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Article/Artigo

Reemergence of yellow fever: detection of transmission in the State of São Paulo, Brazil, 2008

Reemergência de febre amarela: detecção de transmissão no Estado de São Paulo, Brasil, 2008

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ABSTRACT

Introduction: Following yellow fever virus (YFV) isolation in monkeys from the São José do Rio Preto region and two fatal human autochthonous cases from the Ribeirão Preto region, State of São Paulo, Brazil, two expeditions for entomological research and eco-epidemiological evaluation were conducted. **Methods:** A total of 577 samples from humans, 108 from monkeys and 3,049 mosquitoes were analyzed by one or more methods: virus isolation, ELISA-IgM, RT-PCR, histopathology and immunohistochemical. **Results:** Of the 577 human samples, 531 were tested by ELISA-IgM, with 3 positives, and 235 were inoculated into mice and 199 in cell culture, resulting in one virus isolation. One sample was positive by histopathology and immunohistochemical. Using RT-PCR, 25 samples were processed with 4 positive reactions. A total of 108 specimens of monkeys were examined, 108 were inoculated into mice and 45 in cell culture. Four virus strains were isolated from *Alouatta caraya*. A total of 931 mosquitoes were captured in Sao Jose do Rio Preto and 2,118 in Ribeirão Preto and separated into batches. A single isolation of YFV was derived from a batch of 9 mosquitoes *Psorophora ferox*, collected in Urupês, Ribeirão Preto region. A serological survey was conducted with 128 samples from the municipalities of São Carlos, Rincão and Ribeirão Preto and 10 samples from contacts of patients from Ribeirão Preto. All samples were negative by ELISA-IgM for YFV. **Conclusions:** The results confirm the circulation of yellow fever, even though sporadic, in the Sao Paulo State and reinforce the importance of vaccination against yellow fever in areas considered at risk.

Keywords: Yellow fever. Flavivirus. Epizooties. Entomological surveillance.

RESUMO

Introdução: A partir do isolamento do vírus febre amarela (VFA), de macacos, da região de São José do Rio Preto e de dois casos humanos autóctones fatais, da região de Ribeirão Preto, Estado de São Paulo, foram realizadas duas expedições para pesquisa entomológica e avaliação ecoepidemiológica. **Métodos:** Um total de 577 amostras de humanos, 108 de macacos e 3.049 mosquitos foram analisados por um ou mais métodos: isolamento viral, ELISA-IgM, RT-PCR, histopatologia e imunohistoquímica. **Resultados:** De 577 amostras humanas, 531 foram testadas por ELISA-IgM, sendo 3 positivas, 235 foram inoculadas em camundongos, 199 em cultura de células, obtendo-se 1 isolamento viral. Uma amostra foi positiva por histopatologia e imunohistoquímica. Por RT-PCR foram processadas 25 amostras com 4 reações positivas. Os 108 espécimes de macacos foram inoculados em camundongos, 45 em cultura de células, obtendo-se 4 isolamentos de VFA, de *Alouatta caraya*. Um total de 931 mosquitos foram capturados em São José do Rio Preto e 2.118 em Ribeirão Preto e separados em lotes. Um único isolamento de VFA foi derivado de um lote de 9 mosquitos *Psorophora ferox*, coletados em Urupês, região de Ribeirão Preto. Um inquérito sorológico foi realizado com 128 amostras dos municípios de São Carlos, Rincão e Ribeirão Preto e mais 10 amostras de contactantes de pacientes de Ribeirão Preto. Todas as amostras foram negativas por ELISA-IgM para VFA. **Conclusões:** Os resultados confirmam a circulação, mesmo que esporádica, do VFA no Estado de São Paulo e reforça a importância da vacinação antiamarílica nas áreas consideradas de risco.

Palavras-chaves: Febre amarela. Flavivírus. Epizotias. Vigilância entomológica.

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INTRODUCTION

Yellow fever (YF) is an infectious disease, endemic in the tropical forests of Africa and Central and South America[1]. Clinical manifestations in humans range from asymptomatic to mild and severe forms. Yellow fever virus (YFV) has two transmission patterns in the Americas: sylvatic yellow fever (SYF) and urban yellow fever (UYF)[1], but both lead to the same clinical disease. The sylvatic cycle involves mosquitoes and monkeys. Some monkeys are highly susceptible to YFV, such as the howler monkey (genus *Alouatta*), while other species show strong resistance to the virus, such as the capuchin monkey (genus *Cebus*)[2]. The sylvatic yellow fever cycle in the Americas includes vectors from the genus *Haemagogus* and *Sabethes*, and the main species are *Hg. janthinomys*, *Hg. albomaculatus*, *Hg. leucocelaenus*, *Sa. chloropterus*, *Sa. Soperi* and *Sa. Cyaneus*[2,3]. Urban yellow fever involves humans and *Aedes aegypti* mosquitoes[2-4].

