

Research Note—

Application of DNA Barcoding Technique in Avian Influenza Virus Surveillance of Wild Bird Habitats in Korea and Mongolia

Dong-Hun Lee,^A Hyun-Jeong Lee,^A Yu-Na Lee,^A Youn-Jeong Lee,^B Ok-Mi Jeong,^B Hyun-Mi Kang,^B Min-Chul Kim,^B Ji-Sun Kwon,^B Jun-Hun Kwon,^B Joong-Bok Lee,^A Seung-Yong Park,^A In-Soo Choi,^A and Chang-Seon Song^{AC}

^AAvian Disease Laboratory, College of Veterinary Medicine, Konkuk University, 143-701, Seoul, Korea

^BAvian Disease Division, National Veterinary Research and Quarantine Service, Ministry for Food, Agriculture, Forestry, and Fisheries, 430-757, Anyang, Korea

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SUMMARY. In a previous study, we optimized DNA barcoding techniques for avian influenza virus (AIV) isolation and host identification, using fecal samples from wild birds, for high-throughput surveillance of migratory waterfowls. In the present study, we surveyed AIV in Mongolia during the breeding season and, subsequently, in Korea in winter, to compare prevalent AIV subtypes and hosts using DNA barcoding. In Korea, H4 and H5 subtypes were the most abundantly detected HA subtypes, and most AIVs were isolated from the major population (mallards, *Anas platyrhynchos*) of wild bird habitats. On the other hand, in Mongolia, H3 and H4 subtypes were the most abundantly detected HA subtypes, and most AIVs were isolated from a small population of wild bird habitats that were not visible at the sampling site. In conclusion, AIV isolation using fecal samples, accompanied with DNA barcoding techniques as a host bird species identification tool, could be useful for monitoring major and minor populations of wild bird habitats. Further, continuous, and large-scale surveillance could be helpful for understanding the AIV epidemiology, evolution, and ecology in wild waterfowl.

RESUMEN. *Nota de Investigación*—Aplicación de la técnica de código de barras para ADN en los muestreos de vigilancia del virus de influenza aviar en hábitats de aves silvestres en Corea y Mongolia.

En un estudio previo, se optimizaron las técnicas de código de barras de ADN para el aislamiento del virus de influenza aviar y para la identificación del hospedador, utilizando muestras de excretas fecales provenientes de aves silvestres para realizar una vigilancia masiva a gran escala en aves acuáticas migratorias. En el presente estudio, se estudiaron los virus de influenza aviar en Mongolia durante la temporada de apareamiento y subsecuentemente en Corea durante el invierno para comparar la prevalencia de los subtipos del virus de influenza aviar y sus hospedadores utilizando la técnica de códigos de barras de ADN. En Corea, los subtipos de HA que se detectaron con más frecuencia fueron los subtipos H4 y H5 y la mayoría de los virus de la influenza aviar fueron aislados de un hábitat con la población de aves silvestres mas abundante (patos de collar). Por otra parte en Mongolia, los subtipos H3 y H4 fueron los más detectados y la mayoría de los virus de influenza aviar fueron aislados de un hábitat con una población pequeña de aves silvestres que no era evidente al momento del muestreo. En conclusión, el aislamiento de virus de influenza aviar a partir de muestras fecales, acompañada de técnicas de código de barras de ADN como herramientas para la identificación de la especie aviar hospedadora, podría ser útil para el muestreo de grandes y pequeñas poblaciones en los hábitats de aves silvestres. La vigilancia continúa y a gran escala puede ser utilidad para entender la epidemiología, la evolución y la ecología de los virus de influenza aviar en aves acuáticas silvestres.

