Rev. Inst. Med. Trop. Sao Paulo
53(3):133-139, May-June, 2011
doi: 10.1590/S0036-46652011000300004

ISOLATION OF YELLOW FEVER VIRUS (YFV) FROM NATURALLY INFECTED *Haemagogus (Conopostegus) leucocelaenus* (DIPTERA, CULICIDAE) IN SÃO PAULO STATE, BRAZIL, 2009

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SUMMARY

After detecting the death of Howlers monkeys (genus *Alouatta*) and isolation of yellow fever virus (YFV) in Buri county, São Paulo, Brazil, an entomological research study in the field was started. A YFV strain was isolated from newborn *Swiss* mice and cultured cells of *Aedes albopictus* - C6/36, from a pool of six *Haemagogus (Conopostegus) leucocelaenus* (*Hg. leucocelaenus*) mosquitoes (Dyar & Shannon) collected at the study site. Virus RNA fragment was amplified by RT-PCR and sequenced. The MCC Tree generated showed that the isolated strain is related to the South American I genotype, in a monophyletic clade containing isolates from recent 2008-2010 epidemics and epizootics in Brazil. Statistical analysis commonly used were calculated to characterize the sample in relation to diversity and dominance and indicated a pattern of dominance of one or a few species. *Hg. leucocelaenus* was found infected in Rio Grande do Sul State as well. In São Paulo State, this is the first detection of YFV in *Hg. leucocelaenus*.

KEYWORDS: Yellow fever; *Haemagogus leucocelaenus*; Arbovirus.

INTRODUCTION

Yellow Fever (YF) is an endemic disease in South America and Africa, affecting about 200,000 people yearly[16].

In São Paulo State, Brazil, YF first appeared in Santos county. From 1850 onward, the city was hit by constant outbreaks of the disease, introduced by the crew of newly arrived ships, spreading from the port region[23]. In 1889 there was a strong epidemic in Santos county, moving towards the west and reaching as far as the county of Campinas, which was successively affected by epidemics in 1889, 1890, 1892, 1896 and 1897[18]. The 1895-1898 outbreaks affected Araraquara county, and in 1898 and 1904, there were epidemics in cities in the western region of the São Paulo State[18,23]. The last reported case of Sylvan YF (SYF) in São Paulo State was in 1953[23] and it remained so up to 2000, when the disease reemerged in São Paulo State, after a period of 49 years[19]. The last reported case of Urban Yellow Fever (UYF) in Brazil was in 1942, in Sena Madureira county, Acre State[15].

Sylvan Yellow Fever has remained endemic in Northern Brazil ever since, with epidemics and epizootic occurrences sporadically detected in the Midwest region and more rarely in the Southeast[14]. After this period, and considering the epidemics in the years 2008 and 2009, the virus, once considered endemic in only a limited portion of the country, started to be detected in almost all Brazilian territory, and so far only the coastal region is still considered to be free of transmission[26].

The SYF has been gradually expanding its territory, which led to a gradual increase in cases near the traditional boundaries of the enzootic area, as well as in the states of Rio Grande do Sul[9] and Minas Gerais in 2000 and 2001, respectively[8]. The high mobility of human populations in such regions of transmission, associated with the spread of the *Aedes aegypti* in Brazil[25], represents a real risk for the reintroduction of YFV in the urban environment.

In 2000 two autochthonous SYF cases were registered in the State of São Paulo, along the border of Minas Gerais State[19]. The environmental circumstances in which transmission occurred were not elucidated. In 2008 the circulation of YFV in São Paulo State was confirmed in the region of São Jose do Rio Preto county. The YFV was recovered from four primates of the genus *Alouatta* and from a pool of mosquitoes *Ps. ferox*. In the Ribeirão Preto region, two autochthonous human cases were confirmed in Luiz Antônio and São Carlos counties by virus isolation and immunohistochemistry, respectively.

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In 2009, epidemics and epizootics were detected in the Southwest region of São Paulo State, in the counties of Sarutaiá, Avaré, Buri, Piraju and Tejupá. In Buri county, five human cases and one non-human primate positive for YFV were detected (unpublished data). This study describes the results obtained during the entomological survey that followed the detection of YF epidemics in Buri, State of Sao Paulo.

MATERIAL AND METHODS

Study Area: The study was conducted in Enchovias District (23º 42' S 108 - 48º 41' W 174) located in Buri county, São Paulo, Brazil. Buri is located 270 Km from São Paulo city, at an altitude of 590 meters. The climate is temperate and the mean annual temperature is 20 ºC (Fig. 1).