Yellow fever virus is the prototype member of the genus *Flavivirus,* family *Flaviviridae*[1]. The genome is a single strand, positive sense RNA, approximately 11kb in length. The complete genome has 10,862 nucleotides that encode 3,411 aminoacids[1,4-8]. Genetic studies of YFV strains have revealed some variations; the YFV strains isolated in South America and Africa are genetically distinct and are associated with different geographic regions. Thus, in Africa five genotypes have been identified: West Africa I, West Africa II, East Africa, Central and East Africa and Angola[8,9]. In South America two genotypes have been identified: South America I, which involves strains identified from Brazil, Panama, Colombia, Ecuador, Venezuela and Trinidad, and genotype South America II from Peru[8,11].

Yellow fever usually occurs as outbreaks in cycles of 7 to 10 years, alternating with periods with lower numbers of cases[4]. Epizooties are usually registered before human cases are detected.

Brazil has the most YF enzootic areas in continental America. Yellow fever is endemic in the

northern region and appears sporadically as epidemic/endemic in the central-western region[12]. Since 2000, SYF has been spreading progressively, transcending its usual boundaries and reaching other areas formerly known as enzootic[13-15]. The last urban outbreak in Brazil occurred in 1942[4]. The last autochthonous cases were reported in the State of São Paulo 47 years ago[15], but in 2000, two autochthonous SYF cases were notified along the border of the State of Minas Gerais[14].

After YF laboratory confirmation in four monkeys from the region of São José do Rio Preto and two confirmed fatal human autochthonous cases in the region of Ribeirão Preto, two expeditions for entomological studies and eco-epidemiological assessment of the likely sites of infection (LSI) were conducted. This study presents the results of laboratory analysis performed with blood/serum and tissues of humans and monkeys and mosquitoes collected in these regions from January to July 2008.

METHODS

Biological samples from humans

A total of 577 biological samples from individuals with suspected yellow fever infection were processed from January to July 2008. These samples came from networks of private and public healthcare in the State of São Paulo. The human autochthonous cases occurred in April and May 2008.

Serosurvey

Serological surveys to determine the prevalence of IgM antibodies against YFV were conducted in the municipalities of São Carlos, Rincão and Ribeirão Preto, in areas close to the LSI, following the confirmation of human YF cases.

Biological samples-monkeys

Monkeys that were found dead, though still in a suitable state for storage, were submitted to necropsy to remove the liver, spleen, kidneys, heart, lungs and occasionally blood. The samples were sent, in liquid nitrogen, for virus research and in 10% formalin for immunohistochemical investigation of viral antigen.

Vectors

Expeditions to the region of São José do Rio Preto and Ribeirão Preto were conducted in April and June 2008, respectively.

To catch vectors, mobile collection was used, with stops for 15-20min, with the aid of capturing oral suction and dip nets at times of highest light intensity between 9am and 4pm. The expeditions lasted four days. The mosquitoes collected were stored in cryo-resistant tubes, transported in a liquid nitrogen flask and stored at -70°C in the laboratory. Identification of mosquitoes was performed on a cold table and the mosquitoes were separated into batches of the same species or genus.

Entomological area research

São José do Rio Preto region: entomological surveys were conducted in the municipalities of Nova Aliança, Mendonça and Urupês, in areas where monkeys were found dead. These environments consist of small remaining fragments of forest, mostly gallery forests, surrounded by areas of agriculture and pasture. The local vegetation consists of heavily modified savannah s*ensu strictu*, forming small groves of varying density, depending on soil and water availability. The area presents a mesothermal humid climate: a dry and mild winter, with average temperatures during the coldest month above 18°C and an annual average of 25.3°C. The average rainfall during the driest month is less than 60mm[16]

Ribeirão Preto region: an entomological survey was conducted close to the likely sites where the two humans were infected. These areas are located in rural/wild areas in the municipalities of Luiz Antônio, São Carlos and Rincão, surrounding the Jataí Farm Forest Reserve located in Luiz Antônio. The Farm Forest Reserve has an extension of 4,532 hectares, with vegetation consisting primarily of *cerrado sensu latu*, with variations related to forests covering water bodies: riparian forest, gallery forest, semideciduous forest and transition forest riparian/savannah, as well as dry forests: high altitude savannah, high altitude open savannah and low altitude savannah[17].