Key words: avian influenza, DNA barcoding, surveillance, Mongolia, Korea

Abbreviations: AIV = avian influenza virus; COI = cytochrome oxidase I; HPAI = highly pathogenic avian influenza; LPAI = low pathogenic avian influenza; NDV = Newcastle disease virus; RRT-PCR = real-time reverse transcription–polymerase chain reaction

Migratory birds make round-trip migrations of up to 26,000 km each year between their nonbreeding grounds in the northern hemisphere and their nonbreeding areas to the south. These trips are made in several weeks, with long stopovers at staging sites along the way to rest and refuel for the next leg of their journey (1,4,17). In Korea, there are a number of wetlands located along the East Asian–Australasian bird flyway that provide resting and wintering sites for many bird species migrating from the northern hemisphere in winter seasons (17). In contrast, Mongolia and Russia have become representative breeding grounds, where diverse avian hosts from Asia and Europe overlap in a region of intercontinental avifaunal mixing. These regions are hypothesized to be zones of virus

exchanges. In particular, Holbo Lake (45°14'N, 114°06'E) and Ganga Lake (45°15'N, 113°59'E) in Mongolia are stopover and breeding sites for whooper swans (*Cygnus cygnus*) and other waterfowl migrating from Russia to Korea. In these wetlands, waterfowl share feeding and roosting sites in wild bird habitats, resulting in contact and indirect avian influenza virus (AIV) transmission by water contaminated with AIV (3).

Surveillance for AIV infection in wild birds provides information regarding AIV distribution and ecology in moving targets, and global AIV surveillance in wild birds can play a key role in the early recognition of, and preparation for, emergence of new and potentially pandemic AIV strains (7,10). AIV surveillance studies have been conducted with virus isolation procedures using cloacal swabs from live, captured birds (11,16). However, capturing wild birds can result in serious avian injuries. Therefore, in our previous

^CCorresponding author. E-mail: songcs@konkuk.ac.kr

study, we optimized DNA barcoding techniques (8) using fecal samples to identify correct bird species carrying AIV for high-throughput surveillance of migratory waterfowl. In the present study, we applied DNA barcoding techniques to AIV surveillance to understand the bird population, the prevalence and distribution of AIV, and the virus–host ecology at stopover and breeding sites as well as at major southern wintering sites in Korea.

MATERIALS AND METHODS

Collection of samples. In Korea, fresh fecal samples ($n = 1721$) were collected from the wintering sites (Cheonsu-bay A [36°38'N, 126°27'E] and Cheonsu-bay B [36°39'N, 126°21'E]) of mallards (*Anas platyrhynchos*), green-winged teals (*Anas crecca*), bean geese (*Anser fabalis*), white-fronted geese (*Anser albifrons*), and whooper swans around the agricultural fields from September 2007 to February 2008. In Mongolia, fresh fecal samples ($n = 240$) were collected from stopover and breeding sites (Ganga Lake [45°15'N, 113°59'E] and Holbo Lake [45°14'N, 114°06'E]) of whooper swans and swan geese (*Anser cygnoides*) in Sukhbaatar from September to October 2007. There were no domestic waterfowl in the sampling sites in both countries. All fecal samples were kept in coolers until transported to the laboratory.

Virus detection and isolation. Fresh fecal samples were dissolved in phosphate-buffered saline with 400 µg/ml gentamicin, then the suspensions were centrifuged and the top clear liquid was collected. All specimens were inoculated into the allantoic cavity of 9- to 11-day-old specific-pathogen-free chicken embryonated eggs. After 72 hrs of incubation, the eggs were chilled, allantoic fluids were harvested from an axenic medium, and the undiluted allantoic fluids were tested for hemagglutinin activity. RNA was extracted using the RNeasy kit (Qiagen, Valencia, CA) according to manufacturer's instructions. RNA was tested for AIV with real-time reverse transcription–polymerase chain reaction (RRT-PCR) directed to the matrix gene (13). AIV-positive specimens were further characterized for HA and NA subtypes by nucleotide sequencing.

