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Anopheles (Kerteszia) cruzii (DIPTERA: CULICIDAE) IN PERIDOMICILIARY AREA DURING ASYMPTOMATIC MALARIA TRANSMISSION IN THE ATLANTIC FOREST: MOLECULAR IDENTIFICATION OF BLOOD-MEAL SOURCES INDICATES HUMANS AS PRIMARY INTERMEDIATE HOSTS

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SUMMARY

Anopheles (Kerteszia) cruzii has been implicated as the primary vector of human and simian malarias out of the Brazilian Amazon and specifically in the Atlantic Forest regions. The presence of asymptomatic human cases, parasite-positive wild monkeys and the similarity between the parasites infecting them support the discussion whether these infections can be considered as a zoonosis. Although many aspects of the biology of *An. cruzii* have already been addressed, studies conducted during outbreaks of malaria transmission, aiming at the analysis of blood feeding and infectivity, are missing in the Atlantic Forest. This study was conducted in the location of Palestina, Juquitiba, where annually the majority of autochthonous human cases are notified in the Atlantic Forest of the state of São Paulo. Peridomiciliary sites were selected for collection of mosquitoes in a perimeter of up to 100 m around the residences of human malaria cases. The mosquitoes were analyzed with the purpose of molecular identification of blood-meal sources and to examine the prevalence of *Plasmodium*. A total of 13,441 females of *An. (Ker.) cruzii* were collected. The minimum infection rate was calculated at 0.03% and 0.01%, respectively, for *P. vivax* and *P. malariae* and only human blood was detected in the blood-fed mosquitoes analyzed. This data reinforce the hypothesis that asymptomatic human carriers are the main source of anopheline infection in the peridomiciliary area, making the probability of zoonotic transmission less likely to happen.

KEYWORDS: Asymptomatic malaria; Atlantic forest; *Anopheles (Kerteszia) cruzii; Plasmodium malariae; Plasmodium vivax.*

INTRODUCTION

Currently, the Amazon Region concentrates 99.8% of the malaria cases described in Brazil, with approximately 306,000 cases registered in 2009[28]. Malaria outside the Amazon region is situated mainly in Atlantic forest regions, due to the presence of bromeliads where *Anopheles* mosquitoes of the subgenus *Kerteszia* use the axils as larval habitat[28]. *An. (Ker.) cruzii* and *An. (Ker.) bellator* are considered malaria vectors, and the first is the primary vector of human and simian malaria in these regions[6,7,10,12]. Accordingly, since the 1960's there is a discussion whether these infections, termed "forest-malaria" or "bromeliad-malaria", can be considered as a zoonosis, where monkeys possibly act as reservoirs[8].

Some data support the hypothesis that malaria can be a zoonosis in the Atlantic Forest. In relation to the vector, synanthropy of *An. cruzii* has been demonstrated in Atlantic Forest areas of the state of São Paulo[16,20]. These studies have shown that females may fly to the anthropic environment to feed on blood mainly in the domestic and peridomestic areas and then return to the natural environment[20]. Moreover, a high vertical mobility, with distribution from ground level to tree tops, has been shown in *An. cruzii*[36]. In relation to the hosts, simian malaria was shown to frequently occur in the forested coastal mountains of the Southeastern region, where 35% of the examined monkeys were positive for *P. brasilianum* or *P. simian* and the identification of natural accidental human infection due to *P. simium*[9]. The parasitological prevalence of *P. vivax* and *P. malariae* in wild monkeys from the Atlantic Forest has also been described in more recent studies[11,39]. Finally, the simian malaria parasites *P. brasilianum* and *P. simium* are genetically indistinguishable from those responsible for human malaria in the Atlantic forest, *P. malariae* and *P. vivax*, respectively[13,25].

On the other hand, it is also important to note that human cases detected in the Atlantic forest are generally asymptomatic, showing only subpatent levels of parasites. These individuals figure as appropriate reservoir hosts because they are not routinely identified as parasite carriers by typical malaria control programs which monitor only patent malaria cases.

