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Serological evidence for Saint Louis encephalitis virus in free-ranging New World monkeys and horses within the upper Paraná River basin region, Southern Brazil

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ABSTRACT

Introduction: Saint Louis encephalitis virus (SLEV) primarily occurs in the Americas and produces disease predominantly in humans. This study investigated the serological presence of SLEV in nonhuman primates and horses from southern Brazil. **Methods**: From June 2004 to December 2005, sera from 133 monkeys (*Alouatta caraya,* n=43; *Sapajus nigritus*, n=64; *Sapajus cay,* n=26) trap-captured at the Paraná River basin region and 23 blood samples from farm horses were obtained and used for the serological detection of a panel of 19 arboviruses. All samples were analyzed in a hemagglutination inhibition (HI) assay; positive monkey samples were confirmed in a mouse neutralization test (MNT). Additionally, all blood samples were inoculated into C6/36 cell culture for viral isolation. **Results**: Positive seroreactivity was only observed for SLEV. A prevalence of SLEV antibodies in sera was detected in *Alouatta caraya* (11.6%; 5/43), *Sapajus nigritus* (12.5%; 8/64), and *S. cay* (30.8%; 8/26) monkeys with the HI assay. Of the monkeys, 2.3% (1/42) of *A. caraya*, 6.3% 94/64) of *S. nigritus*, and 15.4% (4/26) of *S. cay* were positive for SLEV in the MNT. Additionally, SLEV antibodies were detected by HI in 39.1% (9/23) of the horses evaluated in this study. Arboviruses were not isolated from any blood sample. **Conclusions**: These results confirmed the presence of SLEV in nonhuman primates and horses from southern Brazil. These findings most likely represent the first detection of this virus in nonhuman primates beyond the Amazon region. The detection of SLEV in animals within a geographical region distant from the Amazon basin suggests that there may be widespread and undiagnosed dissemination of this disease in Brazil.

Keywords: Saint Louis encephalitis. Serology. New World monkeys. Horses. Arboviruses.

INTRODUCTION

Saint Louis encephalitis virus (SLEV) belongs to the genus *Flavivirus*, family *Flaviviridae*, which consists of approximately 70 virus species and subspecies distributed worldwide[1]. Most flaviviruses are transmitted between susceptible vertebrates by hematophagous arthropods, in particular mosquitos[1,2]. Flaviviruses are the most import causes of infectious diseases in humans from Brazil[3]; these viruses include Bussuquara, Cacipacore, dengue (serotypes 1, 2, 3, and 4), Iguape, Ilhéus, Rocio, Saint Louis encephalitis, and yellow fever[3,4].

SLEV may have initially originated in Central America[5], but it has now disseminated throughout the Americas, with reports of its presence in the USA[6,7], Canada[7], Argentina[8,9], Uruguay[10], and Trinidad[11]. In Brazil, SLEV was likely first isolated in the 1960s from a pool of *Sabethes belisarioi* mosquitoes captured on the Belém-Brasília Highway[12]. Since then, this virus has been identified predominantly in humans[13-15] and horses[16-18] from Brazil.

Serological confirmation of SLEV in wildlife is very rare; it has been described in the white-tailed deer in the USA[19], while seropositivity has been demonstrated in wild and sentinel animals and arthropods from both the Amazon region[20,21] and the State of São Paulo[22], Brazil. Moreover, at least three epizootic incidents of SLEV have occurred in nonhuman primates,

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including sentinel *Cebus* monkeys in the Brazilian Amazon region[21]. Additionally, a serological survey conducted in French Guiana detected low levels of SLEV antibodies in free-ranging primates[23].

This study presents the findings of a serological investigation of SLEV in free-ranging New World monkeys and farm horses from southern Brazil.

METHODS

Study location

All monkeys used in this study were trap-captured within the Porto Rico County region, located between the northwestern region of the State of Paraná and the southeast region of the State of Mato Grosso do Sul, on the upper Paraná River in Brazil **(Figure 1)**. This region consists of islands and sub-tropical forest reserves (where the animals were captured) that are protected by the *Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis* (IBAMA). The riparian forests of the islands of Mutum, Porto Rico, Gaivota, and Japonesa and the forests on the opposite shore of the Paraná River were included in this study. Porto Rico County is located in the northwestern region of the State of Paraná (22°46'20"S latitude and 53°16'01"W-GR longitude). This sub-tropical region has an average annual rainfall of 1,200-1,300mm and temperatures ranging from 16 to 29°C.