DNA barcoding for species identification of fecal samples. We analyzed both AIV-positive and negative samples from Mongolia to examine bird populations and to correctly identify bird species infected with AIV in Mongolia. In Korea, only AIV-positive samples were used for DNA barcoding to identify those bird species infected with AIV. DNA was extracted from fecal samples using an Accuprep 96-well stool DNA extraction kit (Bioneer, Daejeon, Korea), or the DNeasy stool mini kit (Qiagen), according to manufacturer's instructions. PCR was performed with primers (AvesF: gcatgagcaggaatagtgg and AvesR: aagatgtagactctctgggtg). DNA sequences of PCR products were compared with the sequence database of the cytochrome oxidase I (COI) gene offered by the Barcode of Life Data Systems.

RESULTS

Prevalence of AIV in wild bird habitats. In Mongolia, a total of five different HA and NA subtype combinations, of 16 isolates, were isolated from 240 samples collected (Table 1). The overall prevalence rate of AIV was 6.7% (16/240), but the rate varied depending on the type of bird species: ruddy shelduck (*Tadorna ferruginea*), 100% (3/3); common pochard (*Aythya ferina*), 100% (1/1); lapwing (*Vanellus vanellus*), 100% (1/1); northern pintail (*Anas acuta*), 57.1% (4/7); whooper swan, 5.5% (6/110); swan goose, 0% (0/44); green-winged teal, 0% (0/19); bean goose, 0% (0/8); greylag goose (*Anser anser*), 0% (0/7); white-fronted goose, 0% (0/5); tundra swan, 0% (0/1); and Anseriformes, 0% (0/4). On the other hand, the majority of HA subtypes were H3 (62.5%) and H4 (31.3%), followed by H10 (6.3%). In particular, AIV subtypes H3N1 and H3N8 were obtained from whooper swans; H3N2 and H3N8 from ruddy shelduck; H3N8 and H4N6 from northern pintail; H4N6

from lapwing; H10N6 from common pochard; and H4N6 from an unidentified host.

In Korea, a total of 16 AIVs were isolated from 1721 samples, and the overall prevalence rate of AIV was 0.93% (16/1721; Table 2). Of the 16 AIV isolates, the prevalence rate of AIV in the identified host bird species were as follows: mallard, 56.3% (9/16); white-fronted goose, 18.8% (3/16); northern pintail, 12.5% (2/16); green-winged teal, 6.3% (1/16); and bean goose, 6.3% (1/16). AIV subtypes H4N6, H5N2, H7, and H11N3 were obtained from mallard; H1N2 and H6N1 from white-fronted goose; H4N6 from northern pintail; H1N1 and H3N8 from green-winged teal; and H9N2 from bean goose. The majority of HA subtypes were H4, 25% (4/16) and H5, 25% (4/16). Interestingly, a total of five Newcastle disease viruses (NDV) were isolated from fecal samples. For NDV, the identified host bird species were mallard ($n = 3$) and green-winged teal ($n = 2$).

Differences between observed species and identified species. Although flocks of whooper swans were observed around Ganga Lake in Mongolia, our results showed that in addition to fecal samples from whooper swans, samples from bean geese and swan geese have also been collected and identified. Finally, a total of six low pathogenic avian influenza (LPAI) viruses were isolated from 110 fecal samples from whooper swans and two LPAI viruses were isolated from other species. Around Holbo Lake in Mongolia, flocks of swan geese were observed during sampling. However, identified species from the DNA barcoding of fecal samples also included green-winged teal, northern pintail, whooper swan, and white-fronted goose. Although AIVs were not isolated from the fecal samples of swan geese ($n = 40$), AIVs were detected from the fecal samples of different species with less than 10 samples, including northern pintail, ruddy shelduck, common pochard, and lapwing.

In Korea, the sampling sites were at the pastures and agricultural fields in Cheonsu-bay where geese were observed. However, AIVs were also isolated from northern pintails at those locations. In the northern section of the lakes in Cheonsu-bay, where mallards and spot-billed ducks (*Anas poecilorhyncha*) were observed, most of bird species infected with AIV were mallards and green-winged teals.