Fig. 1 - Map of the Study Area: **01.** Location of Buri county in Brazil. **02.** Capture Site in relation to Buri county. **03.** Capture site, with forest areas near by farmlands and grazing pastures used for cattle ranching. We can see the intensely patchy and irregular distribution of natural vegetation. Images from Google Earth 6.0. ©2011 Inav Geossistemas. US Dept. State Geographer. ©2011 Europa Technologies. ©2011 MapLink/Teleatlas.

The region is characterized by the presence of forested areas covered by the native vegetation of typical Atlantic Rainforest, strongly affected by human action. The forest areas border farmlands and grazing pastures used for cattle ranching, and it is intensely patchy and irregularly distributed.

Mosquito capture: Mosquito capture occurred from 11 to 13 April, 2009. Mosquitoes were collected at ground level, with the aid of dip nets and oral suction capturing devices. The capture was conducted inside forest fragments, along a transect in the area. Technicians (all of whom had been previously immunized against YF) moved for 15 minutes and stopped for one hour to perform the capture and then moved again. The capture process occurred between 09:30 am and 4:00 pm.

The collected mosquitoes were immobilized by freezing and were transported in liquid nitrogen and stored in freezers (-70 ºC) in the laboratory, until species identification and isolation of the virus were conducted.

Virus isolation in mice: The mosquitoes collected in the same place, date and time were processed in pools of one to 50 individuals separated by species or, in cases in which the definition of species was not possible, the same genus. Pool size was determined by the size of the mosquitoes and quantity of specimens. Pools of mosquitoes were grounded and suspended in 2 mL of phosphate buffer with 1.8% bovine serum albumin, containing 100 U/mL of penicillin and 100 µL of streptomycin. Each suspension, after centrifugation, was inoculated by the intracerebral route in six newborn *Swiss* mice at a dose of 0.02 mL/mouse. The animals were observed over the course of 14 days. Brains of sick mice were ground and suspended in 2 mL of phosphate buffer with 0.75% bovine serum albumin, containing 100 U/mL of penicillin and 100 µL of streptomycin and subsequently inoculated into new mice and processed by reverse transcriptase-polymerase chain reaction (RT-PCR)[2,25].

Virus isolation in cell culture: Twenty microliters of suspensions from the mosquito pools (see protocol for virus isolation in mice) were inoculated in tubes seeded with cultured cells of *Aedes albopictus*, clone C6/36[12]. After nine days at 28 °C, the cell cultures were shaken, centrifuged and the pellets of cells were resuspended in PBS pH 7.5. Indirect immunofluorescence assay (IFA) using polyclonal anti-yellow fever antibodies[4] and anti-mouse immunoglobulin conjugated (fluorescein isothiocyanate - Sigma) were performed. Positive samples were typed by IFA with monoclonal antibodies to YFV (Centers for Disease Control and Prevention).

RNA extraction and RT-PCR: Total RNA was extracted from supernatant fluid of C6/36 and tissue (brain and liver) of suckling *Swiss* mice infected with suspension of mosquitoes, using commercial kits QIAamp® Viral RNA and QIAamp® RNA Blood (Qiagen Inc., Ontario, CA), respectively, according to the manufacturer's instructions.

The amplification of viral RNA was carried out as described previously[5] with some modifications. RT-PCR one step was performed with SuperScript™ and Platinum® (Invitrogen/Life Technologies, Carlsbad, CA), followed by a second amplification (semi-nested), from the products of the first reaction, diluted at 1:50. Other conditions, including the cycle program, were identical. The target region was the NS5-3'UTR of the Yellow Fever virus. The amplified products (expected to be approximately 542 bp) were visualized by electrophoresis in 1.7% agarose gel stained with ethidium bromide.

Sequencing: The purified PCR products were directly sequenced on ABI 377 sequencer, using the BigDye terminator sequencing kit v.3.1 (Applied Biosystems, Foster City, CA), following exactly the manufacturer's instructions with the same pair of primers from the one step RT-PCR.

