Widespread occurrence of antibodies against circumsporozoite protein and against blood forms of *Plasmodium vivax*, *P. falciparum* and *P. malariae* in Brazilian wild monkeys

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Abstract

**Background** A survey of malaria antibodies was carried out over 7 years and a total of 777 serum samples from wild monkeys were collected in three distinct ecological areas of Brazil where autochthonous malaria has been reported: the ‘Cerrado’ (similar to savanna), the Atlantic Forest and the Atlantic Semideciduous Forest.

**Methods** We carried out enzyme-linked immunosorbent assay to investigate the presence of IgG antibodies against peptides of the circumsporozoite protein (CSP) repeat region of ‘classic’ *Plasmodium vivax*, *P. vivax* VK247, human *P. vivax*-like/*P. simiovale*, *P. brasilianum/*P. malariae and *P. falciparum*. We also carried out immunofluorescence assay with asexual forms of *P. vivax*, *P. malariae* and *P. falciparum*.

**Results** The high prevalence of antibodies against CSP in all areas indicates that the monkeys had intense contact with sporozoites from infected anophelines. The immune response against asexual forms of *Plasmodium* in the monkeys from the Atlantic Forest indicates the development of the infection.

**Conclusions** We discuss the possibility of monkeys being malaria reservoirs in non-endemic areas.

Introduction

Malaria in Brazilian wild monkeys was widely studied during the 1960s and 1980s. Monkeys were reported to harbor *Plasmodium brasilianum* in the Amazon region, while *P. simium* and *P. brasilianum* were harbored in the southern and south-east regions. These studies were based only on blood smear morphology of parasites. Between 1937 and 1990, a review showed the geographical distribution of *P. brasilianum* and *P. simium* in Brazil. The infection rates varied among different regions. The highest infection rate was recorded in the southeast (35.6%), while it was lower in the southern region (17.9%) and Amazon region (10.1%). In the north-east and west-central regions, the number of samples was very small and few animals were found to be infected [11]. Data on simian malaria in these areas were therefore limited. Only three studies based on malaria
serology in wild New World monkeys have been published. Consequently, very little is known about wild monkey malaria parasites [2, 20, 30].

Similarities in morphological and immunological responses between \textit{P. vivax} and \textit{P. simium} [3, 5, 10, 17, 27] and \textit{P. malariae} and \textit{P. brasilianum} were reported [5–7, 19]. Molecular and phylogeny studies based on 18S ribosomal subunit RNA (ssrRNA), the circumsporozoite protein (CSP) gene and cytochrome b gene of \textit{P. malariae}/\textit{P. brasilianum} [14, 15, 23], \textit{P. vivax}/\textit{P. simium} [14, 18] and human \textit{P. vivax}-like/\textit{P. simiovale} [13] showed that they are not distinct and that \textit{P. falciparum}/\textit{P. reichenowi} (a parasite of \textit{Pan troglodytes}, an Old World ape) are very closely related [13, 14, 21]. These results reinforce the argument that monkeys may act as a reservoir for human malaria [2, 5, 15, 22, 30]. In the context of the human–simian relationship and human–simian transmission, it is important to explain the \textit{Plasmodium} population dynamics and consider its epidemiological implications.

The present study was carried out to investigate the prevalence of serological responses in Brazilian wild monkeys against the CSP repeat region and asexual form antigens of \textit{Plasmodium} in the following three distinct ecological areas: the ‘Cerrado’ (similar to savanna), the Atlantic Semideciduous Forest and the Atlantic Forest.

\textbf{Materials and Methods}

\textbf{Study area and monkey blood samples}

Serum samples were obtained from 777 monkeys from three distinct regions where malaria had previously been reported. The first study area is located along the Tocantins River in the Serra da Mesa highlands in the State of Goiás in the west-central region of Brazil, 250 km north-east of the Brazilian capital (Brasília). This region is a typical ‘Cerrado’, with sparse vegetation and tree canopies <5 m high. A hydroelectric dam has been built in this area (Fig. 1).

