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Epidemiologic Survey of Avian Influenza Virus Infection in Shorebirds Captured in Hokkaido, Japan

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ABSTRACT: There is limited information about virus epidemiology of shorebirds (family Charadriidae and Scolopacidae) in the East Asia-Australasia flyway. We investigated the prevalence of avian influenza viruses (AIVs) in shorebirds in Hokkaido, Japan, the stopover site of the flyway, to understand the ecology of AIV translocation in the flyway from 2006 to 2010. In total, 1,698 shorebirds belonging to 26 species were captured and released into two different sites using mist nets. Tracheal and cloacal swabs were collected from each bird using cotton swabs. The RNA of influenza A viruses was detected using reverse transcription loop-mediated isothermal amplification. One AIV-positive sample was obtained from a Lesser Sand Plover (*Charadrius mongolus*) captured in September 2010 at Lake Komuke. Full lengths of hemagglutinin (HA), neuraminidase (NA), polymerase acidic protein, nucleoprotein, matrix protein 1, and nuclear export protein genes were successfully amplified from the AIV-positive sample. All sequences showed the highest identity with sequences obtained from virus strains from Anseriformes species. Shorebirds migrated to Japan 1 mo earlier than did Anseriformes species. Therefore, the Lesser Sand Plover could have been infected by the virus from Anseriformes species on the breeding grounds. The HA sequence showed the highest identity with the H10 sequence whereas the NA sequence exhibited the highest identity with the N7 sequence. Phylogenic analysis showed that the detected subtype H10N7 belongs to the Eurasia lineage and the related strain might have widely spread in Asia in 2009.

Key words: Avian influenza virus, East Asia-Australasia Flyway, shorebird.

Avian influenza virus (AIV) of the family Orthomyxoviridae has been detected in over 100 bird species belonging to 13 avian orders, with the orders Anseriformes and Charadriiformes constituting the most important reservoirs of AIV (Webster et al. 1992; Olsen et al. 2006; Stallknecht et al. 2007). In particular, shorebirds (families Scolopacidae and Charadriidae) could play an important role in global AIV translocation because they breed in the Northern Hemisphere during summer and migrate to the Southern Hemisphere during winter. There are eight recognized flyways of shorebird species (Boere and Stroud 2006), and various epidemiologic surveys have been performed on the East Atlantic, Mediterranean-Black Sea, West Asia-Africa, and Atlantic America flyways (Gaidet et al. 2012; Hall et al. 2014; Maxted et al. 2016). However, there is limited information about virus epidemiology in the East Asia-Australasia flyway. Although AIV prevalence data have been collected in Alaska (Ip et al. 2008; Winker et al. 2008), a portion of the flyway and breeding ground of shorebirds, no data are available on AIV prevalence at the stopover sites such as Hokkaido, Japan. In summer to autumn, shorebirds migrate to Hokkaido from Siberia and Alaska on their way to wintering grounds, mainly in Oceania (Fig. 1). In spring, they migrate to Hokkaido from the wintering ground on their way to breeding grounds. Sampling during autumn migration could show the highest prevalence of AIV because they migrate from Siberia and Alaska, where there are various kinds of AIV strains in the environment. Hence, we explored the prevalence of these viruses in shorebirds that have migrated to Hokkaido, Japan to gain a better understanding of AIV translocation in this flyway.

Between July and September (during autumn migration) of 2006–10, 1,698 shorebirds belonging to 26 species were captured using mist nets, sampled, and released at two sites in

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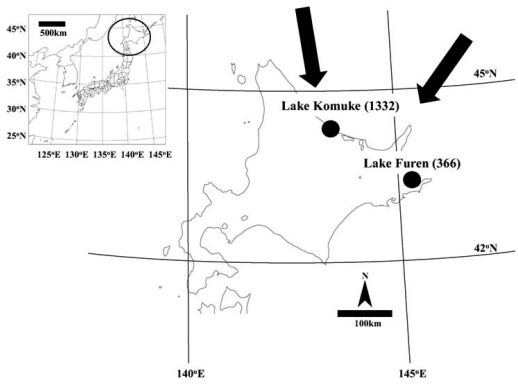


FIGURE 1. Location map of sites in Hokkaido, Japan, where birds were captured for testing for avian influenza virus from 2006 to 2010. The numbers in parentheses indicate the captured bird number in each site. Arrows indicate the direction of autumn migration of shorebirds.

Hokkaido (1,332 individuals in Lake Komuke and 366 individuals in Lake Furen; Table 1 and Fig. 1). The two locations are major monitoring sites for nationwide shorebird populations.