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

Phylogenetic analysis: Sequences representative of the genotypes South American I, South America II, East Africa and West Africa were

Table 1
3'NCR sequences used in this study

Isolate	Sequence ID	Origin/Year of Isolation	Source	Genbank No.
Asibi	Ghana27	Ghana/1927	Human	AY326411
Senegal65	Senegal65	Senegal/1965	Human	U52414
69056	Nigeria46	Nigeria/1946	Human	U52403
H117505	Nigeria87C	Nigeria/1987	Human	AY541410
Serie 227	Ethiopia61A	Ethiopia/1961	Human	AY541407
Uga	Uganda48	Uganda48	Human	U52423
85-82H	IvoryC85	Ivory Coast/1982	Human	U54798
Ar B 8883	CAR77A	Central African Republic/1977	*Aedes africanus*	U52393
Ar B 9005	CAR77B	Central African Republic/1977	*Aedes africanus*	U52396
Trinidad79	Trinidad79	Trinidad/1979	*Hg. spegazzini*	U52420
1899/81	Peru81	Peru/1981	Human	U52411
Peru95	Peru95	Peru/1995	Human	U52407
OBS7687	Bolivia99A	Bolivia/1999	Human	AY541326
OBS8026	Bolivia99D	Bolivia/1999	Human	AY541327
BeH203416	Brazil71	Brazil/1971	Human	AY541344
JSS	Brazil35	Brazil/1935	Human	U52390
SPH188002	Brazil00B	SP/2000	Human	FJ875521
SPH287923	Brazil08A	MT/2008	Human	FJ875524
SPH287992	Brazil08B	MS/2008	Human	FJ875525
SPH288116	Brazil08C	GO/2008	Human	FJ875526
SPH258595	Brazil04	AM/2004	Human	FJ875523
SPAn288183	Brazil08E	SP/2008	*Alouatta* sp	FJ875528
BeAR628124	Brazil00A	Brazil/2000	*Hg. janthinomys*	AY541328
BeAr645693	Brazil01D	Brazil/2001	*Haemagogus sp*	AY541334
BeH605427	Brazil98E	Brazil/1998	Human	AY541398
BeH603325	Brazil98D	Brazil/1998	Human	AY541397
BeAn604552	Brazil98A	Brazil/1998	*Alouatta belzebul*	AY541394
BeAr512943	Brazil92C	Brazil/1992	*Hg. janthinomys*	AY541379
BeH511843	Brazil91B	Brazil/1991	Human	AY541377
BeH422312	Brazil84H	Brazil/1984	Human	AY541369
BeAr424492	Brazil84E	Brazil/1984	*Hg. janthinomys*	AY541366
BeH350698	Brazil78A	Brazil/1978	Human	AY541352
BeH233393	Brazil73D	Brazil/1973	Human	AY541348
BeAr44824	Brazil62B	Brazil/1962	*Haemagogus spp.*	AY541340
BeAn23536	Brazil60	Brazil/1960	*Cebus* spp.	AY541338
BeH111	Brazil54	Brazil/1954	Human	AY541335
614819	Panama74	Panama/1974	Human	AY541412
35720	Venezuela98A	Venezuela/1998	Human	AY541443
SPAr303739		Brazil/2010	*Hg. leucocelaenus*	

retrieved from GenBank and included in the phylogenetic analysis for comparison with the sequence generated in this study (Table 1).

We used the Bayesian inference method available in the software BEAST v. 1.4.6 in order to analyze the phylogenetic relationship of the strains of this study[7].

Sequences were analyzed using a strict molecular clock model and Bayesian skyline demographic models, considering a constant rate of evolution of 1.0 X 10^{-4} nucleotide substitutions per site per year[7].

Statistical analysis: From the captured sample, Diversity and Population Dominance indexes were calculated in order to better characterize the mosquito population in the area[13,20,21].

Margalef's diversity Index:

$$\alpha = \frac{s - 1}{\text{Log} \quad N}$$

Where: S is the number of species sampled and N is the total number of individuals in all species. Values below 2.0 are representative of areas of low diversity and greater than 5.0 are considered as an indicator of high biodiversity.

Shannon diversity Index:

$$H' = -\sum p_i \text{ Log } p_i$$

Where: p_i is the proportion of species in relation to the total number of specimens found in surveys.

The Shannon index varies from 0-1, 0 being an indicator of a sample with zero diversity (few species or marked dominance of a few) and 1 indicates a high diversity, with many species and populations numerically equitable.

Simpson dominance Index:

$$D_s = 1 - l_s$$

being,

$$l_s = \frac{\sum n_i(n_i - 1)}{N(N-1)}$$

Where: n_i is the number of individuals of each species and N is the number of individuals. The Simpson index reflects the probability of two randomly selected individuals in the community belonging to the same species. It varies from 0-1 and higher values indicate a greater probability of individuals being the same species, suggesting great dominance of a single species.

