

Serosurvey of Arbovirus in Free-Living Non-Human Primates (*Sapajus* Spp.) in Brazil

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Abstract

The aim of the present study was to investigate the presence of arboviruses that could potentially cause outbreaks and epidemics and negatively affect public health in free-living non-human primates (NHP). The study was conducted in the municipality of Mundo Novo, on the border between Brazil and Paraguay, Bodoquena, Jardim and Coxim, both in the state of Mato Grosso do Sul. Samples (n=47) of asymptomatic NHP were analyzed using the Hemagglutination Inhibition Test (HIT) and molecular biology techniques. All samples from Mundo Novo were positive for viruses belonging to the genus *Flavivirus* in the serology and negative in the molecular and virological tests. From the samples of the Jardim municipality (n=14), one was positive for viruses belonging to the genus *Flavivirus*, one was positive for viruses belonging to the genus *Alphavirus* and two were positive for Mayaro virus. From the samples of the Coxim municipality (n=8), four were positive for viruses belonging to the genus *Flavivirus* and two were positive for Mayaro virus in the serology. The positive results in the serology demonstrated that the animals were infected by *Flavivirus* and Mayaro viruses at some point in their lives. Conversely, the negative results found by the molecular techniques indicated that the animals were not infected at the time of sample collection.

Keywords: Public health; Non-human primates; Arbovirus infection; Molecular biology

Introduction

Arboviruses are classified according to their antigenic properties. Recently, they have been classified into the following five families, based on their physicochemical characteristics: *Bunyaviridae*; *Flaviviridae*; *Reoviridae*; *Rhabdoviridae* and *Togaviridae* [1]. All viruses of the families exhibit a genome composed of ribonucleic acid (RNA), which may or may not be segmented [2].

Viruses belonging to the family *Flaviviridae* and the genus *Flavivirus* are the most relevant for public health. This genus is composed of approximately 70 viruses, which exhibit cross-reactivity. The Yellow Fever Virus (YFV), the Dengue Virus (DENV), the Japanese Encephalitis Virus (JEV) and the Saint Louis Encephalitis Virus (SLEV) are the most common arboviruses that affect human's worldwide [3]. These viruses can cause fever, encephalitis, hemorrhages and liver disease among vertebrates and present high morbidity and mortality rates [4].

Most of the arboviruses in Brazil are found in the Amazon region and are maintained in nature through wild zoonotic cycles, which involve haematophagous vectors and wild host reservoirs [1]. Humans, who live in close contact with the wild environment, where the ecological niche of arboviruses can be found, are more commonly infected [5].

Interestingly, the interface of tropical forests, which involves wide biodiversity, farming communities, high human population density, domestic animals and agriculture, has been associated with the increase of arbovirus transmission in several areas (e.g., the Amazon region) [6]. In general, free-living NHP are found in several natural habitats and share similar physiological and genetic characteristics with humans. Therefore, they are susceptible to several common pathogens and are considered important wild hosts of arboviruses. These animals can act as models for studies on the dynamics of the natural transmission of pathogens, serving as sentinels for the surveillance of emerging viruses

[7]. Furthermore, the presence of high titers of antibodies and persistent viremia, sufficient to infect vector species, is one of the most important factors to determine the effectiveness of an animal as a reservoir, host and disseminator of arboviruses [8].

Recently, due to the expansion of ecotourism and the invasion of forests, the interaction between NHP and humans has increased, leading to an increase in the risk of transmission of the virus by vectors [7]. Other factors such as ecological changes resulting from economic development, agriculture, population growth, international travel, and trade of wild animals, microbial adaptation and the inefficiency of disease control programs could also contribute to the increase in the transmission of these emergent viruses [2].

Emerging arboviruses could potentially cause significant disease and greatly impact public health services. Surveillance of these arboviruses could enable the early detection of cases and the prevention of large outbreaks. Laboratory tests to diagnose these infections are of great importance given that the diagnosis based on clinical evidence is not easy, especially during the initial phase of the disease when the symptoms are non-specific [8].

The aim of the present study was to investigate the presence of arboviruses in asymptomatic NHP living in the state of Mato Grosso do Sul (municipality of Bodoquena, Coxim, Jardim e Mundo Novo), specifically on the borders with the state of Paraná and Paraguay.