Adjacent areas are used for technified agriculture linked to industrial complexes, with a predominance of cultures of sugar cane and citrus plantations, as well as eucalyptus and pine, and pasture areas[18].

The study areas are located in a climate zone that has two well defined seasons; hot and rainy in the period from October to April, and another cold and relatively dry in the period from May to September. The total estimated average annual rainfall is 1,400mm, and in the dry period, the monthly average is below 20mm. The average annual temperature is 22°C, with an average maximum of 29°C and a minimum of 16°C[19].

The State of São Paulo Environmental Department[20] describes the presence of species of the following monkeys *Callicebus personatus nigrifrons*, *Alouatta caraya* e *Cebus apella* in these regions.

Laboratory diagnosis

Depending on whether the samples were acute or convalescent, they were analyzed by one or more of the following methods: virus isolation (in mice and/or cell culture), capture ELISA IgM antibodies, indirect immunofluorescence, RT-PCR and sequencing. The samples from necropsy were analyzed by histopathology and immunohistochemical.

Capture ELISA IgM

The tests were performed in accordance with the protocol described by Kuno et al[21]. The samples were processed at 1:40 dilution in PBS containing 0.5% bovine albumin. The system used for revelation of the test consisted of a conjugate (anti-flavivirus labeled peroxidase) and substrate (ABTS). Samples with optical density reading of greater than 0.2 were considered positive. Positive and negative controls were added to all assays.

Isolation of virus in mice

To isolate the virus, samples of blood, serum, tissues from the autopsies of monkeys and humans, and mosquitoes were inoculated in 1-3 day-old Swiss mice. Samples of liver and brain of human and monkeys were inoculated separately, and the other viscera were processed in a mixed suspension. The tissues were macerated, suspended in a solution of bovine albumin, 0.75%, with antibiotics (100µg/mL of streptomycin and 100UI/mL of penicillin) and centrifuged at 6,000rpm for 20min. Each suspension was inoculated by the intracerebral route in 6 suckling mice at a dose of 0.02ml/mouse. Blood samples or serum were inoculated separately, pure or diluted (50%) in albumin solution. The animals were observed for 21 days.

Mosquitoes collected in the same place, date and time were processed in batches of 1 to 50 individuals of the same species or, in

cases in which the definition of species was not possible, in batches of the same genus. Mosquitoes were macerated and suspended in a solution 1.8% of bovine albumin and antibiotics (100µg/mL of streptomycin and 100UI/mL of penicillin) and centrifuged at 10,000rpm for 30min. Each supernatant was inoculated into mice in the same manner described above. The mice were observed for 21 days and those showing signs of disease had their brains harvested and subjected to subsequent passages to adapt the virus[22,23].

Virus isolation in cell culture

The same suspensions of tissues from human and monkey tissues, prepared for inoculation in mice, in addition to blood and serum, were also subjected to virus isolation in cell culture. An aliquot of 20µl was inoculated into tubes seeded with cultured cells of *Aedes albopictus*, clone C6/36[24]. The tubes were incubated for 9 days at 28°C and then agitated and centrifuged at 1,500rpm for 5min. The supernatants were stored at -70ºC, the pellets of cells were resuspended in PBS pH 7.5 and placed on slides with 12 parallel holes, for the indirect immunofluorescence assay (IFA) following the technique standardized by Gubler et al[25]. Polyclonal anti-yellow fever antibodies prepared in mice and anti-mouse immunoglobulin conjugated marked with fluorescein isothiocyanate (Sigma) were used. The positive samples were identified by IFA with YFV monoclonal antibodies (Center for Disease Control and Prevention, USA).

RNA extraction and RT-PCR

Total RNA was extracted using commercial kits. For tissue fragments, the QIAamp® RNA Blood (Qiagen Inc., Ontario, CA) was utilized and for serum, the QIAamp® Viral RNA Kit (Qiagen Inc., Ontario, CA) was used, in accordance with the manufacturer's instructions.

