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Review

Presence of antibodies against H5, H7 and H9 influenza A virus in wild birds in the State of São Paulo, Brazil

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ABSTRACT

Although the natural reservoirs of the avian influenza (AI) virus have been extensively studied in many countries, there is a clear lack of information on this subject in South America, particularly in Brazil. The objective of this study was to conduct a serological survey for H5, H7 and H9 antibodies to AI-subtype viruses in wild birds in the state of São Paulo , Brazil. Serum samples were tested using the hemagglutination-inhibition assay. Out of the 31 wild birds sampled between January and December of 2006, seven (22.58%), were seropositive for H5, H7 and H9; four (12.90%) were seropositive for H5 and H7; 13 (41.94%), were seropositive only for H7; three (9.7%), were seropositive only for H9; and four (12.90%) were negative for all three hemagglutinin subtypes. These results indicate that AI viruses belonging to H5, H7 and H9 subtypes circulate among wild birds in the state of São Paulo in the form of either concurrent or consecutive infections. This study contributes to the knowledge of AI epidemiology in Brazil, and stresses the need of further detailed and long-term epidemiological and ecological investigation to determine the current status of this virus.

INTRODUCTION

Influenza virus type A of bird origin, also called avian influenza (AI) virus, has been implicated in endemic infections and outbreaks in poultry and wild birds and in human infection and fatalities, as well as in important economic losses (Martins, 2001; Mcleod, 2008; Moraes *et al*., 2009; Malik, 2009; Lupiani & Reddy, 2009; Kalthoff *et al*., 2010). AI viruses belong to the Orthomyxoviridae family, and are classified into subtypes based on two surface glycoproteins used for host-cell entry: hemagglutinin (H) and neuraminidase (N) (Moraes *et al*., 2009). Different 16 H and 9 N subtypes are currently identified (Halvorson, 2002; Suarez, 2008). AI viruses are further classified into low pathogenic AI (LPAI) or highly pathogenic AI (HPAI) viruses according to their ability to cause illness and death of 4- to 6-week-old chickens infected intravenously and/or the presence of multiple basic amino acids at the cleavage site of the H molecule (World Organisation For Animal Health Avian Influenza, 2009). So far, only H5 and H7 AI viruses have been identified as HPAI, and these viruses have been responsible for outbreaks in many countries, causing the death of many thousands of domestic poultry and wild birds (Capua & Alexander, 2004; Kalthoff *et al.*, 2010). In 1999, an H9N2 AI virus was implicated in human infections in Asia, suggesting a pandemic threat related to this virus (Lin *et al.*, 2000; Peiris *et al.*, 1999).

AI viruses have been isolated in more than 100 wild avian species worldwide (Stallknecht & Shane, 1988; Alexander, 2000; Olsen *et al.*, 2006; Stallknecht & Brown, 2008). Most have been isolated from species present in wetlands and aquatic habitats, particularly Anseriformes and Charadriiformes, which are considered as natural reservoirs of AI viruses (Stallknecht & Brown, 2007; Webster *et al.*, 1992). AI infections in these birds are usually subclinical, and can be produced by a single or multiple AI viruses simultaneously (concurrent infections) or subsequently (consecutive infections) (Sinnecker *et al.*, 1982; Suss *et al.*, 1994). Although the diagnosis of AI is usually based on virus isolation and identification (Martins, 2001; Swayne & Halvorson, 2008), serological tests, such as agar gel immunodiffusion, enzyme-linked immunosorbent assay, and hemagglutination inhibition (HI) test, have been widely used for AI surveillance (Allwinn *et al.*, 2002; Brown *et al.*, 2010; Moraes *et al.*, 2009; Swayne & Halvorson, 2008).

AI is considered an exotic disease in Brazil, and has not been reported in commercial poultry (Martins, 2001; Moraes *et al.,* 2009). Few studies have been conducted in Brazil aiming at either isolating (Couceiro, 1986; Kawamoto *et al.*, 2005) or identifying (Soares, 2002) AI viruses, or at investigating the production of antibodies (Oliveira Junior *et al.*, 2001; Viegas, 2006) in wild or ornamental birds and domestic poultry. The present study reports the results of a serological surveillance of H5, H7 and H9 AI viruses in wild birds in the state of São Paulo, Brazil, and aims at contributing to the current knowledge on AI in South America.

MATERIAL AND METHODS

Birds

Thirty-one wild birds (Table 1) were included in this study. Birds were admitted to the Exotic and Wild Animal Service of Hospital Veterinário Governador Laudo Natel (Faculdade de Ciências Agrárias e Veterinárias, Universidade Estadual Paulista, Jaboticabal, SP, Brazil) between January and December of 2006. Birds had been found injured and had been taken to the hospital by firefighters, forest rangers, or employees of the highway concessionaires in the area. Access to birds and collection of biological samples were authorized by the Brazilian Institute of Environment and Renewable Natural Resources (IBAMA, process # 02027.000775/2005-25; license # 500/2005).

Blood collection and Serology

Blood was collected from the right jugular vein. Plasma was separated by centrifugation, inactivated at 56 °C for 30 minutes, and stored at -20°C.

