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Short Communication

A survey of flaviviruses in birds in green areas of São Paulo, Brazil

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Abstract

Introduction: This study aimed to investigate *Flavivirus* infection in birds captured in green areas of São Paulo. **Methods:** Oropharyngeal swabs, cloacal swabs, and blood samples from 170 birds captured in two green areas in São Paulo, Brazil were subjected to real time-polymerase chain reaction (RT-qPCR) analysis of *Flavivirus* specific NS5 gene fragment. **Results:** All samples were negative for the presence of viral ribonucleic acid (RNA). **Conclusions:** Despite the negative results, *Flavivirus* surveillance must be performed regularly due to favorable ecological conditions for virus circulation and transmission among birds in these areas and their close proximity to humans.

Keywords: Birds. *Flavivirus*. Green areas.

The genus *Flavivirus* comprises of approximately 70 viruses that can cause severe human diseases, such as West Nile fever, tick-borne encephalitis, Zika, and dengue fever[1]. These viruses are maintained in the environment by transmission between hematophagous arthropods (mosquitoes or ticks) and susceptible vertebrate hosts, such as humans and birds[2]. Interactions among these species can take place in green areas due to high biological diversity in these areas. In large cities such as São Paulo, intense urbanization has devastating impact on green areas[3]. According to Almeida & Vasconcellos, small pockets of forests still exist in São Paulo as public parks and conservation units[4]. A fauna survey performed in 81 green areas of the city showed 700 different species of animals, 372 of which were birds[5]. Forty one taxonomic categories of mosquitoes were found in 35 municipal parks of the city, including *Culex* species, a crucial mosquito genus for *Flavivirus* transmission[6].

Migratory wild birds are considered the main hosts for certain flaviviruses, such as the Saint Louis encephalitis virus (SLEV) and West Nile virus (WNV)[7]. It is believed that these birds, specifically the Passeriformes, can carry pathogens during their migration, increasing the risk of *Flavivirus* spread in their staging areas[8]. Many species of migratory birds visit São Paulo annually; three of which arrive in Brazil from the Northern hemisphere between August and September, and return to the USA and Canada between April and May: osprey (*Pandion haliaetus*), peregrine falcon (*Falco peregrinus*), and lesser yellowlegs (*Tringa flavipes*)[5]. Because of the interaction between migratory wild birds and local avifauna that favors the spread of *Flavivirus* and favorable conditions for mosquito infestation, the objective of this study was to investigate *Flavivirus* circulation in birds captured in green areas of São Paulo.

Between March 2012 and January 2013, mist nets were utilized to capture birds in two different green areas in São Paulo **(Figure 1)**: Anhanguera Park, located in the Perus district (coordinates: UTM Córrego Alegre - 23 S X-317.106 Y-7.409.705), and Castanheiras Farm/Environmental Protection Area (APA) Bororé Colônia, located in the Grajaú district, Bororé peninsula (coordinates: UTM Córrego Alegre - 23 S X-331.691 Y-7.365.989). These nets were left open 30 hours per month, at dawns or evenings, over a 12-month period. Nets were set before sunrise (at 05:30) and checked every 30 min. Captured birds were placed inside a cloth bag to minimize stress. Biometry of the birds was evaluated and all birds received a metal ring with individual identification number. A total of 170 birds were captured: 88 at the Anhanguera Park and 82 at the Castanheiras Farm **(Table 1).** The majority of these birds belonged to the order Passeriformes (84.6%), followed by Piciformes (7.7%), Columbiformes (3.8%), and Apodiformes (3.7%). Among the species captured, only *Vireo olivaceus* is considered migratory; other species are resident and/or endemic of the areas.

