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Flavivirus Infection in Wild Birds from Brazilian Amazon

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Abstract

The Amazon region has the greatest biodiversity on earth, including birds and hematophagous arthropods. Birds are hosts and amplifiers of at least 80 species of arthropod-borne viruses (arbovirus) including those belonging to the Flavivirus genus (Flaviviridae family). In Brazil, occur many Flavivirus of the Japanese encephalitis group, including Ilheus virus (ILHV). These Flaviviruses are linked to birds and transmitted by Culex mosquitoes. We show here a study aimed to detect infected birds by Flavivirus in the Amazon region. The birds captures were performed in Alter do Chão, Pará State, Northern Brazil, and birds were also obtained in the Wildlife Refuge Sauim-Chestnut, in Amazonas State, North of Brazil. A total of 189 birds, distributed into 11 orders (Passeriformes, Galbuliformes, Coraciiformes, Accipitriformes, Strigiformes, Psittaciformes, Falconiformes, Anseriformes, Pelicaniformes, Columbiformes and Cathartiformes) were bled and liberated. It was possible to detect Flavivirus genome in 7 sera (3.7% positivity) from *Nystalus maculatus* (n=3), *Tolmomyias flaviventris* (n=1), *Dendroplex picus* (n=1), *Amazona festiva* (n=1) and *Elaenia flavogaster* (n=1), using a RT-PCR specific for Flavivirus genus followed by a species-specific nested-PCR. Based on the size of the amplicons (all having ~ 470 bp) it is suggested that birds were likely infected by Ilheus virus (ILHV), a pathogenic Flavivirus to humans. The importance of infected bird species involved in the natural cycle of ILHV or another related Flavivirus is unknown.

Keywords: Wild birds; Arbovirus; Flavivirus; Ilheus virus; Amazonic region

Introduction

Brazil has the largest diversity of wild birds in the world (1832 species), and 20% of them occur in the Amazon region [1,2]. About 92% of Brazilian birds are resident and only 8% are migrant species [3]. A proportion of 61% of the migrant species to Brazil come from the northern hemisphere (Nearctic). Brazil is regularly visited by thousands of birds that fly seasonally between North and South America [2,3]. Most of these animals migrate to Brazil in the summer, looking for abundant food [4]. Southern migratory species (austral) represent 39% of the migrant species into Brazil. However, their movements and natural histories are less studied than that of northern migratory birds [5].

Birds are hosts and amplifiers of at least 80 species of arthropod-borne viruses (arbovirus) including those belonging to the Flavivirus genus (Flaviviridae family), but only some of these may affect the health of these animals [6]. Introduced by migratory birds, Flavivirus found in the Brazilian Amazon and have been maintained in this area due to suitable ecological and climatic conditions for proliferation of arthropod vectors that feed in birds and may maintain the natural cycles of these arboviruses [7].

In Brazil, mosquito-borne Flaviviruses can be grouped phylogenetically into three main branches: the yellow fever, the dengue (types 1, 2, 3 and 4) and the Japanese encephalitis, which includes Saint Louis encephalitis (SLEV), West Nile Virus (WNV), Cacipacoré, Iguape, Rocio, ILHV and Bussuquara viruses. The viruses in the Japanese encephalitis group are related to birds and mostly transmitted by Culex mosquitoes. Many of these viruses can infect and produce human disease [8].

ILHV was first isolated in 1944, from mosquitoes collected in Ilhéus County, at the northeastern coast of Brazil [9]. ILHV has been isolated from various species of mosquitoes and wild animals. Moreover, antibodies to the virus have been reported in bats, monkeys, rodents and marsupials, but, particularly, in birds. *Psorophora ferox* mosquito is considered as the main vector of ILHV and many wild birds were found infected with the virus. Birds are considered as the principal hosts of ILHV in the Brazilian Amazon [6,10].

ILHV has been reported to cause human acute febrile illness in Brazil, Argentina, Panama, Colombia and Trinidad. Infections by ILHV are commonly associated to the exposition to forested areas. Infections by ILHV have been associated to sporadic human disease but not to outbreaks. ILHV disease has been reported in ecotourists, fishermen, farm workers and truck drivers. The virus can produce moderate or high fever, headache, chills, photophobia, arthralgia, myalgia, and asthenia. The disease takes 3 to 5 days, and patients recover without sequelae. Serological surveys to ILHV have shown a high number of seropositives contrasting with a relatively small number of disease cases [7,11]. It is possible that most of the disease cases by ILHV remain undiagnosed. On this basis, the goal of the study described here was to detect infected birds by Flaviviruses in the Amazon region.

