San-in and Tohoku districts and those flying to Hokkaido originate from different breeding areas.

Isolation of the H3N5 and H11N3 subtypes is uncommon worldwide, and the H3N5 virus obtained in this study is the first isolate of that particular subtype recovered in Japan. The nucleotide sequence of HA and NA genes of A/swan/Shimane/183/1985 (H11N3) isolated from a pintail in San-in district in 1985 was recently released (GenBank accession numbers AB296076 and AB296077, respectively). The H11N3 subtype has not been isolated in Hokkaido [10]. A phylogenetic tree for the HA gene of the H11 subtype showed that A/swan/Shimane/183/1985 belonged to the American lineage [17]. It should be interesting to compare the complete genomic sequences of the 2 H11N3 subtype strains isolated in Japan after 25 years to understand the ecology of this uncommon strain.

In this study, only the M gene was analyzed for all 18 isolates. Phylogenetic analysis revealed that all of the M gene sequences belonged to the Eurasian lineage. Further analyses on other gene segments are required to understand the evolution of AIV. Manzoor *et al*. [10] reported that the H10N8 virus isolated in northern Hokkaido, Japan, was a reassortant with an M gene that belonged to North American non-gull-avian lineages and other genes that belonged to Eurasian non-gull-avian lineages. This result suggests that lands under overlapping migratory waterfowl flyways, such as Japan's islands, may be a mixing bowl for AIV, generating new reassortant viruses. Undoubtedly, surveillance studies should be continued globally to monitor the generation of new, virulent viruses and to prepare for new pandemics.

As a screening method, we used RRT-PCR for the original samples combined with egg inoculation (the gold standard method for AIV isolation). Of the 18 samples from which AIV was subsequently isolated, 2 samples could not be detected in the first RRT-PCR testing of the original samples (Table 3). However, these samples were HA positive on either E1 (KI0184) or E2 (KI0040). Conversely, 1 M-gene-positive sample (KI0041) was HA positive when tested for E2 but not E1. RRT-PCR is a sensitive and reliable quantification method that can provide timely and cost-effective results. RRT-PCR results depend on virus particle numbers in the samples regardless of infectivity. This method may provide the opportunity to examine virus prevalence even if virus infectivity has been lost by environmental factors such as UV. However, RRT-PCR cannot detect the virus when numbers are below the threshold level. In contrast, the egg inoculation method strictly reflects the infectious virus number, and passages of the sample effectively promote virus amplification. The WHO manual suggests repeating the passage twice before reporting an inability to recover virus from the sample, but this procedure requires a weekly timeframe. In this study, we found that the Ct values in RRT-PCR testing of E1 might be useful for AIV isolation. AIV was successfully isolated from all samples with E1 Ct values of 33.61 or lower (Table 3); we could not isolate virus from samples with Ct values of 40.5 or higher (Table 4). The combined method using egg inoculation and RRT-PCR might be applicable for isolating AIV and useful for reducing labor, time, and the number of eggs required for AIV surveillance—especially by using automated devices such as KingFisher Purification Systems.

This is the first report on AIV surveillance in eastern Hokkaido, Japan. Results of this and ongoing studies provide useful, new information on the epidemiology and ecology of AIV.

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