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Source: Journal of Wildlife Diseases, 44(2) : 434-439

Published By: Wildlife Disease Association

URL: https://doi.org/10.7589/0090-3558-44.2.434

Journal of Wildlife Diseases, 44(2), 2008, pp. 434–439

Avian Influenza Surveillance in Hunter-harvested Waterfowl from the Gulf Coast of Texas (November 2005–January 2006)

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ABSTRACT: The objectives of our study were to determine prevalence of avian influenza viruses (AIV) on wintering grounds on the Texas Gulf Coast, USA, and to compare real-time reverse-transcriptase polymerase chain reaction (rRT-PCR) and virus isolation for detection of AIV in cloacal swabs from wild waterfowl. Cloacal swabs were collected from hunter-harvested waterfowl from November 2005 to January 2006 at four wildlife management areas. Seven AIV were isolated from four species of ducks: Green-winged Teal (*Anas crecca*) in November; Blue-winged Teal (*Anas discors*) in November; Mottled Duck (*Anas fulvigula*) in December, and Northern Shoveler (*Anas clypeata*) in January. Prevalence of AIV for each of these species during the sampling period was 1.4, 2, 6, and 0.6%, respectively. The AIV subtypes detected were H1N2, H1N4, H4N6, H6N2, and H10N7, all previously reported in North American waterfowl. Our study identified AIV subtypes not previously reported on the Texas Gulf Coast and provides baseline data for a multiyear surveillance project.

Key words: Avian influenza, ducks, surveillance, Texas, waterfowl, wintering grounds.

Wild waterfowl are considered the natural reservoir of type A influenza viruses (Webster et al., 1992). The migratory nature of many waterfowl species and the persistence of influenza in these populations present a vehicle for dissemination of influenza viruses globally. Understanding the migratory patterns of different waterfowl populations, as well as identifying influenza virus subtypes within these populations, is critical to our understanding of how influenza viruses persist in nature and evolve over time. With the increased concern regarding the spread of highly pathogenic avian influenza (HPAI) H5N1 viruses, and with wild waterfowl considered a vehicle for dissemination of the virus, several international surveillance programs have been implemented in an effort to reduce the potential worldwide spread of Asian-origin H5N1 virus. Real-time reverse-transcription polymerase chain reaction (rRT-PCR) for the matrix gene, a highly conserved and abundantly expressed gene in type A influenza virus, has been the primary tool used for recent wild waterfowl surveillance, although virus isolation is also being employed (Wild Bird Plan: An Early Detection System for Highly Pathogenic H5N1 Avian Influenza in Wild Migratory Birds U.S. Interagency Strategic Plan http://www.usda.gov/wps/portal/usdahome?contentidonly=true&contentid=2006/03/0094.xml, WHO Manual on Animal Influenza Diagnosis and Surveillance WHO/CDS/CSR/NCS/2002.5). In many of these surveillance programs, any samples positive for type A influenza virus are further screened by rRT-PCR specifically targeting H5 and H7 subtypes, the two most commonly associated with potential morbidity and mortality in poultry (Alexander 2000).

Before the implementation of surveillance programs focused on identifying HPAI H5N1 viruses, most studies on the prevalence of influenza viruses within North America were concentrated in

Alaska, USA; Canada; the upper Midwestern USA; and the Northeastern USA, and were conducted primarily during the late summer to early fall when premigration staging occurs (Webster et al., 1992; Krauss et al., 2004). Few studies involved waterfowl on their wintering grounds or nonmigratory waterfowl during the winter, particularly along the Gulf Coast, and most of those studies were limited to a few species (teal [*Anas crecca, Anas cyanoptera, Anas discors*], Gadwall [*Anas strepera*], Mottled Duck [*Anas fulvigula*], Northern Pintail [*Anas acuta*], and Mallard [*Anas platyrhynchos*]) (Stallknecht et al., 1990; Hanson et al., 2005). To develop a more comprehensive understanding of the ecology of influenza viruses in nature, more extensive studies are needed. The objectives of this study were 1) to determine the prevalence of avian influenza viruses (AIV) in both migratory and resident waterfowl along the Texas Gulf Coast, focusing on wintertime sampling; and 2) to compare real-time RT-PCR and virus isolation for the detection of avian influenza (AI) using cloacal swabs collected from wild waterfowl.