In the 1980s, intense malaria transmission was observed in this area due to disorganized gold mining activities that attracted migrants from malaria-endemic regions. In 1983, 163 cases of \textit{P. falciparum} malaria were identified, with sporadic cases of \textit{P. vivax}. After this, transmission continued at a low level. The disease has been kept reasonably well under control for the last 10 years, but there are clandestine mining activities about 70 km from the flooding area. Eighty-five cases were notified between 1995 and 1997, of which 51 were caused by \textit{P. vivax} and 34 by \textit{P. falciparum}. Some cases were among workers on the construction site for hydroelectric dam. The region is currently not considered endemic for human malaria (Brazilian Health Ministry communication).

Monkeys were captured in the canopy woods in flooded areas of the lake at the Serra da Mesa hydroelectric dam during wildlife rescue operations in 1997. Some animals were caged and shipped to the Centro Nacional de Primatas, Ananindeua, Pará, for a number of studies, with the consent of the Brazilian Institute for the Environment and Renewable Natural Resources (IBAMA). A total of 51 samples were collected between January and April 1997. Of this total, 42 were from \textit{Alouatta caraya} (howler monkeys), five from \textit{Callithrix penicillata} (marmosets) and four from \textit{Cebus apella} (capuchin monkeys) (Table 1).

The second area, in the state of São Paulo, is part of Brazilian Atlantic Forest in the south-east region (Fig. 1). In the Atlantic Forest, a few autochthonous human cases were notified and diagnosed as \textit{P. vivax}. Some of these presented atypical, very mild and transitory clinical symptoms with low parasitemia of short duration which often cleared even prior to treatment. These malaria cases are generally concentrated in a few foci in the Vale do Ribeira and Serra do Mar [9].

Injured monkeys and those captured from illegal hunting were transported to the Departamento de Parques e Áreas Verdes, Prefeitura Municipal de São Paulo (DEPAVE) for treatment, screening and
integration into ecological reserves. Between June 1996 and December 2002, we obtained 114 blood samples from monkeys. Of these, 45 were from *A. fusca*, 39 from *Callithrix jacchus* and 30 from *Cebus apella* (Table 1).

The third area is located on the border of the states of São Paulo and Mato Grosso do Sul, along the Paraná River in the Brazilian Atlantic Semideciduous Forest region, and has a 35 m tall arboreal stratum (Fig. 1). This region serves as a corridor for species migration between moist and semideciduous forests and also between Atlantic Forest and ‘Cerrado’ habitats [29].

The Porto Primavera dam area had reported previous autochthonous cases of *P. vivax* and *P. falciparum* malaria (last case in 1993). We believe that this area should receive special attention from sanitary authorities in view of the possibility of malaria being reintroduced with the increase in vector density after the dam is filled.

Monkeys were captured in the canopy of woods in flooded areas of the lake at Porto Primavera dam during wildlife rescue operations. Animals were caged and shipped to the Divisão de Meio Ambiente da Companhia Energética de São Paulo (CESP), with the consent of IBAMA. A total of 612 samples were collected between April 2000 and May 2001. Of these, 590 were from *A. caraya* and 22 from *Cebus apella* (Table 1).

Blood was drawn from venal punctures at the femur of monkeys previously anesthetized with ketamine and was collected in dry vacuum tubes. Sera were separated by centrifugation and samples were transported to the laboratory on dry ice or liquid nitrogen and kept frozen at −20°C until processed.

**Enzyme-linked immunosorbent assay**

Enzyme-linked immunosorbent assay (ELISA) was performed as previously standardized [30, 31] with some modifications. We used synthetic peptides corresponding to CSP repeats of *P. malariae*/*P. brasiliensis* (Pm/Pb) [(NAAG)]₄, ‘classic’ *P. vivax* – type I (Pvc) (GDRADGQPA)₂ (GDIRAGQPA)₂ (GDRADGQPA), *P. vivax* VK 247 – type II (Pvk) (ANGAGNQPG), human *P. vivax*-like/P. simiovale (Pvl) (APGANQEGGA)₃ and *P. falciparum* (Pf) (NANP)₈ [1, 22, 25, 26, 31]. These peptides were synthesized by Invitrogen Corporation (Carlsbad, CA, USA).

