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Surveillance of Avian Coronaviruses in Wild Bird Populations of Korea

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ABSTRACT: We examined the role of wild birds in the epidemiology of avian coronaviruses by studying oropharyngeal swabs from 32 wild bird species. The 14 avian coronaviruses detected belonged to the gamma-coronaviruses and shared high nucleotide sequence identity with some previously identified strains in wild waterfowl, but not with infectious bronchitis viruses.

Coronaviruses (order *Nidovirales*, family *Coronaviridae*, subfamily *Coronavirinae*) contain a single-stranded, nonsegmented RNA, positive-sense genome of 26–31 kb (Cavanagh 1997). Coronaviruses are classified into four genera (*Alpha*, *Beta*, *Gamma*, and *Deltacoronavirus*; Gonzalez et al. 2003) and are important pathogens of birds, mammals, and humans. *Alphacoronavirus* and *Betacoronavirus* have been isolated from mammals (Muradrasoli et al. 2010). Severe acute respiratory syndrome coronavirus, a *Betacoronavirus*, is well known for its pandemic potential. Because it originated from recombination events between mammalian-like and avian-like parental viruses present in wild animal species, it highlights the importance of studying the genetics of coronaviruses of diverse origins (Saif 2004; Stavrinides and Guttman 2004). Avian infectious bronchitis virus (IBV), a *Gammacoronavirus*, is responsible for severe global economic losses in the poultry industry (Cavanagh and Gelb 2008). Chickens (*Gallus galllus domesticus*) are the primary natural host of IBV and, although IBV-like coronaviruses are found in different birds (Cavanagh 2005; Hughes et al. 2009; Woo et al. 2009), further studies are required to determine the exact role of wild birds in the epidemiology of IBV.

In Korea, IBV has caused a variety of syndromes, ranging from respiratory disease to nephropathogenic death, since 1986 (Rhee et al. 1986), and IBV has generated extensive genotypic variations due to the high mutation and recombination rates of coronaviruses during replication (Woo et al. 2008). However, surveillance of wild birds as both reservoirs and long-distance vectors of IBV has not been conducted.

To examine the role of wild birds as natural reservoirs for avian coronaviruses, we performed molecular identification and phylogenetic analyses. Between 2010 and 2012, we collected oropharyngeal swabs from 1,473 wild birds of 32 species (Table 1). The locations sampled are habitats of 41 migratory species located throughout South Korea, which were selected for collection of samples for avian influenza surveillance by the Ministry of Agriculture, Food, and Rural Affairs (Fig. 1).

Phosphate-buffered saline (pH 7.2) containing gentamicin (45 μg/mL) was added to swab samples and frozen at −80 C until processed. Total RNA was extracted from clarified oropharyngeal swabs by using the RNeasy Mini Kit (Qiagen,Valencia, California, USA) according to the manufacturer's instructions. Reverse transcription-PCR was used to detect avian coronaviruses as described by Chu et al. (2011). The primers targeted the 360-base pair (bp) RdRp region of the coronavirus genome, which is highly conserved among all known avian coronaviruses. The amplified DNA fragment was purified using an agarose gel DNA extraction kit (iNtRON Biotechnology Inc., Daejeon, Korea) and subcloned into the vector pGEM-T (Promega Corp., Madison, Wisconsin, USA) according to the manufacturer's instructions. The vector insert was nucleotide sequenced using an ABI 3130XL genetic analyzer (Applied Biosystems, Foster City, California, USA) with the BigDye™ Terminator cycle sequencing kit (Applied Biosystems). Nucleotide identities at all positions were

TABLE 1. Wild bird species tested for coronaviruses in South Korea from 2010 to 2012.