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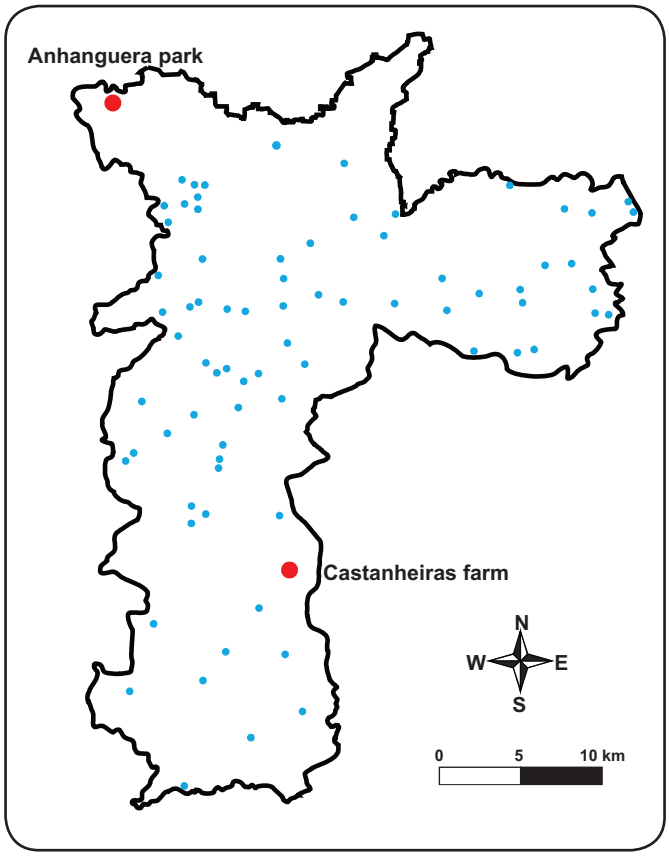

FIGURE 1. Location of the study areas. **Pink:** Anhanguera park and Castanheiras Farm. **Blue:** other green areas of São Paulo. Modified from São Paulo[5]. The identification of the blue dots were removed from the picture.

Cloacal and oropharyngeal swabs were collected from captured birds and placed in cryotubes containing 300µL of viral transport media [0,2% bovine serum albumin (GIBCO™), 0,8% penicillin/streptomycin solution (Sigma-Aldrich, USA), 0,2% amphotericin B (Sigma-Aldrich, USA), and 10% glycerol (Sigma-Aldrich, USA) in phosphate buffered saline (PBS) pH 7.5]. After collection, the tubes were transferred to a liquid nitrogen tank (-196°C) for flash freezing the samples and subsequently stored at -80°C until processing. Birds recaptured in different months were subjected to another swab collection. Deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) were extracted using the NucliSENS® automated platform (EasyMAG®; bioMérieux, France) according to the manufacturer's instructions. The extracted samples were reverse transcribed using the SuperScript VILO kit (Invitrogen, USA) according to the manufacturer's instruction.

The complementary deoxyribonucleic acid (cDNA) was subjected to real time-polymerase chain reaction (qPCR) to amplify a 200-bp fragment of the *Flavivirus* NS5 gene, according to a method described by Johnson et al.[9]. The PCR reactions were performed in a 25µL volume [2.5µL of cDNA, 12.5µL of Power SYBR Green PCR Master Mix (Applied Biosystems, USA), 1µL of each 10µM primer, and 8µL of UltraPure water (Invitrogen)]. The thermocycler parameters were as follows: 95°C for 15 min, 40 cycles of 94°C for 30 sec, and 60°C for 1 min, followed by a dissociation curve at the end. The melting curve analysis showed comparable T_m values to those verified by Moureau et al.[10]. In all analyses, RNA samples extracted from culture supernatant of dengue virus (DENV) types 2, 3, and 4 were included as positive controls and water was used as a negative control.

Complementary deoxyribonucleic acid from positive samples were subjected to a semi-nested *Flavivirus* generic PCR to amplify a 269-272-bp fragment from the NS5 gene. The first reaction was carried out in a final volume of 25µL (2.5µL of cDNA, 16.2µL of UltraPure water, 2.5µL of PCR Buffer, 1.5µL of 50mM $MgCl_2$, 0.5µL of 10mM dNTP mix, 0.75µL of each 10µM primer, and 0.3µL of 5U/µL Platinum *Taq* DNA Polymerase (Invitrogen)]. The thermocycler parameters were 95°C for 10 min; 40 cycles of 94°C for 30 sec, 50°C for 30 sec, and 72°C for 45 sec; and 72°C for 10 min. Subsequently, a 2.5-µL aliquot from the first reaction was used for the second PCR according to a method described by Cook et al.[11] in a final volume of 25µL (2.5µL of amplicon, 16.95µL of UltraPure water, 2.5µL of PCR Buffer, 0.75µl of 50mM $MgCl_2$, 0.5µL of 10mM dNTP mix, 0.75µL of each 10µM primer, and 0.3µL of 5U/µL Platinum *Taq* DNA Polymerase). The thermocycler parameters were: 94°C for 2 min; 30 cycles of 94°C for 30 sec, 50°C for 45 sec, and 72°C for 1 min; and 72°C for 10 min. The amplified product was subjected to electrophoresis in a 1.5% agarose gel. The gel was subsequently stained with ethidium bromide and visualized under ultraviolet (UV) light.