Nonhuman primates

One hundred thirty-three nonhuman primates [43 *Alouatta caraya* (black and golden howler monkey), 64 *Sapajus nigritus,* and 26 *S. cay* (both called capuchin monkeys)] were trap-captured as previously described[24]. The biological data for the captured nonhuman primates are given in **Table 1**; all primates were captured between June 2004 and December 2005 by a team of biologists and veterinarians with permission from IBAMA (license number 104/04). All primates were anesthetized[25], after which blood samples were obtained by jugular or brachial venipuncture, and sera were centrifuged (±1,000g) and stored at -196°C until used. All trapped animals were released after complete recovery at the capture location.

All captured primates were strict forest inhabitants. However, a number of *S. nigritus* specimens (n=13) were trapped in a forest reserve (within the Paraná River basin) close to a farm whose owner reportedly had frequent contact with monkeys.

Horses

Blood samples were obtained by jugular venipuncture from 23 mixed-breed adult horses (16 males and 7 females) located in proximity to where the *S. cay* monkeys were captured. All sampled horses were located on the same property and were representative of the total horse population maintained at this holding. Serum samples were obtained and stored at -196°C until they were used.

Serological assays

All serological assays were performed at the Evandro Chagas Institute (IEC-PA), Department of Arbovirology and Hemorrhagic Fevers, Belém, PA, Brazil. All samples were stored on dry ice and then air-shipped to Belém.

Hemagglutination inhibition test

All samples were initially subjected to a microplate hemagglutination inhibition (HI) test[26] against a panel of standardized antigens for 19 arboviruses, including four from the genus *Alphavirus* (eastern equine encephalomyelitis, western equine encephalomyelitis, Mayaro, and Mucambo),

