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Detection of antibodies against Icoaraci, Ilhéus, and Saint Louis Encephalitis arboviruses during yellow fever monitoring surveillance in non-human primates (*Alouatta caraya*) in southern Brazil

Marco Antonio Barreto de Almeida[1,4] | Edmilson dos Santos[1] | Jáder da Cruz Cardoso[1] | Carlos Alberto Noll[2] | Marcelo de Moura Lima[2] | Franko de Arruda e Silva[3] | Milene Silveira Ferreira[3] | Lívia Carício Martins[3] | Pedro Fernando da Costa Vasconcelos[3] | Júlio César Bicca-Marques[4]

[1]Centro Estadual de Vigilância em Saúde, Secretaria da Saúde do Estado do Rio Grande do Sul, Porto Alegre, Brazil

[2]17ª Coordenadoria Regional de Saúde, Secretaria da Saúde do Estado do Rio Grande do Sul, Ijuí, Brazil

[3]Ministério da Saúde, Instituto Evandro Chagas, Ananindeua, Brazil

[4]Escola de Ciências, Pontifícia Universidade Católica do Rio Grande do Sul, Porto Alegre, Brazil

Correspondence
Marco Antonio Barreto de Almeida, Centro Estadual de Vigilância em Saúde, Secretaria da Saúde do Rio Grande do Sul, Avenida Ipiranga 5400, Prédio Central, Sala 95, Bairro Jardim Botânico, CEP 90610-030, Porto Alegre, Rio Grande do Sul, Brazil.
Email: mabalmeida@gmail.com

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Abstract

Background: Free-ranging non-human primates (NHPs) can host a variety of pathogenic microorganisms, such as arboviruses, which include the yellow fever virus (YFV). This study aimed to detect the circulation of YF and other arboviruses in three wild *Alouatta caraya* populations in forests in southern Brazil.

Methods: We collected 40 blood and serum samples from 26 monkeys captured/recaptured up to four times from 2014 to 2016, searching for evidence of arboviruses by virus isolation, PCR, and neutralization tests.

Results: Viral isolation and genome detection were negative; however, we detected neutralizing antibodies against the Saint Louis, Ilhéus, and Icoaraci viruses in three NHPs.

Conclusions: Saint Louis Encephalitis, Ilhéus, and Icoaraci viruses circulated recently in the region. Future studies should investigate the role of NHPs, other vertebrate hosts and wild vectors in the region's arbovirus circulation and the potential risks of the arboviruses to wildlife, domestic animals, and humans.

KEYWORDS
arboreal primates, emerging diseases, Howler monkey, *Phlebovirus*, virus

1 | INTRODUCTION

Free-ranging non-human primates (NHPs) can host 100s of species of viruses, bacteria, protists, helminths, and arthropods. Approximately 20% of these parasites' recorded diversity is represented by viruses (82 species), of which at least 1/3 has also been found to infect humans.[1,2] The viruses recorded in humans and NHPs are mainly RNA viruses (85%), more than half of which are arboviruses.[1]

Hematophagous (blood feeding) arthropod vectors, such as mosquitoes and biting flies and ticks, transmit arboviruses between vertebrate hosts.[3] Many RNA viruses are arboviruses, including

Orthobunyavirus, *Nairovirus*, and *Phlebovirus* (Peribunyaviridae), *Flavivirus* (Flaviviridae), *Thogotovirus* (Orthomyxoviridae), *Orbivirus* (Reoviridae), *Vesiculovirus* (Rhabdoviridae), and *Alphavirus* (Togaviridae).[3] Among the 500+ arbovirus species suspected to be pathogenic, 150+ cause disease in humans, most of which are zoonotic.[4]

Caused by the yellow fever virus (YFV; genus *Flavivirus*, family Flaviviridae), the yellow fever (YF) is a febrile hemorrhagic disease of great importance to public health, that is, endemic to tropical regions of Africa and South America, where it periodically expands and retracts geographically.[5] Neotropical NHPs are highly susceptible to YF and are considered "sentinels" of virus circulation in forested areas.[6] Efforts to detect YFV exposure in free-living NHPs in the Americas have demonstrated increased arbovirus diversity. Since the 1930s, Brazil has conducted serological surveys on NHPs.[7,8] A similar strategy has been adopted in other American countries, such as Panama, after human YF outbreaks in the 1950s.[7-11]

