Transboundary and Emerging Diseases

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ORIGINAL ARTICLE

Avian Influenza Survey in Migrating Waterfowl in Sonora, Mexico

M. Montalvo-Corral, G. López-Robles and J. Hernández

From the Laboratorio de Inmunología, Centro de Investigación en Alimentación y Desarrollo, A.C. Hermosillo, Sonora, Mexico

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Correspondence:
Jesús Hernández. Laboratorio de Inmunología, Centro de Investigación en Alimentación y Desarrollo A.C., Carretera a la Victoria Km. 0.6. PO Box 1735, CP 83000, Hermosillo, Sonora, México. Tel.: +52 6622800010; Fax: +52 6622892400; E-mail: jhdez@ciad.mx

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Summary

A two-year survey was carried out on the occurrence of avian influenza in migrating birds in two estuaries of the Mexican state of Sonora, which is located within the Pacific flyway. Cloacal and oropharyngeal swabs were collected from 1262 birds, including 20 aquatic bird species from the Moroncarit and Tobari estuaries in Sonora, Mexico. Samples were tested for type A influenza (M), H5 Eurasian and North American subtypes (H5EA and H5NA respectively) and the H7 North American subtype (H7NA). Gene detection was determined by one-step real-time reverse transcription polymerase chain reaction (RRT-PCR). The results revealed that neither the highly pathogenic avian influenza virus H5 of Eurasian lineage nor H7NA were detected. The overall prevalence of avian influenza type A (M-positive) in the sampled birds was 3.6% with the vast majority in dabbling ducks (*Anas species*). Samples from two birds, one from a Redhead (*Aythya americana)* and another from a Northern Shoveler *(Anas clypeata)*, were positive for the low-pathogenic H5 avian influenza virus of North American lineage. These findings represented documented evidence of the occurrence of avian influenza in wintering birds in the Mexican wetlands. This type of study contributes to the understanding of how viruses spread to new regions of North America and highlights the importance of surveillance for the early detection and control of potentially pathogenic strains, which could affect animal and human health.

Introduction

Avian influenza virus (AIV) type A, is commonly found in waterfowl and shorebirds. These birds are a natural reservoir where low pathogenic AIV (LPAIV) viruses are maintained and genetically diversified (Webster et al., 1992). The gene pool of influenza viruses is well represented in birds from the Anseriformes (ducks, swans and geese) and Charadriiformes (gulls, waders and terns) orders (Alexander, 2000). Although host-range restrictions have limited the transmission of AIV to human hosts (Suzuki, 2005; Neumann and Kawaoka, 2006), mammals – including humans – can still be infected with specific subtypes. In 1997, the world was alarmed with the onset of the first human infections with the highly pathogenic avian influenza virus (HPAIV) subtype H5N1 of Asian origin (Yuen et al., 1998; Tran et al., 2004). This strain can also affect wild birds, which have been described to cause some massive mortality events in Southeast Asia and Europe (Ellis et al., 2004; Liu et al., 2005; Fink et al., 2010). In response to the zoonotic and pandemic potential, surveys have been implemented throughout the world to track the movement of this Asian strain and to increase knowledge about AIV in new regions. In addition, different multiyear studies have been conducted in North America over the last thirty years to investigate AIV ecology and epidemiology. These studies reported that influenza prevalence ranged from 0.7% in southern US latitudes to 55% in Canadian upper latitudes (Rosenberger et al., 1974; Hinshaw et al., 1980; Krauss et al., 2004). Although the HPAIV H5N1 virus has not been detected in North America, migration of waterfowl through flyways is one of the likely sources of entry of H5N1 and other important AIV. The aim of this study was to determine the presence of AIV in migratory waterfowl wintering in Sonora wetlands. The emphasis was on the detection of HPAIV H5N1 and LPAIV North American strains H5 and H7, which would be important for poultry production and human health in nearby areas.

Materials and Methods

Sampling strategy

The study area included Moroncarit (N 2,953940, E 646,307 UTM; 12 zone) and Tobari estuaries (N 2,988249, E 608,768 UTM), which are some of the most important wetlands within the Pacific flyway for avian migration in Mexico. Sampling size $n = 300$ birds per location and year, was calculated considering a 95% confidence interval and an expected influenza prevalence of 1% (U.S, Interagency Working Group, 2006; Guberti and Newman, 2007). The feasibility of sampling birds nationally sanctioned for hunting and other constraints were also taken into account.