Cloacal swabs were collected from hunter-harvested waterfowl collected during the 2005–2006 hunting season (November–January) at four state wildlife management areas (WMA) along the Gulf Coast of Texas: Peach Point WMA in Brazoria County (28°56′55″N, 095°26′17″W), Mad Island WMA in Matagorda County (28°41′13″N, 096°48′46″W), Guadalupe Delta WMA in Calhoun County (28°30′51″N, 096°48′46″W), and Matagorda Island WMA in Calhoun County (28°20′40″N, 096°27′34″W). Trained personnel identified waterfowl species. Data from all four WMAs were combined for analysis. The sex and age of the waterfowl were not consistently recorded, so were excluded from analysis.

Trained personnel collected cloacal swabs within 6 hr of harvest using sterile Dacron swabs (Fisher Scientific, Houston Texas, USA) and placed them in 1.5 ml tryptose phosphate broth (TPB; Becton Dickinson, Franklin Lakes, New Jersey, USA) supplemented with antibiotics (penicillin G [2×10^3 U/ml], streptomycin [200 μg/ml], gentamicin [250 μg/ml], and amphotericin B [2×10^3 U/ml]) (Sigma, St. Louis, Missouri, USA) (Rosenberger et al., 1974; WHO Manual on Animal Influenza Diagnosis and Surveillance WHO/CDS/CSR/NCS/2002.5). Samples were transported from the field on wet ice (<10 hr collection and transport time) and stored at −80 C until processed.

Samples were processed for virus isolation and rRT-PCR as follows: samples were thawed and vortexed, swabs discarded, and the remaining fluid centrifuged 1,500 × G for 10 min. The supernatant was then diluted 1:2 in TPB containing antibiotics as listed above, and 100 μl was dispensed into 96-well plates for RNA isolation. Diluted samples and 96-well plates were frozen at −80 C until processed for virus isolation or rRT-PCR.

For rRT-PCR, 96-well plates were thawed, and RNA was extracted from the samples using the MagMax™-96 Viral RNA Isolation Kit (Ambion, Austin, Texas, USA) according to the manufacturer's instructions. Extracted RNA was transferred to nuclease-free 96-well plates for immediate use. rRT-PCR was performed using the AgPath-ID™ AIV-M Reagent Kit (Ambion), a one-step rRT-PCR for the detection of AI matrix-gene RNA per manufacturer's instructions, and an ABI 7900HT (Applied Biosystems, Inc., Foster City, California, USA) thermocycler in a 384-well format using a 15-μl final reaction volume. Primers and probe for the *M* gene, H5, and H7 were those described by Spackman et al. (2002).

For virus isolation, diluted samples were thawed and 0.2 ml was inoculated via the allantoic route into two 9-day-old embryonated chicken eggs. Eggs were incubated at 37 C for 72 hr, amnioallantoic fluid (AAF) was collected and subsequently tested for hemagglutination (HA) activity. Fluids negative for HA

activity were reinoculated into two 9-day-old embryonated chicken eggs. Positive (HA) fluids were further analyzed for the presence of influenza virus by rRT-PCR or by FluDetect® (Synbiotics Corporation, San Diego, California, USA) or both. Fluids testing positive for influenza virus by rRT-PCR or FluDetect were sent to the National Veterinary Services Laboratory in Ames, Iowa, USA, for typing via classical methods (hemagglutination inhibition [HI] and neuraminidase inhibition [NI] tests). Isolates that could not be typed by classical methods were typed by sequencing RT-PCR amplified HA genome segments as described by Hoffmann et al. (2001).

From November 2005 to January 2006, 1,460 waterfowl were sampled and 86 were positive for AIV by rRT-PCR. Twenty-three hemagglutinating agents were identified from 896 samples processed for virus isolation. Seven of the hemagglutinating agents (isolates) were determined to be AIV (Table 1), and the remaining 16 are presumed to be paramyxoviruses. Five of the seven (71%) AIV isolates were obtained on first passage and the other two (29%) upon second passage in embryonated chicken eggs. Of the influenza-positive samples, whether tested by rRT-PCR or virus isolation, none were positive for H5 or H7. We were unable to determine the hemagglutinin subtype from two isolates (one Green-winged Teal and one Mottled Duck) using conventional HI testing. These isolates were determined to be H1 by RT-PCR amplification and sequencing of the PCR products. Waterfowl species collected and areas sampled reflect hunters' choices and personnel available to collect swabs on sampling days. More dabbling ducks were sampled than diving ducks—1,270 dabbling ducks representing 13 species as opposed to 145 diving ducks representing seven species (Table 1). Overall prevalence of AIV based on rRT-PCR and virus isolation was 5.9 and 0.8%, respectively. Prevalence for the four species (Green-winged Teal, Blue-winged Teal, Mottled Duck, and Northern Shoveler) from which viruses were isolated were 1.4, 2, 6, and 0.6%, respectively, and by rRT-PCR were 8.5, 10, 7, and 7.3%, respectively (Table 1).