Family	Common name	Scientific name	No. birds screened		
			2010	2011	2012
Anatidae	Northern Pintail	*Anas acuta*	4	34	58
	Green-winged Teal	*Anas crecca*	171	74	38
	Mandarin Duck	*Aix galericulata*	53	19	46
	Mallard	*Anas platyrhynchos*	58	168	201
	Indian Spot-billed Duck	*Anas poecilorhyncha*	75	165	121
	European Wigeon	*Anas penelope*	72	2	3
	Greater White-fronted Goose	*Anser albifrons*	0	3	0
Ardeidae	Intermediate Egret	*Mesophoyx intermedia*	5	0	0
	Great Bittern	*Botaurus stellaris*	1	0	0
Charadriidae	Lesser Sand Plover	*Charadrius mongolus*	0	2	0
	Kentish Plover	*Charadrius alexandrinus*	0	1	0
Columbidae	Oriental Turtle Dove	*Streptopelia orientalis*	1	4	0
Corvidae	Eurasian Jay	*Garrulus glandarius*	6	0	0
	Eurasian Magpie	*Pica pica*	0	1	0
Emberizidae	Yellow-throated Bunting	*Emberiza elegans*	1	2	0
Gruidae	Red-crowned Crane	*Grus japonensis*	0	0	3
Laridae	Black-tailed Gull	*Larus crassirostris*	0	10	0
Muscicapidae	Daurian Redstart	*Phoenicurus auroreus*	1	0	0
Picidae	Grey-faced Woodpecker	*Picus canus*	1	0	0
	Great Spotted Woodpecker	*Dendrocopos major*	0	3	0
Pycnonotidae	Brown-eared Bulbul	*Hypsipetes amaurotis*	4	2	0
Rallidae	Eurasian Coot	*Fulica atra*	39	0	3
Scolopaciade	Grey-tailed Tattler	*Tringa brevipes*	0	1	0
	Terek Sandpiper	*Xenus cinereus*	0	3	0
	Sharp-tailed Sandpiper	*Calidris acuminata*	0	1	0
Strigidae	European Scops Owl	*Otus scops*	3	1	0
	Brown Boobook	*Ninox scutulata*	1	0	0
Sylviidae	Vinous-throated Parrotbill	*Paradoxornis webbianus*	0	2	0
Turdidae	Grey-backed Thrush	*Turdus hortulorum*	1	2	0
	Scaly Thrush	*Zoothera dauma*	1	0	0
	Eyebrowed Thrush	*Turdus obscurus*	1	0	0
	Pale Thrush	*Turdus pallidus*	1	0	0
		Total	500	500	473

confirmed by three or more independent, bidirectional sequencing reactions.

Viral sequences were analyzed using BioEdit software (Ibis Biosciences, Carlsbad, California, USA), and phylogenetic trees were generated by the neighbor-joining method by using MEGA 4.0 (Tamura et al. 2007) with 1,000 bootstrap replications. Reference sequences were obtained using the Basic Local Alignment Search Tool (National Center for Biotechnology Information 2014). The cut-off point for bootstrap replication was 70%.

Fourteen of the 1,473 samples tested (0.95%) were positive. We found coronaviruses in two species of waterfowl; viz., one of 96 Northern Pintails (*Anas acuta*; 1.0%) and 13 of 361 Indian Spot-billed Ducks (*Anas poecilorhyncha*; 3.6%). None of the other 30 species examined were positive. All coronavirus-positive samples were collected in 2012.

The partial viral RdRp sequences were determined from 14 coronaviruses and compared with those of 32 other reference coronaviruses (Fig. 2). All the coronaviruses we identified were phylogenetically classified as *Gammacoronavirus*, along with two Korean IBV strains (SNU8067 and KM91), and the RdRp segment of the 14 Korean coronaviruses demonstrated more than 93.0% sequence homology.

