Malaria outside the Amazon region: Natural Plasmodium infection in anophelines collected near an indigenous village in the Vale do Rio Branco, Itanhaém, SP, Brazil

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ABSTRACT

A few cases of Plasmodium vivax malaria in which anophelines of subgenus Kerteszia were incriminated as vectors have been reported outside the Amazon region, in the Atlantic Forest. This study was carried out near an indigenous Guarani village in the Curucutu reserve, an environmental protection area in the municipality of Itanhaém in the state of São Paulo, Brazil, on November 30, 2009, February 18, 2010, April 29, 2010 and May 26, 2010. Mosquitoes were collected along the route to the Guarani village where the edge of the Branco river floodplain meets the forests on the mountain slopes. Adult forms were collected with CO2-baited CDC traps and Shannon traps from twilight to 10:00 P.M. Anopheles cruzii predominated in both traps. The other species collected in the CDC traps were An. pseudomaculipes/maculipes, An. fluminensis and An. mediopunctatus/forattinii/costai. In addition to the latter three species, An. apicimacula/intermedius and An. strodeii were also found in the Shannon traps. All but An. cruzii and An. strodeii belong to subgenus Anopheles. A total of 306 mosquitoes were assayed by PCR to detect natural infection by Plasmodium species. In the CDC traps, An. fluminensis and An. pseudomaculipes/maculipes were positive for Plasmodium malariae, while in the Shannon traps An. pseudomaculipes/maculipes was positive for Plasmodium vivax and Plasmodium malariae and An. cruzii was positive for P. malariae, resulting in a minimum infection rate of 0.24%. Our findings suggest that An. cruzii may be incriminated in the transmission of malaria between monkeys and humans, as this species was found to be infected by P. malariae. They also highlight the need for an understanding of the role of anophelines from outside subgenus Kerteszia in the transmission of malaria in the Atlantic Forest, as these were also found to be naturally infected by P. vivax and P. malariae.

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1. Introduction

Cases of residual malaria have been reported in areas of Brazil outside the Amazon region (Yamasaki et al., 2011; Branquinho et al., 1997; Curado et al., 1997). These occurred in the Atlantic Forest along the Serra do Mar mountain range in the east of the country, where the disease is known as bromelial malaria and its vectors are anophelines belonging to subgenus Kerteszia (Zavortink, 1973). Serological studies of the inhabitants, including the indigenous population, of some regions of the Atlantic Forest showed that these individuals have antibodies against Plasmodium vivax and Plasmodium malariae (Carvalho et al., 1988; Curado et al., 2006; Cerutti et al., 2007).

Located in the southeast of Brazil, the state of São Paulo lies partly in a region where malaria associated with bromeliads occurs, a mountainous coastal area dominated by the Atlantic Forest. Indigenous tribes from the Tupi branch can be found along the coast, where they live in reservations established by the federal government. According to data supplied by the São Paulo state department of health, 46 cases of autochthonous malaria were reported in the state of São Paulo with one case reported in Itanhaém in 2007 (http://www.cve.saude.sp.gov.br/htm/zoo/malaria07_cmes.htm).

The present study was carried out in the environmental protection area known as Curucutu reserve, which covers an area of 2856.10 ha in the Branco River valley, about 30 km from the town of Curucutu.
of Itanhaém on the southern coast of the state of São Paulo. There is an indigenous Tupi-Guarani village nearby where 19 families (70 people) were recorded in 2005 (http://www.cve.saude.sp.gov.br/htm/2009/malaria_cautocote.htm), of which 11 were in the indigenous population living in the Branco river valley. The outbreak affected seven children up to nine years old, a 12-year-old adolescent and three adults working in banana fields. As well as the indigenous people, two workers in the team that provides health services in the village were also infected (http://oglobo.globo.com/cidades/mat/2009/08/19/desequilíbrio-ambiental-pode-ser-causa-de-sulto-de-malaria-em-itanhame-litoral-de-sp-757473057.asp).

The aim of this study was to gain a better understanding of epidemiological aspects of the malaria outbreak in the Branco River valley by identifying the species of anophelines and plasmodia circulating in the area and characterizing their habitat.

