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Investigation of Influenza A, West Nile and Newcastle Disease Viruses in Birds from the Pantanal Wetlands of Mato Grosso, Brazil

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ABSTRACT

The Pantanal is the world's largest wetland biome with a seasonal flood pulse that attracts a great diversity of birds, many of which are migratory. Birds can be natural reservoirs *Influenza A*, *West Nile* and *Newcastle Disease* viruses. However, the occurrence of carriers for these viruses in the Pantanal was not verified yet. The present study evaluated the occurrence of natural infection by Influenza A, WN and ND virus of birds in the municipality of Poconé, a subregion of the Pantanal in the state of Mato Grosso, Brazil. A total of 76 birds belonging to 11 orders and 20 families were captured using mist nets. The most representative order was Passeriformes, followed by the other nine orders, which included Columbiformes, Psittaciformes, Charadriiformes and Anseriformes. The most representative family was Thamnophilidae, with 16 individuals (21.0%), followed by the family Tyrannidae with 10 individuals (7.6%) and the family Furnariidae, with eight individuals (10.5%). The bird species were identified, and cloacal and tracheal swab samples were collected. The samples were subjected to RNA extraction and tested for the presence of the three agents by real-time polymerase chain reaction (qRT-PCR). All the sampled birds were considered healthy, had no clinical sign of infection, and were tested negative for the three viruses. Based on our findings, we can conclude that *Influenza*, *West Nile* and *Newcastle Disease* viruses were absent from the samples in this region of the Pantanal wetlands during the period of this study.

INTRODUCTION

The Pantanal has a wide diversity of bird species and, compared to other similar floodplains around the world, it is considered the most diverse. Most of these species depend on multiple habitats to survive throughout the year, and the annual flood pulse of the Pantanal attracts many migratory birds, further contributing to the diversity of birds in this region (Pinho, 2005, Signor & Pinho, 2011).

According to the first bird survey in the Brazilian Pantanal wetlands performed in 1986 by Brown Jr. (1986), and more recent surveys, the birds occurring most frequently in the Pantanal belong to the family Tyrannidae with 48 species, followed by the family Emberizidae with 21 species, including both resident and migratory birds (Nunes *et al.,* 2008; Signor & Pinho, 2011). Many of these birds, being they migratory, wild or domestic, are natural hosts and transmit various infectious viruses, such as Influenza A, West Nile and Newcastle Disease (Fernandes *et al*., 2010, Flores & Weiblen, 2009).

The Avian Influenza virus belongs to the family *Orthomyxoviridae* and the genus *Influenza A*. The RNA of this virus comprises eight segments that encode several proteins, including hemagglutinin (H)

and neuraminidase (N). These proteins are responsible for the characterization of the subtype viral strain of Influenza A. This virus, which is highly mutable and therefore presents high levels of virulence, has so far been isolated in birds, pigs, horses and mammals in general, including humans (Carneiro *et al*., 2009). Surveys of Influenza virus in bird specimens collected in the state of São Paulo, Brazil revealed high viral loads, emphasizing the need to monitor birds as reservoirs of *Avian Influenza* (Kawamoto *et al*., 2005). The first case of *Avian Influenza Virus* H11N9 isolated from migratory birds in South America was recently reported in the Amazon region (Araujo *et al*., 2014). However, the pathogenicity of those H11N9 subtypes was considered low.

The *West Nile virus* (WNV) belongs to the family *Flaviviridae* and the genus *Flavivirus*. This family also includes other very important pathogens such as *Dengue virus* and *Yellow Fever virus* (Flores & Weiblen, 2009). There are two known WNV strains. The first was described in Africa in 1937, and present in Europe and Asia. The second strain circulates in North America since 1999; it was first described in New York and has subsequently spread throughout the United States, infecting wild and domestic birds, reptiles, mammals, and humans. The viral isolation case closest to Brazil was reported in Argentina in 2006, in an outbreak of equine neurologic disease. The ecological conditions of South and Central America, i.e., climate, vegetation and fauna, favor the introduction and maintenance of this virus in this region (Morales *et al*., 2006). Anti-WNV antibodies in horses have also been reported in the Pantanal region of Mato Grosso and its surroundings (Ometto *et al*., 2013, Pauvolid-Corrêa *et al*., 2011). In addition, the Brazilian Ministry of Health of Brazil recently reported a case of West Nile Virus (WNV) in the State of Piauí. This was the first detection of a human case of WNV infection in Brazil (Vieira *et al*., 2015).

