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# CIRCULATION OF ANTIBODIES AGAINST YELLOW FEVER VIRUS IN A SIMIAN POPULATION IN THE AREA OF PORTO PRIMAVERA HYDROELECTRIC PLANT, SÃO PAULO, BRAZIL

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## SUMMARY

Yellow fever (YF) is an acute viral infectious disease transmitted by mosquitoes which occurs in two distinct epidemiological cycles: sylvatic and urban. In the sylvatic cycle, the virus is maintained by monkey's infection and transovarian transmission in vectors. Surveillance of non-human primates is required for the detection of viral circulation during epizootics, and for the identification of unaffected or transition areas. An ELISA (enzyme-linked immunosorbent assay) was standardized for estimation of the prevalence of IgG antibodies against yellow fever virus in monkey sera (*Alouatta caraya*) from the reservoir area of Porto Primavera Hydroelectric Plant, in the state of São Paulo, Brazil. A total of 570 monkey sera samples were tested and none was reactive to antibodies against yellow fever virus. The results corroborate the epidemiology of yellow fever in the area. Even though it is considered a transition area, there were no reports to date of epizootics or yellow fever outbreaks in humans. Also, entomological investigations did not detect the presence of vectors of this arbovirus infection. ELISA proved to be fast, sensitive, an adequate assay, and an instrument for active search in the epidemiological surveillance of yellow fever allowing the implementation of prevention actions, even before the occurrence of epizootics.

**KEYWORDS:** Yellow fever**;** Arbovirus; ELISA; Monkeys; Reservoirs; Epidemiological surveillance.

## INTRODUCTION

Yellow fever (YF) is an infectious viral disease that remains endemic or enzootic in rainforests of both South America and sub-Saharan Africa, periodically causing isolated outbreaks or epidemics of major public health impact. YF virus is the prototype of the genus *Flavivirus,* family *Flaviviridae*, a single, positive-stranded, enveloped RNA virus. It is transmitted to humans through bites of haematophagous insects of the family *Culicidae*, principally of the genera *Aedes* and *Haemagogus*[17,23].

There are two main cycles of transmission of yellow fever: urban and sylvatic. They have different vectors, vertebrate host and areas of occurrence[17,18].

In the urban yellow fever, the domestic mosquito *Aedes aegypti* carries the infection from person to person. Infected humans experiencing the viremic phase of the illness act as amplifiers. After the virus penetrates an urban environment, those infected will develop viremia and can manifest the disease and be a source of infection for new mosquitoes[25].

The sylvatic yellow fever is maintained by virus circulation between monkeys and diurnally active mosquitoes that breed in tree holes in the forest canopy (*Haemagogus* spp. in South America and *Aedes* spp. in Africa). In Brazil, the YF virus main vector is the *Haemagogus janthinomys*, but other species of this genus and also of the genus Sabethes play a role in the maintenance cycle, acting as secondary vectors.

Cases of sylvatic yellow fever in humans are incidental and they occur when a non-vaccinated individual penetrates into areas where the virus is circulating and is bitten by mosquitoes that had acquired infection from monkeys[24].

Many species of non-human primates are hosts of the sylvatic transmission cycle. Species most commonly involved in virus transmission belong to the genera *Cebus*, *Alouatta* and *Callithrix* [2, 8, 9]. While in Africa the majority of simian species has greater resistance to yellow fever virus infection, and rarely develops disease, in the Americas some neotropical species of monkeys are more susceptible to develop lethal infections[28].

The principal exception is genera *Cebus* (tufted capuchin monkey) which is more resistant to yellow fever virus infection. Infected monkeys do not die but develop antibodies against the virus[28].

Monkey susceptibility to the yellow fever virus in the Americas has been considered a major indicator for enzootic disease outbreaks in forest areas[28].

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In recent years, sylvatic cases of yellow fever in humans in Brazil were often preceded by epizootics in animals. Epizootics are considered a sentinel event for potential circulation of sylvatic yellow fever virus among monkeys.