Material and Methods

Bird captures

Bird captures were performed in a peninsula of 3 km long and 1-2 km wide, located at the right bank of Tapajós River, in Alter do Chão (2 "31'S, 55" 00'W), County of Santarem, Pará State (Figure 1). The region has a well-defined dry season, which runs from July to December and

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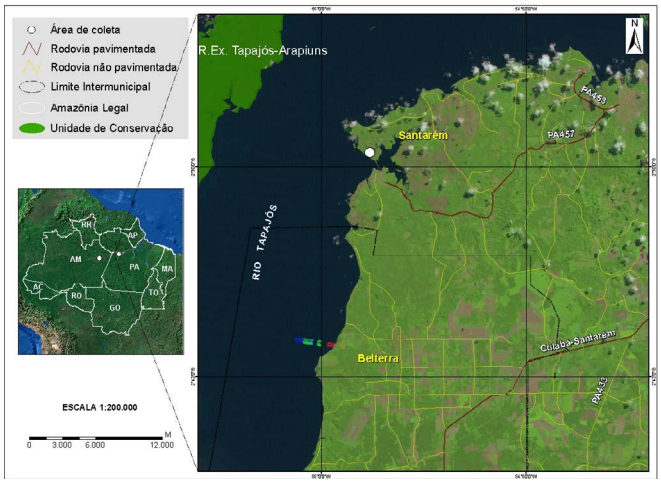

Figure 1: Geographic location of different study sites used for sample collection in Pará (Alter do Chão county) and Amazonas (RVSSC, Manaus county). Map elaborated based on the digital cartographic platform. Scale of 1: 250.000. SIPAM/IBGE/IBAMA, 2009. Image from LANDSAT TM5 scene 227/62 de 01/07/2005. Source: INPE (National Institute of Spatial Research).

a rainy season from January to June. The traps were set near to the center of an area of savanna surrounded by a deciduous forest. This area is used by migratory arctic birds where they rest after the long trip. Two bird captures were performed in Alter do Chão, one in October 2009 (dry season) and another in May 2010 (rainy season). Captures with mist-nets measuring 2.5 meters high by 12 meters long and 36 mm mesh were performed before sunrise until late morning, and again in the late afternoon until dusk. Nets were checked every 30 minutes. Species of the captured birds were identified and the animals were weighed and bled. Birds were also marked with a ring of the Center for the Study of Bird Migration (CEMAVE / IBAMA; http://www.ibama.gov.br/). Finally, the animals were released at the same site of capture.

Birds were also obtained, April-May 2010, in the Wildlife Refuge Sauim- Chestnut (RVSSC), a protected area administered by the Environment Secretary of the City of Manaus (SEMMA), that receive birds from rescues and seizures. These animals, that were also bled, belong to the orders Strigiformes, Psittaciformes and Falconiformes and are rarely captured in mist nets.

Blood samples

The approach for blood collection was determined according to the body weight of the birds. For those weighing over to 200 grams, blood collections were performed in jugular, wing or metatarsal veins, using heparinized insulin syringes. For birds weighing less than 200 grams, few drops of blood were harvested in capillary tubes. Blood samples were maintained in cryotubes, which were properly identified. Samples were transported into the laboratory in liquid nitrogen barrel and stored at -70˚C.

Virus genome amplification

RNA was extracted from bird serum using the QiAampViral RNAMini Kit, according to the manufacturer's instructions (QIAGEN, Germany). This RNA extract was tested for the presence of genomes of Flavivirus by using a generic RT-PCR protocol. FG1 (+) primers (forward TCAAGGAACTCCACACATGAGATGTACT and FG2 (-reverse, GTGTCCCATCCTGCT GTGTCATCA), that allow amplification of approximately 980 bases (bp) of the NS5 protein gene were used in the RT-PCR. The RT-PCR was followed by a species-specific multiplex-nested-PCR, using the 14 inner primers shown in Table 1, as previously reported Bronzoni et al. [12]. Each species-specific primer was used in combination with the FG1 primer for Flavivirus. All RNA extracts showing amplicons in the multiplex-nested-PCR had a confirmation of the positive result using the virus-specific primer alone in a Hemi-Nested-PCR.