All procedures were conducted by licensed bird banders and were authorized by the Ministry of Environment, Japan and the Yamashina Institute for Ornithology, Japan, and all operations were permitted by the Ministry of Education, Culture, Sports, Science and Technology, Japan for academic research purposes. Cloacal and tracheal swabs were collected from the captured birds. The swabs were preserved in the BD™ Universal Viral Transport medium (Becton Dickinson, Franklin Lakes, New Jersey, USA), transported to the National Institute for Environmental Studies, Tsukuba, and stored at –80 C until total nucleic acid extraction. Total nucleic acid was extracted from the viral transport medium using the EZ1 Virus Mini Kit v2.0 (Qiagen, Hilden, Germany) following previously reported procedures (Onuma et al. 2017) and subjected to reverse transcription loop-mediated isothermal amplification (RT-LAMP; Eiken Chemical Co., Ltd., Tokyo, Japan), which is used as the standard method for nationwide AIV survey in Japan. The RT-LAMP primer set was designed for the M gene (Yoshida et al. 2011). The reported sensitivity of RT-LAMP for fecal samples is $10^{2.5}$ copies of viral RNA and that for allantoic fluid of embryonated chicken (*Gallus gallus domesticus*) eggs is $10^{2.9}$ copies of viral RNA (Yoshida et al. 2011).

Full-lengths of the hemagglutinin (HA) and neuraminidase (NA) genes were amplified in RT-LAMP–positive samples for virus subtyping. One-step reverse transcription-PCR (RT-PCR) was performed to prepare the templates for HA and NA gene amplification using the primers Uni12 and Uni13 (Skehel and Hay 1978; Desselberger et al. 1980; Hoffmann et al. 2001). A one-step RT-PCR reaction mixture was prepared using a Takara PrimeScript High Fidelity RT-PCR Kit (Takara Bio, Inc., Shiga, Japan). One-step RT-PCR was conducted with a Gene Amp PCR System 9700 (Applied Biosystems, Waltham, Massachusetts, USA) using the following conditions: one cycle of 42 C for 30 min and 94 C for 2 min; 40 cycles of 98 C for 10 s, 30 C for 30 s, and 72 C for 7 min; one cycle of 72 C for 7 min, and a holding step at 4 C. The reaction mixtures were diluted 1:50 in tris-ethylenediaminetetraacetic acid buffer and the diluted PCR reaction mixtures were prepared using KOD-Plus-Ver.2 (Toyobo Life Science, Tokyo, Japan) and used for PCR amplifications of the HA and NA using the reported primer sets (Tseng et al. 2014). We conducted PCR amplifications with a Gene Amp PCR System 9700 (Applied Biosystems) using the following conditions: 94 C for 2 min; 35 cycles of 98 C for 10 s, 50 C for 30 s, and 68 C for 2 min, followed by a hold step at 4 C. The PCR products were purified with the QIAquick PCR Purification Kit (Qiagen) and direct sequencing was performed using the BigDye® Terminator v3.1 cycle sequencing kit (Applied Biosystems) and the 3130 Genetic Analyzer (Applied Biosystems). The sequences were analyzed by BLAST in GenBank (National Center for Biotechnology Information 2016) for virus subtyping.

Among the 1,698 swab samples collected between July and September of 2006–10, one AIV-positive sample was detected from a Lesser Sand Plover (*Charadrius mongolus*) captured in Lake Komuke in September 2010.

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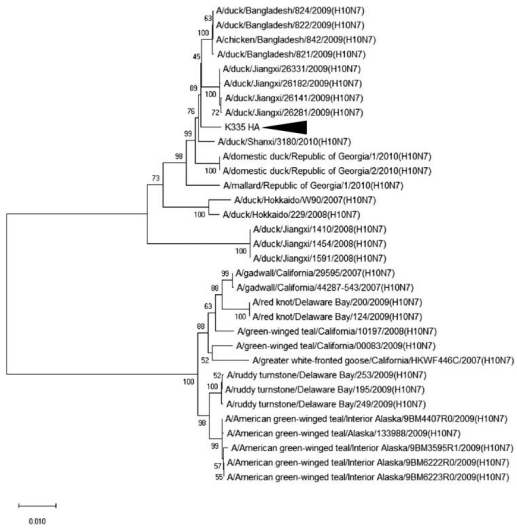


FIGURE 2. Phylogenic tree of avian influenza virus subtype H10N7 sequences using the hemagglutinin gene sequence obtained from the Lesser Sand Plover (*Charadrius mongolus*) captured in Lake Komuke, Hokkaido, Japan in 2010 and the sequences of the H10N7 isolated during the study period in Asia and North America. The sequence of the present study is indicted by a black arrow.