In Brazil, there have been repeated epizootics since 2001, and the first cases reported were in the southern state of Rio Grande do Sul. Between 2003 and 2004, other epizootics were reported in several Brazilian states within areas outside those identified as endemic. A major southward extension of the epizootic was underway in 2007-2008, from southern Brazil into Paraguay and Argentina.

The objectives of the present study were: a) to standardize immunoenzymatic assay (ELISA) procedures for the detection of IgG antibodies in monkey sera samples, and b) to estimate the prevalence of IgG antibodies against yellow fever virus in serum samples of *Alouatta caraya* monkey species captured within surrounding areas of flooded lands after the construction of the Porto Primavera dam and hydroelectric plant, São Paulo State, Brazil.

## MATERIALS AND METHODS

**Study area:** The study area is located at the right margin of a lake formed by the Rio Paraná dam in the reservoir area of Engenheiro Sérgio Motta Hydroelectric Plant (HEP), also known as Porto Primavera HEP, in the city of Presidente Epitácio, southwest region of the State of São Paulo, Brazil[11].

**Serum samples:** Before inundation, monkeys of the *Alouatta caraya* species and other wild animals living in riparian forest areas adjacent to Porto Primavera HEP reservoir were rescued for an extensive research program with financial support from the São Paulo State Energy Company (CESP) with allowance of the Brazilian Institute of Environment and Renewable Natural Resources (IBAMA). Captures were carried out in May 2000 and between March and May 2001[11]. Monkeys were preanesthetized with ketamine, following the Canadian Council on Animal Care ethical recommendations (1980-1984)[5] and blood was drawn from their femoral veins using vacutainer tubes containing an anticoagulant agent (EDTA). A total of 570 serum samples were tested.

### Serological tests

*Reagent preparation:*

a) Immune ascitic fluid: Immune ascitic fluid (IAF) was prepared in young adult mice (*Mus musculus*) by inoculations of brain macerates from newborn mice infected with yellow fever (17D) vaccine virus. They were inoculated with 0.2 mL of this suspension diluted to 1:30 in phosphate-buffered saline (PBS) intraperitoneally, four times weekly. Five days following their last inoculation, the mice were inoculated with sarcoma 180/TG cells[3]. They developed voluminous ascites within a week indicating disease development and formation of antibodies against yellow fever virus[12].

b) Yellow fever antigen: Yellow fever antigens were prepared at the Centre of Research in Virology, School of Medicine of Ribeirão Preto, University of São Paulo (USP), Ribeirão Preto, São Paulo, Brazil, following BARROS protocol[1]. In brief, newborn mice were inoculated with the yellow fever (17 D) vaccine virus intracerebrally and followed up for two weeks for the development of encephalitis, which occurred between day 4 and day 6 post-inoculation.

The dying animals were sacrificed and frozen and stored in a freezer at -70º C. After thawing, their brains were suctioned, mixed and crushed using a mortar and pestle and diluted 1:20 (p/v) in RPMI-1640 cell culture media and stored at -70º C as viral seeds for subsequent use. The antigen was not inactivated.

c) Positive control: The positive control was provided by Laboratory of Flavivirus, Department of Virology, Oswaldo Cruz Foundation (FIOCRUZ), Rio de Janeiro, Brazil from Rhesus macaques.

d) Conjugate: Since there was not a specific conjugate for New World monkeys, at the time of the study, a commercially available conjugate produced in an Old World monkey (anti-Rhesus - *Macaca mulatta, infra-ordem Catarrhini*) was used in order to detect the IgG-class anti-YF (17D) antibodies. Additionally, this reagent was used because of the evolutionary proximity among the monkeys' species.

The same conjugate was used in malaria studies and was able to detect IgG antibodies against sporozoite proteins (infective forms of *Plasmodium* species) in neotropical monkey blood samples[7,11,27]. Despite a possible loss in the test sensitivity, since it was used in a high concentration, the conjugate was competent to discriminate positive and negative sera, allowing its use in epidemiological studies.