For the species *Hg. leucocelaenus,* the minimum infection rate (MIR) was calculated, obtained from the division between[11, 28]:

$$\frac{\textbf{Positive Pools X 1,000}}{\textbf{Tested Specimens}}$$

RESULTS

The captured mosquitoes were identified and separated in species, number of the specimens, frequency and polls to study (Table 2). Although these data represent a single collecting event, ecological indexes were calculated to better characterize diversity and dominance among the species present in the sample.

Margalef's Diversity Index: $\alpha = 4.0$
Shannon Diversity Index H '= 0.63
Simpson's Dominance Index: D = 0.67

Table 2
Species of mosquitoes captured, April 11-13, Buri, São Paulo, 2009

Captured Species	Specimens	Frequency %	Pools No.
Ochloretatus serratus	166	52.7	6
Psorophora ferox/melanota	24	7.6	2
Coquiletidia justamansonia	4	1.3	3
Sabethes purpureus	12	3.8	3
Culex Mel. Sp	13	4.1	4
Ochloretatus scapularis	1	0.3	1
Psorophora ferox/pseudo	88	28	4
Haemagogus leucocelaenus	6	1.9	1
Psorophora albipes	1	0.3	1
TOTAL	315	100	25

Virus isolation (SPAR 303739 strain) was obtained in both *Swiss* mice, after six days following inoculation, and cell culture from a pool of six specimens of *Hg. leucocelaenus* mosquitoes. All other pools were negative both in mice and cell culture models. The minimum infection rate (MIR) was calculated at 0.17 (MIR = 0.17).

It was amplified a product of about 542 nucleotides (NS5-3'UTR region) with semi-nested RT-PCR in 1.7% agarose gel, ethidium bromide stained. The material was positive to Yellow Fever (Fig.2).

The phylogenetic relationship among this strain and Yellow Fever Virus circulating in South America were reconstructed by Bayesian analysis. The analysis generated a phylogenetic tree (Fig. 3).

The isolated strain sequenced in this study was located in South American I genotype, subclade 1E, coupled with samples of 2008 and 2009 from Brazil and other regions (Fig. 3). The group is strongly supported (posterior probability of 1.00). Internal relations within the subclade, however, are less supported, a factor likely generated by high homology of the samples, which hinders the separation of subgroups.

DISCUSSION

These data represent a single collection, focusing on a method that facilitates the capture of diurnal species which are attracted by noise or the presence of primates.

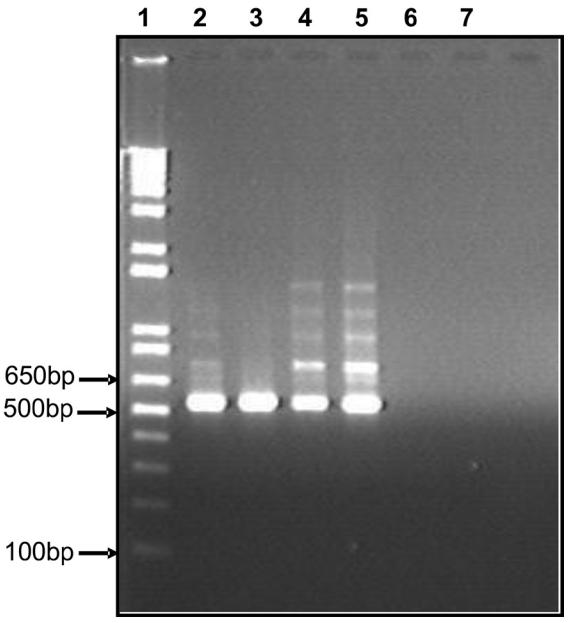

Fig. 2 - Identification of YF amplified product of about 542 nucleotides (NS5-3'UTR region) with semi-nested RT-PCR in 1.7% agarose gel, ethidium bromide stained. Lane 1 = 1kb plus ladder (Invitrogen); lane 2 = SPAr 303739, lanes 3 to 5 = positive control, lanes 6 and 7 = negative control (H2O).

Although the methodology may represent a bias in ecological analysis, a single collection in a restricted area was performed to try to capture mosquito species involved in transmission of YFV in the region, aiming the virus isolation.