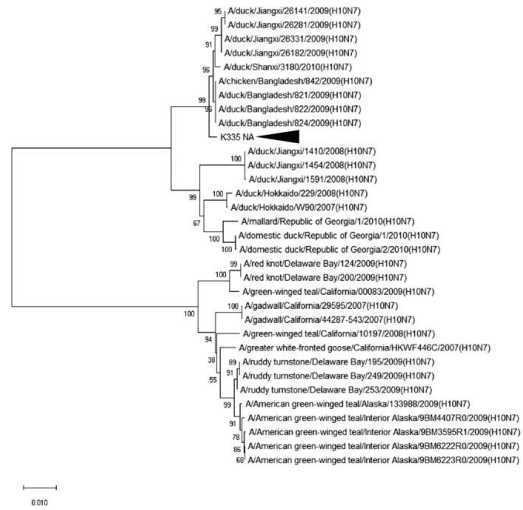


FIGURE 3. Phylogenic tree of avian influenza virus subtype H10N7 sequences using the neuraminidase gene sequence obtained from the Lesser Sand Plover (*Charadrius mongolus*) captured in Lake Komuke, Hokkaido, Japan in 2010 and the sequences of the H10N7 isolated during the study period in Asia and North America. The sequence of the present study is indicted by a black arrow.

Full lengths of the HA and NA genes, as well as polymerase acidic protein (PA), nucleoprotein (NP), matrix protein 1 (MP), and nuclear export protein (NS) genes were successfully amplified from the AIV-positive sample (see Supplementary Material Table 1). However, RNA-directed RNA polymerase catalytic subunit 1 and polymerase basic protein 2 genes could not be amplified. Information on the highest homology of each gene is presented in Table 2. All the sequences showing the highest homology were isolated from Anseriformes species. Shorebirds migrate to Japan 1 mo earlier than did Anseriformes. Therefore, the Lesser Sand Plover could have been infected by virus from Anseriformes species at the breeding ground and not in Japan.

The HA gene sequence showed the highest identity with the H10 sequence, and the NA gene sequence exhibited the highest identity with the N7 sequence. There were 17 complete genome sequences of subtype H10N7 isolated in Asia during the study period (National Institute of Allergy and Infectious Diseases 2017). The phylogenic trees for the HA and NA genes were constructed using the 17 sequences obtained in Asia and 15 sequences obtained in North America using the neighbor-joining method (Saitou and Nei 1987) with a bootstrap test of 1,000 replicates (Felsenstein 1985; Figs. 2, 3). We used MEGA X (Kumar et al. 2018) to construct the trees. Moreover, phylogenetic trees for the PA, NP, MP, and NS genes were constructed using 50 sequences with high homology (see Supplementary Material Figs. 1–4). The phylogenic analysis results showed that the HA and NA detected in the present study were related to H10N7 isolated in Bangladesh and China in 2009. Moreover, sequences of the other four genes, PA, NP, MP, and NS, detected from the Lesser Sand Plover had high homology with sequences found in Asia. Thus, the subtype H10N7 detected in the present study belongs to the Eurasian lineage and the related virus strain existed in Asia in 2009.

The H4N8 subtype of avian influenza virus was reportedly isolated from Red-necked Stint (*Calidris ruficollis*) captured at Lake Komuke,

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TABLE 1. List of shorebird species captured in Hokkaido, Japan, from 2006 to 2010, at Furen and Komuke lakes. Cloacal and tracheal swabs were collected from the captured birds to detect the virus RNA of avian influenza virus (AIV) by reverse transcription loop-mediated isothermal amplification. The "+" symbol represents positive for AIV detection.

	AIV detection		2006		2007		2008		2009		2010		Total		
			August–September	August	July–August	August–September	July	August–September	August	August–September	No sampling	August–September			
Shorebird species	Reported (references)	Present study	Furen	Komuke	Furen	Komuke	Furen	Komuke	Furen	Komuke	Furen	Komuke	Furen	Komuke	All
Scolopacidae															
Ruddy Turnstone (*Arenaria interpres*)	+[a, b, c, d, f]		2		4		1	1		1		2	7	4	11
Dunlin (*Calidris alpina*)	+[a, f]			4		21		7		12		10		54	54
Red Knot (*Calidris canutus*)	+[a, b, d, h]			1	1			2					1	3	4
Curlew Sandpiper (*Calidris erruginea*)					1								1		1
Red-necked Stint (*Calidris ruficollis*)	+[e]		18	91	7	88	14	104	9	212		422	48	917	965
Long-toed Stint (*Calidris subminuta*)			2	5	1	4		3		6		37	3	55	58
Temminick's Stint (*Calidris temminckii*)								1						1	1
Great Knot (*Calidris tenuirostris*)												2		2	2
Common Snipe (*Gallinago gallinago*)				4		1		1		1		4		11	11
Latham's Snipe (*Gallinago hardwickii*)				14	3					2		6	3	22	25
Broad-billed Sandpiper (*Limicola falcinellus*)				1		7		8	1	9		12	1	37	38
Bar-tailed Godwit (*Limosa lapponica*)	+[g]					3	1					3	1	6	7
Black-tailed Godwit (*Limosa limosa*)				1								4		5	5
Whimbrel (*Numenius phaeopus*)										1		1		2	2
Red-necked Phalarope (*Phalaropus lobatus*)						1		13		2				16	16
Ruff (*Philomachus pugnax*)				6				1						7	7

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TABLE 1. Continued.