The *Newcastle Disease virus* (NDV), which belongs to the family *Paramyxoviridae* and genus *Avulavirus*, has various pathogenic strains (Arns *et al*., 2007; Farkas *et al*., 2009; Fernandes *et al*., 2010). The history of Newcastle Disease began in 1926 with the description of a highly pathogenic disease in two geographical areas on opposite sides of the world, Newcastle-on-Tyne in England and the island of Java in Indonesia (Kraneveld, 1926; Doyle, 1927; Alexandre, 2001). NDV infections have been detected in at least 241 avian species, representing 27 of the 50 orders of the class (Alexander & Manvell, 2004), and many serotypes have been isolated from asymptomatic wild aquatic birds (Alexander, 1995). Today this disease is distributed around the globe, since the lack of specific legislation on international trade in birds in the 90s enabled the worldwide dissemination of the virus (Farkas *et al*., 2009, Fernandes *et al*., 2010). Phylogenetic analysis has separated NDV into two sister clades, class I and II, which contain several genotypes each. The majority of reported virulent viruses belong to class II, while low virulence NDV, characteristic of wild birds, predominate in class I (Kim *et al*., 2008).

Considering the importance of the Pantanal wetlands of Mato Grosso as a region of agglomeration of resident and migratory birds, the aim of this study was to investigate the presence of Influenza, West Nile and Newcastle Disease viruses in birds collected in this region of Brazil.

MATERIALS AND METHODS

The study was carried out in the region of Porto Cercado, located in the municipality of Poconé, state of Mato Grosso (Figure.1). The studied area contains four forest types known regionally as Cambarazal, Landizal, Pombeiro and Cordilheiras. These phytophysiognomies are forests seasonally flooded from January to June, except for Cordilheiras, which is flood-free throughout the year. The climate is characterized by a pronounced dry season from May to September and a wet season from October to April, with an average rainfall of 1400 mm in the rainy season, with maximum rainfall in January and minimum in July (Nunes & Junk, 2004).

The birds were captured in July and November, 2013, and February, 2014. Captures were carried out using ten 10m long, 2.75m tall mist nets (36mm mesh) set up consecutively in three sampling points. The collection campaign covered the three seasonal cycles of the Pantanal, *i.e.*, the flood, ebb and dry phases. The nets were left open to capture specimens only in the morning, from 06:00 to 10:00h. Species were identified based on the field guide of Qwynne *et al*. (2010), and, after completing the capture and sample collection processes, the birds were released. The sampling effort involved 1200 mist net/hours (Signor & Pinho, 2011) and encompassed a total area of 100m at each collection point.

Secretions of the cloaca and orotracheal mucosa of the captured birds were sampled using sterile disposable swabs. Samples were stored in 2mL cryotubes in transportation media containing antifungals, antibiotics, and glycerol, according to the protocol