Sample collection

Oropharyngeal and cloacal Dacron-tipped sterile swabs of birds were collected from an opportunistic sampling of hunter-killed aquatic migratory birds ($n = 1262$) during November 2007 through March 2008 and December 2008–February 2009. These samples were collected in collaboration with hunting clubs (duck hunters) from two management coastal areas. The age, collection date, site and sex of the birds were recorded before sampling. Dacron swabs were placed into cryovials containing transport medium (Hank's solution with 10% glycerol supplemented with 10 000 u/ml penicillin G, 2 mg/ml streptomycin, 1 mg/ml gentamicin and 0.02 mg/ml amphotericin B), packed with frozen gel packs and shipped to the Laboratory of Immunology from Centro de Investigación en Alimentación y Desarrollo, A.C., (within the next 6–24 h). Samples were pooled and stored at −80°C until use. The swabs were processed by pooling five cloacal or oropharyngeal samples of the same species, age and date of collection. Pools positive for AIV were separated and individual samples reanalysed.

RNA extraction

After the samples were thawed (pools or individual) and centrifuged, total RNA was manually extracted with an RNEasy mini kit, (Qiagen, Inc., Valencia, CA, USA) following the manufacturer's instructions. The RNA was eluted in 50 μl of nuclease-free water.

Real-time RT-PCR (RRT-PCR)

Real-time RT-PCR was carried out using QIAGEN® One-Step RT-PCR kit, (Qiagen, Inc.). Briefly, 8 μl of RNA was added to a 25-μl reaction mixture containing 0.8 μl of 10 mM dNTP mix, 1.25 μl of 25 mM MgCl2, 5 μl of 5 × One-Step RT-PCR buffer, 1 μl of One-Step Enzyme Mix, type A (matrix gene) and subtype specific H5 (Eurasian and North American lineages) and H7 (North American lineage) oligonucleotides and Taq Man probes (Spackman et al., 2002). In addition, another set of published oligonucleotides were used to detect the HPAIV H5N1 Asian strain (Payungporn et al., 2006). RRT-PCR conditions were one cycle at 50°C for 30 min, one cycle at 95°C for 10 min, forty cycles at 95°C for 15 s and 60°C for 10 s. RT-PCR products for M, H5 and N1 genes from viral RNA A/Vietnam/1204/2003 (H5N1) of a strain kindly provided by MS Irma López (National Institute for Diagnosis and Epidemiologic Reference, INDRE, Mexico City) were cloned and used as plasmidic positive controls (Montalvo-Corral et al., 2009). No template controls were included in each assay. A Mexican LPAIV H5N2 from chicken was also included as an H5 North American lineage positive control.

Results

To determine the sensitivity of our assay, standard curves were constructed according to a previous report (Montalvo-Corral et al., 2009). Results showed that the cut-off Ct value for a positive sample (M) was 37. Samples less than or equal to the cut-off value were retested to confirm the results. The populations sampled included dabbling and diving ducks, geese and shorebirds, which represented 20 species of the migratory and resident avifauna richness of two wildlife management areas in Sonora, Mexico (Table 1).

The analyses of field samples showed that 46/1262 cloacal samples were positive for the M gene with a mean Ct value of 33.2 (the range was from 26 to 36.8) (Table 1). These results represented an overall two-year prevalence of 3.6% and an annual prevalence of 2.93% and 4.3% in the 2007–2008 and 2008–2009 seasons respectively (Table 2). Interestingly, no oropharyngeal sample was positive and no significant differences were found in AIV occurrence due to the sampling location. After analysis, all of the positive samples ($n = 46$) were screened for subtyping. We tested for H5EA (Eurasian lineage), H5NA and H7NA (North American lineage) strains, and only positive samples to H5 were tested for N1. The results showed that all samples were negative to the HPAIV H5N1 as well as the H7NA subtype. Viral isolation attempts in embryonated chicken eggs for positive

Table 1. Detection of avian influenza virus (AIV) by Real-time RT-PCR (RRT-PCR) in species sampled during the 2007–2009 season at estuaries of Sonora, Mexico