NUNC Maxisorp plates (NUNC GmbH & Co., Wiesbaden, Germany) were coated with each peptide at optimal concentrations determined by checkerboard titrations (10 μg/ml). The sera were diluted at 1:50. Peroxidase conjugated antimonkey (rhesus – *Macaca mulatta*) IgG immunoglobulin (Sigma-Aldrich, St. Louis, MO, USA; cod A-2054) at 1:3000 and 2,2′-azino-bis [3-ethylbenzothiazoline-6-sulfonic acid (ABTS)] (Kierkegaard and Perry, Gaithersburg, MD, USA) were used. Absorbance was read at 414 nm in a Multiskan EX (Thermo Labsystems, Waltham, MA, USA). Positive and negative controls were included in each assay. The positive control sera consisted of ‘classic’ *P. vivax*, *P. falciparum* and *P. malariae* sera taken from hyperimmune monkeys (*Aotus* and *Saimiri* genus). The sera were kindly furnished by A.A. Lal and W.E. Collins from the Center for Disease Control and Prevention/CDC, Atlanta, GA, USA. Positive *P. vivax*-like and *P. vivax* VK247 sera were selected from monkey sera from the study area that had a high reactivity. The checkerboard titrations were performed for each peptide with the positive and negative sera (from monkeys in captivity) at 1:25, 1:50, and 1:100 dilutions. The peptide concentrations were 2.5, 5.0, 10.0, and 20.0 μg/ml. The peptide concentrations and serum dilutions chosen were those that gave maximum absorbance with positive sera and minimal values with negative sera. Cut-off values were determined for each peptide by adding three standard deviations to the

### Table 1 Sera samples collected in the three study areas

<table>
<thead>
<tr>
<th>Area</th>
<th><em>Alouatta</em> (howler monkeys)</th>
<th><em>Cebus</em> (capuchin monkeys)</th>
<th><em>Callithrix</em> (marmosets)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>‘Cerrado’ (similar to savanna)</td>
<td>42</td>
<td>4</td>
<td>5</td>
<td>51</td>
</tr>
<tr>
<td>Atlantic Forest</td>
<td>45</td>
<td>30</td>
<td>39</td>
<td>114</td>
</tr>
<tr>
<td>Atlantic Semideciduous Forest</td>
<td>590</td>
<td>22</td>
<td>0</td>
<td>612</td>
</tr>
</tbody>
</table>

1Serra da Mesa Hydroelectric Dam, State of Goiás (January–April 1997). *Alouatta caraya* species.
2Departamento de Parques e Áreas Verdes, Prefeitura Municipal de São Paulo/DEPAVE (June 1996 to December 2002). *Alouatta fusa*, *Cebus apella*, and *Callithrix jacchus* species.
3Porto Primavera Dam, States of São Paulo and Mato Grosso do Sul (April 2000 and May 2001). *Alouatta caraya* and *Cebus apella* species.
mean absorbance of negative sera. This was done in two steps: the first step was with 50 negative sera of monkeys bred in captivity (Cebus, Callithrix and Saimiri specimens from the Núcleo de Procriação de Macaco-Prego, Universidade Estadual Paulista, Araçatuba, and the Centro Nacional de Primatas, Ananindeua, Pará); the second step used 50 randomly chosen sera with negative absorbances of monkeys from the three study areas. Sera were considered negative depending on their initial cut-off value. We have used the term ‘area cut-off value monkeys’ to refer to the latter group of monkeys. Sera were tested in duplicate and were considered positive if the average absorbance was equal to or greater than that of ‘area cut-off value monkeys’ for the corresponding peptide.

**Indirect immunofluorescence assay**

Indirect immunofluorescence assay (IFA) was performed as previously standardized [16, 30]. For antigen, we used erythrocytes of *P. vivax* (from primo-infected patients), *P. falciparum* (from culture), and *P. malariae* (obtained from an experimentally infected Saimiri monkey) washed in phosphate-buffered saline pH 7.2. Negative controls (sera of monkeys in captivity) and positive controls (sera of an experimentally infected monkey furnished by W.E. Collins) were included in all tests. The antimonkey (rhesus) IgG fluorescent isothiocyanate conjugate (Sigma, F-3893) was tested at several dilutions with positive and negative sera. A 1:40 serum dilution was determined as the cut-off point for the three antigens, and the ideal conjugate dilution was 1:200. Each antigen was tested with 50 sera samples from monkeys in captivity, and the results were negative.