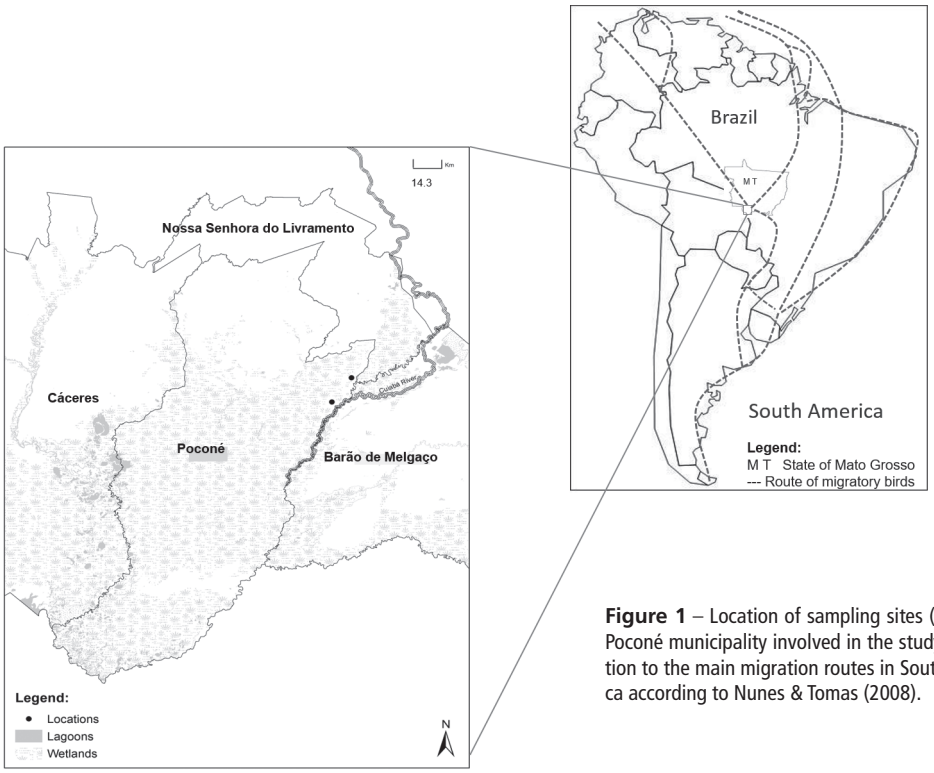

Figure 1 – Location of sampling sites (●) in the Poconé municipality involved in the study in relation to the main migration routes in South America according to Nunes & Tomas (2008).

described in the Manual on Animal Influenza Diagnosis and Surveillance of the World Health Organization (Webster *et al*., 2002). The duly identified cryotubes were immediately frozen in liquid nitrogen at -196ºC until the moment of analysis.

Cloacal and orotracheal samples from each individual bird were pooled. The genetic material in each pool was extracted using a semi-automated MagMAX™ Express-96 Pathogen RNA/DNA kit (5X) (Ambion, Inc., Austin, TX, USA), according to the protocol described by Ometto *et al*. (2013). The RNA sample extracted from each pool was analyzed by One-Step Quantitative Real-Time PCR (qRT-PCR) in an ABI 7300 Real-Time PCR System® (Applied Biosystems, Foster City, CA, USA). The cDNA was synthesized from the extracted viral RNA, which amplifies the genetic material in a single step using specific probes and primers for each agent.

A TaqMan AIV-M Matrix Reagents kit (Applied Biosystems) was used for the detection of Influenza virus, using specific primers for the matrix gene (Curd *et al*., 2011). For the detection of WNV, specific primers for Gene E, SEQ1F (GCGATCTCTCCACCAAAGCT) and SEQ1R (TGGGTCAGCACGTTTGTCAT) were applied with the SEQ1M1 [FAM]-CCATGGGAGAAGCTCACA [NFQ] probe (Ometto *et al*., 2013). Lastly, to detect both classes of NDV, a specific class I NDV pair of primers, forward for the matrix gene M+4100F (AGTGATGTGCTCGGACCTTC) and reverse for M-4220R (CCTGAGGAGAGGCATTTGCTA), covering 120 pb, were applied with an M4169 [FAM] TTCTCTAGCAGTGGGACAGCCTGC [MGB] probe (Wise *et al*., 2004); and class II NDV with same cycling conditions, allowing a multiplex format, whereupon the polymerase gene L+8738 sense primer (TGTTGAAAAGAAGCTGCTAGGC) and reverse L-8847 (TGGACCATGAAGAGTGGAACC), were applied with an L8762 [TET] TGCCTGGTCACACAAGATCCGCCG [MGB] probe (Kim *et al*., 2008).

The samples that showed an amplification curve in the qRT-PCR for any of the three viruses under study were then subjected to conventional RT-PCR. Samples that had real-time PCR amplification for Influenza virus were retested using the conventional PCR method on another region of interest of the matrix gene, using specific forward M52CF (CTTCTAACCGAGGTCGAAACG) and reverse M253R (AGGGCATTTTGGACAAAKCGTCTA) primers that amplify approximately 245 bp. Samples amplified by one-step RT-PCR are considered positive only after amplification by standard RT-PCR and confirmation by sequence analysis.