Amplification of viral RNA was performed by one-step RT-PCR, followed by a second amplification (semi-nested)[26] of the products of the first reaction, diluted at 1:50. The amplified products were visualized by electrophoresis in 1.5% agarose gel stained with ethidium bromide. Positive samples were those that had a band compatible with the expected.

Sequencing

Positive samples were sequenced directly in an ABI 377 sequencer by the method of dideoxy chain-termination cycle sequencing using the BigDye terminator sequencing kit v.3.1 (Applied Biosystems, Foster City, CA), in accordance with the manufacturer's instructions, with the same pair of primers of the one step RT-PCR.

For the edition of the nucleotide sequences, the Chromas Lite v.2.01 (Technelysian Pty Ltd.) was used, excluding the sequences of primers.

Histopathology and immunohistochemistry

Viscera samples for pathological and immunohistochemical study were collected and immediately placed in 10% formaldehyde, subjected to histological procedures for paraffin embedding. Slides with histological sections obtained from specimens were submitted to histological (hematoxylin-eosin staining) and immunohistochemical analysis. For the immunohistochemical investigation of viral antigen, polyclonal anti-YFV antigen, amplified in reaction with enzyme-conjugated polymer (EnvisionTM System- HRP, Dako Cytomation, USA) was used and revealed with diaminobenzidine[27,28].

RESULTS

Monkeys

A total of 108 specimens of monkeys were analyzed: 76 (70.5%) *Callithrix penicillata*, 13 (12%) *Alouatta caraya*, 13 (12%), *Cebus apella* and 6 (5.6%) other species. Tissue samples and/or blood of 108 monkeys were inoculated in mice and 45 in cell culture. YFV isolation was obtained from four *Alouatta caraya*, two were collected in Mendonça, one in Urupês and the other in Nova Aliança on January 14, 2008, using mice and cell culture. Two of them were found dead in Mendonça and Nova Aliança. The third one, a sick infant, was found with its dead mother in Mendonça, and the fourth was found dead in Urupês, on February 14, 2008. All isolates were analyzed by RT-PCR, with positive results for SYF (**Table 1**).

Humans

A total of 577 samples from humans with clinical suspicion of YF infection were analyzed. Among them, five were positive (two autochthonous and three allochthonous) by at least one of the techniques (**Table 1**). The serological survey was performed with 128 human samples from São Carlos (70), Rincão (22) and Ribeirão Preto (36). Besides these, another 10 serum samples from individuals who had been in contact with the patient from Ribeirão Preto were analyzed. All samples presented negative ELISA-IgM for YFV.

TABLE 1 - Laboratorial tests for yellow fever virus in the State of São Paulo, from January to July 2008.

Species	Mac ELISA	RT-PCR	Cell isolated	Mice isolated	Histopathol/ Immunohist	LSI
Human	negative	positive	positive	positive	Nd	autochthonous
Human	inconclusive*	Nd	Nd	Nd	positive	autochthonous
Human	positive	positive	negative	negative	Nd	allochthonous
Human	positive	positive	negative	negative	Nd	allochthonous
Human	positive	positive	negative	negative	Nd	allochthonous
Alouatta caraya	Nd	positive	positive	positive	Nd	autochthonous
Alouatta caraya	Nd	positive	positive	positive	Nd	autochthonous
Alouatta caraya	Nd	positive	positive	positive	Nd	autochthonous
Alouatta caraya	Nd	positive	positive	positive	Nd	autochthonous
Psorophora ferox	Nd	positive	positive	positive	Nd	autochthonous

Mac ELISA: IgM antibody capture Enzyme-linked immunosorbent assay, RT-PCR: Reverse transcription polymerase chain reaction ,Histopathol/Immunohist: Histopathology/Immunohistochemistry, LSI: Likely site of infection, Nd: not done. *Absorbance reading next to the value of the cutoff test

A total of 235 human tissue fragments and blood/serum, submitted for virus isolation in mice, resulted in the isolation of one strain of YFV, later characterized by RT-PCR and sequencing. The same isolation was obtained in cell culture among 194 samples inoculated. Among the 25 samples tested by RT-PCR, four were positive, one autochthonous and three allochthonous.