Shorebird species	AIV detection		2006		2007		2008		2009		2010		Total		
			August–September	August	July–August	August–September	July	August–September	August	August–September	No sampling	August–September			
	Reported (references)	Present study	Furen	Komuke	Furen	Komuke	Furen	Komuke	Furen	Komuke	Furen	Komuke	Furen	Komuke	All
Grey-tailed Tattler (*Tringa brevipes*)			94	6	127		31	6	42	19		15	294	46	340
Wood Sandpiper (*Tringa glareola*)						2		1				2		5	5
Common Sandpiper (*Tringa hypoleucos*)				8				3				7		18	18
Greenshank (*Tringa nebularia*)				15		3		2		1		5		26	26
Marsh Sandpiper (*Tringa stagnatilis*)				1		2						4		7	7
Terek Sandpiper (*Xenus cinereus*)			1	1	3	2		1	1			5	5	9	14
Charadriidae															
Little ringed Plover (*Charadrius dubius*)				1				1		2				4	4
Great Sand Plover (*Charadrius leschenaultii*)							1						1		1
Lesser Sand Plover (*Charadrius mongolus*)		+	1	11		6		7		11		38	1	73	74
Grey Plover (*Pluvialis squatarola*)						1				1				2	2
Total			118	170	147	141	48	162	53	280	0	579	366	1,332	1,698

[a] Gaidet et al. 2012.
[b] Stallknecht et al. 2012.
[c] Maxted et al. 2016.
[d] Maxted et al. 2012.
[e] Bui et al. 2012.
[f] Pearce et al. 2012.
[g] Ip et al. 2008.
[h] Johnson et al. 2014.

TABLE 2. The result of homology search with BLAST (National Center for Biotechnology Information 2016) in hemagglutinin (HA), neuraminidase (NA), polymerase acidic protein (PA), nucleoprotein (NP), matrix protein 1 (MP), and nuclear export protein (NS) genes obtained from the Lesser Sand Plover (*Charadrius mongolus*) captured in Lake Komuke, Hokkaido, Japan, on 3 September 2010.

	Gene information	Maximum score	Total score	% Query cover	E value	% Identity	Accession no.
HA	A/mallard/Korea/1242/2010(H10N6)	3040	3040	100	0.0	99.17	JN817576.1
NA	A/common teal/Hong Kong/MPM1740/2011(H7N7)	2604	2604	100	0.0	99.86	KF259638.1
	A/common teal/Hong Kong/MPM1670/2011(H7N7)	2604	2604	100	0.0	99.86	KF259636.1
PA	A/duck/Guizhou/888/2006(H6N5)	3890	3890	100	0.0	99.35	CY109281.1
NP	A/wild waterfowl/Hong Kong/MPM2121/2011(H7N7)	2748	2748	100	0.0	99.80	KF259824.1
	A/common teal/Hong Kong/MPM1645/2011(H7N1)	2748	2748	100	0.0	99.80	KF259819.1
MP	A/wild bird/Korea/A344-2/2009(H5N1)	1797	1797	100	0.0	99.69	JX236015.1
	A/wild duck/Korea/SNU50-5/2009(H5N1)	1797	1797	100	0.0	99.69	JX497771.1
NS	A/muscovy duck/Vietnam/LBM348/2013(H3N8)	1537	1537	100	0.0	99.76	LC028124.1
	A/duck/Zhejiang/D486/2013(H9N2)	1537	1537	100	0.0	99.76	KF357835.1

Hokkaido (Bui et al. 2012). Our result showed that the Lesser Sand Plover is the second shorebird to have tested positive for AIV in Japan. Other possible hosts are found in Table 1, such as Ruddy Turnstone (*Arenaria interpres*) and Red Knot (*Calidris canutus*). However, the number of these species that was captured was relatively low in the present study. Thus, further evaluation of AIV prevalence in these species is essential to understand AIV translocation in the East Asia-Australasia flyway.

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SUPPLEMENTARY MATERIAL

Supplementary material for this article is online at http://dx.doi.org/10.7589/2019-02-052.

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