The resulting amplifications were subjected to an electrophoretic run in 1% agarose gel and examined

using a transilluminator under UV light (Webster *et al*., 2002). The products of the conventional RT-PCR amplification were purified according to the protocol of Applied Biosystems, using ExoSAP-IT (GE) reagent on 5 µl of cDNA sample, followed by purification using BigDye® XTerminator reagent (Applied Biosystems). Lastly, the purified samples were subjected to direct double-stranded sequencing using a BigDye Terminator Cycle Sequencing Ready Reaction kit and AmpliTaq DNA polymerase kit (Applied Biosystems) (Thomazelli *et al*., 2012). The samples were sequenced in an ABI PRISM 3100 Genetic Analyzer and the resulting sequences were aligned with other corresponding sequences available in GenBank, using the NCBI Nucleotide BLAST Search program (Altschul *et al*., 1990).

All the above described laboratory procedures were performed in the Level 3+ Biosafety Laboratory (BSL3+) of the Institute of Biomedical Sciences II at the University of São Paulo (USP). This project was registered on March 23, 2013 in Brazil's Biodiversity Authorization and Information System – SISBIO, under No. 10698-3, and was granted a permanent license to collect zoological material. The project is also filed at the Ethics Committee on Animal Use (CEUA), under No. 23108.041148/13-0, in accordance with the ethical principles of animal experimentation adopted by the National Council for the Control of Animal Experimentation (CONCEA).

RESULTS

Seventy-six endemic, migratory, domestic and wild birds were recorded, distributed among 11 orders and 20 families. The most representative order was Passeriformes (10 families, representing 50% of the total), followed by the other nine orders, which included Columbiformes, Psittaciformes, Charadriiformes, and Anseriformes, each represented by only one family. The most representative family was Thamnophilidae, with 16 individuals (21.0%), followed by the family Tyrannidae with 10 individuals (7.6%) and the family Furnariidae, with eight individuals (10.5%) (Table 1).

No amplification curves were observed in the qRT-PCR for either WNV or NDV. In the case of *Influenza A* virus, 14 samples showed an amplification curve in the initial qRT-PCR results. Then, using the conventional PCR method, 10 samples presented amplicons of approximately 245 bp. The amplified suspected samples were sequenced and analyzed in the database. However, none of the samples presented a sequence similar to that of *Influenza A* virus.

DISCUSSION

Despite the considerable number and diversity of bird species sampled in this study, none was tested positive for the target agents. According to Steininger *et al*. (2002), the most suitable tests for effectively diagnosing avian viral diseases, including avian Influenza, WNV and NDV, are RT-PCR and qRT-PCR owing to their high sensitivity and specificity. Based on these data, we analyzed the samples from the captured birds for the three viruses, using molecular methods. Nguyen *et al*. (2013) found that the use of multiplex qPCR can lead low detection sensitivity, which is why they tested the three viruses separately.

Pauvolid-Correa *et al*. (2011) described the Pantanal as a favorable location for studies on infectious diseases in birds, considering that natural infection foci have already been described in wetlands around the world. Moreover, the abundant movement of migratory birds in Brazilian Pantanal wetlands explains the recent detection of arboviral infection in humans, horses and reptiles in the region, and of antibodies against WNV in horses. On the other hand, crocodilians captured in the Pantanal, coexisting with several species of water birds, tested negative for WNV (Pauvolid-Corrêa *et al.,* 2011), which is consistent with the findings of this study. Ometto *et al*. (2013), who also evaluated birds in the same region, did not detect any positivity for WNV. Negative results in birds and crocodilians, coupled with the serological detection of antibodies in horses, reinforces the need for further studies and intensive surveillance of WNV in the Pantanal region.