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Despite the fact that many aspects of the biology of *An. cruzii* have been elucidated[26], studies conducted in settings of asymptomatic malaria transmission, aiming at the analysis of vector blood feeding and infectivity, are missing for the Atlantic Forest. Using this approach, this study tried to screen individual mosquitoes for the presence of non-human blood, mainly monkey blood, in order to support or reject the hypothesis of zoonotic transmission of malaria in this region. The city of Juquitiba was chosen to investigate these aspects, as thirteen cases, almost one third of all autochthonous cases in the Atlantic Forest of the state of São Paulo, were notified there in 2007[5]. The location of Palestina was specifically chosen for being responsible for the identification of the largest number of cases in the region and because dwellings are in the vicinity of woods, where monkeys are frequently seen. The mosquitoes were analyzed with the purposes of (i) molecular identification of blood-meal sources; (ii) to examine the prevalence of *Plasmodium;* (iii) to collect information on seasonality; and (iv) to evaluate methods of *Kerteszia* sampling, with the final aim of contributing to the design of new strategies for asymptomatic malaria prevention and control in Atlantic Forest.

MATERIAL AND METHODS

Study Area: The city of Juquitiba is located in the metropolitan area of São Paulo, state of São Paulo, Brazil, and covers an area of 521.6 km^2, with a population of 28,961 inhabitants, of whom 22.6% live in the rural area[32]. Juquitiba has an ecotourism economic activity that comprises activities such as canoeing, camping and fishing, due to the mountainous landscape and rivers. Concerning exposure to malaria vectors in peridomiciliary habitats, the local population consists mainly of employees who look after the properties and live near the cottages, as well as people who come from the city of São Paulo to spend the weekend at holiday cottages for recreation. The climate in this region is humid and subtropical[24], with the coldest mean temperature below 18 °C in a dry winter (June-August) and the warmest month above 22 °C in the wet summer season (December-February). The annual rainfall is about 1300 mm and the average altitude is 685 m. In 2006 and 2007, 16 and 13 mainly asymptomatic malaria cases were reported in Juquitiba, respectively[5].

Mosquito sampling and handling: Adult female mosquitoes were collected from January 2006 to September 2007 in the wet and dry seasons at eight sites in the peridomiciliary environment (Fig. 1). The collection sites have the same landscape features; they are rural human settlements with anthropic modifications and variable distances from patches of forest, allowing frequent contact of man-forest and man-mosquitoes. The dwellings are scattered along Palestina Road, usually located near the forest, and the anthropic environment is constituted by extensive agricultural activities. The collection sites are connected by Embratel Road, Palestina Road and Olaria Road (Fig. 1B).

Mosquitoes were collected from January 2006 to September 2007 by: (i) Nasci aspirator[27]; (ii) Shannon trap[34]; and (iii) UV-CDC (Centers for Disease Control) light traps baited with CO_2[35]. These methods were used from nine to 12 am, seven to nine pm and six pm to seven am, respectively, during wet (January to March) and dry (July to September) seasons, for three consecutive days per month, totaling 36 days.

Collections with Nasci aspirator were made to supply representative samples of blood-engorged resting females[33]. The Nasci aspirators were used in the early morning, in the proximity of bromeliads (*Kerteszia* breeding sites), to collect resting females at four peridomiciliary sites where malaria cases were identified. One collector went towards the canopy trees to aspirate near the bromeliads, for intervals of 15 minutes, to complete the three-hour period.

Collections with CDC light traps and Shannon traps were used to sample *Plasmodium* infected females. Ten CDC light traps with UV light baited with CO_2 were distributed on site 4, specifically in the peridomiciliary outskirts of the cottage. This site was chosen