Entomological research

A total of 3,049 mosquitoes were captured, 931 assembled in 148 lots, collected in the São Jose do Rio Preto region and 2,118 assembled in 172 lots, collected in the Ribeirão Preto region. Species richness is presented in **Tables 2** and **3**. A single isolation of YFV was derived from a batch of nine samples of *Psorophora ferox* collected in Urupês, Ribeirão Preto region, on March 14, 2008. Viral isolation was achieved in mice and identification was achieved by RT-PCR and sequencing.

Histopathological analysis

Histopathological findings in both humans and monkeys consisted in lesions, predominantly mediozonal, sometimes extending from the hepatic parenchyma. Abundant apoptosis in hepatocytes, focal necrosis, steatosis and micro macrogoticular, hyperplasia and hypertrophy of Kupffer cells and space-port with mild lymphocytic infiltrate and no evidence of lesion interface were found (**Figure 1**).

TABLE 2 - Mosquito species collected in the São José do Rio Preto region, State of São Paulo, March 2008.

	Pools	Females	
Species	**n**	**n**	**%**
Aedes serratus group	14	295	31.7
Psorophora ferox	18	145	47.3
Ochlerotatus scapularis	27	117	12.6
Culex (Melanoconion) spp.	14	96	10.3
Aedeomyia squamipennis	1	39	4.2
Psorophora albigenu	7	39	4.2
Culex declarator affinis	6	38	4.1
Sabethes chloropterus	10	28	3.0
Sabethes tridentatus	5	21	2.3
Psorophora discrucians	5	19	2.0
Aedes terrens	3	18	1.9
Culex (Culex) spp.	6	18	1.9
Haemagogus leucocelaenus	7	16	1.7
Coquillettidia albicosta	3	7	0.8
Uranotaenia geometrica	1	6	0.6
Anopheles triannulatus	3	5	0.5
Howardina argyrothorax	2	3	0.3
Coquillettidia juxtamansonia	2	3	0.3
Haemagogus janthinomys/capricornii	3	3	0.3
Sabethes shannoni	1	3	0.3
Culex bidens	1	2	0.2
Limatus durhamii	2	2	0.2
Culex ameliae affinis	1	2	0.2
Anopheles parvus	1	1	0.1
Culex declaratory	1	1	0.1
Culex (Microculex) spp.	1	1	0.1
Wyeomyia aporonoma	1	2	0.1
Aedes albopictus	1	1	0.1
Total	**148**	**931**	**100.0**

TABLE 3 - Mosquito species collected in the Ribeirão Preto region, State of São Paulo, March/2008

	Pools	Females	
Species	**n**	**n**	**%**
Aedes serratus group	34	1,298	61.3
Psorophora ferox	19	359	16.9
Ochlerotatus scapularis	22	249	11.8
Culex (Melanoconion) spp.	9	40	1.9
Culex (Culex) spp.	8	25	1.2
Haemagogus leucocelaenus	5	17	0.8
Sabethes chloropterus	4	14	0.7
Aedes albopictus	8	14	0.7
Coquillettidia juxtamansonia	7	11	0.5
Ochlerotatus fulvus	7	9	0.4
Sabethes intermedius	1	9	0.4
Wyeomyia spp.	3	8	0.4
Anopheles triannulatus	5	7	0.3
Mansonia titillans	4	6	0.3
Howardina fulvithorax	4	6	0.3
Culex restuans/declarator	2	4	0.2
Mansonia wilsoni	2	4	0.2
Psorophora albigenu	3	4	0.2
Sabethes quasicyaneus	3	4	0.2
Sabethes undosus	2	4	0.2
Sabethes identicus	1	4	0.2
Anopheles albitarsis s.l.	3	3	0.1
Anopheles mediopunctatus group	1	3	0.1
Culex habilitator affinis	3	3	0.1
Haemagogus janthinomys/capricornii	2	2	0.1
Sabethes belisarioi	1	2	0.1
Sabethes tridentatus	2	2	0.1
Aedes terrens	1	1	0.0
Anopheles galvaoi	1	1	0.0
Culex bidens	1	1	0.0
Culex coronator	1	1	0.0
Sabethes glaucodaemon	1	1	0.0
Psorophora lanei affinis	1	1	0.0
Total	**172**	**2,118**	**100.0**