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In addition, the epidemiological consequences of this study were discussed, providing important data that could lead to better prevention and control of these zoonoses.

Materials and Methods

Study area

The present study was conducted during November 2012 in two locations of the municipality of Mundo Novo, during October 2013 in the municipality of Bodoquena and Jardim and during April 2014 in the municipalities of Coxim, Both in the state of Mato Grosso do Sul (Figure 1).

The municipality of Mundo Novo is located in the southern Midwest region of Brazil; specifically in the Southwest of the state of Mato Grosso do Sul, close to the border towns of Guaira (state of Parana) and Salto Del Ghaira (Paraguay). The former area is a fragment of forest near the BR 163 highway (23°54'15.7"S and 54°18'9.4"W). This highway links the municipality of Santarem (state of Para) to Tenente Portela (state of Rio Grande do Sul). Truckers from many locations use this highway and may come into contact with animals and increase the risk of viral transmission. The latter site is situated in a fragment of forest in the center of Mundo Novo (23°56'16.3"S and 54°17'8.9"W),

where free-living NHP are adapted to living in close contact with humans (Figure 1). In the municipality of Mundo Novo, there is a predominance of the *Cerrado* ecosystem, which is characterized by low, sloping and twisted trees. The *Cerrado* has well-defined climatic seasons: a rainy season during the summer (from October to April) and a dry season in the winter (from June to August).

The Bodoquena municipality is located in the Southwest region of the State of Mato Grosso do Sul (20°32'19" S, 56°42'54" W) within the Pantanal region, which is a seasonal tropical wetland with an area of approximately 140,000 km². This area is considered to be one of the largest freshwater ecosystems in the world.

Jardim municipality is located in the southwest of the state (21°28'49"S and 56°08'17"W). It has a territorial extension of 2,207.6 km², with a humid subtropical climate and temperatures of 15-39°C.

The fourth study area comprised a fragment of forest in the Coxim municipality. It is located in the North of Mato Grosso do Sul (18°30'25"S and 54°45'36"W). It is located at the confluence of the Coxim and Taquari rivers. Its economy is based on tourism, fishing and animal husbandry.