Ethic statements

Bird captures were authorized by the Biodiversity Information System - SISBIO (No: 21722-1) for activities with scientific purpose of collection and transportation of biological material *in situ* (Alter do Chão, PA) and *ex-situ* (Manaus, AM). All procedures involving animal manipulation (e.g. blood collection) were performed according to national biosafety rules.

Results

Captured bird species

A total of 189 birds, distributed into 11 orders (Passeriformes, Galbuliformes, Coraciiformes, Accipitriformes, Strigiformes, Psittaciformes, Falconiformes, Anseriformes, Pelicaniformes, Columbiformes and Cathartiformes), 23 families and 50 species, were captured. A proportion of 58.7% of these animals were Passeriformes of the family Tyrannidae (n=111) and 46 of them were identified as *Elaenia cristata* (23), *Thamnophilidae sp* (22) and *Formicivora grisea* (15). In Alter do Chão, *Elaenia cristata* was more frequently captured in the dry season (n=14) while *Formicivora grisea* was in the rainy season (n=8), as shown in Table 2. Nine species of non-passerine Psittacidae were also obtained, representing 24.9% (n=47) of the analyzed samples.

Virus genome detection

It was possible to detect a virus genome in 2 of 189 sera. The obtained amplicons had approximately 980 bp in the RT-PCR for Flavivirus, representing 1% positivity, as shown in Figure 2A. Virus genome detection increased into 7 amplicons of approximately 470 bp, in the multiplex-nested-PCR (3.7% positivity), as shown in Figure 2B and 2C. These 7 amplicons were obtained from *Nystalus maculatus* (3), *Tolmomyias flaviventris* (1), *Dendroplex picus* (1), *Amazona festiva* (1) and *Elaenia flavogaster* (1). All birds infected by Flavivirus lived free with the only exception was *Amazona festiva* which was captive, as shown in Table 3.

As previously reported by Bronzoni et al. [12], based on the size of

Primer	Sequence (5'- 3')	Amplification Step	Amplicon (bp)
nDENV1	CGTTTTGCTCTTGTGTGCGC	MN-PCR	472
nDENV2	GAACCAGTTTGTTTDRTTTCATAGCTGCC	MN-PCR	316
nDENV3	CCCATTGGTTCTCCTCTGTG	MN-PCR	628
nYFV	TCAGAAGACCAAGAGGTCATGT	MN-PCR	253
nDENV4	GCAATCGCTGAAGCCTTCTCCC	N-PCR	222
nSLEV	ATTCTTCTCTCAATCTCCGT	N-PCR	232
nROCV	TCACTCTTCAGCCTTTCG	N-PCR	230
nILHV	TCCACCGCTGATCTGAGCCCGTGA	N-PCR	474
nBSQV	AAGTGACACCTGTTCAGGGTA	N-PCR	388

Table 1: Species-specific inner primers used for detection of distinct flavivirus genomes in the Nested-PCR reactions. DENV-1: Dengue Virus Serotype 1; DENV-2: Dengue Virus Serotype 2; DENV-3: Dengue Virus Serotype 3; DENV-4: Dengue Virus Serotype 4; YFV: Yellow Fever Virus; SLEV: Saint Louis Encephalitis Virus; ROCV: Rocio Virus; ILHV: Ilheus Virus; BSQV: Bussuquara Virus; N: Nested PCR Step; MN: Multiplex-Nested PCR; bp: Base Pairs.

Order	No. of individuals	Locality	Month / Year
Passeriformes	111	Alter do Chão (Santarém)	10/2009 and 05/2010
Galbuliformes	6	Alter do Chão (Santarém)	10/2009 and 05/2010
Coraciiformes	1	Alter do Chão (Santarém)	05/2010
Psittaciformes	47	RVSSC (Manaus)	04/2010
Strigiformes	7	RVSSC (Manaus)	04/2010
Accipitriformes	4	RVSSC (Manaus)	04/2010
Cathartiformes	3	RVSSC (Manaus)	04/2010
Columbiformes	2	RVSSC (Manaus)	04/2010
Pelicaniformes	2	RVSSC (Manaus)	04/2010
Falconiformes	2	RVSSC (Manaus)	04/2010
Anseriformes	4	RVSSC (Manaus)	04/2010
	Total= 189		

Table 2: Total of bird species collected during the study.