Increased detection and monitoring efforts for YFV have increased the known arbovirus richness of NHPs. For example, researchers have detected black howler monkeys (*Alouatta caraya*) infected with the Saint Louis Encephalitis virus (SLEV) in Argentina[12] and red howler monkeys (*Alouatta macconnelli*), white-faced sakis (*Pithecia pithecia*) and golden-handed tamarins (*Saguinus midas*) with serological signs of YFV and Mayaro virus (MAYV) infection in French Guiana.[13] In Argentina, recent serological findings for the West Nile virus (WNV) and dengue 1 and 3 viruses (DENV) yielded increased reports of arboviruses infecting NHPs.[14]

The Brazilian Ministry of Health (BMH) adopted NHP surveillance as a tool for detecting YFV in 1999.[15] The Rio Grande do Sul State (RS) Health Department in the extreme south of Brazil followed this regulation and began monitoring YFV and other arboviruses circulating in *A caraya* and *Alouatta guariba clamitans* (southern brown howlers) in 2002.[16] Since then, arbovirus surveillance initiatives in NHPs have been adopted in other Brazilian states, which have indirectly evidenced circulation of SLEV in *A caraya*, *Sapajus nigritus*, and *Sapajus cay* in Paraná in 2004-2005[17]; Ilhéus virus (ILHV), MAYV, SLEV, Rocio virus (ROCV), Oropouche virus (OROV), and Mucambo virus (MUCV) in *Sapajus* spp in Alagoas, Paraíba, Pernambuco, Piauí, and Rio Grande do Norte in 2008-2010[18]; Cacipacoré virus (CPCV), MAYV, and OROV in *A caraya* and *Sapajus* spp in Mato Grosso do Sul in 2010-2013[19]; and Bussuquara virus (BSQV), Icoaraci virus (ICOV), ILHV, and SLEV in *Leontopithecus chrysomelas* in Bahia in 2006-2014.[20]

In RS, arbovirus surveillance in NHPs evidenced YFV,[6,16,21,22] SLEV,[22-24] and OROV[22,24] circulation, indicating that the environment, hosts, and vectors are suitable for these pathogens to circulate. The present study aimed to determine the arbovirus circulation in three *A caraya* populations in forests in the region where most of the previously described findings occurred, during YFV monitoring. We used a 2-year capture-recapture strategy to detect changes in the animals' arboviral immunity.

2 | MATERIALS AND METHODS

2.1 | Human care guidelines

This study followed the Code of Best Practices for Field Primatology (International Primatological Society and American Society of Primatologists) and the Guidelines for the Ethical Treatment of Primates (IACUC protocol 09267). It was also approved by the Ethics Committee on the Use of Animals of the Pontifical Catholic University of Rio Grande do Sul (CEUA registration # 15/00450) and conducted in accordance with Brazilian legislation under the SISBIO/ICMBio/MMA authorizations for activities with scientific purpose, # 13016-4, 13016-5 and 13016-6. All samples were analyzed in an official laboratory of the Brazilian Ministry of Health, whose procedures are part of routine techniques approved and supervised by the institution's ethics committee.

2.2 | Study area

We conducted this study in three forested areas in the municipality of Santo Antônio das Missões, northwestern RS. Two of these areas have a history of arbovirus circulation in *A caraya*: *S1* (28°23'54.65"S, 55°26'34.02"W; circulation in 2001 and 2008-2009) and *S2* (28°24'3.03"S, 55°28'1.64"W; circulation in 2008-2009). The third area (*N*), also inhabited by *A caraya*, was not previously investigated (28°29'40.10"S, 55°22'10.95"W). We selected these areas during routine capture by the Health Surveillance Coordination, Rio Grande do Sul State Health Department, in November 2014 (capture A).

2.3 | Capture and sample collection

We conducted three field trips of 5 days each (captures B: June 2015; C: November 2015; and D: June 2016) to obtain samples in periods before and after possible arbovirus contact. We assumed the possibility of YF re-emergence during the seasonal period (October to May) proposed by Romano et al.[15]

We captured the howler monkeys using a dart riffle to induce sedation by ketamine hydrochloride, midazolam, and levomepromazine hydrochloride, per the protocol adopted by BMH.[25] We then transported the howler monkeys to a field laboratory, where we evaluated their health status, weighed and measured them to estimate their ages and detect possible signs of disease. We marked each individual with a numbered metal ear tag, and implanted a microchip subcutaneously in the back. We collected blood by puncturing the femoral vein in the inguinal arteriovenous plexus. We placed aliquots of blood (0.5-1.0 mL) in duplicate in cryotubes and immediately froze them in N_2 until viral detection.[25] We centrifuged the remaining volume to obtain the serum and then froze them in duplicate in N_2 for antibody detection. We released the howler monkeys at their place of capture after they had recovered from sedation.[25] A team member accompanied the released animal until it reached a safe tree height to reduce the risk of attack by terrestrial predators.