Common Name	Species	M[a]	%	H5EA[b]	H5NA[c]	H7NA[d]
Green-winged Teal	*Anas crecca*	16/387	4.1	–	–	–
Northern Shoveler	*Anas clypeata*	12/160	7.5	–	1/12	–
Gadwall	*Anas strepera*	2/54	3.7	–	–	–
Ring-necked Duck	*Aythya collaris*	0/6	0	–	–	–
American Wigeon	*Anas americana*	8/124	6.4	–	–	–
Northern Pintail	*Anas acuta*	2/171	1.2	–	–	–
Blue-winged Teal	*Anas discors*	2/40	5	–	–	–
Cinnamon Teal	*Anas cyanoptera*	0/31	0	–	–	–
Redhead	*Aythya americana*	3/93	3.2	–	1/3	–
Lesser-scaup	*Aythya affinis*	0/9	0	–	–	–
Black Brant	*Branta bernicla*	0/156	0	–	–	–
Laughing Gull	*Larus atricilla*	0/1	0	–	–	–
Heerman's Gull	*Larus heermanni*	0/2	0	–	–	–
Wilson's Plover	*Charadrius wilsonia*	0/2	0	–	–	–
Mexican Duck	*Anas diazi*	1/20	5	–	–	–
White-winged Scoter	*Melanita fusca*	0/2	0	–	–	–
Mallard	*Anas platyrhynchos*	0/1	0	–	–	–
Long-billed Curlew	*Numenius americanus*	0/1	0	–	–	–
Marblet Godwit	*Limosa fedoa*	0/1	0	–	–	–
Double-crested Cormorant	*Phalacrocorax auritus*	0/1	0	–	–	–
Overall AIV prevalence	*3.6%*					

[a]Avian influenza virus matrix gene (M) detection; (%) percentage of positive birds.
[b]H5EA: Eurasian highly pathogenic H5 subtype.
[c,d]North American H5 and H7 subtypes.

Table 2. Type A (matrix segment) Real-time RT-PCR (RRT-PCR) detection by date and family

	Date					
	Nov 2007–Mar 2008 (*n* = 648)			Dec 2008–Feb 2009 (*n* = 614)		
Family	No. species	RRT-PCR[a]	H5[b] (LPAI)	No. species	RRT-PCR[a]	H5[b] (LPAI)
Anatidae						
Dabbling ducks	6/8	17/578 (2.9)	(-)	5/9	26/566 (4.7)	(1/26)
Anas crecca		6/231 (2.6)	(-)		10/156 (6.4)	(-)
Anas discors		2/22 (9.0)	(-)		0/18 (0)	(-)
Anas acuta		2/100 (2.0)	(-)		0/71 (0)	(-)
Anas americana		3/50 (6.0)	(-)		5/74 (6.7)	(-)
Anas diazi		0 (0)			1/20 (5.0)	(-)
Anas clypeata		2/25 (8.0)	(-)		10/135 (7.4)	(1/10)
Anas strepera		2/50 (4.0)	(-)		0/4 (0)	(-)
Diving ducks	1/2	2/66 (3.0)	(1/2)	1/4	1/44 (2.2)	(-)
Aythya americana		2/65 (3.0)	(1/2)		1/28 (3.5)	(-)
Charadridae	1	0/2 (0)	(-)		0 (0)	
Laridae	1	0/2 (0)	(-)	1	0/1 (0)	(-)
Scolopacidae		0 (0)		2	0/2 (0)	(-)
Phalacrocoracidae		0 (0)		1	0/1 (0)	(-)
One-year Prevalence		19/648 (2.93)			27/614 (4.3)	
Overall 2-year		46/1262 (3.6)				

[a]Avian influenza virus matrix gene (M) detection; number of positives/number of tested; (%) percentage of positive birds.
[b]North American H5 virus subtype.

samples were not successful. In addition, AIV were not detected in the shorebird species analysed in this study. Two H5 North American lineage viruses were identified, one sample came from a Redhead (*Aythya americana*) in the 2007–2008 season, and the other from a Northern Shoveler *(Anas clypeata)* during the 2008–2009 season.

Discussion

This study was conducted to increase knowledge on the occurrence of AIV type A in waterfowl and other migratory birds arriving in Sonora estuaries, which are located close to agricultural areas and human peri-urban settlements. The overall prevalence of AIV in migratory birds arriving at estuaries located in Sonora, Mexico was 3.6%; the prevalence in 2007–2008 was of 2.93% and 4.3% for season 2008–2009. Previous reports of the prevalence of influenza in different populations of free-living ducks and other aquatic birds in California were low (1–2%) (Slemons et al.,1974; Siembieda et al.,2010). However, comparison is difficult because variations in influenza infections could be influenced by geographical and temporal factors (Table 1). This has been observed in massive monitoring studies in Alaska, where the prevalence of influenza ranged from 1.7 to 25% (Runstadler et al., 2007; Ip et al., 2008).