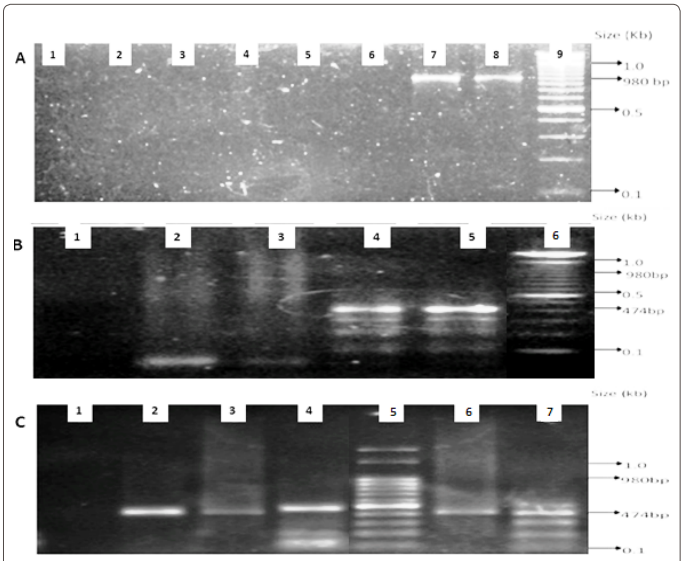

Figure 2: Electrophoretic gel showing the flavivirus genome amplification. (A) RT-PCR amplicons. Column 1: Negative control (RNAse free water); columns 2-6: samples RT-PCR negative; columns 7 and 8: positive RT-PCR samples (amplicons of ~980 bp) obtained from birds captured in Alter do Chao, PA suggestive of flavivirus gemome amplification; column 9: 100 bp molecular weight marker (Fermentas Life Sciences); (B) Agarose gel photographs showing amplicons obtained by nested-PCR species-specific to Flavivirus: column 1: negative control (RNAse free water); columns 2 and 3 samples negative; columns 4 and 5: samples A-41 and A-42 respectively (amplicons ~474 pb) RT-Nested-PCR samples; column 6: 100 bp molecular weight marker (Fermentas Life Sciences). (C) Column 1: Negative control (RNAse free water); column 2: sample A93; column 3: sample A36; column 4: sample R36; column 5: 100 bp molecular weight marker (Fermentas Life Sciences); column 6: sample A58; column 7: sample A-111.

the amplicon, approximately 470 bp, it is probable that the Flavivirus infecting the birds would be ILHV, a bird-related virus, and not dengue virus serotype 1, that produces amplicons at approximately the same size, but is not associated with these animals. Unfortunately, the obtained volume of amplicons was not suitable for nucleotide sequencing.

Discussion

Birds are, probably, the main reservoirs for some Flavivirus of the Japanese encephalitis group: ILHV, SLEV, WNV and ROCV. Infected birds can achieve a high and prolonged viremia, thus serving as a source for vector infection. However, most of time, it is not possible to confirm what wild species of bird (migratory or resident), could be a reservoir and transmitter of the arbovirus. The vector competence of arthropods which parasitizes birds is directly linked to their abundance on site and the transmission of Flavivirus to man is related to their eclectic feeding habits that include humans [13,14]. Many bird-related flaviviruses can infect humans.

Some birds, depending on the infecting Flavivirus may have severe and fatal disease while others become viremic but asymptomatic or develop disease after migration, or when going through stress, reproductive activity, or acquiring concurrent diseases that alter their immune defenses [13]. Migratory birds could spread into densely populated urban areas (in places like urban parks) allowing introduction of a Flavivirus that could infect local *Culex* mosquitoes and produce disease after feeding on humans. Human disease due to Flavivirus infections could also be related to some birds that are of great interest due to the beauty of its colors or beautiful singing. Pet birds in Brazil are the main trafficked animals being traded in free markets or transported to other cities and countries [15]. Pets carrying zoonotic viruses (including pathogenic arboviruses) could be introduced into urban areas (e.g. houses) where *Culex* are abundant allowing transmission of the virus to man. In this context, as same as observed to domestic birds (chickens) in United States of America, the potential risk of arbovirus (in this case flavivirus) transmission to humans could be increased when inadequate management of the environment (ranch or shop) where birds are commercialized allows the breading of large amounts of mosquitoes [16].

In the current work, a total 189 birds were obtained in two places of Amazon region. Seven of the studied wild birds were infected with a Flavivirus. This diagnosis was made based on amplicons of approximately 470 bp obtained from their sera by nested-RT-PCR. The amplicon size suggests that these birds could be infected by ILHV [12]. It is noteworthy that the risk of false-positive results in the reaction was avoided by measures that include the separation of pre- and post-PCR areas, the use of unique pipettors for each area, and the use of tips with barriers and negative controls in each reaction. However, an important difficulty in the study was related to substantial loss of bird serum volumes, which were consumed in unsuccessful viral isolation attempts in cell culture (data not shown). Besides, the volume of amplicons was also lost in purification procedures impairing nucleotide sequencing.