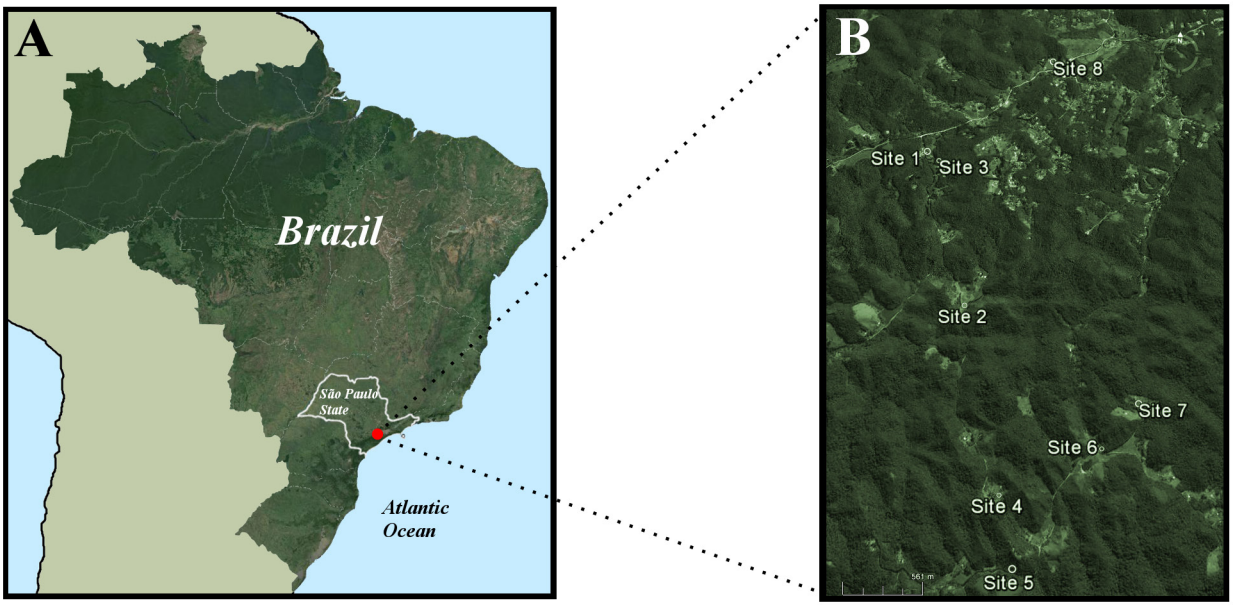

Fig. 1 - A. Location of the County (Municipality) of Juquitiba, São Paulo State, Brazil. **B.** Sites of collection at the domiciliary environment in Juquitiba County. Modified from Google Earth and Wikimedia Commons.

because the first malaria case detected in the parasitological survey done in January 2005 lives in this cottage. The complete data of the parasitological survey will be published elsewhere. Site 4 is a remnant of the forest in a steep relief with higher altitudes situated 40 m away from the cottage. The peridomiciliary area corresponds to open man-made fields with isolated trees without bromeliads. Samples were collected at site 4 to verify the abundance of *Kerteszia* subgenus in a high forest cover in a steeper area in the proximity of the dwelling. The traps were distributed in an altitudinal range of 711-778 m (above sea level) with 10 m of distance between each other. Altitudinal positions of mosquito traps were measured using portable GPS. The seasonal abundance of *An. cruzii* with the overall collections at site 4 (Fig. 1B) was estimated by using the William's mean[21], and the altitudinal distribution of *An. cruzii* abundances was compared in dry and wet seasons by the non-parametric Friedman test.

A comparison of the effectiveness of CDC light traps and the Shannon traps at sampling *An. cruzii* was carried out in the peridomiciliary habitats of the sites 1, 2 and 6, where collections were conducted simultaneously. The collections were compared by standardizing their number to obtain the monthly Williams geometric mean. The comparison of the anopheline abundances was verified by Wilcoxon's matched-pairs test.

Adult mosquitoes were killed with chloroform steam and transported to the laboratory, where their specimens were identified on chilled tables with a stereomicroscope and using identification keys[18,40]. All mosquitoes with fresh or visible blood remnants were individually placed in 1.5-mL microcentrifuge tubes, sealed with parafilm, labeled according to species, collection site and stored at -20 °C.