Avian influenza virus was detected during both years of the survey in some species and no significant differences were observed due to location (Table 2). Similar to other reports, most of the species positive for AIV were from the *Anas* genus (Olsen et al., 2006; Wallensten et al., 2006; Munster et al., 2007). Although most surveillance studies in North America have included Mallards (*Anas platyrhynchos*), which are well-known carriers of influenza virus, other species have also been shown to be susceptible to infection with different subtypes of AIV (Olsen et al., 2006). Anseriformes (mainly *Anas* genus), are intrinsically related to water resource, an abiotic reservoir for AIV, in a low to middle salinity range. The circulation of AIV in these populations could be maintained for the bird behaviour patterns and by the continuous exposition of susceptible birds to viral particles. In addition, the high prevalence observed in Mallards could be result of a higher susceptibility to viral infection compared with other species. But also a sampling bias should not be ruled out, since during epidemiological surveys, it is the species mostly targeted. Our results agree with the vast evidence of the occurrence of AIV in the *Anas* group and also it is supported by our findings that diving ducks particularly the *Aythya* genus, was a carrier of LPAIV H5 subtype. We included a comprehensive list of representative migratory species arriving to coastal estuaries of Sonora, Mexico but Mallards were less represented because they were rare in our sampling area. We did not identify any influenza-positive samples from Black Brants or the few shorebirds, seagulls and diving ducks, except for the positive male Redhead (*Aythya americana*), which has been a lowly represented species in influenza surveys. Salinity, pH and temperature are factors that can limit the presence of influenza virus in aquatic environments (Stallknecht et al., 1990; Brown et al., 2009) and these birds typically live in marine or brackish habitats, which have been suggested as one of the reasons for the low prevalence of AIV.

The epidemiological status of AIV in feral aquatic migratory and resident bird populations in Mexico and Latin American countries has been poorly studied. Recently, an H7N3 virus was isolated from a Cinnamon Teal (*Anas cyanoptera*) in central Mexico (Cuevas-Domínguez et al., 2009). In another report, we described the analysis of two North American LPAIV H5 subtype, one H6 subtype and one H9 subtype. In addition, the phylogenetic relationships and molecular determinants of low pathogenicity were confirmed by partial sequencing of hemagglutinin, including the cleavage site (Montalvo-Corral and Hernandez, 2010). Previous studies showed that the occurrence of the H5 subtype was not uncommon in AIV monitoring (Hanson et al., 2003; Wallensten et al., 2007; Dusek et al., 2009). Other studies have provided information about the AIV in migratory and resident birds in Argentina, Bolivia and Caribbean countries, and these studies identified H1, H3, H4, H10 and H13 subtypes (Douglas et al., 2007; Spackman et al., 2007a; Pereda et al., 2008; Ghersi et al., 2009; Alvarez et al., 2010). This kind of research can provide valuable information about the species involved in the introduction and circulation of AIV to new areas.

In Mexico, AIV reports have been incorporated into surveillance activities performed by sanitary officials at the poultry farms as part of the campaign for early detection and prevention of HPAI influenza outbreaks (Villarreal-Chávez and Rivera-Cruz, 2003). During the outbreak of an HPAI in 1995, the responsible virus was characterized as the H5N2 subtype, and it was determined to have arisen from a LPAIV precursor (Horimoto et al., 1995). However, the origin of this strain remains unclear because it genetically shifted apart from other H5 North American water bird-isolated strains (Spackman et al., 2007b). This subtype has continued to circulate in some central and southern Mexican states; however, other regions (including the Sonora State in Northwestern Mexico) are free of AIV. The introduction of LPAIV H5 strains from waterfowl into wetlands is an interesting issue that requires further analysis in our region and other regions with similar agro-ecological characteristics because of the ability of this subtype to possibly become highly pathogenic in poultry (Horimoto et al., 1995). Therefore, an improvement in poultry farm biosecurity should be considered to prevent potential AIV outbreaks. Such measures may be the design of a biosecurity plan that includes traffic control in the farm and ways to prevent interactions between free-flying waterfowl and domestic poultry populations, as well as the potential introduction of AIV by trade. Further, a critical component of the plan to achieve the goals is the training of poultry farmers through an educational programme.

To address AIV subtype diversity in waterfowl, researchers need to examine seasonal variation, virus persistence in the environment, inter-relationships with resident birds and the risk of potential transmission to domestic animals (e.g. poultry and swine) in nearby agricultural areas.

This exploratory study contributed to the knowledge of the occurrence of AIV in wintering birds passing through the Mexican wetlands. In addition, this study provided baseline information for future research and highlighted the importance of surveillance for the early detection and control of potentially pathogenic strains that could affect animal and human health.

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