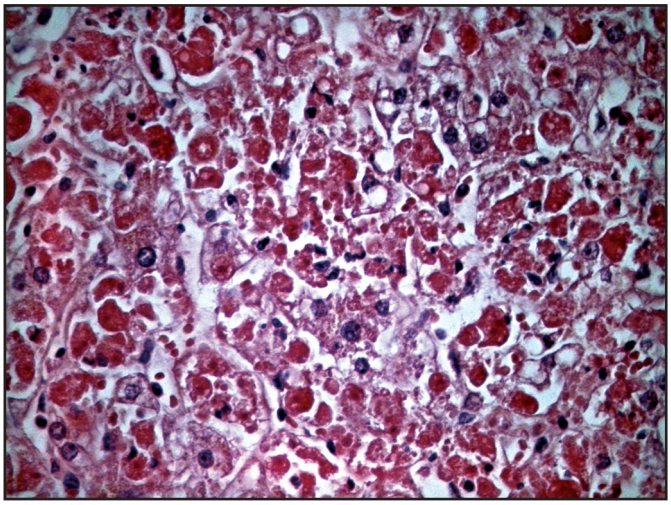

FIGURE 1 - Histological features of yellow fever, midzone lesions in liver with Councilman bodies the zone 2 (HE-x200).

Immunohistochemistry

The presence of viral antigen was verified by a brownish color, present in the cytoplasm of hepatocytes and Kuppfer cells (**Figure 2**).

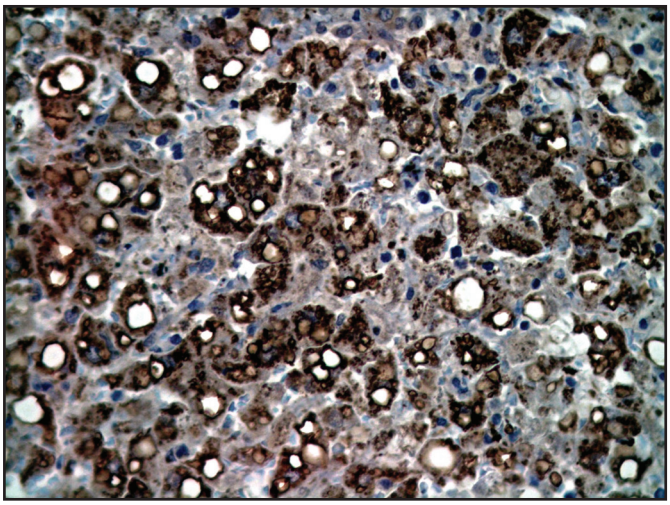

FIGURE 2 - Hepatocytes positively imunostained with anti-anti-antigen of yellow fever virus amplified in reaction with enzyme-conjugated polymer (X400).

DISCUSSION

Monkey deaths have been registered in the area of transition to YF in the State of São Paulo since January 2008.

All the monkeys from which YFV was isolated belonged to the species *Alouatta caraya*, which originated from cities outside the region of Sao José do Rio Preto. Due to their high susceptibility to YF, when found dead, infection in the *Alouatta caraya* monkey species can indicate the emergence of YF[12]. Continuous monitoring of these communities makes it possible to detect viral activity in the environment. The most abundant species, among the 108 monkeys found sick or dead, was *Callithrix penicillata* (over 70%).

The presence of three principal species of YFV vector in the region of São José do Rio Preto was observed: *Haemagogus janthinomys*, *Haemagogus leucocelaenus* and *Sabethes chloropterus*. However, these mosquitoes were identified in small numbers, which could be explained by the fact that the collections were made at ground level and these species favor the upper strata of trees[29]. However, it should be noted that the forests studied did not have the same exuberance observed in the Amazon rainforest.

This is the first report of the isolation of YFV from *Psorophora ferox*, although it had previously been found naturally infected by the arbovirus Ilheus[30] and Rocio[31]. However, these findings do not necessarily mean that the species is a vector for these viruses. *Psorophora ferox* was the second most abundantly caught species, corresponding to 47.3% in the region of São José do Rio Preto (**Table 2**) and 16.9% in the region of Ribeirão Preto (**Table 3**) from the total number of female specimens. Virus isolation could be facilitated by this abundance, since a dense population of mosquitoes is more susceptible to entering in contact with a circulating arbovirus and more easily accessed. *Ps. ferox* females are persistent and painful biters and they are not likely to be overlooked in biting collections. This aggressive behavior is the primary reason for its abundance in the collection. The virus isolation in a mosquito captured in the field is an indication that this species could be naturally infected, but this occurs only in certain circumstances and, up to now, no evidence of transmission associated with *Psorophora* had been verified. Additional studies of vector competence and capacity are required to evaluate the possibility of this species acting as a vector, but it is most probable that the YF infection in this species is accidental. Most isolations of YF from mosquitoes come from *Haemagogus* or *Sabethes*, with occasional evidence of other species infected with YFV, such as *Aedes fulvus*, *Psorophora*, *Aedes scapularis* and *Psorophora albipes*, each presenting one isolation[2].