2. Materials and methods

2.1. Field work

2.1.1. Capture of adult mosquitoes

Four collections were carried out on the following dates: November 30, 2009 (S24° 04’07.9/046° 47’51.1); February 18, 2010 (S24° 04’48.6/046° 46.776); April 29, 2010 (S24° 01’41.1/046° 41.907); and May 26, 2010 (S24° 04’35.3/046° 46.363). Captures were made along the access road to the indigenous Guarani village and started at dusk and finished at around 10:00 P.M. Fig. 1 shows the places where the captures took place. The straight-line distances from the village to the nearest (G) and furthest (A) points are 0.66 km and 13.0 km, respectively.

2.1.2. Traps

Automatic CDC light traps baited with dry ice (CO2) were used at all the capture points. The traps were placed inside and at the edge of the forest and in areas occupied by humans, such as domiciliary environments and plantations. The total of CDC traps were 7, 9, 9, and 8 respectively for the first, second, third and fourth collections. Shannon traps were also placed close to the CDC traps and the number ranged from 2 to 3.

2.1.3. Storage, transportation and preservation of specimens

The anophelines were sorted immediately after capture and kept in insect boxes lined with silica, cotton and filter paper. The specimens were sent to the Public Health Entomology Laboratory, LESP, at the Faculty of Public Health, University of São Paulo, where they were identified by comparing them with others in the LESP collection and by reference to the literature (Zavortink, 1973; Forattini, 1973; Faran and Linthicum, 1981; Consoli and Lourenço-de-Oliveira, 1994; Forattini, 2002). Once identified, the specimens were preserved in isopropanol and sent to the Protozoology Laboratory at the Institute of Tropical Medicine, University of São Paulo, to determine whether they were infected with plasmodia.

2.1.4. Tests to detect Plasmodium infection

2.1.4.1. Extraction of DNA from anophelines. DNA was extracted using a pool of at most 10 anophelines/tube and the QIAamp DNA Mini kit from Qiagen. Care was taken to record capture date and time and trap type and location. The DNA precipitate was resuspended in 50 μL of TE buffer (0.01 M Tris–HCl and 1 mM EDTA pH 8.0) and stored in a freezer until use for PCR analysis.

Table 1

<table>
<thead>
<tr>
<th>Species</th>
<th>CDC + CO2</th>
<th>Shannon</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>%</td>
</tr>
<tr>
<td>Anopheles (Kerteszia) cruzii</td>
<td>112</td>
<td>78.9</td>
</tr>
<tr>
<td>Anopheles (Anopheles) pseudomalaculipes/maculipes</td>
<td>26</td>
<td>18.3</td>
</tr>
<tr>
<td>Anopheles (Anopheles) fluminensis</td>
<td>3</td>
<td>2.1</td>
</tr>
<tr>
<td>Anopheles (Anopheles) mediopunctatus/forattinii/costai</td>
<td>1</td>
<td>0.7</td>
</tr>
<tr>
<td>Anopheles (Anopheles) sp.</td>
<td>–</td>
<td>0</td>
</tr>
<tr>
<td>Anopheles (Anopheles)</td>
<td>–</td>
<td>0</td>
</tr>
<tr>
<td>Anopheles (Nyssorhynchus) strodei</td>
<td>–</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td>142</td>
<td>100</td>
</tr>
</tbody>
</table>

2.1.4.2. Description of the technique. The protocol used for the PCR was developed by Kimura et al. (1997) and modified by Win et al. (2002). The following oligonucleotides were used for the genus-specific amplification: P1UP – 5′ TTC ATT AAT CAA GAA CAA CAG TTA AG 3′ and P2 – 5′ GAA CCC AAA GAC TTT GAT TTT CTA T 3′; for the species-specific amplification, the following primers were used: P1 (genus-specific) – 5′ AGC ATG ACA TAC CGT CTT AAT CTT 3′; and the species-specific reverse primers V1 (for P. vivax) – 5′ CAA TCT AAT AAT CAA CTC CGA AGA A 3′; F2 (for P. falciparum) F2 – 5′ CAA TCT AAT AAA AGT CAC CTC GAA AGA TG 3′ and M1 (for P. malariae) – 5′ GAA AGC TAT CTA AAA GAA ACA CTC ATA T 3′.