Our data support previous reports that dabbling ducks have a higher prevalence of infection with influenza viruses than other birds, including diving ducks (Olsen et al., 2006). Our sampling was probably biased to some unknown degree in that the variety of ducks sampled was in part a reflection of hunter's choices and not simply the relative abundance of each species. By targeting hunter-harvested waterfowl, however, we were able to estimate the prevalence of various AI subtypes carried by waterfowl in the Gulf Coast of Texas to which humans are likely to be exposed. After all, hunters are much more likely to come in direct contact with waterfowl than most other humans living in this region.

The 0.8% prevalence of AIV based on virus isolation reported here is consistent with previous reports of 0.4 to 2.0% on duck wintering grounds in the southern USA (Stallknecht et al., 1990; Olsen et al., 2006). Hanson et al. (2005), during an earlier study conducted at Peach Point WMA, Texas, USA, reported an AIV prevalence of >10%, which was considered unusually high by the authors. Perhaps the time of year or the year samples were collected could account for the discrepancy. Variables such as weather conditions and population densities could affect virus prevalence. Most samples collected by Hanson et al. (2005) were taken in February and included only a few species (teal [*A. crecca*, *A. cyanoptera*, *A. discors*] and Mottled Duck and Northern Pintail [*A. acuta*]), whereas our study focused on the wintering months (November to January) and included many more species (Table 1). One other study, conducted on the Gulf Coast of Louisiana, USA, reported results similar to ours (Stallknecht et al., 1990). These authors reported an overall prevalence of 2.0% in

TABLE 1. Results for avian influenza virus (AIV) virus isolation and real-time reverse transcriptase-polymerase chain reaction (rRT-PCR) from cloacal swabs obtained from waterfowl along the Texas, USA, Gulf Coast.

Species	rRT-PCR[a]	Virus isolation[a]	Isolate[b]
American Coot *Fulica americana*	0/13	0/10	
American Wigeon *Anas americana*	2/109 (1.8%)	0/83	
Bufflehead *Bucephala albeola*	0/1	0/0	
Canada Goose *Branta canadensis*	0/4	0/1	
Canvasback *Aythya valisineria*	0/7	0/7	
Common Snipe *Gallinago gallinago*	0/1	0/0	
Black-belly Tree Duck *Dendrocygna autumnalis*	0/10	0/0	
Gadwall *Anas strepera*	16/340 (4.7%)	0/149	
Greater White-fronted Goose *Anser albifrons*	1/10 (10.0%)	0/4	
Hooded Merganser *Lophodytes cucullatus*	0/4	0/3	
Lesser Scaup *Aythya affinis*	1/50 (2.0%)	0/19	
Mallard *Anas platyrhynchos*	1/4 (25.0%)	0/2	
Mottled Duck *Anas fulvigula*	3/42 (7.1%)	1/17 (5.9%)	H1N4[c]
Northern Pintail *Anas acuta*	1/38 (2.6%)	0/36	
Northern Shoveler *Anas clypeata*	17/234 (7.3%)	1/158 (0.6%)	H10N7
Redhead *Aythya americana*	1/47 (2.1%)	0/40	
Ring-necked Duck *Aythya collaris*	1/27 (3.7%)	0/13	
Ross's Goose *Chen rossii*	0/2	0/0	
Ruddy Duck *Oxyura jamaicensis*	0/9	0/7	
Teal unidentified	2/38 (5.3%)	0/5	
Teal, Blue-winged *Anas discors*	16/154 (10.4%)	2/96 (2.1%)	H4N6 (n=2)
Teal, Cinnamon *Anas cyanoptera*	0/2	0/2	
Teal, Green-winged *Anas crecca*	24/284 (8.5%)	3/218 (1.4%)	H4N6 H6N2 H1N2[c]
Sandhill Crane *Grus canadensis*	0/4	0/4	
Snow Goose *Chen caerulescens*	0/24	0/22	
Wood Duck *Aix sponsa*	0/2	0/0	
Total	86/1460 (5.9%)	7/896 (0.8%)	

[a] No. positive/No. tested (prevalence).