Attempts to isolate the virus in *Aedes albopictus*, collected in the Ribeirão Preto region, were all negative. This species is considered a potential vector of YF[32]or a potential link between urban and rural YF[33], since it is disseminated in the periurban, rural and sylvan environments.

The Jataí Farm Forest Reserve and fragments of forests in the region of São José do Rio Preto, in principle, are ideal habitats for the circulation of YFV, since susceptible host monkeys and species of mosquitoes that are vectors of YF were found in this area. However, the forested environment is deteriorated and largely fragmenting this area, which may not be sufficient for the development of a large group of monkeys. As a result, the region does not favor the concentration of vector species in the dry season in order to maintain the cycles of YFV for long periods. In addition to the collection points located within the Jataí Forest Reserve Farm, mosquitoes were also collected in rain forest areas near the Anhanguera Highway, a forest habitat rather modified by human action. Nevertheless, it was possible to capture species known to be vectors of YF, often found in preserved forested environments[21]. This suggests possible differences in the dynamics of YF in fragmented landscapes due to adaptations to their hosts and reservoirs, specific to each region.

Further research is needed to assess the vector ability of other species of mosquitoes present in the region to identify possible alternative vectors and, in such cases, to determine the importance of these vectors in the maintenance of YFV in the environment.

In the expedition to the region of Ribeirão Preto, *Haemagogus leucocelaenus* and *Sabethes chloropterus*, known vectors of YF, were captured. Several reports exist of YFV isolation from *Haemagogus lecocelaenus* mosquitoes in the Amazon region[34]. More recently, in 2003, these mosquitoes have been found naturally infected with YFV in Rio Grande do Sul[33].

No monkey positive for YFV was detected in the Ribeirão Preto region. Through information from local people, the members of the expedition learned that groups of monkeys that had been previously seen or heard of in the region were no longer observed, suggesting the death of most or all of these groups.

Prompt laboratory diagnosis and the investigation of cases, whether autochthonous or allochthonous, is very important because of the environmental conditions favorable for the spread and establishment of the transmission of the agent in unaffected areas of the state. This determines the implementation of control measures related to urban vectors (preventing the risk of urban YF) or of blockage by anti-yellow fever vaccination. The importance of highways that connect the Amazon region to other states of Brazil should be highlighted, since these help disseminate different strains of YFV[33].

Regarding laboratory diagnosis, it is essential to highlight the issue of the sensitivity of the techniques and methods used. ELISA IgM capture was positive for samples collected from day

four following the onset of symptoms. The RT semi-nested PCR permitted confirmation of 50% of SYF cases in this period. Through necropsy, it was possible to correlate morphological findings and the immunopathology of infection, confirmed by immunohistochemical research positive for viral antigen.

Another important aspect concerns the role that the human population exercises in the maintenance of YFV in fragmented landscapes. Within a given location, for each case diagnosed as typical SYF, numerous asymptomatic cases also exist that usually go unnoticed. In Brazil, serological investigations have shown that 90% of infections are asymptomatic[34]. Thus, in primary infections of wild YF, the symptoms are atypical, requiring the existence of a cycle *Haemagogus* - Man - *Haemagogus* for the appearance of secondary or tertiary cases, the only ones likely to be diagnosed clinically[34]. It is important to assess whether the dynamics of YF is influenced by human populations living and working near forest fragments with small populations of monkeys.

The present study findings confirm the circulation of YFV, even if sporadic, in the State of São Paulo and reinforce the importance of maintaining vaccination coverage for YF in areas considered at risk. The high mobility of the human population to transmission areas, individuals in the course of viremia to areas still without established transmission, associated with high rates of infestation by *Aedes aegypti* in Brazil represent a risk for the reemergence of YF.

The findings indicate the need for eco-epidemiological studies, with a multidisciplinary focus, to elucidate the dynamics of transmission and geographical distribution of YFV in the state. Equally relevant is the impact of disease on wildlife endangered species.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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