Positive samples were purified with the Invisorb Fragment CleanUp reagent (Stratec Biomedical, Germany) according to the manufacturer's protocol for agarose gel DNA fragment purification. The BigDye Terminator Ready Reaction Mix (Thermo Fisher Scientific, USA) was used for sequencing in a final volume of 20µL [8µL of the Big Dye Reaction Mix, 5µL of amplicon, 5.9µL of water, and 1.1µL of each primer (3.2µM) described by Cook et al.[11]]. The thermocycler parameters were: 96°C for 1 min; and 25 cycles of 96°C for 10 sec, 50°C for 5 min, and 60°C for 4 min. After precipitation using 75% isopropanol, samples were sequenced using the ABI PRISM®3500 Genetic Analyzer automatic sequencer (Applied Biosystems). Sequences were analyzed using the BLAST tool (Basic Local Alignment and Search Tool; https://blast.ncbi.nlm.nih.gov/Blast.cgi) and BioEdit software (Ibis Biosciences, USA).

In total, 347 samples were collected: 163 oropharyngeal swabs, 165 cloacal swabs, and 19 blood samples. A sample was considered positive when the amplification curve reached over the defined threshold and had a melting temperature between 79.4 and 83.7°C. Samples were considered negative when a) no cycle threshold (Ct) value was obtained, or b) Ct value was obtained but the melting temperature was outside of the defined range. Among all analyzed samples, four were considered positive by RT-qPCR **(Table 2)**.

Among the four samples, blood samples from two birds (ID 230 and 232) did not show the expected 250-bp band of the *Flavivirus* NS5 gene following a semi-nested PCR. Oropharyngeal samples from the other two birds (ID 87 and 211) displayed the expected band (data not shown), which suggested viral genome amplification. These samples were subjected to sequencing analysis. However, a Basic Local Alignment Search Tool (BLAST) analysis showed sequence similarity between nucleotides

TABLE 1

Species of birds captured from March 2012 to January 2013 at the Anhanguera Park and Castanheiras Farm.

		Anhanguera Park		Castanheiras Farm		Total	
Scientific name	**Popular name**	**n**	**%**	**n**	**%**	**n**	**%**
Basileuterus culicivorus (Deppe, 1830)	Golden-crowned warbler	1	100.0	-	-	1	0.6
Celeus flavescens (Gmelin, 1788)	Blond-crested Woodpecker	3	17.6	14	82.4	17	10.0
Chiroxiphia caudata (Shaw & Nodder, 1793)	Blue Manakin	-	-	1	100.0	1	0.6
Coereba flaveola (Linnaeus, 1758)	Bananaquit	7	100.0	-	-	7	4.1
Cyclarhis gujanensis (Gmelin, 1789)	Rufous-browed Peppershrike	3	60.0	2	40.0	5	2.9
Dacnis cayana (Linnaeus, 1766)	Blue-dacnis	-	-	4	100.0	4	2.3
Lanio melanops (Vieillot, 1818)	Black-goggled Tanager	1	90.9	10	9.1	11	6.6
Leptopogon amaurocephalus (Tschudi, 1846)	Sépia-capped Flycatcher	1	25.0	3	75.0	4	2.3
Leptotila rufaxilla (Richard & Bernard, 1792)	Grey-fronted Dove	-	-	3	100.0	3	1.8
Pachyramphus castaneus (Jardine & Selby, 1827)	Chestnut-crowned Becard	-	-	1	100.0	1	0.6
Phaetornis eurynome (Lesson, 1832)	Black-billed Hermit	1	100.0	-	-	1	0.6
Pipraeidea melanonota (Vieillot, 1819)	Fawn-breasted Tanager	1	100.0	-	-	1	0.6
Pitangus sulphuratus (Linnaeus, 1766)	Great Kiskadee	-	-	3	100.0	3	1.8
Ramphastus dicolorus (Linnaeus, 1766)	Green-billed Toucan	-	-	2	100.0	2	1.2
Tachyphonus coronatus (Vieillot, 1822)	Ruby-crowned Tanager	24	75.0	8	25.0	32	18.8
Tangara cayana (Linnaeus, 1766)	Burnished-buff Tanager	2	100.0	-	-	2	1.2
Tangara ornata (Sparman, 1789)	Rufous-chested Tanager	3	60.0	2	40.0	5	2.9
Tangara sayaca (Linnaeus, 1766)	Blue Tanager	18	62.1	11	37.9	29	17.1
Tolmomyias sulphurescens (Spix, 1825)	Yellow-olive Flycatcher	-	-	1	100.0	1	0.6
Troglodytes musculus (Naumann, 1823)	Southern House Wren	1	100.0	-	-	1	0.6
Turdus albicollis (Vieillot, 1818)	White-necked Thrush	1	25.0	3	75.0	4	2.3
Turdus leucomelas (Vieillot, 1818)	Pale-breasted Thrush	3	50.0	3	50.0	6	3.6
Turdus rufiventris (Vieillot, 1818)	Rufous-bellied Thrush	6	46.2	7	53.8	13	7.6
Vireo olivaceus (Linnaeus, 1766)	Red-eyed Vireo	3	100.0	-	-	3	1.8
Xiphorhynchus fuscus (Vieillot, 1818)	Lesser Woodcreeper	-	-	1	100.0	1	0.6
Zonotrichia capensis (Statius Muller, 1776)	Rufous-collared Sparrow	7	70.0	3	30.0	10	5.9
Total		**88**	**51.8**	**82**	**48.2**	**170**	**100.0**