[b] Isolates typed by National Veterinary Services Laboratory (NVSL), Ames, Iowa, USA.

[c] The hemagglutinin of these isolates could not be subtyped by hemagglutination inhibition (HI) test and, therefore, were subtyped by sequencing.

November and 0.4% for December–January as compared with our 1.7% in November and 0.3% for December to January. Year-to-year and day-to-day variations in subtype and prevalence have been reported in other surveillance studies (Sharp et al., 1993; Runstadler et al., 2007).

The subtypes we identified are consistent with previous studies and are common North American subtypes. H3, H4, and H6 are considered the most common, whereas H1, H2, H7, H10, and H11 are less common. Krauss et al. (2004), in a 26-yr study of wild ducks in Canada, found that the most frequent subtypes isolated from ducks were H3N8 (22.8%), H6N4 (20.8%), and H4N6 (12.5%). In our study, three of the seven isolates were H4N6; interestingly, this subtype had previously been reported in Louisiana, USA, but not in Texas, USA (Stallknecht et al., 1990; Hanson et al., 2005).

The discrepancy between rRT-PCR results and virus isolation is not surprising. It is generally accepted that rRT-PCR is more sensitive than virus isolation because of its ability to detect both infectious and noninfectious viral particles (Kraft et al., 2005; Runstadler et al., 2007). It is possible the two freeze-thaw cycles our samples underwent, might have lead to negative isolation results for samples with low levels of virus. Several samples, however, underwent additional freeze-thaw cycles for reisolation attempts without problems, so we expect two freeze-thaw cycles to have had minimal affect. Another possible explanation for this discrepancy could be the length of time between hunter-harvest and sampling. During the teal season, hunters typically leave their blinds and pass through the check station within 2 hr of harvesting their teal; whereas, during the regular waterfowl season, most hunters remain in their blinds for the duration of hunting hours (sometimes up to 6 hr after shooting time) (Ferro, pers. obs.). It is also possible that the use of embryonated chicken eggs for virus isolation limits the isolation to those viruses capable of replicating in this system. The use of commercial eggs as opposed to specific-pathogen–free eggs could be a concern; however, because of the extensive AI surveillance in US poultry and the lack of vaccination of US poultry flocks, the concern is minimal.

Much information is available on the prevalence of AIV in ducks on premigration staging grounds (Sharp et al., 1993; Krauss et al., 2004). With current technology, large-scale sequence analysis of AIV isolates is possible and can provide valuable information on the evolution and persistence of influenza viruses in nature (Hatchette et al., 2004; Obenauer et al., 2006). Further studies involving molecular characterization and comparison of the same influenza virus subtype from different regions along a flyway will, therefore, provide significant information regarding what changes within AIV occur in nature. Similarly, studies following target species (those identified as having a high prevalence) throughout their migration, could provide valuable information regarding persistence of AIV in these species. Finally, studies covering consecutive years in the same wintering grounds will help us understand the ecology and evolution of influenza viruses and how these viruses persist in nature over winter. This study contributes to the knowledge base of influenza virus prevalence on waterfowl wintering grounds in Texas, USA, and provides baseline information for a multi-year surveillance project. Information gained over the next few years will assist in the elucidation of subtype prevalence, evolution, and persistence of AI in wild waterfowl, including migratory and non-migratory species on wintering grounds.

We greatly appreciate the cooperation and patience of the waterfowl hunters along the Texas Gulf Coast who graciously allowed us to sample their harvested waterfowl. We thank those who assisted in collecting cloacal swabs: V. Lowry, T. Raabe, T. J. Klein, S. Jester, S. Byrne, K.

Foley, S. Stevens, J. Cole, B. Jacklitsch, M. Ferro, R. Rollo, I. Rollo, J. Rollo, and K. Wilcox. We also appreciate the help of Texas Parks and Wildlife Department biologists D. Hailey, J. Oetgen, M. Nelson, M. Hensley, R. Korenek, K. Kriegel, G. Sheguit, L. Reinecke, M. Ealy, K. Hartke, and K. Kraai. For assistance with molecular testing, we thank the Animal Health Solutions Group at Ambion, Inc.: Q. Hoang, W. Xu, A. Burrell, M. Bounpheng, and C. Willis. This research was funded by the CSREES AICAP Prevention and Control of Avian Influenza in the US (AUP 2005-59).

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Received for publication 13 August 2007.