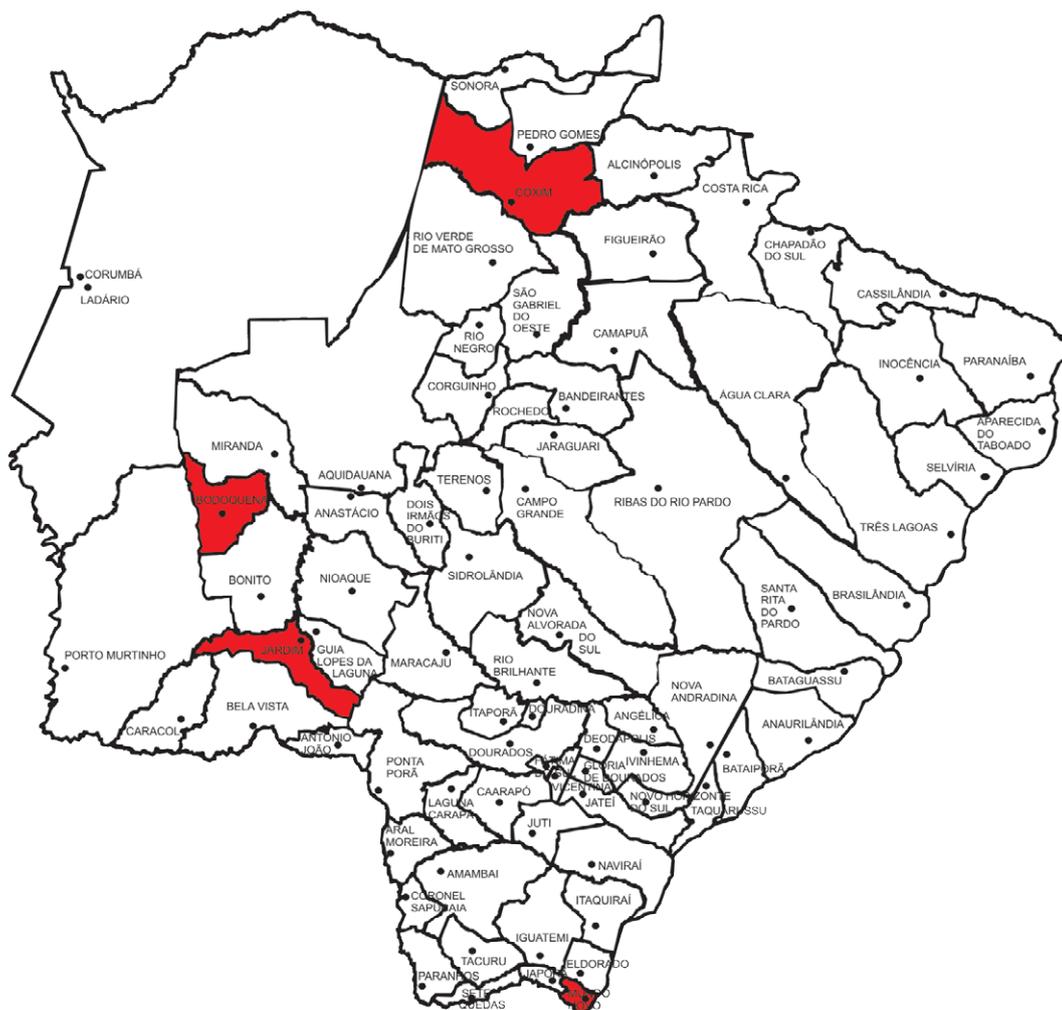


Figure 1: Survey conducted in the municipality of Bodoquena and Jardim and during April 2014 in the municipalities of Coxim, Both in the state of Mato Grosso do Sul.

Sample collection

Blood samples (43) were collected from free-living NHP (*Sapajus* spp.). The animals were captured using Tomahawk traps [9]. They were anesthetized (chlorhydrate tiletamine plus zolazepam) [10] and the intramuscular dose was adjusted based on the weight of the animal. The blood samples were frozen in liquid nitrogen and subsequently stored at -70°C until processing. All procedures were performed by a team of biologists and veterinarians, and were authorized by the *Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis* (IBAMA) under protocol number 21808-1.

Serological test

Serum samples were analyzed in the Hemagglutination Inhibition Test (HIT), using a panel containing 19 different arboviruses, distributed as follows: *Alphavirus* (WDDD, VWEE, VMAY and Mucambo virus), *Flavivirus* (VFA, Ilheus virus, VESL, Cacipacore virus VROC and Bussuquara virus), *Orthobunyavirus* (Guaroa virus, Maguari virus, Tacaiuma virus, Utinga virus, Bethlehem virus, Caraparu virus, VORO and Catu virus) and *Phlebovirus* (Icoaraci virus). The HIT used herein was standardized in the section of Arbovirology and Hemorrhagic Fevers (SAARB) of the *Instituto Evandro Chagas* (IEC) [11], according to the protocol adapted for micro-plates [12].

RT-PCR

The samples from Mundo Novo and Bodoquena municipalities were analyzed through RT-PCR in order to identify the arboviruses. A protocol previously described, with some modifications, was followed [13]. In particular, the primers were replaced by degenerated primers for the *Flavivirus* genus (FG1 and FG2), which amplify the NS5 genomic region [14].

The viral RNA was extracted using a commercial kit (QIAmp Viral Mini kit, Qiagen, USA), following the manufacturer's instructions. The reverse transcription to obtain the cDNA was conducted using 2000 ng of viral RNA and 1 μL of primer (FG1), before placing the material in a thermocycler for 5 min at 70°C . Subsequently, 5 μL of 5 x buffers [250 mM Tris-HCl (pH 8.3); KCl 375 mM; MgCl_2 15 mM], 0.5 μL of dNTPs and 20 U of AMV reverse transcriptase enzymes (Promega, USA) were added. Finally, samples were put in a thermocycler for 90 min at 42°C and for 15 min at 70°C .