Mosquito gDNA extraction and PCR amplification of *Plasmodium* SSU fragment: DNA from each mosquito was extracted as described[31], and the assay to detect *Plasmodium* infection was performed in pools of ten mosquitoes. The pools were separated by species, day of capture and type of trap. PCR amplification was carried out according to a full-nested protocol[38], which uses oligonucleotides in conserved sequences, in the small subunit (SSU) ribosomal RNA of human *Plasmodium* species in a first reaction. The second amplification was carried out with specific primers for three human *Plasmodium* species circulating in Brazil (*P. falciparum*, *P. vivax* and *P. malariae*). PCR products were electrophoresed in 2% agarose gels, stained with ethidium bromide and visualized under UV-light. The minimum infection rate (MIR) was calculated as the ratio of the number of positive pools to the total number of mosquitoes tested[37].

gDNA extraction from blood-fed mosquitoes and blood meal identification: Genomic DNA of blood fed mosquitoes was obtained using PureLink™ Genomic DNA Purification Kit (Invitrogen). PCR was used to amplify host DNA from the mosquito blood meal using primers L14841 and H15149[23] or B1 and B6[30] designed to amplify, respectively, fragments with ~300 bp and ~1 kb of the mitochondrial *cytb* gene from a wide array of animals, including mammals, birds, amphibians, reptiles and fishes. This methodology was successfully used to identify the blood meal sources in mosquitoes from São Paulo Zoo[2].

Amplified fragments were purified from gels and sequenced directly using the corresponding flanking primers. Sequences were identified by comparison to the GenBank DNA sequence database (www.ncbi.nlm.nih.gov/blast/Blast.cgi). Positive identification and host species assignment were made when exact or nearly exact matches (> 98%) were obtained. Human *cytb* fragments show less than 86% of sequence identity with those from different primate species normally described in the Atlantic Forest of the state of São Paulo (*Callithrix jacchus*, *Alouatta guariba* and *Cebus apella*) (data not shown).

RESULTS

Anopheline abundance, distribution and seasonal variation: A total of 13,462 *Anopheles* females were collected, including 13,441 of *An. (Ker.) cruzii*, 13 of *An. (Nys.) evansae*, six of *An. (Nys.) lutzii*, one of *An.(Nys.) galvaoi* and one of *An. (Ker.) bellator.* Since the study of *An. (Ker.) cruzii* is the objective of this work, and this species corresponded to 99.8% of the collected individuals, only these mosquitoes were used for infection rate determination and blood meal identification. From the 13,441 of *An. (Ker.) cruzii* collected, 55.2% were obtained in CDC, 42% in Shannon traps and 2.8% in Nasci aspirators (Table 1).

Table 1
Females of *An. (Ker.) cruzii* collected by different methods, at eight peridomiciliary collection sites, in Juquitiba, State of São Paulo, from January 2006 through September 2007

Site	Position according GPS#	Malaria case*	Method	Mosquitoes collected
1	24°00'01.2"S 47°06'12.9"W	19/05/2005	CDC	1
			Shannon	51
2	24°00'46.8"S 47°05'57.2"W	19/05/2005	CDC	304
			Aspirator	361
			Shannon	1759
3	24°00'03.8"S 47°06'09.0"W	02/06/2005	CDC	143
			Aspirator	1
4	24°01'31.7"S 47°05'44.5"W	08/12/2005	CDC	5925
5	24°01'46.6"S 47°05'40.4"W	02/06/2005	CDC	996
6	24°01'20.8"S 47°05'18.4"W	--	CDC	57
			Shannon	3374
7	24°01'10.1"S 47°05'08.4"W	--	Shannon	349
8	23°59'28.7"S 47°05'33.7"W	--	Aspirator	9
			Shannon	111
Total				13441

Global Positioning System. *Date (DD/MM/YY) of the last malaria case detected by thick blood smear before the beginning of the entomological survey.

To verify the abundance of *An. cruzii* in a high forest cover in a steeper area in the proximity of the dwelling samples were collected at site 4 using CO_2-baited UV-CDC traps. Most mosquitoes were captured in the wet season (January-March), as shown in Table 2. The comparison made in the varying altitudinal positions of CDCs showed statistical support (Fr = 45.3, $p < 0.0001$), meaning that there was a statistically

significant difference between the number of females collected according to the season.