**Statistical analysis**

The prevalence of positive reactions obtained in ELISA and IFA tests was compared by chi-square test. Absorbance was compared by non-parametric Kruskall–Wallis test, and geometric mean titers (GMT) by ANOVA.

**Results**

For ELISA reaction standardization, we carried out preliminary tests with positive sera samples from experimentally infected monkeys against antihuman IgG conjugate (Sigma, A-8419), and no reactivity was observed. When antimonkey (rhesus) IgG conjugate was used, however, we detected reactivity against the sera tested (Table 2).

In order to enhance the specificity and to avoid false-positive ELISA, so as to show the real seroepidemiological profile of the results for these areas, the cut-off value used was calculated from the screening of the negative sera samples from the study area. Table 3 illustrates cut-off values obtained in the ELISA reaction both for negative sera of monkeys bred in captivity and for negative sera of monkeys from the three study areas. ‘Area cut-off value monkeys’ were used to determine the prevalence of positive sera from the study areas.

### Table 2

<table>
<thead>
<tr>
<th>Positive sera samples</th>
<th>ELISA</th>
<th>Anti-human conjugate</th>
<th>Anti-monkey conjugate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1:1500</td>
<td>1:3000</td>
</tr>
<tr>
<td>'Classic' <em>P. vivax</em> (type I)</td>
<td>0.156</td>
<td>0.071</td>
<td>0.014</td>
</tr>
<tr>
<td><em>P. falciparum</em></td>
<td>0.111</td>
<td>0.087</td>
<td>0.023</td>
</tr>
<tr>
<td><em>P. malariae</em></td>
<td>0.062</td>
<td>0.126</td>
<td>0.012</td>
</tr>
</tbody>
</table>

Results expressed in absorbance.

<table>
<thead>
<tr>
<th>Peptides</th>
<th>In captivity (N = 50)</th>
<th>Study area (N = 50)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean absorbance</td>
<td>SD</td>
</tr>
<tr>
<td>Pvc</td>
<td>0.062</td>
<td>0.018</td>
</tr>
<tr>
<td>Pvk</td>
<td>0.092</td>
<td>0.048</td>
</tr>
<tr>
<td>Pvl</td>
<td>0.150</td>
<td>0.039</td>
</tr>
<tr>
<td>Pm/Pb</td>
<td>0.066</td>
<td>0.042</td>
</tr>
<tr>
<td>Pf</td>
<td>0.104</td>
<td>0.049</td>
</tr>
</tbody>
</table>

Pvc, ‘classic’ *P. vivax* (type I); Pvk, *P. vivax* VK247 (type II); Pvl, human *P. vivax*-like; Pm/Pb, *P. malariae*/*P. brasilianum*; Pf, *P. falciparum*.
These three regions have distinct ecological and epidemiological characteristics. We did not, therefore, compare the data obtained from sera samples of monkeys from these three areas but instead compared the distribution of positive reactions among the Plasmodium species. Despite the fact that these three study areas had low endemicity or no malaria transmission, we detected IgG antibodies against CSP in ELISA and against blood forms in IFA tests in sera of wild monkeys.

Overall positivity for A. caraya from the ‘Cerrado’ was significantly higher for human P. vivax-like/ P. simiovale (Pvl) (45.2%, 19/42) and P. falciparum (Pf) (35.7%, 15/42) compared with that of ‘classic’ P. vivax (Pvc) (26.2%–11/42), P. vivax VK247 (type II) (Pvk) (2.4%, 1/42) and P. malariae/P. brasilianum (Pm/Pb) (14.3%, 6/42) (P < 0.001) (Fig. 2A).

Of all the positive sera from Alouatta specimens from the ‘Cerrado’ (29 sera), 13 reacted with only one peptide (three sera were positive for Pvc, five for Pvl, two for Pm/Pb, and three for Pf (data not shown), 16 were positive for more than one peptide (Table 4), and 13 were negative for all the peptides in ELISA.