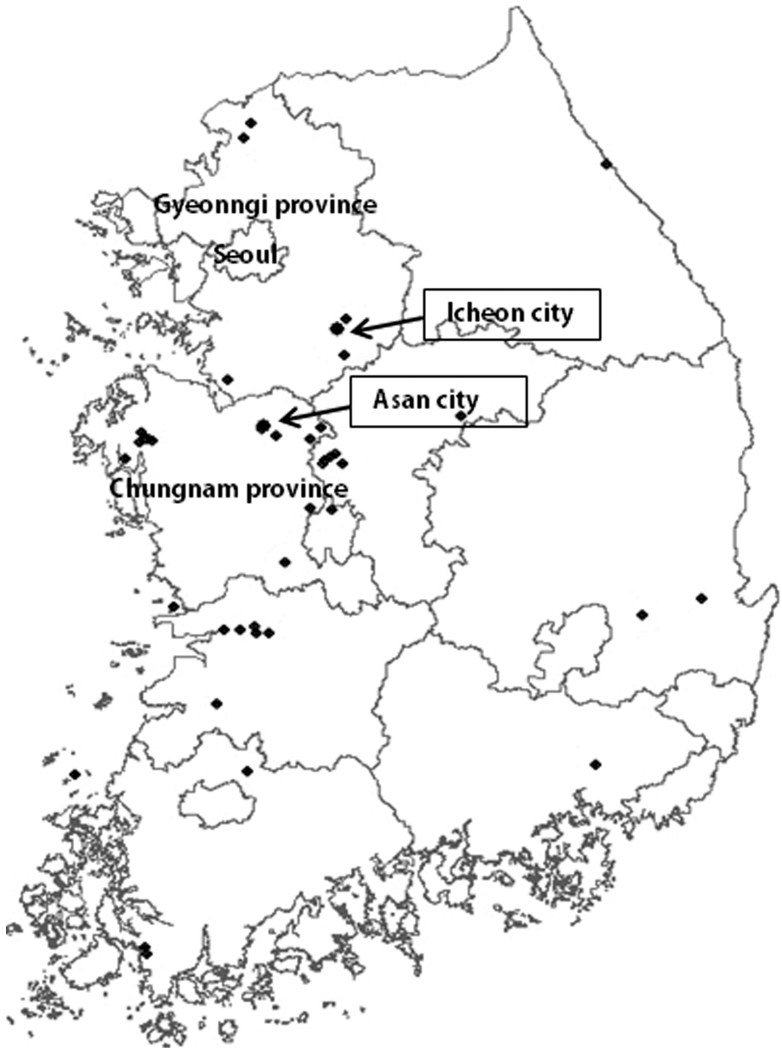

FIGURE 1. Locations in South Korea where wild bird samples were collected. Avian coronaviruses were found only in Icheon and Asan.

The genetic difference within the same *Gammacoronavirus* was 2.8–21.4%, and these viruses were genetically heterogeneous compared to *Deltacoronavirus* (58.8–61.9%). The sequence differences between the 14 coronaviruses and the two IBV strains were 18.9–22.8%, and they were located in different subclades, suggesting that these 14 coronaviruses detected in wild waterfowl were genetically distinct from IBVs. Further surveillance of wild bird populations and a full-genome analysis of avian coronaviruses are required to better understand the evolution of coronaviruses and the relationship between avian coronaviruses in wild waterfowl and IBVs in chickens. However, some coronavirus sequences from wild birds shared high nucleotide sequence identity (97.2–100%) with the sequence of the IBV vaccine strain (Hughes et al. 2009). This finding indicates that continuous surveillance is required for coronaviruses in wild bird populations.

