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Identification of Avian Coronavirus in Wild Aquatic Birds of the Central and Eastern USA

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ABSTRACT: Coronaviruses (CoVs) are worldwide in distribution, highly infectious, and difficult to control because of their extensive genetic diversity, short generation time, and high mutation rates. Genetically diverse CoVs have been reported from wild aquatic birds that may represent a potential reservoir for avian CoVs as well as hosts for mutations and recombination events leading to new serotypes or genera. We tested 133 pooled samples representing 700 first-passage (in eggs) and 303 direct cloacal swab transport media samples from wild aquatic birds in the US that were avian influenza-negative. We isolated RNA from frozen samples and performed reverse transcriptase-PCR using a published universal CoV primer set. Of the samples tested, one from a Ruddy Turnstone (*Arenaria interpres*) was positive for CoV, showing nucleotide sequence similarity to a duck coronavirus (DK/CH/HN/ZZ2004). These data indicate a possible low prevalence of CoVs circulating in wild aquatic birds in the eastern half of the US.

Key words: Cloacal swab, duck coronavirus, reservoir, RT-PCR, wild bird.

Coronaviruses (CoVs) are enveloped, single-stranded, positive-sense RNA viruses in the family *Coronaviridae*; they are classified within three genera, *Alpha-*, *Beta-*, *and Gammacoronavirus* (Gonzalez et al. 2003), with a possible fourth genus, *Deltacoronavirus* (Chu et al. 2011) being recently proposed. Avian CoVs are in the *Gamma*-CoV genus, though debate has arisen around some isolates being classified as *Delta*-CoVs (Chu et al. 2011). Genetic evidence of *Gamma*-CoV infection has been reported from a variety of wild bird species in Europe, Beringia, China, and other regions of Asia (Cavanagh et al. 2002; Jonassen et al. 2005; Liu et al. 2005; Sun et al. 2007; Hughes et al. 2009; Woo et al. 2009; Muradrasoli et al. 2010; Chu et al. 2011), and it has been suggested that wild bird populations may be a natural reservoir for these viruses. They may also be infected with CoVs found in commercial poultry, as viruses with high genetic similarity to the common vaccine strain H120 of avian infectious bronchitis virus (IBV) and a Massachusetts type IBV have been identified in waterfowl, shorebirds, and peafowl (Sun et al. 2007; Hughes et al. 2009). Other isolates identified in songbirds are much more distantly related to IBV-type CoVs (Woo et al. 2009) and more closely related to mammalian CoVs (Woo et al. 2012). This genetic disparity indicates wild bird populations could be a possible host for CoVs relevant to disease in commercial poultry as well as a mixing vessel for CoVs that attain the ability to cross species (Woo et al. 2012).

Original samples used in this study were collected by our group as part of an avian influenza surveillance program in the US between 2010 and 2011 (Chander et al. 2013). A total of 1,003 samples were tested in this study and were collected from 33 species in five orders; Anseriformes ($n=675$), Charadriiformes ($n=189$), Gruiformes ($n=82$), Pelicaniformes ($n=50$), and Podicipediformes ($n=7$) (Table 1). These samples represented multiple locations in the US: Anseriformes in Minnesota ($n=557$), New Jersey ($n=57$), Texas ($n=41$), and Louisiana ($n=20$); Charadriiformes in Minnesota ($n=16$), New Jersey ($n=162$), and New York ($n=11$); Gruiformes in Tennessee ($n=54$) and Minnesota ($n=28$); Pelicaniformes in Minnesota ($n=50$); and Podicipediformes in Minnesota ($n=7$). In the first trial of the study, allantoic fluid (AAF) samples from the first embryonated egg passage of avian

TABLE 1. Numbers of individuals for each wild bird species examined for avian coronaviruses in the central and eastern USA, 2010–11.