Table 2
Seasonal activity of *An. cruzii* (William' s mean) collected with CO_2 baited UV-CDC, at different altitudes at site 4, Juquitiba, during summer (Jan-Mar) and winter (Jul-Sep), 2006-2007, São Paulo

Position (m)	Altitude (m)	Jan	Feb	Mar	Jul	Aug	Set
10	711	2.1	1.0	1.6	0.1	0.0	0.3
20	722	3.1	0.6	1.5	0.2	0.1	0.6
30	729	2.6	0.9	0.6	0.3	0.1	0.2
40	742	3.7	1.9	0.9	0.0	0.1	0.4
50	740	4.3	1.0	2.5	0.5	0.2	0.5
60	741	4.6	1.2	3.4	0.2	0.2	0.6
70	755	4.3	2.1	2.0	0.4	0.2	1.2
80	773	2.0	1.6	2.4	0.0	0.1	0.4
90	779	4.1	2.1	2.1	0.2	0.1	0.8
100	799	2.3	0.7	1.6	0.3	0.0	0.0

The efficacy of CDC and Shannon traps as collection methods was compared by standardizing the number of collections and collectors, and also evaluated in the sites, where collections were conducted simultaneously (sites 1, 2 and 6). The Williams mean of *An. (Ker.) cruzii* collected with Shannon traps suggested higher values than those obtained by CDC-CO_2 traps (Fig. 2). The Wilcoxon test (W = 20.0, p = 0.19) showed no significant differences in the numbers of captured *An. (Ker.) cruzii* collected by the two methods in the peridomiciliary sites.

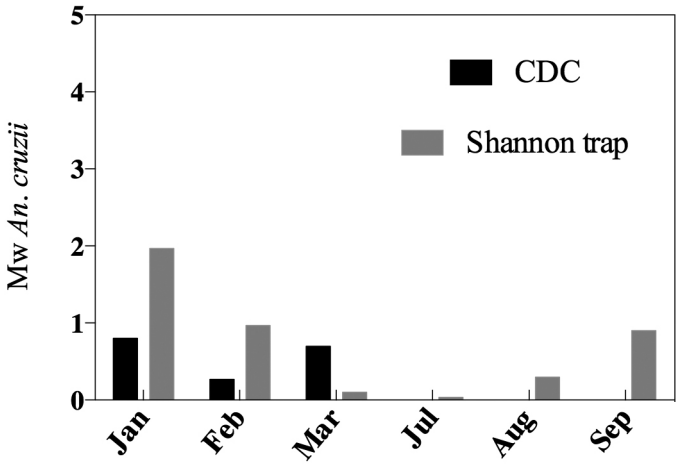

Fig. 2 - Comparison of collections with CDC and Shannon traps in the peridomiciliary sites 1, 2 and 6, in Juquitiba, São Paulo. The values were shown as William's mean of *An.* (*Ker.*) *cruzii* collected in the wet (Jan-Mar) and dry (Jul-Sep) seasons in 2006 and 2007.

Infection rate: Considering the large number of *An. (Ker.) cruzii* females obtained, only 67.5% of the total (9,072 mosquitoes from 13,441 females) was tested for *Plasmodium* infection (1,119 and 7,953, collected in 2006 and 2007, respectively). From mosquitoes collected in 2006, two pools, both from January 16th, were positive for *P. vivax*: one with females sampled from site 4 and another one with females obtained from site 5. From those collected in 2007, one pool from site 4, also from January 16th, was positive for *P. vivax*, and one pool sampled from site 6 on January 17th, was positive for *P. malariae*. All samples were collected with CDCs. The minimum infection rate (MIR) was 0.03% and 0.01% for *P. vivax* and *P. malariae*, respectively.