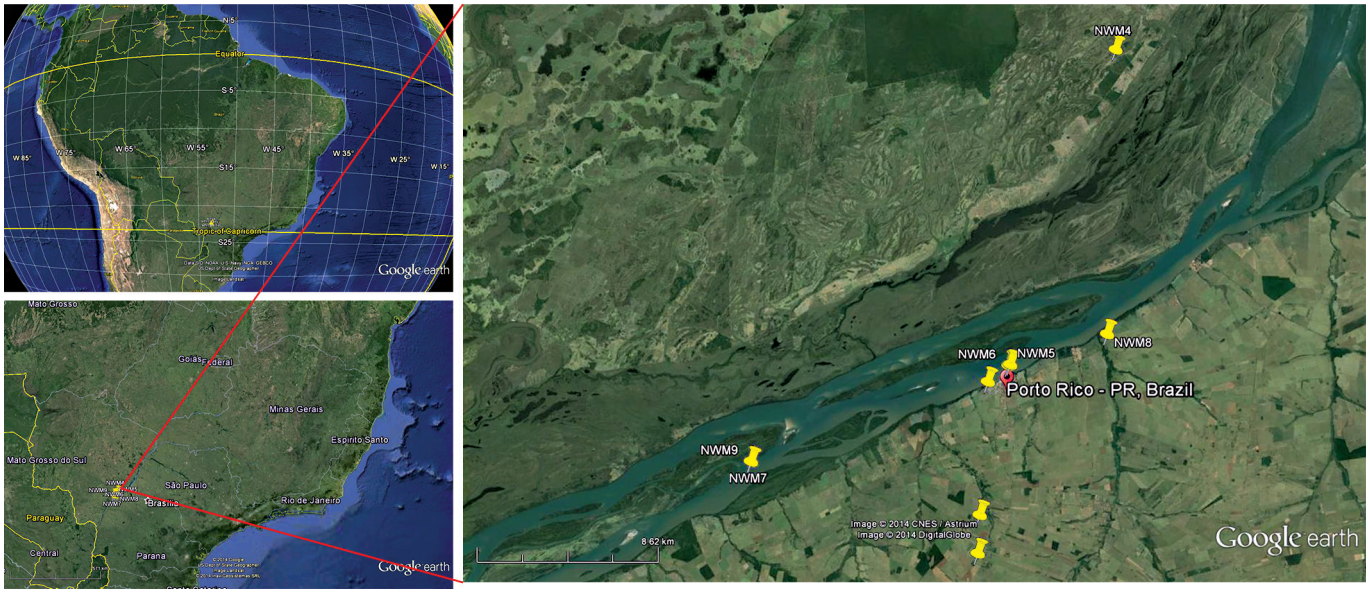

FIGURE 1 - Google earth images illustrating the region where the research was conducted. The yellow pins indicate the coordinates where monkeys seropositive for Saint Louis encephalitis virus were trap-captured.

TABLE 1 - The species, sex, and age distributions of free-ranging New World monkeys captured within the Porto Rico County region, Southern Brazil.

	Female (F)				Male (M)				
Monkey species	infant	juvenile	sub-adult	adult	infant	juvenile	sub-adult	Adult	Total (F, M)
Alouatta caraya	1	3	—	16	—	5	6	12	43 (F=20, M=23)
Sapajus nigritus	—	4	2	13	5	16	3	21	64 (F=19, M=45)
Sapajus cay	—	5	2	4	1	2	2	10	26 (F=11, M=15)
Total	1	12	4	33	6	23	11	43	133

six *Flavivirus* (yellow fever, Saint Louis encephalitis, Rocio, Ilhéus, Cacipacoré and Bussuquara), eight *Orthobunyavirus* (Oropouche, Caraparu, Catú, Guaroa, Maguari, Tacaiuma, Utinga and Belém) and one from the genus *Phlebovirus* (Icoaraci). Animals were considered seropositive when a titer of ≥ 20 was observed with the HI assay.

Mouse neutralization test

All positive monkey sera with HI titers >20 were subjected to a mouse neutralization test (MNT), a confirmatory assay that was used to characterize the virus associated with the infection and performed according to a previously described protocol[26]. The results were calculated using the neutralization logarithmic index (NLI)[27]. Sera from horses were not evaluated by MNT.

Virus isolation

All blood samples were inoculated into *Aedes albopictus* cell culture (clone C6/36); immunofluorescence was used for viral identification[28].

Statistical analysis

Statistical significance was analyzed using the Chi-square test (Yates corrected) to establish differences between the characteristics evaluated (species, gender, age, and the presence of horses). Associations between variables and positivity were determined by odds ratios (ORs) with 95% confidence levels. The results were considered statistically significant when the ρ-value was 5%.

Ethical considerations

This study was approved by the Animal Experimental Ethics Committee, *Universidade Estadual de Londrina* (Protocol number 34/05).

RESULTS

Serological assay and virus isolation from nonhuman primates

According to the HI assay, SLEV antibodies were present in primates, specifically in 11.6% (5/43) of *A. caraya*, 12.5% (8/64) of *S. nigritus*, and 30.8% (8/26) of *S. cay* animals (**Table 2**). However, confirmation of SLEV infection by MNT was obtained for only 2.3% (1/43) of *A. caraya*, 6.3% (4/64) of *S. nigritus* and 15.4% (4/26) of *S. cay* primates **(Table 2)**. Arboviruses were not isolated from any of the 133 primate-derived

TABLE 2 - Results of hemagglutination inhibition assays and mouse neutralization tests for free-ranging New World monkeys captured within the Porto Rico County region, Southern Brazil.

	Alouatta caraya		*Sapajus nigritus*		*Sapajus cay*		Total
HI result*(antibody titer)	male	female	male	female	male	female	(male/female)
Negative	21	17	40	16	10	8	112 (71/41)
20	—	2	2	1	2	2	9 (4/5)
40	2	$1^{(2.7)}$	1	—	$2^{(1.8/2.7)}$	—	6 (5/1)
80	—	—	$1^{(1.9)}$	$1^{(2.1)}$	—	$1^{(2.7)}$	3 (1/2)
160	—	—	—	$1^{(2.8)}$	—	—	1 (—/1)
320	—	—	—	—	—	—	—
640	—	—	$1^{(3.5)}$	—	$1^{(3.1)}$	—	2 (2/—)
Total	23	20	45	19	15	11	133 (83/50)

HI: hemagglutination inhibition. *HI test results: positive HI ≥ 20; $^{(n)}$neutralization logarithmic index (NLI): positive NLI ≥ 1.8.