Order	Species, *n*
Trial 1	
Anseriformes	Wood Duck, *Aix sponsa*, 39
	Northern Pintail, *Anas acuta*, 9
	American Wigeon, *Anas americana*, 3
	Green-winged Teal, *Anas carolensis*, 64
	Northern Shoveler, *Anas clypeata*, 11
	Blue-winged Teal, *Anas discors*, 100
	Mallard, *Anas platyrhynchos*, 200
	American Black Duck, *Anas rubripes*, 15
	Mallard/Black Duck Hybrid, *Anas (rubripes* × *platyrhynchos)*, 12
	Gadwall, *Anas strepera*, 51
	Redhead, *Aythya americana*, 29
	Ring-necked Duck, *Aythya collaris*, 19
	Canvasback, *Aythya valisineria*, 5
	Canada Goose, *Branta canadensis*, 2
	Bufflehead, *Bucephala albeola*, 3
	Pied-billed Grebe, *Podilymbus podiceps*, 5
	Ruddy Duck, *Oxyura jamaicensis*, 1
Charadriiformes	Ring-billed Gull, *Larus delawarensis*, 27
Gruiformes	American Coot, *Fulica americana*, 1
	Sandhill Crane, *Grus canadensis*, 26
	Sora, *Porzana carolina*, 1
Pelicaniformes	American White Pelican, *Pelecanus erythrorhynchos*, 50
Podicipediformes	Red-necked Grebe, *Podiceps grisegena*, 7
Trial 2	
Anseriformes	Green-winged Teal, *Anas carolensis*, 2
	Northern Shoveler, *Anas clypeata*, 16
	Blue-winged Teal, *Anas discors*, 41
	Gadwall, *Anas strepera*, 6
	Mallard, *Anas platyrhynchos*, 42
Charadriiformes	Ruddy Turnstone[a], *Arenaria interpres*, 150
	Rod Knot, *Calidris canutus*, 2
	Herring Gull, *Larus smithsonianus*, 1
	Laughing Gull, *Leucophaeus atricilla*, 9
Gruiformes	Sandhill Crane, *Grus canadensis*, 54

[a] Indicates positive identification of an avian coronavirus.

influenza-negative cloacal swabs were tested. Seven hundred individual samples were pooled in groups of 10, using 100 μL per sample for a total pooled volume of 1 mL. Samples in pools that showed amplification at ~250 base pairs (bp) in size on an agarose gel were then tested in the same way individually, again using a 100 μL of sample. The chicken CoV IBV replicates efficiently in eggs, but the ability of avian CoVs from wild birds to replicate is currently unknown. We tested AAF first to determine if this approach would enhance CoV detection in wild bird samples. In the second trial, we tested transport media directly inoculated with cloacal swabs. In Trial 2, we pooled 303 samples in groups of five, again using 100 μL of sample for a total volume of 500 μL, except in the last pool where only three samples were grouped. As in the previous trial, samples from pools showing amplification were tested individually. Samples were pooled by species when possible. We extracted RNA from pools and individual samples using the High

Pure RNA Isolation Kit (Roche Diagnostics, Mannheim, Germany) and it was eluted in a 50-μL volume per the manufacturer's instructions. Transport medium from original cloacal swabs was filtered through a 0.22-μm spin column filter (Corning Inc., Corning, New York, USA) prior to RNA isolation. Infectious bronchitis virus Mass41 (Mass/Mass41/41) was used as a positive control and extracted individually in the same manner.