The seropositivity in A. caraya, from the Atlantic Semideciduous Forest region (Porto Primavera dam) was very similar for the five peptides. The prevalence of CSP for Pf was 8.3% (49/590), for Pvc 8.1% (48/590), for Pvk 8.0% (47/590) and Pvc and Pm/Pb peptides 7.6% (45/590) (P < 0.001) (Fig. 2A).

Of all the positive sera from Alouatta specimens from the Atlantic Semideciduous Forest (150 sera), 85 reacted with only one peptide (18 sera were positive for Pvc, six for Pvk, 10 for Pvl, 24 for Pm/Pb and 27 for Pf) (data not shown), 65 reacted with more than one peptide (Table 4), and 440 were negative for all the peptides in ELISA.

Sera of A. fusca from the Atlantic Forest had the highest prevalence of positivity for Pvc (15.6%, 7/45). The positivity for Pm/Pb was 6.7% (3/45) and for Pf 4.4% (2/45) (P < 0.001). No reactivity was found for Pvk and Pvl in these sera (Fig. 2A).

Of all the positive sera from Alouatta specimens from the Atlantic Forest (n = 39), 9 reacted with only one peptide (six sera were positive for Pvc and one for Pm/Pb) (data not shown), two were positive for more than one peptide (Table 4), and 36 were negative for all the peptides in ELISA.

Only Cebus apella specimens from the Atlantic Forest presented antibodies against CSP peptides, while those from other areas were negative. The prevalence of positivity for Pvl was higher (36.7%–11/30) than for Pvc (30.0%–9/30). The prevalence of positivity for Pvc was 10.0% (3/30), for Pf 6.7% (2/30) and for Pm/Pb 3.3% (1/30) (P < 0.001) (Fig. 2B).

Of all the positive sera from Cebus specimens from the Atlantic Forest (17 sera), 11 reacted with only one peptide (three sera were positive for Pvc, seven for Pvl and one for Pm/Pb) (data not shown), six sera were positive for more than one peptide (Table 4), and 13 were negative for all the peptides in ELISA.

All the specimens from the genus Callithrix from the Atlantic Semideciduous Forest (n = 39) and the Atlantic Forest (n = 5) were negative with five peptides in ELISA. The absorbance distribution in ELISA for positive and negative sera samples from monkeys from the ‘Cerrado’, Atlantic Semideciduous Forest and Atlantic Forest is shown in Fig. 3A–D. Figure 4 illustrates the prevalence and corresponding GMT of the
Table 4  Number of positive sera of monkeys (IgG antibodies) from the three study areas that reacted with more than one Circumsporozoite Protein (CS) synthetic peptide in enzyme-linked immunosorbent assay

<table>
<thead>
<tr>
<th>CS synthetic peptides</th>
<th>'Cerrado' (42)</th>
<th>Atlantic Forest (590)</th>
<th>Atlantic Semideciduous Forest (45)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alouatta specimens</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N = 42</td>
<td>1</td>
<td>28</td>
<td>0</td>
</tr>
<tr>
<td>Vivax complex¹</td>
<td>3</td>
<td>15</td>
<td>0</td>
</tr>
<tr>
<td>Vivax complex¹ + Pm/Pb</td>
<td>11</td>
<td>16</td>
<td>0</td>
</tr>
<tr>
<td>Vivax complex¹ + Pf</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Pm/Pb + Pf</td>
<td>0</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>Total (%)</td>
<td>16 (38.1)</td>
<td>65 (11.0)</td>
<td>2 (4.4)</td>
</tr>
<tr>
<td>Cebus specimens</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N = 4</td>
<td>0</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>Vivax complex¹ + P. falciparum</td>
<td>0</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Total (%)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>6 (20.0)</td>
</tr>
</tbody>
</table>

Pm/Pb, P. malariae/P. brasilianum; P. falciparum.
¹Vivax complex: classic P. vivax (type 1) (Pvc) and/or P. vivax VK247 (type 2) (Pvk) and/or human P. vivax-like (Pvl).