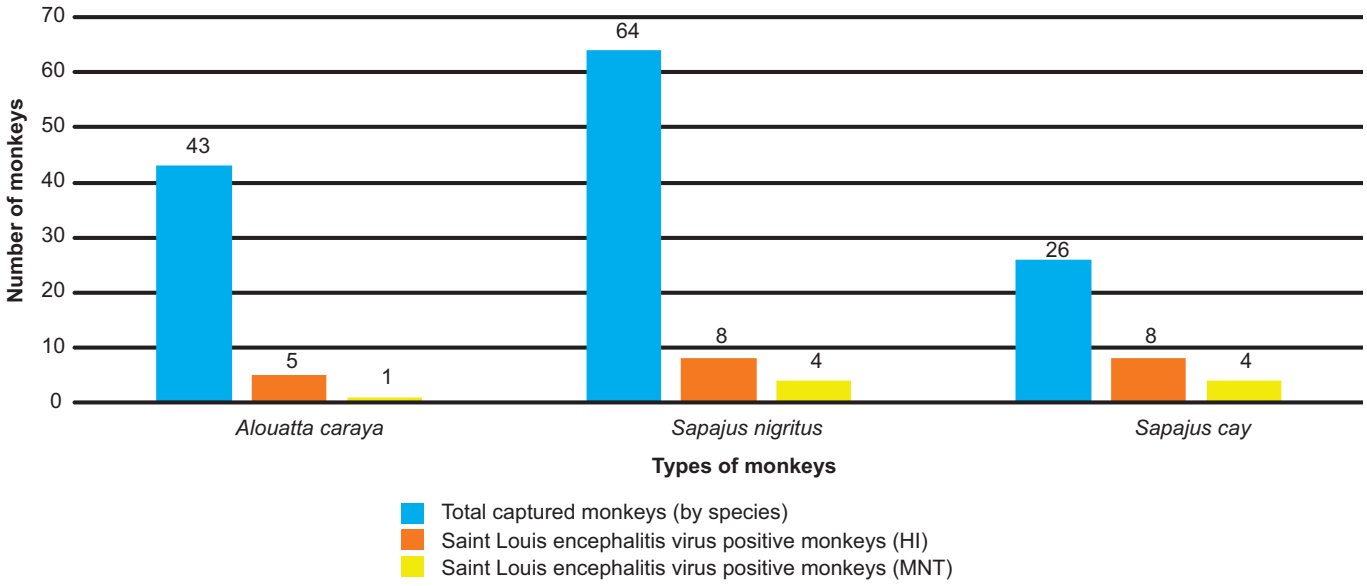

FIGURE 2 - Relationships between the total numbers of captured monkeys (by species) and positive results in the hemagglutination inhibition (HI) and mouse neutralization test (MNT) assays.

blood samples, although SLEV antibodies were detected by HI in 15.8% (21/133) of serum samples, with HI titers to SLEV ranging from 20 to 640 (**Table 2** and **Figure 2**). Negative seroreactivity was observed for all other arboviruses.

The association of the results obtained based on NMT analysis of characteristics evaluated (species, sex, age, and the presence of horses within the same habitat) with the neutralization logarithm index (NLI) is summarized in **Table 3**. The prevalence of anti-SLEV antibodies was elevated in *S. cay* (15.4%; 4/26) relative to *S. nigritus* (6.3%; 4/64) and *A. caraya* (2.3%; 1/43) primates. Interestingly, negative seroreactivity of HI to SLEV antibodies was observed in infant and juvenile primates, while SLEV antibody serum prevalence was identified in sub-adult (13.3%; 2/15) and adult (9.2%; 7/76) animals, most likely due to their more extensive contact with this pathogen. Additionally, primates that live within proximity to farm horses demonstrated elevated seroreactivity to SLEV antibodies (15.4%; 4/26) compared to those living without potential contact with horses (4.8%; 5/107). However, no significant differences were observed based on species (ρ=0.1090), sex (ρ=0.7281), age (ρ=0.1876), and the presence of horses within the same habitat (ρ=0.0727).