*Technique standardization*

A modified enzyme-linked immunosorbent assay (ELISA) was performed based on the protocol of the Centers for Disease Control and Prevention (CDC), in Atlanta, GA, U.S.A., as follows:

Polystyrene microtiter plates (Maxisorp/Nunc) containing 96 wells were sensitized with 100 µL of IAF diluted in carbonate buffer solution (0.1 M and pH 9.6). Each well was sensitized in duplicate. The plates were incubated at 4º C overnight and then washed with a PBS-Tween 20 solution five times.

A blocking solution (250 µL) was added (PBS-Tween 20, 300 mL, and skim milk, 5.0 g) to each well and incubated for an hour at 37 ºC. No washings were carried out in this step. Seventy-five µL of antigen were diluted in the blocking solution and 100 µL were dispensed at each well. The plate was incubated again for an hour at 37 ºC and underwent a new cycle of washings with PBS-Tween 20. Serum samples (100 µL) for antibody detection were diluted in the blocking solution to 1:40 and dispensed followed by a new incubation and a cycle of five washings.

Next, 100 µL of Rhesus monkey conjugate (Sigma A-2054) with peroxidase, diluted at 1:300 in the blocking solution, were added to each well. The plates were incubated for an hour at 37 ºC and then washed five times.

In the last step, 100 µL of ABTS substrate (peroxidase substrate and peroxidase) were added. The plates were left to sit at room temperature in a place with no light for the development of color. Fifteen minutes later they were read in a Labsystems Multiskan (Multiskan) analyzer at a wavelength of 405 nm.

To prevent false-positive results, samples were processed in duplicate and positive and negative controls, as well as milk without serum were added to each step of the reaction. A positive reaction was defined as absorbance equal to or greater than the cut-off value.

*Cut-off setting*

There were 19 negative sera of *Cebus apella* species used, provided by the Tufted Capuchin Monkey Procreation Center of the University of the State of São Paulo (UNESP), in Araçatuba, São Paulo, Brazil. Results were expressed as absorbance and, for higher specificity, the cut-off value was set by adding up three standard deviations to the arithmetic mean of serum absorbances[7,11,16].

## RESULTS

**1. Standardization of the enzymatic reaction for detection of IgG antibodies in monkey sera:** The results of positive and negative control sera standardizations as well as titrations of anti-monkey IgG conjugate are shown in Tables 1 and 2. Tables 3 and 4 show the results of block titration of antigen and IAF.

Dilutions at 1:320, 1:40, and 1:3000 for antigen, serum and conjugate, respectively, showed the highest reactivity.

For cut-off setting, mean absorbance of 19 negative sera was 0.060

**Table 1**
Titration of anti-monkey (IgG) conjugates with monkey sera positive for yellow fever

| Dilution of positive monkey serum | Conjugate dilution* | | |
|---|---|---|---|
| | 1:1000 | 1:2000 | 1:3000 |
| 1:20 | 2.662 | 1.444 | 1.254 |
| 1:40 | 1.298 | 1.210 | 0.987 |
| 1:80 | 1.185 | 1.139 | 0.803 |
| 1:160 | 1.177 | 1.106 | 0.589 |
| 1:320 | 1.150 | 1.005 | 0.497 |
| 1:640 | 1.028 | 0.934 | 0.391 |

* Anti-monkey conjugate (IgG) Sigma A:2054 with peroxidase; ascitic fluid: 1:200; antigen 1:320. Substrate: ABTS. Absorbance readings: 405 nm. Multiskan analyzer.

**Table 2**
Titration of anti-monkey conjugates (IgG) with monkey serum negative for yellow fever

| Dilution of negative monkey serum | Conjugate dilution* | | |
|---|---|---|---|
| | 1:1000 | 1:2000 | 1:3000 |
| 1:20 | 0.087 | 0.070 | 0.055 |
| 1:40 | 0.078 | 0.062 | 0.043 |
| 1:80 | 0.064 | 0.050 | 0.037 |
| 1:160 | 0.051 | 0.045 | 0.028 |
| 1:320 | 0.039 | 0.031 | 0.019 |
| 1:640 | 0.023 | 0.014 | 0.001 |

* Anti-monkey conjugate (IgG) Sigma A:2054 with peroxidase; ascitic fluid: 1:200; antigen 1:320. Substrate: ABTS. Absorbance readings: 405 nm. Multiskan analyzer.