In this study, none of the birds collected in the Pantanal was tested positive for NDV. On the other hand, Demetrio (2002) reported the detection of NDV in wild birds in the state of São Paulo by RT-PCR, and emphasized the significant sensitivity of this test and the importance of monitoring Newcastle Disease in wild birds in Brazil. The negative results found in this study for Influenza viruses are similar to those reported by Buscaglia (2012), who captured 157 birds in Argentina, all of which were tested negative for the presence of the avian influenza virus. On the other hand, Kawamoto *et al*. (2005) and Soares *et al*. (2005) detected the presence of *Influenza A* viruses in wild and migratory birds in the state of São Paulo. The first subtype H11N9 isolated from migratory birds collected in the Amazon region was recently reported, and was found to present a very high similarity with viral subtypes described in North

Table 1 – Order, Family and species of birds collected in the Pantanal region of Poconé, that resulted negative for Influenza A, West Nile and Newcasttle viruses.

Order	Family	Species	Collected
Passeriformes	*Emberizidae*	*Arremon flavirostris*	1
		Paroaria capitata	2
	Tyrannidae	*Basileuterus flaveolus*	3
		Camptostoma obsoletum	1
		Hemitriccus striaticollis	1
		Inezia inornata	1
		Myiarchus ferox	2
		Platyrinchus mystaceus	1
		sp.	1
	Troglodytidae	*Campylorhynchus turdinus*	2
		Pheugopedius genibarbis	1
	Thamnophilidae	*Cercomacra melanaria*	4
		Herpsilochmus longirostris	1
		Thamnophilus doliatus	1
		Thamnophilus pelzelni	1
		Taraba major	3
		Hypocnemoides maculicauda	3
		Myrmotherula axillaris	3
	Furnariidae	*Cranioleuca vulpina*	5
		Synallaxis albilora	1
		Furnarius leucopus	2
	Thraupidae	*Lanio penicillata*	2
		Ramphocelus carbo	1
		Tachyphonus luctuosus	2
	Pipridae	*Pipra filicauda*	1
	Rhynchocyclidae	*Poecilotriccus latirostris*	3
	Dendrocolaptidae	*Sittasomus griseicapillus*	2
		Xiphorhynchus guttatus	1
		Xiphorhynchus picus	2
	Turdidae	*Turdus leucomelas*	1
Apodiformes	*Trochilidae*	*Amazilia fimbriata*	1
Psittaciformes	*Psittacidae*	*Amazona aestiva*	1
		Psittacara leucophthalmus	1
Charadriiformes	*Charadriidae*	*Vanellus chilensis*	1
Anseriformes	*Anatidae*	*Amazonetta brasiliensis*	2
Strigiformes	*Strigidae*	*Athene cunicularia*	1
Accipitriformes	*Accipitridae*	*Buteogallus urubitinga*	1
		Spizaetus melanoleucus	1
Columbiformes	*Columbidae*	*Columbina sp*	1
		Leptotila rufaxilla	3
Galbuliformes	*Galbulidae*	*Galbula ruficauda*	1
Galliformes	*Phasianidae*	*Galus galus*	6
Rheiformes	*Rheidae*	*Rhea americana*	1

America (Araujo *et al*., 2014). Therefore, the lack of detection of the viruses in the birds of present study may be associated with the captured species, which were mostly resident species with terrestrial habits. Cui *et al*. (2011) stated that only aquatic birds of the orders Charadriiformes and Anseriformes are natural hosts of Influenza virus, and in our study, these two orders of aquatic birds accounted for only 18% of the total sample.

Based on the findings of this study, it can be concluded that there was no evidence of the circulation of *Influenza A*, WN, or ND viruses in the birds studied between 2013 and 2014 in the northern Pantanal wetlands. Despite the low incidence of NDV and Influenza virus in Brazil, it is essential to monitor these virus in scientific studies because Brazil is one of the world's largest exporters of poultry products. In North America and most European countries, the Avian

Influenza virus is constantly monitored in wild birds, whereas there still is a significant gap in the knowledge on those viruses in wild birds in South America, and particularly in Brazil (Araujo *et al*., 2014).

In summary, this study highlights the need for further in-depth research involving the continuous monitoring of the birds in the Pantanal region, and in particular, to expand sampling during every season in order to include a wider variety of avian species, which are considered potential reservoirs of infectious diseases. The purpose of such an epidemiological strategy is to minimize possible risks to public health resulting from the local introduction of these viruses.

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