Serological assay and virus isolation from horses

The overall seroprevalence of SLEV antibodies in horses was 39.1% (9/23) and was elevated in females (57.1%; 4/7) relative to male horses (31.3%; 5/16). SLEV antibody titers in seropositive horses were as follows: 160 (three males; two females), 320 (one male and female) and 640 (one male and female). However, significant differences were not observed based on sex [ρ=0.3630; OR = 0.34 (0.04 < OR < 2.91)], and these results were not confirmed with the MNT assay. Additionally, arboviruses were not isolated from any of the 23 horse-derived blood samples, but as previously observed in primates, specific seropositivity only to SLEV antibodies was observed with the HI assay.

DISCUSSION

The results of this study reveal that we have identified the presence of SLEV in several species of nonhuman primates and horses from southern Brazil. The classical HI and MNT methodologies used during this investigation are based on the conventional serologic diagnosis of flavivirus due to the presence of virus-specific antibodies in the serum. Similar serological strategies have been used to detect SLEV in primates[23], white-tailed deer[19,29], livestock[11], mules[19], and horses[8,10,11,17,18]. The importance of these results lies primarily in the observation of SLEV circulation in a completely new habitat within Brazil, considering that most cases of SLEV have been described within the Amazon region[4,20-22]. However, SLEV was recently isolated from a horse in the State of Minas Gerais[16], and SLEV has been serologically identified in both horses from Corumba, Central West Brazil[17] and in patients from the State of São Paulo[14]. These results suggest a southern drift of SLEV from the Amazon region to other parts of continental Brazil, most likely attributable to migratory birds[16,21,22].

In the USA, SLEV causes encephalitis in approximately 100 human cases annually[30] and is one of the most common causes of arbovirus-induced disease[31], with sporadic epidemics[6,30,31]. However, this arboviral disease has different epidemiological features in Brazil, largely in terms of the relatively few cases of SLEV-induced encephalitis identified in humans[20,22]. Additionally, most flaviviruses that occur in Brazil, with the exception of dengue virus, are predominantly maintained

TABLE 3 - Associations between the characteristics studied (species, sex, age, and the presence of horses within the same habitat) and the presence of anti-Saint Louis encephalitis virus antibodies (neutralization logarithm index) in serum samples of free-ranging New World Monkeys.

Variables	Neutralization logarithmic index test							
	positive		negative		total			
Species	n	%	n	%	n	%	OR[a]	ρ-values
Alouatta caraya	1	2.32	42	97.68	43	32.33	*Ac* x *Sn*	0.63
							0.36 (0.01-3.6)	
Sapajus nigritus	4	6.25	60	93.75	64	48.12	*Ac* x *Sc*	0.12
							0.13 (0.01-1.38)	
Sapajus cay	4	15.38	22	84.61	26	19.55	*Sn* x *Sc*	0.38
							0.37 (0.07-1.95)	
Sex								
male	5	6.02	78	93.98	83	62.41	0.74	0.72[c]
female	4	8.00	46	92.00	50	37.59	0.16 < OR < 3.48	
Age								
infant	—	—	7	100.00	7	5.27		
juvenile	—	—	35	100.00	35	26.31	NC	0.18[b]
sub-adult	2	13.33	13	86.67	15	11.28		
adult	7	9.21	69	90.79	76	57.14		
Presence of horses in the same habitat								
yes	4	15.38	22	84.62	26	19.55	3.71	0.07[c]
no	5	4.67	102	95.33	107	80.45	0.76 < OR < 17.79	
Total (%)	9	6.77	124	93.23	133	100.00		

Ac: *Alouatta caraya;* ***Sn:*** *Sapajus nigritus;* ***Sc****: Sapajus cay;* **NC:** not calculated; **OR[a]**: odds ratio (inferior and superior limits); [b]chi-square; [c]Fisher's exact test.