TABLE 2

Birds positive for flavivirus by quantitative reverse transcription polymerase chain reaction.

Bird ID	Species	Collected samples	Positive sample type	Ring number	Area
87	*Pitangus sulphuratus* (Linnaeus, 1766)	Cloacal and oropharyngeal swabs	Oropharyngeal swab	G93147	Castanheiras Farm
211	*Pitangus sulphuratus* (Linnaeus, 1766)	Cloacal and oropharyngeal swabs	Oropharyngeal swab	G109512	Castanheiras Farm
230	*Leptopogon amaurocephalus* (Tschudi, 1846)	Cloacal and oropharyngeal swabs and blood sample	Blood sample	-	Anhanguera Park
232	*Zonotrichia capensis* (Statius Muller, 1776)	Cloacal and oropharyngeal swabs and blood sample	Blood sample	E130524	Anhanguera Park

of the oropharyngeal samples of bird IDs 87 and 211 and the RNA pseudouridylate synthase (RPUSD2) gene of *Ficedula albicollis*, a migratory bird species in the order Passeriformes (data not shown). Both samples were obtained from one bird species, *Pitangus sulphuratus*, which also belongs to the order Passeriformes.

Despite favorable conditions for *Flavivirus* transmission, the lack of positive results in this study was expected because typically PCR can only detect virus in viremic animals[12]. According to Komar et al., the viremic period in birds lasts approximately seven days[8]. Even when flaviviruses are circulating, it was unlikely to capture viremic birds, especially when sample collection was only performed once. Semi-nested PCR was performed in this study to confirm positive results obtained by SYBR Green-based RT-qPCR. Despite the cost benefit of SYBR Green-based PCR, a significant number of false positive results may be obtained due to non-specific SYBR Green binding to dsDNA, resulting in non-specific amplicons and primer dimers. In the current study, the gene encoding for the RPUSD2 enzyme was amplified by SYBR Green-based PCR in samples from three different birds. A high degree of similarity between amplified sequences with only few mismatches can be explained by genomic differences among bird species. Based on results of the current study, primers used in this study may not be suitable for bird samples due to sequence similarity with endogenous DNA sequences of birds.

In 2015, among major arboviruses circulating in Brazil, four were flaviviruses (SLEV, DENV, WNV, and Zika virus), with Zika virus and DENV found in São Paulo[13]. In average, 16 birds were captured per month in each area during the study period, which may not be a representative of the local avifauna population. Many important species were not captured during the period, presumably due to the need for other trapping methods or unsuitable site and/or time to set up mist nets. All captured birds are considered resident and/or endemic. These birds may travel short distance in search for territory or food, but they do not cross countries and continents, reducing the chance of introducing new pathogens. However, the presence of other flaviviruses in endemic species was previously described, for example, Rocio virus (ROCV), which was detected in a *Zonotrichia capensis* specimen[14]. This species can be found in green areas of São Paulo, suggesting *Flavivirus* circulation among local avifauna species. Therefore, despite the negative results, our study could not rule out the presence of *Flavivirus* species circulating among birds in these areas. These findings supported those reported in another study, in which the presence of *Flavivirus* of medical importance was not detected in mosquitoes captured in several municipal parks in São Paulo[15]. Due to their potential for harboring *Flavivirus* species, it would be necessary to capture more birds for better *Flavivirus* surveillance. The surveillance should be performed frequently in these and other areas in São Paulo, Brazil.

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Conflict of Interest

The authors declare that there is no conflict of interest.

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