Fig. 3  Results of ELISA (absorbance) using circumsporozoite protein (CSP) repeat peptides of the ‘classic’ Plasmodium vivax (Pvc), P. vivax VK247 (Pvk), human P. vivax-like (Pvl), P. malariae/P. brasilianum (Pm/Pb) and P. falciparum (Pf) with Alouatta caraya sera from ‘Cerrado’ (A) and Atlantic Semideciduous Forest (B), Alouatta fusca (C) and Cebus apella (D) from Atlantic Forest. ‘Area cut-off values monkeys’ (mean+3SD) are indicated by horizontal lines (Pvc = 0.148; Pvk = 0.244; Pvl = 0.251; Pm/Pb = 0.223; Pf = 0.261). Positive sera are above the bars.
IFA-positive sera with the asexual forms of *Plasmodium* antigens.

For samples from the ‘Cerrado’, we only detected IgG antibodies against *P. falciparum* in *A. caraya* specimens (33.3%-14/42, GMT = 65.6), with negative results for *P. vivax* and *P. malariae* antigens (Fig. 4). *Alouatta caraya* specimens from the Atlantic Semideciduous Forest showed a prevalence of 10.5% (62/590, GMT = 82.7) for *P. malariae*, 9.7% (57/590, GMT = 49.2) for *P. vivax* and 4.9% (29/590, GMT = 72.7) for *P. falciparum* antigen (*P* = 0.0017).

Of all the positive sera from *Alouatta* specimens from the Atlantic Semideciduous Forest (135 sera), 118 reacted with only one antigen (47 sera were positive for *P. vivax* antigen, 50 for *P. malariae* and 21 for *P. falciparum*) (data not shown), only 17 were positive for more than one antigen (Table 5), and 445 were negative for all the antigens in IFA.

Seropositivity of *A. fusca* from the Atlantic Forest was 37.8% (17/45, GMT = 1509.0) for *P. vivax*, 20.0% (9/45, GMT = 274.3) for *P. malariae* and 28.9% (13/45, GMT = 1671.0) for *P. falciparum* antigen. No significant differences in the mean values were observed (Fig. 4).

Of all the positive sera from *Alouatta* specimens from the Atlantic Forest (26 sera), 15 reacted with only one antigen (six sera were positive for *P. vivax* antigen, six for *P. malariae* and three for *P. falciparum*) (data not shown), 11 were positive for more than one antigen (Table 5), and 19 were negative for all the antigens in IFA.

**Table 5** Number of positive sera of monkeys (IgG antibodies) from the three study areas that reacted with more than one asexual *Plasmodium* forms in immunofluorescence assay

<table>
<thead>
<tr>
<th>Study areas – <em>Alouatta</em> specimens</th>
<th>Atlantic Semideciduous Forest (N = 590)</th>
<th>Atlantic Forest (N = 45)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asexual form antigen</td>
<td>‘Cerrado’ (N = 42)</td>
<td></td>
</tr>
<tr>
<td><em>P. vivax</em>/<em>P. malariae</em></td>
<td>0</td>
<td>9</td>
</tr>
<tr>
<td><em>P. vivax</em>/<em>P. falciparum</em></td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td><em>P. malariae</em>/<em>P. falciparum</em></td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td><em>P. vivax</em>/<em>P. malariae</em></td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>/ <em>P. falciparum</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total (%)</td>
<td>0 (0)</td>
<td>17 (2.9)</td>
</tr>
</tbody>
</table>

*Cebus* and *Callithrix* specimens from the three study areas were negative in the IFA test with all three antigens.

**Discussion**

The presence of antibodies against CSP in monkey blood samples indicates contact with infected anophelines. ELISA reaction with synthetic peptides allowed us to make an epidemiological assessment of malaria in the monkey populations studied, and, furthermore, to assess the variation in the responses to variants within the ‘vivax complex’ (classic, VK247 and human-like). The reaction was adapted to detect IgG-class anti-CS antibodies using a commercially available conjugate produced in an Old World monkey (anti-rhesus – *Macaca mulatta*, infra-ordem Catarhini) and previously tested in similar research [30].