Blood meal identification: All engorged mosquitoes collected by CDC and Nasci aspirator (10 and 16 mosquitoes, respectively), in both years, were tested for blood meal identification (Table 3). Blood-meal sources were successfully identified by DNA sequencing of the *cytb* fragment in 73% of engorged mosquitoes analyzed. In a total of 27% of engorged mosquitoes, no DNA from blood was detected, since the amplification of *cytb* gene was not obtained. All detected blood meals were identified as human-derived. In a more detailed analysis carried out using the PCR fragment sequence obtained from a mosquito (collected at site 2 in 2006 using Nasci aspirator), 730 bp were submitted to BLASTN to GenBank and only two substitutions were found in relation to a *cytb* sequence from *Homo sapiens* haplotype I [GenBank:EU091245].

Table 3
Number of blood-fed mosquitoes analyzed for blood meal identification, according to site, method and year of collection

Collection site	Method	Blood meal source			Total
		2006		2007	
		Human	ND	Human	
2	CDC	-	-	01	01
	Aspirator	02	-	03	05
3	CDC	-	-	-	-
	Aspirator	01	-	-	01
4	CDC	-	05	02	07
5	CDC	-	-	01	01
6	CDC	-	-	01	01
8	Aspirator	08	02	-	10
Total		**11**	**07**	**08**	**26**

ND = not determined.

DISCUSSION

This study reports molecular identification of blood-meal sources, seasonal abundance and malaria infectivity in *Anopheles (Kerteszia) cruzii* (Diptera: Culicidae) from a small focus of malaria transmission in a non-endemic area in the Atlantic Forest, São Paulo, Brazil. To the authors' knowledge, this is the first report using a molecular methodology based on cytochrome b sequences to identify the blood-meal source carried out with mosquitoes collected during malaria transmission in Brazil.

During the sampling period, a good amount of mosquitoes were obtained, with the vast majority (97.2%) collected by CDC and Shannon traps. Although an excessive number of *Kerteszia* females were collected

at the three sites in the wet season of 2007 with the Shannon trap, the Wilcoxon test results showed no significant difference between CDC and Shannon traps. CDC collection frequencies compensated negative collections with Shannon traps in the dry season. Negative collections must have occurred due to unidentified specific environmental conditions.

Human malaria cases in the Atlantic Rain Forest, as well as infected mosquitoes, were described by several authors[3,4,12,31], and the finding of natural infections of *P. vivax* and *P. malariae* in anophelines from this study match those detected by these authors. Also, the herein used molecular identification method is able to identify one naturally infected mosquito in a pool of ten mosquitoes and has been used in many other studies[3,12]. Here, the minimum infection rate (MIR) was 0.03% and 0.01% for *P. vivax* and *P. malariae*, respectively. Similar rates were found by using ELISA methodology in anophelines collected in the same city[1] or by using PCR-based techniques, in Parelheiros, a locality at a distance of 70 km from Juquitiba and also located in Atlantic Forest regions[12]. Altogether, these rates are well around ten times lower than in endemic regions in the Amazon[19]. It is important to note that the positive mosquitoes were collected with CDCs on January 16th, 2006, at sites 4 and 5, and on February 17th, 2006, one additional malaria case was detected at site 5. In 2007, the positive mosquitoes were also collected in January, at sites 4 and 6. Perhaps not coincidentally, in February and March, five malaria cases were detected in the region by thick blood smear. The complete data of the epidemiological investigation will be published elsewhere.

Studies of feeding preferences of *An. cruzii* in the state of São Paulo using precipitin tests showed the presence of equine blood and rodent blood, but mainly human blood among the engorged mosquitoes[14,15]. However, the methods used in these studies did not allow the differentiation of human blood and simian blood. Here, a molecular approach capable of discriminating, at the species level, the source of the blood meal was used. Notably, mosquitoes were collected during the transmission season, in an area where malaria can be considered a zoonosis. Notwithstanding, only human blood was identified in the mosquitoes. Importantly, although the blood meal source was not identified for some of the mosquitoes examined, this result was also found in previous studies, using another[14] or the same methodology[2]. This probably occurs due to the fact that the blood begins to be digested soon after ingestion by the mosquito and, as a consequence, the DNA is being degraded. Consequently, the longer the post-ingestion period, the lower the possibility of DNA detection[29]. Ultimately, it is important to note that the methodology used here for blood meal identification is one of the most widely used all over the world and is able to reliably detect blood from all vertebrates with the same performance[22].