We captured 26 howler monkeys (area *N* = 10, *S1* = 8, *S2* = 8). Of these, 17 (65%) were captured only once; 5 (19%) twice; 3 (12%) three times; and 1 (4%) four times. In total, we obtained 40 blood and serum samples.

We labeled samples individually with a number for each NHP and the letter of the corresponding capture (eg, 1A = NHP N° 1 caught in capture A). Recaptured animals retained the original number, but we identified the new capture by its corresponding letter (eg, 1B = NHP N° 1 recaptured in capture B). We sent samples to the Arbovirology and Hemorrhagic Fevers Section, Evandro Chagas Institute, BMH (SAARB/IEC) in the state of Pará, Brazil.

2.4 | Laboratory procedures

We attempted to isolate the viruses by inoculating the samples in cell cultures (C6/36-clone *Aedes albopictus* cells) followed by indirect immunofluorescence (IFI) using polyclonal antibodies[26] for the main arbovirus antigenic groups in Brazil. We used real-time quantitative PCR (RT-qPCR)[27] when attempting to detect YFV and DENV in captures B, C, and D and Zika and Chikungunya in capture C. These arboviruses were all widely circulating in humans in Brazil during the study period.

Antibody screening consisted of an initial screening using the hemagglutination inhibition (HI) method[28] adapted for microplates.[29] We tested the sera by HI against a panel of 19 arboviruses of the genera *Alphavirus* (Eastern Equine Encephalitis-EEEV, Mayaro, Mucambo, and Western Equine Encephalitis virus-WEEV), *Flavivirus* (Bussuquara, Cacipacoré, Ilhéus, Rocio, Saint Louis Encephalitis, West Nile, and Yellow Fever), *Orthobunyavirus* (Belém virus-BLMV, Caraparú virus-CARV, Catu virus-CATV, Maguari virus-MAGV, Oropouche, Tacaiuma virus-TACV, and Utinga virus-UTIV) and *Phlebovirus* (Icoaraci). We confirmed the HI results using the serum neutralization (NT) test in newborn mice.[30] We serially diluted (10^{-2}-10^{-10}) brain suspensions of mice infected with the viruses with a positive HI. We diluted (1:10) the tested anti-virus sera (positive control), heterologous (samples to be analyzed), and negative control sera in PBS and incubated them at 37°C for 60 minutes. Then, we inoculated 0.02 mL of the dilutions in newborn mice via the intracerebral route (IC). We monitored mice during 21 days. The animals that survived presented viral neutralization. We calculated the lethal dose—$LD_{50\ (0.02\ mL)}$ by the Reed & Muench's method.[31] We considered samples with a logarithmic neutralization index (LNI) ≥1.7 as positive.

3 | RESULTS

We did not detect viral genetic material directly by isolation or real-time quantitative PCR. Likewise, we found no serological evidence of arbovirus contact in most howler monkeys (21/26 = 81%), including the individual captured on all four expeditions.

The other howler monkeys (5/26 = 19%), captured in the *S1* and *S2* areas in November 2014 and June 2015, presented serological signs of contact with arboviruses from the genera *Flavivirus*, *Orthobunyavirus*, and *Phlebovirus* on the HI tests, including one individual captured three times (Table 1). We confirmed antibodies specific to the *Phlebovirus* ICOV (LNI = 2.5) and the *Flavivirus* ILHV (LNI = 2.4) and SLEV (LNI = 1.8) in three (60%) of these monkeys by the neutralization method. The SLEV-positive monkey was captured twice (Table 1) and showed increased neutralizing antibody titers to the virus between captures, changing from negative (LNI = 0.1) to positive (LNI = 1.8).

We found an arbovirus prevalence determined by HI of 0% (0/10) in area *N*, 25% (2/8) in area *S1* and 38% (3/8) in area *S2*. On the NT test, we found prevalence rates of 0% (0/10) in *N*, 12% (1/8) in *S1* and 25% (2/8) in *S2*.