The presence of IgG antibodies in sera obtained from monkeys, and in particular from genus *Alouatta*, against synthetic peptides corresponding to CSP of ‘classic’ *P. vivax*, *P. vivax* VK247, human *P. vivax*-like, *P. malariae*/*P. brasiliensis* and *P. falciparum* indicated that they had been challenged by malaria-infected mosquitoes. The presence of antibodies against the crude antigens of malaria parasites in some of the monkeys showed that the infection might have been successful in these animals. The differences in positivity observed in the various *Alouatta* species may be due to epidemiological differences inherent to each region. Further research is required to clarify this.

The highest prevalence of CSP antibodies against PvI, Pf and Pvc was detected in sera from *A. caraya* living in the ‘Cerrado’, but only low GMT *P. falciparum* crude antigen responses were observed. Blood samples were negative by polymerase chain reaction (PCR) and thin/thick smears (data not shown).
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specific part of the ‘Cerrado’ area monkeys with only \textit{P. malariae} were reported [12]. In the 1980s, there were foci of human \textit{P. vivax} and \textit{P. falciparum} malaria. The results observed in monkeys may be related to this situation in the past.

The prevalence of CSP antibodies against the Pvc, Pvk, Pvl and Pm peptides in sera from genus \textit{Alouatta} from the Atlantic Semideciduous Forest was very similar, as well as the response against crude \textit{P. vivax} and \textit{P. malariae} antigens. A low positivity was observed with \textit{P. falciparum} crude antigen. The presence of parasites of \textit{P. vivax}-like morphology was observed in seven slides (thick smears) and PCR reactions were positive for \textit{P. vivax} and \textit{P. malariae} parasites (data not shown). We have demonstrated here the first findings of simian malaria in this ecological area. As the region had endemic human malaria in the past, this suggests that the results seen in monkeys may reflect interaction between human and simian malaria.

A different epidemiological situation was observed in the antibody profile of specimens from the Atlantic Forest compared with those from the ‘Cerrado’ and Semideciduous Forest areas. The highest CSP antibody positivity, against Pvc was detected in \textit{A. fusca}. No CSP antibodies were detected against Pvk or Pvl peptides, and low positivity was detected for CSP antibodies against Pm/Pb and Pf. Both the prevalence and GMT of antibodies against the asexual forms of \textit{P. vivax}, \textit{P. malariae}, and \textit{P. falciparum}, detected by IFA were both high and significant. Thick smear tests were negative, but PCR reactions were positive for \textit{P. vivax} and \textit{P. malariae} infections (data not shown).

The CSP responses against Pvl and Pvc in the genus \textit{Cebus} from the Atlantic Forest were significant compared with those against Pvk, Pm/Pb, and Pf. No responses against crude antigen of the three species were observed. These results suggest that while there is exposure to sporozoite infection, the disease occurs at low frequency. ELISA and IFA did not detect antibodies in \textit{Cebus} from the other two areas, or even in monkeys of the genus \textit{Callithrix}. We believe that, unlike monkeys from the genus \textit{Alouatta}, these monkeys do not play a role in the epidemiological situation.

Several specimens of \textit{Alouatta} monkeys living in the ‘Cerrado’ and Atlantic Semideciduous Forest were positive for more than one CSP peptide in ELISA. Within the genus \textit{Cebus}, only one serum cross-reacted with Pvc and Pf. This is quite interesting, as reactions with CSP repeats are considered very specific [2, 30]. In our experience of human serology with the same peptides, a serum would only react infrequently with more than one peptide [9]. The hypothesis of cross-reaction cannot be discarded, despite the low frequency of occurrence, as it has been observed between Pm/Pb and Pvk peptides in sera from Indian communities in Brazil [8].

Furthermore, humans living in endemic areas can be infected by different species [4]. The occurrence of several malaria episodes signifies a high rate of sporozoite infections by anopheline bites and, instead of cross-reactions, we may be detecting different challenges represented by distinct species of sporozites. The fact that in IFA some sera reacted with more than one antigen could also be due to the presence of antibodies against populations of parasites from distinct malaria episodes rather than to cross-reaction.