Frequently used ecological indexes were calculated to characterize the sample in relation to diversity and dominance. The Margalef's Diversity Index ($\alpha = 4.0$) and the Shannon Diversity Index ($H' = 0.63$) indicate a sampling of diversity slightly above average, which is expected in a sample from an anthropical environment, in a situation where efforts were focused on capturing specific niches of the population. The absence of mosquitoes typically nocturnal or of crepuscular habits corroborates to the lower diversity. Also the Simpson's Dominance Index ($D = 0.67$) indicates a pattern of dominance of one or a few species.

This pattern could be observed for the species *Ochlerotatus serratus* which represented 52.7% of the species caught and *Psorophora ferox / pseudo,* accounting for 28% of sampled specimens (Table 1). The relative dominance of these species can be explained by human disturbance of the environment or the effect of the used method, which favors the capture of those mosquito species.

Viral isolation from a pool of *Hg. leucocelaenus* mosquitoes indicates the natural circulation of YFV in the Buri county, confirming the presence of the virus in São Paulo State. The isolated strain sequenced in this study was located in South American I genotype, coupled with samples of 2008

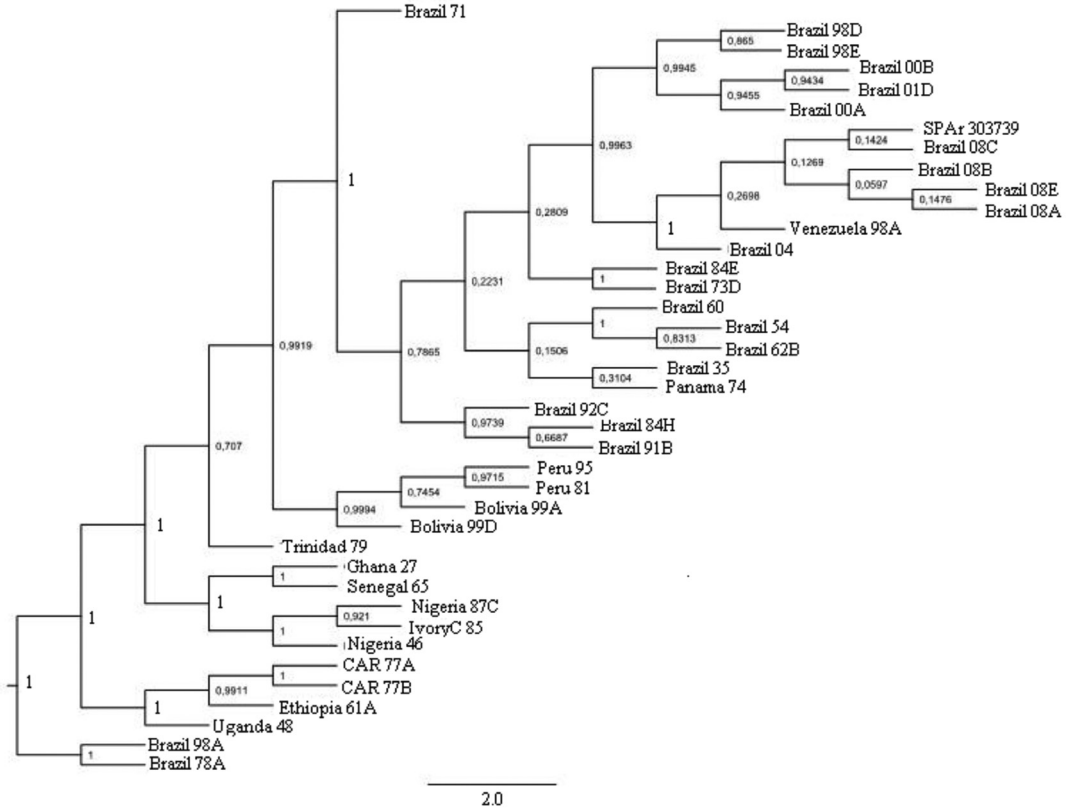

Fig. 3 - Bayesian analysis of the NS5-3'UTR region. This analysis used the Bayesian Skyline model. The consensus of post-burning trees is shown, with nodes with less than 70% support collapsed. Numbers in front of the nodes represent posterior probabilities of those nodes.

and 2009 from Brazil and other regions, representing the continuity of the Epizootic and Epidemic observed elsewhere in Brazil[22]. The isolated strain is strongly associated with the newly described Subclade E1[22].