**Table 3**
Dilution standardization of anti-yellow fever ascitic fluid using positive monkey serum control at 1:40 dilution

| Ascitic fluid dilution | Antigen dilution | | | | | |
|---|---|---|---|---|---|---|
| | 1:20 | 1:40 | 1:80 | 1:160 | 1:320 | 1:640 |
| 1:200 | 1.533 | 1.444 | 1.344 | 1.313 | 1.108 | 0.964 |
| 1:400 | 1.002 | 0.987 | 0.886 | 0.765 | 0.605 | 0.505 |
| 1:800 | 0.601 | 0.503 | 0.440 | 0.313 | 0.277 | 0.231 |
| 1:1600 | 0.235 | 0.189 | 0.156 | 0.127 | 0.099 | 0.075 |

Anti-monkey conjugate (IgG) Sigma A:2054 with peroxidase, concentration: 1:3,000. Substrate: ABTS. Absorbance readings: 405 nm. Multiskan analyzer.

**Table 4**
Dilution standardization of anti-yellow fever immune ascitic fluid using negative monkey serum control at 1:40 dilution

| Ascitic fluid dilution | Antigen dilution | | | | | |
|---|---|---|---|---|---|---|
| | 1:20 | 1:40 | 1:80 | 1:160 | 1:320 | 1:640 |
| 1:200 | 0.083 | 0.080 | 0.071 | 0.049 | 0.035 | 0.016 |
| 1:400 | 0.071 | 0.054 | 0.052 | 0.044 | 0.039 | 0.010 |
| 1:800 | 0.052 | 0.046 | 0.040 | 0.016 | 0.022 | 0.005 |
| 1:1600 | 0.037 | 0.035 | 0.022 | 0.008 | 0.002 | 0.001 |

Anti-Monkey (IgG) conjugate Sigma A:2054 with peroxidase, concentration: 1:3,000. Substrate: ABTS. Absorbance readings: 405 nm. Multiskan analyzer

and the standard deviation (SD) was 0.030. The reactivity cut-off value (mean + 3 SD) was 0.150.

**2. Detection of yellow fever antibodies in monkey sera:** No IgG antibodies against yellow fever virus were detected in the 570 sera samples of *Alouatta caraya* monkeys assayed using the ELISA previously standardized for this purpose.

## DISCUSSION

**1. Standardization of immunoenzymatic assay (ELISA):** Immunoenzymatic assays for arbovirus studies were introduced in Brazil in 1984 for the diagnosis of human Rocio virus infections in sera collected in 1975 and 1976 from patients with clinical encephalitis[14,15] , and later for the diagnosis of yellow fever virus infection[6,24,25].

Among laboratory methods for detection of antibodies and viral antigens in humans and arthropod vectors, ELISA is remarkable as a fast and sensitive assay. This test performance is comparable to those assays traditionally used for detection of arboviruses, such as neutralization test (N), hemagglutination inhibition (HI), immunofluorescence (IF), and complement fixation (CF)[10,19,21,26].

The results of the present study show that ELISA was a fast, effective and adequate assay for detecting IgG antibodies in monkeys. Besides, a marketed anti-Rhesus conjugate could be used because of its reactivity with the New World's monkey sera, which made the detection process easier.

A second advantage of ELISA is that it enables to process and analyze a large number of samples, which is fundamental in epidemiological investigations. Thus, ELISA adjusted to studies for detection of IgG antibodies can be used as an alternative method by reference laboratories or primate research centers for epidemiological surveillance of yellow fever in animal reserves.