Reverse transcriptase-PCR (RT-PCR) was performed using two pancoronavirus primers; 4Bm Mod 5′-TCACATTT(A/T)GGATAGTCCCA-3′ and 2Bp Mod 5′-ACTCAG(A/T)TGAATTTGAAATA(C/T)GC-3′ (Stephensen et al. 1999; Jonassen et al. 2005). Primers were designed against a highly conserved region of the polymerase gene in open reading frame 2 from 11 different coronaviruses, and they have been used to identify coronaviruses in geese (*Anser anser*), pigeons (*Columbia livia*), and mallards (*Anas platyrhynchos*) (Jonassen et al. 2005). The RT-PCR was performed in a two-step reaction using the Takara RNA LA PCR Kit (AMV) ver 1.1 (Takara Bio Inc., Shiga, Japan) per the manufacturer's instructions. The RT reaction conditions with primer 4Bm Mod were 55 C for 45 min followed by an inactivation step at 70 C for 15 min. The PCR reaction conditions were 95 C for 2 min followed by 40 cycles of 95 C for 15 s, 55 C for 30 s, and 72 C for 60 s. A final step of 72 C for 10 min was followed by chilling to 4 C.

The PCR products were electrophoresed on a 1% (w/v) agarose gel and viewed with an ultraviolet light source. Bands visible on the gel equal to or larger than the approximate 250-bp product were excised and purified using the GeneJet Gel Extraction Kit (Thermo Scientific, Waltham, Massachusetts, USA). Purified products were sequenced at the Georgia Genomics Facility (Athens, Georgia, USA) using an Applied Biosystems 3730×l 96-capillary DNA analyzer (Applied Biosystems, Grand Island, New York) with primers 4Bm Mod and 2Bp Mod. Sequences were assembled using the SeqBuilder program (DNAStar, Madison, Wisconsin, USA) and were compared to previously published sequences using the Basic Local Alignment Search Tool (National Center for Biotechnology Information 2014). Samples were considered positive if sequence comparison was confirmed to be CoV.

The RT-PCR assay we used was validated by performing the experiment on 10-fold dilutions of the Mass41 (Mass/Mass41/41) virus used as the positive control. Virus was stored at −80 C for a similar time period as the samples tested in the experiment, and 10-fold dilutions were made starting with an undiluted sample with a titer of $1\times10^{6.4}$ embryo infectious doses 50 (EID_{50})/mL. The RNA purification, RT-PCR, and gel electrophoresis were performed as described. The limit of detection was observed at a titer of $1\times10^{1.4}$ EID_{50}/mL, as this was the highest dilution where amplification was seen on the gel.

Eight pools but no individual samples showed amplification from the single egg passage allantoic fluid used in the first trial when analyzed by RT-PCR. This supports unpublished data from our laboratory suggesting that viral isolates from wild birds may not replicate efficiently in embryonated domestic chicken eggs. Because pools were made prior to RNA isolation, thus concentrating total RNA during purification, pooled samples may have been positive because of higher levels of CoV RNA from pooling multiple positive samples, making the viral RNA level above the detection threshold, while individual samples would be below the level of detection of the RT-PCR assay. From Trial 2, six pools showed amplification but only one individual sample was positive for CoV. The CoV-positive sample showed sequence similarity, 121/127 nucleotides (95%), to a Duck Coronavirus (DK/CH/HN/ZZ2004). This sample was taken from a Ruddy Turnstone at Kimble's Beach, Cape May County, New Jersey, USA.

Duck species (Anseriformes) are predominantly thought to be carriers of many

avian viral diseases, mainly because of their association with avian influenza viruses (Olsen et al. 2006), and this has been supported by evidence from other laboratories identifying avian CoVs in multiple duck species (Hughes et al. 2009; Muradrasoli et al. 2010; Chu et al. 2011; Chen et al. 2013). However, we did not find any evidence of avian CoVs in 14 duck species covering 665 samples (65% of total samples analyzed). We did find one positive sample collected from a Ruddy Turnstone, a small wading bird in the sandpiper family, in the New Jersey shore area. Although not isolated from a duck species, the sample showed the highest sequence similarity to an IBV-like duck CoV originally isolated in China (DK/CH/HN/ZZ2004, Accession JF705860.1). With only one wild bird sample being positive for CoV from over 1,000 samples tested, our data suggest that waterfowl and shorebirds are not a significant natural reservoir for avian CoVs in the US although sample size, collection method, collection location, and bird age may impact prevalence.

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