4 | DISCUSSION

Although we did not detect viral presence via molecular tests, we found serological evidence of circulating ICOV, ILHV, and SLEV among black howler monkeys via monitoring. The first two represent new records of these arboviruses in the extreme south of Brazil. In addition, we found evidence of arboviruses from the genus *Orthobunyavirus* (Oropouche) on HI tests, although this was not confirmed by neutralization, including in the area where previous findings occurred.[22]

Detecting genetic material from viruses circulating in NHPs is common during YF epizootics[22,32-35] given that these animals are highly susceptible to the virus.[16] In these situations, dead animals may be widely available for collecting viscera for molecular diagnosis. In turn, new studies with more samples from live animals are needed to determine antibody prevalence to these arboviruses,[36] and it is rare to find animals during the short viremia period (YF = 6 days).[37] In addition, low infection loads, pathogen migration from the blood to the viscera, and latent infections may make it difficult to detect the virus in the peripheral blood.[36] Detecting active or latent infection by direct testing methods such as viral isolation and PCR is only possible if the pathogen is circulating in the blood, is excreted in the urine or feces, or is colonizing an accessible mucosal surface or superficial lymph node. Otherwise, dead animal tissue is required.[36] The low viral detection frequency by PCR and isolation in healthy free-ranging animals is common in this type of study.[13,14,17,19,20,38]

Saint Louis Encephalitis virus antibodies have been historically detected in NHPs in Brazil, including in RS,[16,22,23] Paraná,[39] Bahia,[20] and other states in northeastern Brazil.[18] The virus has been isolated from vectors, birds, humans, and other mammals (including NHPs) in the Amazon region,[40,41] although only one human outbreak is known. It occurred inland in the state of São Paulo in 2006.[42] The virus is widely distributed in the Americas from Canada to Argentina,[41,43] where human outbreaks were recorded in 2005[44] and 2010.[45] The natural SLEV cycle is predominantly bird-mosquito, but viral antibodies have been found in reptiles, marsupials, rodents, armadillos, horses, and primates.[18,37,41,46,47] The high antibody prevalence to this virus, as well as its ability to infect many vertebrate hosts, explains its finding

TABLE 1 Results of hemagglutination inhibition tests and neutralization tests in primates-by capture, NHP identification, capture site, and number of times the individual was captured.

Capture A Nov/2014					Capture B Jun/2015			
Id	**Area**	**Status**	**HI**	**NTLNI**	**Id**	**Area**	**Status**	**HI**
1A	S1	1st capture	**Flavi 1:20**	**BSQV 0.8 CPCV 0.7 YFV <0.4**	1B	S1	2nd capture	**Phlebo 1:40**
2A	S1	1st capture	ND	–	2B	S1	2nd capture	ND
3A	S1	1st capture	ND	–	–	–	–	–
4A	S1	1st capture	**Flavi 1:20 Ortho 1:20**	**ILHV 2.4** OROV 0.3	–	–	–	–
5A	S1	1st capture	ND	–	–	–	–	–
6A	N	1st capture	ND	–	–	–	–	–
7A	N	1st capture	ND	–	–	–	–	–
8A	N	1st capture	ND	–	–	–	–	–
9A	S2	1st capture	ND	–	9B	S2	2nd capture	ND
10A	S2	1st capture	**Flavi 1:20 a 1:160**	**CPCV 1.5 SLEV 0.1**	10B	S2	2nd capture	**Flavi 1:20 a 1:160**
11A	S2	1st capture	**Phlebo 1:640 Ortho 1:40 Flavi 1:20**	**ICOV 2.5** OROV 1.4 CPCV 1.2 BSQV ≤0.3	–	–	–	–
12A	N	1st capture	ND	–	12B	N	2nd capture	ND
13A	N	1st capture	ND	–	–	–	–	–
–	–	–	–	–	14B	S1	1st capture	ND
–	–	–	–	–	15B	S1	1st capture	ND
–	–	–	–	–	16B	S2	1st capture	ND
–	–	–	–	–	17B	N	1st capture	ND
–	–	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–	–

Id, NHP identification; Area, capture site; Status, number of times the individual was captured; HI, Hemagglutination Inhibition; Flavi, Flavivirus; Ortho, Orthobunyavirus; Phlebo, Phlebovirus; LNI, Logarithmic Neutralization Index of each tested virus; ND, Not Detected.
Viruses identification: BSQV, Bussuquara; CPCV, Cacipacoré; ICOV, Icoaraci; ILHV, Ilhéus; OROV, Oropouche; ROCV, Rocio; SLEV, Saint Louis Encephalitis; WNV, West Nile; YFV, Yellow Fever.
Each table row corresponds to one individual.
Bold values indicates results considered positive in neutralization tests.

in humans in the study areas. Similarities in symptoms between the non-neurological disease caused by SLEV and dengue fever, coupled with the limited knowledge on its transmission cycle and the lack of knowledge on its prevalence in much of the country, make it difficult to diagnose infections and human cases of the disease.[42]