Therefore, we hypothesized that birds are infected by Flavivirus (ILHV), although live virus was not detected in additional techniques.

In the present study, *Nystalus maculatus*, *Tolmomyias flaviventris*, *Dendroplex picus*, *Amazona festivva* and *Elaenia flavogaster* were found

Sample	Location	Date	Species	Amplicon size	Virus compatible
A-36	Alter do Chão (PA)	10/2009	*Elaenia flavogaster*	~474	ILHV
A-41	Alter do Chão (PA)	10/2009	*Nystalus maculatus*	~474	ILHV
A-42	Alter do Chão (PA	10/2009	*Nystalus maculatus*	~474	ILHV
A-58	Alter do Chão (PA)	10/2009	*Dendroplex picus*	~474	ILHV
A-93	Alter do Chão (PA)	05/2010	*Nystalus maculatus*	~474	ILHV
A-111	Alter do Chão (PA)	05/2010	*Tolmomyias flaviventris*	~474	ILHV
R-36	Manaus (AM)	04/2010	*Amazona festiva*	~474	ILHV

Table 3: Information about the seven birds which RNA extracts were possible to obtain amplified products suggestive of Flavivirus, which were identified based on the size of the amplicon.

infected with Flavivirus. Although no information regarding migratory events involving any of the bird species above referred have been found, it is important to note that *Elaenia flavogaster* species is largely distributed in the Americas and found from Mexico to Bolivia and Argentina, as well as in all regions of Brazil (http://www.wikiaves.com.br/guaracava-de-barriga-amarela) and could potentially contribute for ILHV or other virus spread throughout Brazil.

Nystalus maculatus lives in low forests and dry savannas of the Amazonic region, Northeast, Midwest, and Southeast of Brazil [17]. It is also found in Argentina, Bolivia and Paraguay. The bird has approximately 18 cm long, red beak and brown plumage in the back. It makes his nest in soil and can catch food in flight, feeding on insects, spiders, scorpions, fruits and small vertebrates. The reproductive period of the *Nystalus maculatus* is from September to December (http://www.wikiaves.com/rapazinho-dos-velhos#), and matches with the season of highest mosquito activity in Brazilian Amazon region as observed during a study conducted in an important Brazilian highway linking the cities of Cuiaba, Mato Grosso State, Central of Brazil, and Santarem, Para State, Northern Brazil [18-22]. It is possible that *Nystalus maculatus* could participate as a virus-reservoir in the natural cycle of ILHV or of another related Flavivirus.

Tolmomyias flaviventris, a tyrant flycatcher, is a tropical species of passerine that feed primarily on insects and lives at trees of the Brazilian northeastern caatinga, *Mauritia flexuosa* agglomerates and forests. This bird has a yellow plumage that is more intense on the ventral side [3,17].

Dendroplex picus is an insectivorous bird that lives in mangroves and flood forests located at river margins of all Brazilian regions except in Southern areas of the country. This bird is also found in Guyana, Venezuela, Colombia, Ecuador, Peru Bolivia and Panama [2,3] (http://es.wikipedia.org/wiki/Dendroplex_picus).

Amazona festiva is a parrot that lives in forests located at river margins in Brazil, Colombia, Ecuador, Bolivia, Guyana, Peru, and Venezuela. This bird can be 35 cm long, with a dark red plumage and blue spot behind the eyes. *Amazona festiva* feeds on cocoa, fruits, nuts, leaves, berries, seeds and occasionally, eggs and insects. Due to its beauty, intelligence, docility and capacity to imitate human voice these animals are commonly victims of wildlife traffic [20]. *Amazona festiva* commonly become pets that could bring arboviruses to vectors living in close contact with humans.

Elaenia flavogaster is a common and noisy tyrant flycatcher of 6.5 cm long that feeds berries and insects in semi-open woodlands, gardens and farms of southern Mexico, Central and South-America [17].

Conclusively, even not knowing the real importance of the species of infected birds on the natural cycles and human infections by ILHV or another related Flavivirus, it is important to emphasize that this same virus was found infecting birds in two places and possibly, occurs in most of the Amazonic region.

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