The samples were subjected to 1.5% agarose gel electrophoresis in TBE buffer. After the run, the ethidium bromide-stained gel was visualized and photographed using a Stratagene Eagle Eye II Video Imaging System.

2.1.4.3. Minimum infection rate. As we used a pool of mosquitoes to detect natural infection by PCR, we estimated a minimum infection rate (MR = no. of positive pools × 100/total number of insects), as described by Paiva et al. (2006).

3. Results

3.1. Captures with CDC+ CO2 traps and Shannon traps

Table 1 shows all the anopheline species identified in the CDC and Shannon traps. For each species the number of specimens and frequency in percent is shown.

Anopheles (Ker.) cruzii was the predominant species and had a frequency of 78.9% and 81.8% in the CDC and Shannon traps, respectively. Among the other anophelines, An. (Ano.) pseudomalaculipes/maculipes were present in intermediate frequency in the CDC traps (18.3%); this species, together with An. (Ano.) fluminensis, was also present in intermediate frequency in the Shannon traps (11.23% and 3.63%, respectively). The species found least frequently in the CDC traps were An. fluminensis (2.1%) and An. (Ano.) mediopunctatus/forattinii/costai (0.7%), and in the Shannon traps, An. mediopunctatus/forattinii/costai (0.78%), An. (Ano.) apicimacula/intermedius (0.26%) and An. (Nys.) strodei (0.26%).

3.2. Molecular study

A total of 506 anophelines were assayed by PCR to detect Plasmodium vivax, P. malariae and P. falciparum. The total number of specimens for each species was 419, 67, 15, 3, 1 and for An. cruzii, An. pseudomalaculipes/maculipes, An. fluminensis, An.
mediopunctatus/costai/forattinii, *An. apicimacula/intermedius* and *An. strodei*, respectively.

Table 2 shows the data for the captures and the respective PCR results. This shows that we obtained positive results for *P. malariae* infection in *An. pseudomaculipes/maculipes* and *An. fluminensis* in the CDC trap at point 3 on May 26, 2010. On the same date we also obtained positive results for *P. malariae* infection in *An. cruzii* collected between 6:30 P.M. and 7:30 P.M. and *An. pseudomaculipes/maculipes* collected between 8:30 P.M. and 9:30 P.M. Only *An. pseudomaculipes/maculipes* were positive for *P. vivax* infection. These specimens were caught in a Shannon trap on April 29, 2010, between 5:30 P.M. and 6:30 P.M.

The minimal natural infection rate for *An. cruzii* as determined by PCR was 0.24%.

4. Discussion

Analysis of the adult mosquitoes caught in Shannon and CDC traps in the Branco River valley, where the Guarani indigenous village is located, revealed the presence of three subgenera: *Kerteszia*, *Anopheles* and *Nyssorhynchus*. Factors that contribute to the presence of anophelines in the region include the presence of forests, the high diversity of vertebrates and the high rainfall. The Atlantic Forest close to the Brazilian coast is rich in bromeliads, plants that are associated with anophelines from subgenus *Kerteszia* (Marrelli et al., 2007).

The correlation between reported cases of malaria and the predominance of *An. cruzii* in some areas of the Atlantic Forest has already been described by some authors (Marques et al., 2008), and the ability of this species to act as vectors has been proven (Wilkerson and Peyton, 1991; Marrelli et al., 2007). *An. cruzii* has a short life expectancy, and its ability to act as a vector is dependent on its population density (Kakitani, 1992). Other authors have shown that these anophelines have acrodendrophilic and anthropophilic behavior (Forattini et al., 2000; Ueno et al., 2007) and a vertical distribution, increasing the chances of their being an effective vector for simian plasmodia.

Cases of infection in humans and monkeys have been reported in the geographic area in which *An. cruzii* is distributed. In addition, the flow of *Plasmodium* carriers from endemic regions renders states where the plant cover consists of Atlantic Forest more vulnerable to malaria (Portes et al., 2010).