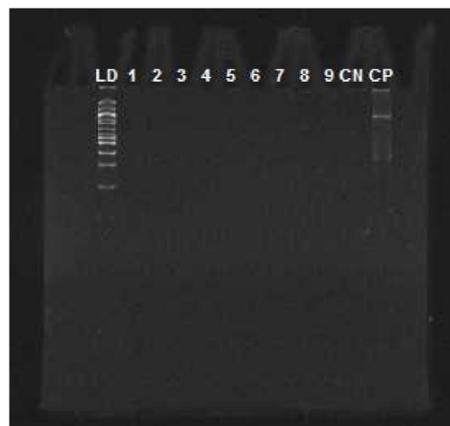
The amplification reaction was conducted using 3 μL of cDNA, 2.5 μL of 10x buffer [200 mM Tris-HCl (pH 8.4); 500 mM KCl], 1 μL of each primer (FG1 and FG2), 1.5 μL of MgCl_2 , 0.5 μL of dNTPs and 3 U of AmpliTaq Gold DNA polymerase (Applied Bio systems, USA). Finally, the samples were amplified in a thermocycler for 35 cycles: 30 sec at 94°C , 1 min at 55°C , and 2 min at 72°C . A positive (Yellow Fever virus, 17D strain) and negative control were used in all reactions.

The amplicons were subjected to electrophoresis, using a horizontal polyacrylamide gel (10%), and then visualized under an ultraviolet light device. All molecular biology tests were carried out in partnership with the Laboratory of Medical and Veterinary Entomology - Department of Biological Sciences, *Universidade Federal do Parana*.

Results

Figures 2, 3 and 4 showed that none of the samples exhibited typical bands related to the *Flavivirus* in the electrophoresis with 10% polyacrylamide gel. Therefore, these samples were considered negative for the presence of arboviruses of this genus.

In all of the free-living NHP assessed from Mundo Novo (22),



Note: LA (LADDER): 100pb, NC: negative control, CP: Positive Control.

Figure 2: Samples (1-9) PNH from the municipality of the New World, Mato southern Gross.



Note: LA (LADDER): 100pb, NC: negative control, CP: Positive Control.

Figure 3: Samples (10-17) PNH from the municipality of the New World, Mato southern Gross



Note: LA (LADDER): 100pb, NC: negative control, CP: Positive Control.

Figure 4: Samples (10-17) PNH from the municipality of the New World, Mato southern Gross.

antibodies against viruses belonging to the genus *Flavivirus* were detected, with antibody titers ranging from 20 to 40. Of the 8 non-

humans primates assessed from Coxim municipality, four (50%) exhibited antibodies to viruses belonging to the genus *Flavivirus* (titers from 20 to 640) and two (25%) exhibited a monotypic reaction to Mayaro virus (titers 80 to 160). Of the 10 non-humans primates assessed from Jardim municipality, one (10%) exhibited antibodies to viruses belonging to the genus *Alphavirus* (titer 320), one (10%) exhibited a monotypic reaction to Mayaro virus (titer 160) (Table 1).

Discussion

The presence of antibodies against *Flavivirus* and *Alphavirus* indicates the circulation of these viruses among the wild NHP investigated herein [14]. This result demonstrates a cross-reaction between the viruses belonging to the genus *Flavivirus* and *Alphavirus*, although it was difficult to identify the virus species and the precise time of infection.

In these studies, the detection of antibodies against *Flavivirus*, *Alphavirus* and *Mayaro* virus in sentinel animals could indicate virus circulation. For example, antibodies against arboviruses have been detected in Spider monkeys (*Ateles geoffroyi*) [15] and in *Callithrix argentata* [16]. Similar findings were reported in a sero-epidemiological survey with 150 sentinel primates in French Guiana, indicating that this species of primate was the main amplifying host of VMAY during an outbreak in that country [17], and sero-epidemiological survey primates in the state of Mato Grosso do Sul. These studies confirmed the circulation of the following arboviruses: Mayaro (VMAY); Oropouche (VORO); Cacipacoré and other *Flavivirus* [18,19].

Epidemiological studies conducted in other regions of Brazil suggest the involvement of free-living NHP in the maintenance of viruses in nature. Indeed, an investigation with 133 NHP, carried out from June 2004 to December 2005 on the border between of the states of Parana and Mato Grosso do Sul, reported the presence of 21 animals that were positive for SLEV, an agent of neurological diseases in humans [20]. A similar study was carried out in Northern Argentina, where four samples were positive for SLEV [21]. In addition, during active surveillance of YFV in the state of Rio Grande do Sul, 181 NHP were analyzed and antibodies against VORO virus and SLEV were detected in one and 16 animals, respectively [22].