HI test was used for the detection of influenza antibodies. In order to remove nonspecific hemagglutination inhibitors, 0.2 mL of plasma were added to 0.8 mL of 25% kaolin and 1.0 mL of phosphate buffered saline solution (PBS) at pH 7.2. The mixture was homogenized, incubated overnight at 37°C, and centrifuged at 600g for 30 minutes. The supernatant transferred to clean tubes. In order to remove nonspecific hemagglutinating agents, plasma was then incubated in 0.2mL suspension of chicken erythrocytes at 50% in PBS for one hour at room temperature (25°C). After incubation, erythrocytes were removed by centrifugation at 600g for 10 minutes at 8°C and the sample was stored at -20°C. The influenza virus strains A/RT/DE/244/94 (H5N2); A/Equine/Prague/56 (H7N7) and A/Hong Kong/1073/99 (H9N2) were inactivated in β-propiolactone and used as viral antigens.

HI was performed in 96-well U-shaped microtiter plates. Treated plasma was diluted in 50μL aliquots in PBS, and mixed with four hemagglutination units of viral antigen. Plates were incubated at room temperaturefor 30 minutes, and 50μL of a suspension of washed SPF chicken red blood cells (1% in PBS) was added to each well. After 30 minutes incubation at room temperature, the microtiter plates were evaluated for the presence of hemagglutination inhibition. Samples with HI titers < 40 for H5, < 64 for H9, and < 256 for H7 were considered negative. Analyses were performed at the Laboratory of Clinical and Molecular Virology of the Institute of Biomedical Sciences II, University of São Paulo (São Paulo, Brazil).

RESULTS AND DISCUSSION

Between January and December of 2006, a total of 31 wild birds (11 species and 7 orders) were tested (Table 1). Twenty-seven (87.0%) of the 31 samples analyzed were positive for AI by the HI test (Table 1). Antibodies against one, two or all three AI subtypes tested were present in a single bird, indicating previous concurrent or consecutive infections with different AI virus subtypes. Overall, 24 birds (77.4%) had detectable antibody titers against H7, 11 birds (35.5%) against H5, and ten (32.3%) against H9. Seven birds (22.6%) were positive for the presence of antibodies against H5, H7 and H9; four (12.9%) for the presence of antibodies against H5 and H7; 13 (41.9%) for the presence of antibodies against H7 only; three (9.7%) for the presence of antibodies only against H9; and four (12.9%) were negative for all three H subtypes

Table 1 - Hemagglutination inhibition (HI) antibody titers against H5, H7 and H9 avian influenza viruses in wild birds, São Paulo state, Brazil.

Avian Species (n)	HI antibody titer[a]			Month of Sample Collection
	H5	H7	H9	
Burrowing owl (*Speotyto cunicularia*) (7)	320	1280	160	August
	40	320	80	September
	160	1280	80	September
	160	1280	-	April
	-	640	-	August
	-	-	80	September
	-	-	80	October
Barn owl (*Tyto alba*) (3)	80	80	-	November
	-	1280	-	January
	-	80	-	September
Rock pigeon (*Columba livia*) (5)	160	320	80	July
	40	80	80	February
	-	640	-	March
	-	80	-	September
	-	-	-	April
Ruddy ground dove (*Columbina talpacoti*) (2)	-	1280	-	August
	-	-	-	June
Chalk-browed mockingbird (*Mimus saturninus*) (6)	160	1280	80	September
	-	1280	-	September
	-	320	-	October
	-	320	-	October
	-	320	-	December
	-	-	-	April
Great kiskadee (*Pitangus sulphuratus*) (1)	-	-	-	December
Campo flicker (*Colaptes campestris*) (1)	-	-	80	October
Toco toucan (*Ramphastos toco*) (1)	-	1280	-	March
Red-legged seriema (*Cariama cristata*) (3)	80	640	80	May
	-	2560	-	May
	-	640	-	November
Black-crowned night-heron (*Nycticorax nycticorax*) (1)	80	640	-	June
Guira cuckoo (*Guira guira*) (1)	80	320	-	February

[a] Samples with HI titer < 40 for H5, < 256 for H7, and < 64 for H9 were considered negative.

analyzed. The lack of data on the prevalence of type-specific antibodies against AI in wild birds in Brazil limits the interpretation and comparison of the results of the present study .

Virus isolation was not attempted in any of the birds in this study. Therefore, it is not known whether these birds were actively shedding AI virus at the time of admittance, and it is not possible to make any statements relating the type of injury birds presented with their AI status. Due to the relatively small number of samples analyzed each month, it is also not possible to make any statistical correlations relative to seasonal variation with AI status.

The results of this study evidenciate that AI viruses belonging to H5, H7 and H9 subtypes circulate among wild birds in the state of São Paulo. Moreover, 11 birds included in this study were previously infected with more than one subtype of AI virus, either concurrently or consecutively. This is an evidence of the complex natural history of AI in wild avian populations (Dugan *et al.*, 2008). Avian influenza, particularly in wild birds, has received little attention in Brazil. Although some studies report the isolation and identification of this agent in Brazil (Couceiro, 1986; Kawamoto *et al.*, 2005), the AI virus is still considered exotic in this country. More detailed and long-term AI serosurveillance studies in wild birds, associated with attempts to isolate the virus, are needed to determine the status and the epidemiological characteristics of this disease in Brazil.

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