Natural *P. vivax* infection in *An. cruzii* has already been reported by various authors (Branquinho et al., 1997; Curado et al., 2006; Resende et al., 2009. Branquinho et al. (1997), using ELISA, found infection rates of 0.086% for the VK217 CSP variant of *P. vivax* and 0.179% for *P. vivax*. In our study the minimal infection rate was 0.24%.

In Brazil, studies of malaria in indigenous peoples are normally undertaken in the Amazon region, where malaria is endemic (Arruda et al., 1989). Little is known about malaria in indigenous populations outside the Amazon region. One such study was undertaken in the municipality of Peruíbe, neighboring on Itanhaém, in a focus known as the “Aldeia dos Índios” (Indian Village), where the presence of anti-*Plasmodium vivax* antibodies was detected using serological techniques (Carvalho et al., 1988). The study found that seropositive individuals were commonest among males over 15 years of age, an age range that includes productive males who work on the land and are in close contact with the forest.

Some authors showed that simian plasmodia are similar to human plasmodia and that there is no difference in molecular terms between *P. simium* and *P. vivax* or between *P. brasilianum* and *P. malariae* (Qari et al., 1993; Agoramoorthy and Rudran, 1994; Fonseca, 1951; Deane, 1992).

Our findings corroborate the hypothesis that residual malaria in this region may be a zoonosis transmitted to Indians and other individuals exposed to monkeys, as *An. cruzii* was positive for *P. malariae/P. brasilianum*, a common parasite among monkeys.

In addition to *An. cruzii*, two other species, *An. pseudomaculipes/maculipes* and *An. fluminensis* were positive for *P. vivax* and *P. malariae* by PCR. Although studies of anophelines in the Atlantic Forest suggest that other species can sometimes be naturally infected (Resende et al., 2009), the result for the other two species was unexpected as there is no record in the literature of such a finding in this ecosystem.

Because of the difficulty in identifying certain species of anophelines, some authors have reported that species identified as *Anopheles* sp. near *An. fluminensis* and others such as *An. (Nys.) trinkae*, *An. (Ano.) pseudopunctipennis*, *An. (Nys.) oswaldoi*, *An. (Nys.) nunezovari* and *An. (Nys.) rangeli* may play a role in malaria transmission since sporozoites were found in their salivary glands (Hayes et al., 1987). Forattini (2002) reported that *An. fluminensis* can sometimes be a vector, but only locally or regionally, and that when it coexists with other species it can help the main vector. Environmental changes and their influence on the adaptation of some species of Culicidae to the domiciliary environment have been studied by various researchers (Rubio-Palis and Zimmerman, 1997). One study reported that species such as *An. fluminensis* are...
not adapted to anthropogenic environments where there has been extensive devastation (Lopes and Lozerei, 1995) and another one registered An. mediopunctatus almost exclusively in forested areas (Guimarães et al., 2000). This supports the finding of An. fluviensis infected by P. malariae in the canopy in regions where the forest is preserved.

It should be stressed that the mosquitoes that were positive were found in or at the edge of the forest, close to the forest floor or in the canopy and that all of them came from the Branco River basin, which is home to the Guarani Indian village and other local dwellers whose main form of agriculture is banana growing. It should also be pointed out that both the Indians and local dwellers work in close contact with the natural environment and make incursions into the forest. These findings suggest that the malaria observed in this study may be a sylvatic zoonosis involving monkeys and other vectors. This hypothesis is supported by the fact that mosquitoes that were positive for infection were collected both in Shannon traps, placed at ground level, and in the canopy, the habitat of monkeys. Further studies with a greater sampling effort covering more specimens of the local anopheline fauna are required to confirm this.

Our finding that plasmodia circulate in the study region provides further evidence that, as has already been shown (Curado et al., 2006; Cerutti et al., 2007), malaria persists outside the Amazon region and is associated with areas where the plant cover consists of Atlantic forest. Continuous epidemiologic surveillance is therefore required in this Brazilian ecosystem.

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