Ilhéus virus-neutralizing antibodies were detected in NHPs in Argentina.[14] The virus, discovered in 1944 in mosquitoes in the city of Ilhéus, state of Bahia, Brazil,[48] continues to circulate among golden-headed lion tamarins (*Leontopithecus chrysomelas*) in that region.[20] Few ILHV isolates exist from humans in Latin America. The clinical presentation ranges from a febrile, oligosymptomatic disease to severe cases. Non-specific symptoms, short viremia, lack of adequate in situ confirmation methods, and high levels of cross-reactivity with other flaviviruses are some barriers that prevent its

	Capture C Nov/2015					Capture D Jun/2016				
NTLNI	**Id**	**Area**	**Status**	**HI**	**NTLNI**	**Id**	**Area**	**Status**	**HI**	**NTLNI**
ICOV 0.7	–	–	–	–	–	1D	S1	3th capture	**Phlebo 1:20 Flavi 1:20**	**CPCV 1.6 ICOV 1.0 ILHV 0.3**
–	2C	S1	3th capture	ND	–	2D	S1	4th capture	ND	–
–	–	–	–	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–	–	–	–
–	–	–	–	–	–	5D	S1	2nd capture	ND	–
–	–	–	–	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–	–	–	–
–	8C	N	2nd capture	ND	–	8D	N	3th capture	ND	
–	–	–	–	–	–	9D	S2	3th capture	ND	–
SLEV 1.8 ILHV 1.3 CPCV 1.2 ROCV 1.0 WNV 0.8 YFV 0.5 BSQV 0.1	–	–	–	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–	–	–	–
–	15C	S1	2nd capture	ND	–	–	–	–	–	–
–	16C	S2	2nd capture	ND	–	–	–	–	–	–
–	–	–	–	–	–	–	–	–	–	–
–	18C	S1	1st capture	ND	–	–	–	–	–	–
–	19C	N	1st capture	ND	–	–	–	–	–	–
–	20C	N	1st capture	ND	–	–	–	–	–	–
–	–	–	–	–	–	21D	S2	1st capture	–	–
–	–	–	–	–	–	22D	S2	1st capture	–	–
–	–	–	–	–	–	23D	S2	1st capture	–	–
–	–	–	–	–	–	24D	S2	1st capture	**Phlebo 1:20 Flavi 1:20**	**ICOV 1.3 CPCV 1.0 ILHV 0.2**
–	–	–	–	–	–	25D	N	1st capture	–	–
–	–	–	–	–	–	26D	N	1st capture	–	–

diagnosis in areas where arbovirus infection is enzootic.[49] As with SLEV, suspect cases of human ILHV infection are rare.

The ICOV antibody detection extends the occurrence of the virus to the south, confirms the possibility of infecting NHPs[20] and *A caraya's* susceptibility. ICOV has been minimally studied, although it is commonly isolated from wild rodents (*Proechimys guyannensis*) in the Brazilian Amazon.[50,51] Its pathogenicity in humans is unknown.

Specific antibodies in wild, free-ranging animals, as mentioned above, only demonstrate that these animals were exposed to an antigen. This evidence usually does not allow to determine infection timing, intensity, or frequency. In population terms, data on antibody prevalence can represent the population's history of cumulative exposure. However, they can be inaccurate relative to infection status.[36] Additionally, their interpretations are limited

by the possibility of cross-reaction or the lack of definition or standardization of the thresholds that define a positive antibody result.[36]

Nevertheless, situations occur where such evidence is more informative. An example is the increased neutralizing antibodies detected in a howler monkey in the present study between successive captures. Its antibody titles changed from negative to positive values. This result is consistent with the host having recent contact with SLEV, suggesting regional circulation.

In other situations, a lack of evidence is also informative. For example, the lack of serological evidence of YFV-neutralizing antibodies in all howler monkeys captured in an area with extensive YFV circulation during the 2008-2009 epizootic, including previous positive results in area *S1*,[22] signals that the virus is not currently circulating after the regional epizootic. This lack of YFV immunity in most (or all) howler monkeys in the study populations is concerning because of the species' high susceptibility to YF, making it potentially vulnerable in cases of YF re-emergence.[14] However, it is possible that the SLEV and ILHV contact detected in 8% of the howler monkeys will confer some degree of protection against future YFV re-emergences.[14,22]

Finally, long-term studies are essential to increase our understanding on the role of NHPs and other vertebrate hosts in arbovirus circulation and to assess the risk that these viruses pose to NHP conservation and public health.[14,18-20,22,52] Providing these answers depends on addressing several challenges, such as developing more specific diagnostic methods for analyzing blood and serum samples from healthy animals, which will allow assessing whether NHPs or other vertebrates are the best choices for actively monitoring viral presence. Such development would yield better results and conserve resources.

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ORCID

Marco Antonio Barreto de Almeida https://orcid.org/0000-0002-9718-2634

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