as sylvatic zoonotic diseases that occasionally produce infections in humans and domestic animals that have entered the ecosystems where these viruses occur[4]. Consequently, it can be speculated that this epidemiological difference might possibly be attributable to specific environmental and biological conditions within Brazil that alter the virulence or pathogenicity of SLEV in humans. Although the factors that are actively or otherwise associated with this phenomenon have not been fully elucidated, at least two theories should be considered. First, the elevated endemicity of other closely related flaviviruses that elicit cross-protection in humans, such as that occurring with dengue and yellow fever immunization, might easily result in the underdiagnosis of SLEV; this occurred in a study of 519 patients who were initially diagnosed as having dengue fever, but later molecular investigation confirmed the presence of SLEV in eight of them[14]. Secondly, the difficulty in efficiently recognizing and/or diagnosing SLEV encephalitis in patients at both public and private health services might also contribute to the reduced number of cases in Brazil, considering that patients may either be asymptomatic[4,16] or present with flu-like disease syndromes that can progress to acute or subacute meningeal and focal neurological manifestations[32]. Moreover, three patients from an outbreak of SLEV in northwestern São Paulo demonstrated hemorrhagic manifestations typical of dengue fever virus[33]. Taken together, the confirmation of SLEV in several states in Brazil[13,14,16] in addition to the Amazon region may suggest dissemination due to migratory birds[16,22], as previously postulated. Furthermore, *Culex declarator* and *Culex coronator* are known vectors, while monkeys, sloths, armadillos, and marsupials are reservoirs of this virus in Brazil[4]. Therefore, the seropositivity of nonhuman primates and horses to SLEV demonstrated in this study in a region geographically distant from the Amazon region is of great concern, and additional investigation must be conducted to understand the dynamics associated with this virus in a new environment but in conventional hosts.

During this investigation, the majority (57.1%; 12/21) of seropositive monkeys demonstrated titers of SLEV according to HI that were ≥ 40, suggesting that these antibodies are attributable to the presence of SLEV and not cross-reactivity

from contact with similar related viruses. Alternatively, in a seroepidemiological survey conducted in French Guiana, primates had low antibody titers to SLEV (HI <40) but elevated titers to yellow fever (HI >320), and the authors suggested that that SLEV antibodies identified could have been due to cross-reactions with yellow fever virus[23]. Low titers of SLEV were also identified in horses, livestock, and wildlife from the island of Trinidad[11]. However, during this investigation, seropositivity was only identified for SLEV, and negative results were obtained with all other viruses by HI, indicating that there was no cross-reaction with any similarly related virus.

In Argentina, SLEV studies have focused primarily on populations of mosquitoes[34,35], humans[36], and horses[8], but the importance of monkeys in the sylvatic SLEV cycle has not been investigated. However, during this study, serological results confirmed the participation of nonhuman primates in the maintenance cycle of SLEV in southern Brazil. Consequently, these results most likely represent the first identification of SLEV in nonhuman primates beyond the Amazon region of Brazil.

Horses are commonly found in the region where sample collection was performed, largely on farms that are close to forested areas, and the relationships contributing to the presence of SLEV antibodies in these animals should be considered. Elevated prevalence rates of SLEV antibodies in horses from the Amazon[18] and Pantanal[17,18] regions have been described, but this report is the first description of SLEV in southern Brazil within an ecosystem that is very distant and quite different from the Amazon and Pantanal regions. Therefore, these results suggest that horses may participate as vertebrate hosts in the dissemination of SLEV and most likely should be considered amplifying sources of SLEV for primates, or vice versa. However, additional investigations must be performed to confirm this theory.

This report is the first describing the seroprevalence of specific SLEV antibodies in free-ranging monkeys within the State of Paraná, southern Brazil, and implicates nonhuman primates in the natural maintenance cycle of SLEV in the Southern Cone region, where an SLEV encephalitis outbreak was identified in northern Argentina[35]. Future research will aim to identify local mosquito and human populations to establish their roles in the SLEV life cycle.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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