The pool of mosquitoes that led to the virus isolation consisted of six specimens. The minimum infection rate (MIR) was 0.17. The MIR index is the ratio between the number of positive pools over the total number of mosquitoes tested of each species, by area and period. One limitation of this method[10] is that the values obtained are valid only for small pools, since the premise of the MIR is the existence of only one infected mosquito per pool. For samples with few individuals, as in this case, and considering that the arboviral infections are rare, the MIR provides the accuracy for this analysis.

The MIR index indicates a probability of about 17% of *Hg. leucocelaenus* being infected with YFV. This value is an indicator of the presence of viral activity in the environment. An increase in this index would indicate an impending transmission cycle.

Although the sequence genomic obtained was relatively small and restricted to a highly conserved site at 3' UT region of the genome were identified, the analysis allowed its initial alignment and recognition among the circulating strains of YFV in Sao Paulo State in 2008. More detailed studies should be the subject of future publications.

Hg. leucocelaenus had previously been found infected in Rio Grande do Sul State[27] and Colombia[4]. The species was also nominated as a vector in epidemics in Argentina and Bolivia[16]. Their role in the transmission of YFV has been considered secondary up to now in these areas.

This isolation supports the role of this species in the circulation of YFV, as a vector in southern and southeastern Brazil.

The behavior of this species has been described as opportunistic, using multiple sources of food, with most predominantly primatophilic and ornithophilic habits, with capacity for adaptation, including marsupials and rodents in their diet, in places where primates and birds were not available[1]. Although they have been previously described as primarily acrodendrophilic[9], the studied mosquitoes were captured on the ground level.

The population stratification of different species of *Haemagogus* has been observed, pointing to *Hg. leucocelaenus* as a common species in the ground level of Caxiuanã National Forest, Pará, Brazil, mainly in the months of the heaviest rains[17].

This is the first detection of YFV in *Hg. leucocelaenus* in São Paulo State. This study indicates the need for continuing surveillance activities, to better understand the behavior of *Hg. leucocelaenus* and their role in the transmission cycle of YFV in São Paulo State.

RESUMO

Isolamento do vírus da Febre Amarela de mosquitos naturalmente infectados *Haemagogus (Conopostegus) leucocelaenus* (Diptera, Culicidae), São Paulo, Brasil, 2009

Após a detecção de morte de macacos Bugios (gênero *Alouatta*) e isolamento do vírus da Febre Amarela (YFV) no município de Buri, Estado de São Paulo, Brasil, foi iniciada uma investigação entomológica em campo. Uma cepa de YFV foi isolada em camundongos recém-nascidos e cultura de células de *Aedes albopictus* - C6/36, a partir de um lote de seis mosquitos *Haemagogus (Conopostegus) leucocelaenus (Hg leucocelaenus)* Dyar & Shannon coletados no local de estudo. RNA do vírus foi amplificado por RT-PCR e seqüenciado. A topologia gerada indica que a cepa isolada está relacionada ao genótipo South American I, em clado monofilético englobando isolados recentes de epidemias e epizootias entre 2008 e 2009. Análises estatísticas geralmente usadas caracterizaram a amostra em relação à diversidade e dominância, indicando dominância relativa de uma ou poucas espécies. *Hg. leucocelaenus* foi detectado infectado também no Rio Grande do Sul. No Estado de São Paulo trata-se da primeira detecção do YFV em *Hg leucocelaenus*.

ACKNOWLEDGMENTS

The authors wish to thank the directors of the *Instituto Adolfo Lutz*, *Superintendência de Controle de Endemias (SUCEN)* and *Centro de Vigilância Epidemiológica "Prof. Alexandre Vranjac"* of the *Secretaria de Estado da Saúde do Estado de São Paulo* for their financial and logistical support. The authors wish to thank the *Secretaria de Vigilância em Saúde, Ministério da Saúde* by the support in field work and the staff of the *Núcleo de Doenças de Transmissão Vetorial - Instituto Adolfo Lutz* for their support in the completion of all phases of this research. We extend our thanks to Resolina Pereira Santos of the *Núcleo de Cultura de Células - Instituto Adolfo Lutz*, for technical assistance in the cell cultures, to Lucy T.A. dos Santos and Regina Celi W.F. Mosetic of the *Núcleo de Biotério* and our colleagues in the *Grupos de Vigilância Epidemiológica (GVEs)* who have contributed to the epidemiological surveillance of YF.

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Received: 24 August 2010
Accepted: 25 April 2011.

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