DISCUSSION

Highly pathogenic avian influenza (HPAI) was not found in any of the samples examined, but overall LPAI prevalence of AIV was higher in Mongolia (6.7%) than in Korea (0.93%). Higher prevalence in Mongolia could be explained by the presence of juvenile birds; juvenile birds are immunologically naïve and, thus, more prone to infection with AIV (6,14,15). There were various juvenile and adult waterfowls sharing breeding sites in wild bird habitats, potentially promoting circulation of AIV among those species. Therefore, juvenile birds are closely monitored during the premigration period at breeding sites in order to predict the spread of AIV along the migratory bird flyway.

In Mongolia, we investigated bird populations using fecal samples, and the results indicated that AIVs were isolated from a very small sampling of bird species (ruddy shelduck, lapwing, and common pochard) regardless of the major flocks observed at each site. These data suggest that using fecal samples based on DNA barcoding to identify the correct host bird species could be useful, as there could be the risk of misidentifying the host if based solely on observation of the major population(s) in the sampling region. In AIV surveillance, capturing wild birds only allows examination of the major populations of bird species. Therefore, for monitoring

Table 1. Prevalence of avian influenza virus in migratory birds in Mongolia.

Region	Primary observed species	Identified bird species			AIV isolation		
		Scientific name (n)	Common name	Subtypes (n)	No. of virus isolated/no. of fecal samples (%)	Prevalence (%)	
Ganga Lake	Whooper swan	<i>Cygnus cygnus</i> (110)	Whooper swan	H3N1 (2) H3N8 (4)	6/110 (5.5)	8/140 (5.7)	
		<i>Anser fabalis</i> (5)	Bean goose	–	–	–	–
		<i>Anser cygnoides</i> (4)	Swan goose	–	–	–	–
		<i>Cygnus colymbianus</i> (1)	Tundra swan	–	–	–	–
		<i>Anser anser</i> (1)	Greylag goose	–	–	–	–
		<i>Anas crecca</i> (2)	Green-winged teal	–	–	–	–
		<i>Tadorna ferruginea</i> (1)	Ruddy shelduck	H3N8 (1)	1/1 (100)	–	–
		Anseriformes (4)	–	–	–	–	–
		Unidentified (2)	–	–	–	–	–
		NDA ^a (10)	–	–	–	–	–
		<i>Anser cygnoides</i> (40)	Swan goose	–	–	–	–
		<i>Anas crecca</i> (19)	Green-winged teal	–	–	–	–
		<i>Anas acuta</i> (7)	Northern pintail	H4N6 (1)	–	–	–
				H3N8 (1) H4N6 (3)	4/7 (57.1)	–	–
Holbo Lake	Swan goose	<i>Cygnus cygnus</i> (6)	Whooper swan	–	–	–	
		<i>Anser anser</i> (6)	Greylag goose	–	–	–	
		<i>Anser albifrons</i> (5)	White-fronted goose	–	–	–	
		<i>Anser fabalis</i> (3)	Bean goose	–	–	–	
		<i>Tadorna ferruginea</i> (2)	Ruddy shelduck	H3N2 (1) H3N8 (1) H4N6 (1) H10N6 (1)	2/2 (100)	–	–
		<i>Vanellus vanellus</i> (1)	Lapwing	–	–	–	–
		<i>Aythya ferina</i> (1)	Common pochard	–	–	–	–
		NDA ^a (10)	–	–	–	–	–
		240	–	–	–	–	–
				16	–	–	–
		Total					16/240 (6.7)

^aFecal samples were used for virus isolation, but species identification was not done (ND).

Table 2. Prevalence of avian influenza virus in migratory birds in Korea.