Although samples were collected from wild bird populations from numerous and

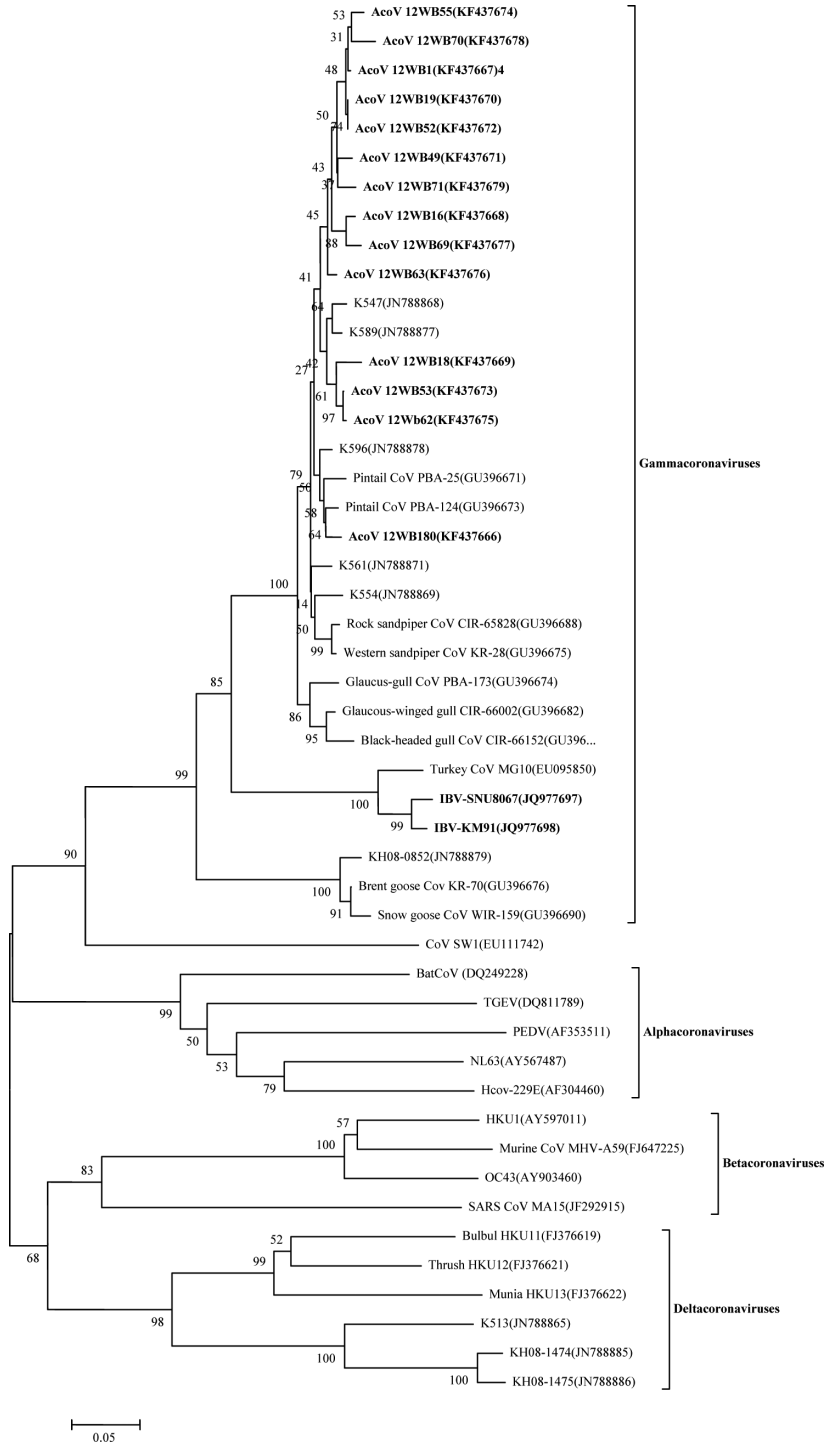

FIGURE 2. Phylogenetic tree of the RNA-dependent RNA polymerase (RdRp) genes showing the genetic relationship among avian coronaviruses. GenBank accession numbers: AcoV12WB180 (KF437666), AcoV12WB14 (KF437667), AcoV12WB16 (KF437668), AcoV12WB18 (KF437669), AcoV12WB19 (KF437670), AcoV12WB49 (KF437671), AcoV12WB52 (KF437672), AcoV12WB53 (KF437673), AcoV12WB55 (KF437674), AcoV12Wb62 (KF437675), AcoV12WB63 (KF437676), AcoV12WB69 (KF437677), AcoV12WB70 (KF437678), and AcoV12WB71 (KF437679). AcoV12WB180 strain was isolated from Northern Pintails and the other strains were isolated from Indian Spot-billed Ducks. Bootstrap values based on 1,000 bootstrap replicates are given at the relevant nodes.

diverse habitats, coronavirus RNA was detected only in the Indian Spot-billed Duck and Northern Pintail. It has been suggested that some avian coronaviruses could persist in a specific bird species and could be carried by these migrating birds to other locations (Chu et al. 2011). Our results support the hypothesis that specific species of birds can be reservoirs of avian coronaviruses.

As partially demonstrated (Fig. 1), some avian coronaviruses similar to those detected in this study have also been identified in wild waterfowl by other investigators (Hughes et al. 2009; Muradrasoli et al. 2010; Chu et al. 2011), indicating that these avian coronoviruses likely circulate widely in wild waterfowl. A recent study further suggested that similar avian coronaviruses may also circulate in domestic ducks, and these avian coronaviruses detected in waterfowl may constitute a novel species of coronavirus distinct from IBVs (Chen et al. 2013).

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