Curiously, an animal in Canada (*Macaca Sylvanus*) which exhibited clinical neurological signs was positive for West Nile Virus (WNV) in the RT-PCR, HIT, virus isolation and immunohistochemistry. This case was diagnosed during the same period in which an epidemic of encephalitis and meningitis occurred in Canada. In the same area, out of another 33 NHP tested, three were positive for WNV in the HIT and two of these three were also positive in the serum neutralization (SN) technique [23].

It is known that the occurrence of arboviruses in humans is associated with its occurrence in NHP. Therefore, since 1999, new measures of virus surveillance have been adopted in Brazil. For example, during the re-emergence of YFV between the years 2007-2009, 1,971

cases of the disease were reported, with 209 confirmed cases of YF in NHP in 19 states of the country [24]. After the occurrence of two fatal human cases of YF in the state of São Paulo in the same period, 108 samples of NHP were analyzed, resulting in four positive animals in the virus isolation and RT-PCR [25].

Although the HIT is frequently used in serological surveys because it can detect antibodies for a long period after the natural infection, it is common to observe the occurrence of cross-reactivity with related viruses. The positivity in this test indicates the exposure of the host to a number of arboviruses and the production of antibodies against them [26]. Therefore, it is a good method of detecting antibodies in animals caught in their natural habitat [27].

A study was conducted in Mato Grosso do Sul to detect arboviruses using the HIT test and serum-reduction by neutralization in plate (SRNP). The results suggest that horses can serve as sentinel animals to indicate the circulation of arboviruses. In this study, animals were positive for SLEV, the Ilheus virus (ILHV) and the Rocio virus (VROC) [28]. Other serological surveys conducted in the same area (the Pantanal in Mato Grosso do Sul) used SN and found three animals positive for Cacipacoré virus, five for WNV, nine for SLEV and 18 for ILHV [29], thereby demonstrating the virus circulation in this area [30].

The negative results in the viral isolation and RT-PCR indicate that recent infections in NHP had not been observed during the study period.

The detection of viral genomes by RT-PCR could confirm the presence of the virus in the animal at the time of collection. This finding would provide evidence of the enzootic activity of the arbovirus in that area, as well as the risk of transmission to humans. However, based on the negativity in the RT-PCR and the positivity in the HIT of 22 animals, the municipality of Mundo Novo can be considered as an area of silent activity for *Flavivirus*. Similar findings have been reported in another study conducted in the Nhecolandia region (Pantanal, Mato Grosso do Sul, Brazil). In this study, 135 horses were tested by viral isolation, RT-PCR and SN, exhibiting positive results in the HIT and negative results in the RT-PCR. However, in the same area a high positivity was observed for the Eastern (VEEL) and Western Equine Encephalitis (VEEW) viruses in the SN, demonstrating that the sub-region of the Pantanal is an important silent area for these arboviruses [31].

From December 2003 to June 2004, in a study performed in Colombia, the viral genome YFV was detected by RT-PCR in liver samples of NHP. The results confirmed the presence of the virus at the time of collection of dead animals, representing a risk of transmission to humans [32].

Sero-epidemiological studies of wild animals conducted through cross-sectional surveys can indicate whether the host was exposed to the antigen and can be considered a sentinel for the active surveillance of arboviruses of public health interest. In general, animal infection

Arbovirus	Location of capture							
	Jardim (n=10)		Coxim (n=8)		Mundo Novo (n=22)		Bodoquena (n=3)	
	Nº	%	Nº	%	Nº	%	Nº	%
<i>Flavivirus</i>	-	-	4	50	22	100	-	-
<i>Alphavirus</i>	1	10	-	-	-	-	-	-
<i>Mayaro</i>	1	10	2	25	-	-	-	-
Total	2	20	6	75	22	100	-	-

Table 1: Arbovirus identified in samples from forty-three non-human primates by capturing municipality, Mato Grosso do Sul, Brazil.

precedes human infection. Therefore, these data could be useful in terms of preventing the occurrence of epidemics and reducing the negative impacts on health, tourism and trade in the regions affected [33,20].

In conclusion, although all samples were negative in the RT-PCR, the positive serological results suggest that arboviruses of the genus *Flavivirus* are present in the region studied. Further studies to monitor viral activity are essential in order to prevent infection in humans, especially considering those locations exhibits favorable conditions for the occurrence of arbovirus outbreaks. These studies will be of great importance, especially for public health policies.

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