Sampling period	AIV isolation		Identified bird species	
	No. of virus isolated/ no. of fecal samples (%)	Subtypes (<i>n</i>)	Scientific name	Common name
October 2007	1/393 (0.25)	H3N8 (1)	<i>Anas crecca</i>	Green-winged teal
November 2007	9/441 (2.04)	H1N2 (1)	<i>Anser albifrons</i>	White-fronted goose
		H4N6 (2)	<i>Anas acuta</i>	Northern pintail
		H5N2 (3)	<i>Anas platyrhynchos</i> ^A	Mallard
		H6N1 (1)	<i>Anser albifrons</i>	White-fronted goose
		H11N3 (2)	<i>Anas platyrhynchos</i> ^A	Mallard
December 2007	3/443 (0.68)	H1N2 (1)	<i>Anser albifrons</i>	White-fronted goose
		H5N2 (1)	<i>Anas platyrhynchos</i> ^A	Mallard
		H9N2 (1)	<i>Anser fabalis</i>	Bean goose
February 2008	3/444 (0.68)	H4N6 (2)	<i>Anas platyrhynchos</i> ^A	Mallard
		H7 (1)	<i>Anas platyrhynchos</i> ^A	Mallard
Total	16/1721 (0.93)			

^A*Anas platyrhynchos* (mallard) has a COI gene sequence identical to that of *Anas poecilorhyncha* (spot-billed duck).

smaller populations in wild bird habitats, capturing wild birds needs to be accompanied with fecal sampling and host identification.

In Korea, of the 16 AIVs isolates obtained, four LPAI subtype H5 samples were identified, and all identified host bird species were mallards. The high incidence of LPAI subtype H5 viruses in mallards suggests that this species may transmit H5 viruses to poultry, and LPAI could evolve into HPAI viruses (2). Recently, antibodies of subtype H5 have been sporadically detected in terrestrial poultry flocks in Korea, and the H5-seropositive flocks were stamped out. The high prevalence of H5 viruses found in wild ducks poses the question of whether these wild ducks were the source of infection in H5-seropositive terrestrial poultry flocks.

In Mongolia, H3 (62.5%) and H4 (31.3%) were the predominant AIV subtypes. These results are supported by the fact that H3 and H4 viruses have been predominantly detected in wild waterfowls in Mongolia (12), suggesting that H3 and H4 subtypes are maintained in the wild bird habitats of Mongolia and that the viruses could infect migratory birds during resting or breeding seasons. In contrast, H4 (25%) and H5 (25%) were the predominant AIV subtypes in Korea. These data indicate that H4 subtypes are the predominant AIV subtypes in both countries. Furthermore, most of the H4N6 viruses were isolated from northern pintails in both countries. Similarly, Jahangir *et al.* (9) reported that H4N6 was also detected in northern pintails in northeast Japan. These data suggest that the northern pintail could be hypothesized as an important host of subtype H4N6 in the East Asian–Australasian flyway. In addition, molecular characterization of H4N6 viruses isolated in these countries is needed to determine the relatedness of these viruses.

In many parts of Southeast Asia, wild and domestic birds are often allowed to mix freely on many wetlands (5). DNA barcoding technique can be effective in discriminating bird species, but it has a limitation in discriminating some sister species, either within genera or between domestic versus wild species such as mallard, spot-billed duck, and the domestic duck (*Anas platyrhynchos domesticus*) (8,18). Therefore, before any sampling procedure, the presence of resident waterfowls in sampling sites should be considered. In addition, it is impossible to identify individual birds with DNA barcoding. In this case, we could not determine whether AIV-positive samples were from the same individual or from different birds. For this reason, the number of AIV-positive samples does not refer to the number of AIV-infected individual birds in the population, but can only refer to number of AIV-positive fecal samples.

In conclusion, we applied DNA barcoding techniques for AIV surveillance to study the role of migratory birds in the spread of AIV.

Breeding sites need to be closely monitored during the premigration period in order to predict the spread of AIV along the bird flyway. Moreover, AIV isolation using fecal samples should be paired with DNA barcoding techniques to achieve effective monitoring of major and minor populations of wild bird habitats. We believe that continued, large-scale surveillance can create a strong foundation for future studies of AIV epidemiology, evolution, and ecology in wild bird habitats, as well as for exploring the role of migratory birds in the spread of AIV during their migration.

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