Some sera reacted to the same species of \textit{Plasmodium} in ELISA and IFA. We cannot, however, assume that there is a correlation, as the immunological responses may relate to exposure to sporozites at different times, so that the episodes of infection are not related. Little information about plasmodia vectorial transmission under natural conditions is available. To date, the only vector that has been incriminated in the transmission of simian malaria in Brazil is \textit{Anopheles (Kerteszia) cruzii}, which was found to be infected by \textit{P. brasilianum} and \textit{P. simium} [11].

An entomological survey of species captured in the ‘Cerrado’ identified a high frequency of \textit{An. (Nyssorhynchus) triannulatus}. \textit{Anopheles oswaldoi} [12] and \textit{An. albitalis} s.l were found in high density in the Atlantic Semideciduous Forest [28]. It is not known if these anophelines can transmit simian malaria, but both areas could be a potential focus of human malaria.

Previous serological studies of humans living in the Atlantic Forest, which is considered a non-endemic area for malaria with sporadic cases of nonspecific symptomatology, showed a prevalence of antibodies against CSP and blood forms of the \textit{P. vivax} complex and \textit{P. malariae}/\textit{P. brasilianum}. The area also has a high simian population density [9]. We believe that there may be human–simian malaria interactions in this area.

No human serological survey which might be compared with our results has been carried out in the ‘Cerrado’. There was a high rate of human \textit{P. vivax} and \textit{P. falciparum} malaria transmission among gold miners in the 1980s, but this is now under control. The few cases that have occurred recently were originated by migrants from other malarial areas of the country. A human serological survey in the Semideciduous Atlantic Forest carried out by us revealed the presence of CSP antibodies against \textit{P. vivax} and its variants, \textit{P. malariae}/\textit{P. brasilianum} and \textit{P. falciparum} peptides.
in the human population surrounded by the forest and near the area where monkeys were captured (data not shown).

Despite differences in ecological settings, our results agree with the observations reported by other researchers investigating malaria in New World monkeys in the Amazon Forest. A study with monkeys from an indigenous area of the Xingu River, in the Amazon region, showed high antibody levels against sporozoites and blood stages of *P. falciparum* and *P. malariae/P. brasilianum*. Low levels of *P. vivax* anti-sporozoite and anti-blood stage were also detected [2].

Antibodies against *P. falciparum* merozoite surface protein (MSP1), SPf66 and CSP were detected in sera of squirrel monkeys (*Saimiri sciureus macrodon*) from the Amazon Forest in Peru. The hypothesis of asymptomatic malaria was raised in view of the fact that monkeys did not present malaria symptoms [20].

Similar results were observed in a survey carried out in Amazon region. Sera from *A. seniculus, Saginus midas* and *Pithecia pithecia* specimens, captured during filling of the Sinnamary River Hydroelectric Dam (French Guyana) showed high levels of CSP antibodies against the vivax complex, *P. malariae/P. brasilianum* and *P. falciparum*. These monkeys may be reservoirs for human malaria in those areas [30].

Our finding of anti-CSP antibodies against *P. vivax* in sera of monkeys from the Amazon Forest [30] suggests that *P. simium* may be present in this region, in spite of the fact that it has not been detected so far [11]. We also observed responses to *P. vivax* variants in the three areas, but with differences among the responses. This may indicate the presence of distinct epidemiological situations, such as strain differences, as well as differences between competent malaria vectors. These findings corroborate previously reported results, where DNA of *P. simium* was amplified with CSP primers of *P. vivax*, and the clones obtained from the PCR products showed sequences identical to that of classic *P. vivax* and *P. vivax* VK247 [18].

In addition, a recent phylogeny study showed that *P. vivax* from Central America and Colombia form a distinct group from that of Asian parasites, and that *P. simium* is a remnant of the Old World *P. vivax* lineage, suggesting that *P. vivax* most likely entered the New World on two separate occasions and from different geographic locations [24]. If monkeys can be a natural reservoir for human and simian malaria, this evidence raises epidemiological implications due to the possibility that the simultaneous occurrence of different transmission situations could compromise the control of human malaria in these study areas.

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References


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5 Coatney GR: The simian malarias: zoonoses, anthropo-