In relation to the collection method used to obtain engorged females, very few mosquitoes were captured with the Nasci aspirator, although this was properly used. Females of the *Kerteszia* subgenus are deliberately exophilic, resting in natural outdoor shelters, but it was assumed that the mosquitoes were rare or flew away with the proximity of the collectors. On the other hand, using CDC traps, other studies also showed a lower probability of collecting engorged mosquitoes, since they collect females that are actively host-seeking[33]. Thus, the analysis of the results showed that the methods for collecting mosquitoes employed here are reliable tools to sample *Kerteszia* females and showed regularity in accordance with seasonal periods of *Kerteszia* activity. However, it would be desirable to improve and standardize the collection methods to enable comparisons of results of different studies.

The participation of *An. cruzii* as a vector of *Plasmodium* in Atlantic Forest regions is recognized in Brazil, as well as the strong dependence of this species on the forest[17]. The predominance of this vector in the peridomiciliary area was confirmed, as well as the capacity of transmitting *P. malariae* and *P. vivax*. However, only human blood was recorded in the engorged mosquitoes. Given the significant sampling reached herein, the complete absence of detection of monkey blood does not favor the hypothesis of considering malaria cases as a zoonosis, and the occurrence of asymptomatic infections can possibly explain the maintenance of the circulation of parasites in humans, in the peridomiciliary habitat. Complementary studies testing the infection rates of monkeys in the study area may elucidate what real risk these animals play in terms of apparently very rarely occurring interspecies transmission of malaria.

CONCLUSION

In the present study, the predominance of *An. (Ker.) cruzii* was confirmed in the peridomiciliary area, as well as the capacity of transmitting *P. malariae* and *P. vivax* and the first molecular study of identification of the blood-meal source carried out with mosquitoes collected during asymptomatic malaria transmission in the Brazilian Atlantic Forest was reported. As only human blood was identified in the engorged mosquitoes, this data reinforce the hypothesis that asymptomatic human carriers are the main source of anopheline infection in the peridomiciliary area.

RESUMO

Anopheles (Kerteszia) cruzii (Diptera: Culicidae) em área peridomiciliar durante transmissão de malária assintomática na Mata Atlântica: identificação molecular das fontes de repasto sanguíneo indica humanos como hospedeiros intermediários primários

Anopheles (Kerteszia) cruzii é o vetor primário das malárias humana e simiana fora da Amazônia Brasileira e especificamente nas regiões de Mata Atlântica. A presença de casos humanos assintomáticos, macacos silvestres positivos para *Plasmodium* e a similaridade entre os parasitas que os infectam suportam a discussão se essas infecções podem ser consideradas como zoonoses. Embora muitos aspectos da biologia de *An. cruzii* já tenham sido abordados, estudos conduzidos durante surtos de transmissão de malária, visando a análise de repasto sanguíneo e infectividade, são ausentes na Mata Atlântica. Este estudo foi conduzido na localidade de Palestina, Juquitiba, Mata Atlântica do Estado de São Paulo, onde anualmente a maioria dos casos humanos autóctones é notificada. Locais em peridomicílio foram selecionados para coleta de mosquitos em um perímetro de até 100 m em torno das residências de casos humanos de malária e da floresta circundante. Os mosquitos foram analisados com o objetivo de identificação molecular das fontes de repasto sanguíneo e para examinar a prevalência de *Plasmodium*. Um total de 13.441 fêmeas de *An. (Ker.) cruzii* foi coletado. A taxa de infecção mínima foi calculada a 0,03% e 0,01%, respectivamente, para *P. vivax* e *P. malariae* e somente sangue humano foi detectado nos mosquitos analisados que se alimentaram com sangue. Nossos dados reforçam a hipótese de que

os portadores humanos assintomáticos são a principal fonte de infecção para os anofelinos na área do peridomicílio, tornando a transmissão zoonótica improvável.

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