**2. Detection of yellow fever antibodies in monkey sera:** In the present study, no IgG antibodies against yellow fever virus were detected in simian sera. The serological test results corroborate the epidemiology of sylvatic yellow fever in the studied area. Although the Brazilian Ministry of Health has considered this place as an area of transition for yellow fever, to date, no epizootic or yellow fever outbreaks were reported in humans within this area.

In addition, entomological studies conducted in this area did not show the presence of yellow fever vectors. Culicid insect investigations in the region of Presidente Epitácio in neighboring areas of Porto Primavera HEP detected very low frequency of mosquitoes of the genera *Sabethes* and none of the *Haemagogus*[20].

Another entomological study aimed to describing vector species within the western and northwestern region of the state of São Paulo[4]. Collections were carried out in 39 localities in four different areas. The most abundant mosquito species were *Haemagogus leucocelaenus* and *Aedes albopictus*. *Haemagogus leucocelaenus* was the most abundant species in the municipalities of Santa Albertina and Ouroeste, where there were reported cases of sylvatic yellow fever in 2000. In Presidente Epitácio, where monkeys were captured for the present study, 16 mosquitoes of the genus *Sabethes* were found. However, there were no mosquitoes found of the genera *Haemagogus* or *Aedes albopictus*.

Non-human primates can act, in some circumstances, as sentinel animals of certain sylvatic zoonotic cycles. These cycles can be identified when epizootics occur, such as those caused by arbovirus and especially yellow fever virus infection.

Although they are extremely susceptible to the yellow fever virus, monkeys of the genus *Allouata* have been investigated for disease surveillance[13]. Yellow fever virus was detected in an immunohistochemical assay conducted in an *Alouatta fusca* monkey found dead in Santo Antonio das Missões, in 2001, after the occurrence of an epizootic in the southern state of Rio Grande do Sul. The cause of the monkey's death was confirmed as yellow fever virus infection[22].

This finding elicited a reassessment of yellow fever status in Rio Grande do Sul. The state's northwestern region, previously with no signs of disease occurrence, became an area of transition and the whole population of neighboring municipalities, as well as those traveling to these areas were vaccinated[13].

Because of their susceptibility to yellow fever virus, these monkeys are good indicators of viral circulation. Their susceptibility has not been interpreted yet as a warning sign for the implementation of preventive actions, such as vaccination.

Based on the results in the present study, it is proposed the use of ELISA in animals less susceptible to yellow fever virus fatal infection, for instance *Cebus apella* species (tufted capuchin monkey), as an instrument for active search in the epidemiological surveillance of yellow fever, allowing the implementation of preventive actions even before the occurrence of epizootics.

## RESUMO

### Circulação de anticorpos contra o vírus amarílico em população simiana da região da usina hidrelétrica de Porto Primavera, São Paulo, Brasil

A febre amarela (FA) é doença infecciosa aguda de origem viral transmitida por mosquitos. No ciclo silvestre, o vírus é mantido por meio da infecção de macacos e da transmissão transovariana nos vetores. A vigilância sobre populações de primatas não humanos torna-se necessária para detectar a circulação viral, quando ainda está restrito a epizootias, e para determinar sua presença em regiões indenes ou de transição para a doença. Padronizou-se a técnica ELISA (Enzyme Linked Immunosorbent Assay) para determinar a prevalência de anticorpos da classe IgG contra o vírus da FA em soros de bugios (*Alouatta caraya*) da região do reservatório da Usina Hidrelétrica de Porto Primavera, SP. Foram testados soros de 570 macacos sendo que nenhuma amostra mostrou-se reativa para a presença de anticorpos contra o vírus da FA. Os resultados são coerentes com a epidemiologia da FA na região. Mesmo sendo área de transição, não se conhece, até o momento, ocorrência de epizootia ou surto de FA em humanos e investigações entomológicas não apontaram a presença de vetores para esta arbovirose. A técnica mostrou-se sensível, rápida e útil à vigilância epidemiológica como instrumento de busca

ativa permitindo desencadear ações preventivas, como vacinação, antes mesmo do surgimento de epizootias.

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