Revista da Sociedade Brasileira de Medicina Tropical
Journal of the Brazilian Society of Tropical Medicine
Vol.:52:e20180537: 2019
doi: 10.1590/0037-8682-0537-2018

Major Article

Asymptomatic *Plasmodium* infection in a residual malaria transmission area in the Atlantic Forest region: Implications for elimination

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Abstract

Introduction: Elimination of malaria in areas of interrupted transmission warrants careful case assessment to avoid the reintroduction of this disease. Occasional malaria cases are reported among visitors of the Atlantic Forest area of Brazil, while data on residents of this area are scarce. **Methods:** A sectional study was carried out to examine 324 individuals living in a municipality where autochthonous cases were detected. **Results:** Asymptomatic *Plasmodium* infections were detected in 2.8% of the individuals by polymerase chain reaction (PCR), with one case of *P. falciparum* (0.3%), two cases of *P. vivax* (0.6%), and six cases of *P. malariae* (1.9%). The thick blood smears were negative in all individuals. Serological tests performed in 314 subjects were reactive in 11.1%, with 3.5% for *P. falciparum* and 7.7% for *P. vivax*. A subsample of 42 reactive individuals for any *Plasmodium* species showed *P. malariae* in 30.9% of specimens. Individuals who entered the Atlantic Forest region were 2.7 times more likely to exhibit reactive serology for *P. vivax* compared with individuals who did not enter this region ($p<0.05$). Children <15 years had a higher chance of reactive serology for *P. falciparum* and *P. vivax* than individuals ≥15 years of age ($p<0.05$). Individuals living in the Paraiso district had a higher chance of reactive serology for *P. vivax* compared to other districts ($p<0.05$). No associations were found between sex, past exposure to malaria, or serological response to antibodies of any *Plasmodium* species. **Conclusions:** The implications of these results for the elimination of malaria were discussed.

Keywords: Malaria. *Plasmodium* infection. Extra-Amazonian region. Elimination. Rio de Janeiro State.

INTRODUCTION

Malaria endemic areas are restricted to the Amazonian region of Brazil where *Anopheles darlingi* Root 1926 is the main vector implicated in transmission[1]. An average of 1,296 cases are reported annually outside of this region, most of which (89%) have been imported from elsewhere[2]. While 50% of the malaria cases in the extra-Amazonian region of Brazil were autochthonous in the middle of the 20th century, this proportion has dropped to only 0.05% to date[2]. Rio de Janeiro state, located in Southeastern Brazil, used to be considered a highly endemic area for malaria with the reported presence of *An. darlingi*[3]. Cases of infection by *P. falciparum* and *P. vivax* occurred particularly in the lowlands (Baixada Fluminense region) in the proximity of the capital of the state[4]. The Global Malaria Eradication Campaign supported the implication of control measures against vectors and the discovery of new drugs for infected people, leading to the interruption of transmission by 1968 with *An. darlingi* now rarely found in this area[5]. In recent years, a new malaria epidemiological scenario is emerging in Rio de Janeiro, with the presence of cases imported from endemic areas of Brazil and outside the country and occasional autochthonous cases including outbreaks[6,7,8,9]. In 2016, the World Health Organization (WHO) launched the "Global Technical

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Received 17 December 2018
Accepted 19 February 2019

Strategy for Malaria 2016-2030", with the goal of "preventing a resurgence of malaria in all countries and areas that are malaria-free"[10]. A recent malaria outbreak caused by *P. simium* was described in tourists visiting some regions of the dense and protected Atlantic Forest in Rio de Janeiro state[9]. Malaria cases in this area are detected predominantly among visitors but it is unclear whether the local residents are also affected. We therefore carried out a cross-sectional study to verify the status of *Plasmodium* infections in residents of the Atlantic Forest area of Rio de Janeiro state. The novel epidemiology of malaria in interrupted transmission areas must be carefully assessed, particularly considering worldwide efforts to eliminate the disease[2,10].

METHODS

A cross-sectional study was carried out during the fall of 2011 in Guapimirim, a municipality located 86 km away from Rio de Janeiro, Brazil. Six localities were eligible for participant recruitment because nearby autochthonous cases were detected, the rural areas Garrafão, Orindi, and Paraíso and the peri-urban areas Barreira, Caneca Fina, and Monte Olivete (**Figure 1**). The required sample size was estimated using an expected prevalence of 4% for *Plasmodium* infection as a variable with an acceptable error of 2%. The final sample size was calculated as at least 312 individuals. Residents of both sexes older than 5 years of age who gave their informed consent (for adults) or received consent from the legal guardian (for children under 18 years) were randomly recruited. A semi-structure questionnaire on personal data, exposure to malaria, daily habits, knowledge about disease, clinical information, and history of febrile symptoms in the month preceding the study period was administered. Thick and thin blood smears were collected from each participant according to standard protocols of the Brazilian Ministry of Health. Total blood (5 mL) was collected in Vacutainer® tubes (Becton Dickinson New Jersey, USA) containing ethylenediaminetetraacetic acid (EDTA) for DNA extraction for parasitological diagnosis of malaria by polymerase chain reaction (PCR). Ten milliliters of blood were collected without anticoagulant for serological studies.

Parasitological diagnosis

Thick blood smears were stained and 100 fields examined at 1,000× magnification[11]. DNA was extracted for molecular diagnosis from 200 µL of whole blood using a commercial DNA Purification Kit (Illustrablood genomicPrep MiniSpin Kit), according to the manufacturer's instructions (GE Healthcare, Pittsburgh,USA). PCR was performed following the nested PCR protocol by Snounou et al. with minor modifications[12,13]. Agarose gel electrophoresis was performed, gels stained with GelRed™nucleic acid gel stain, Biotium, Fremont, CA, USA and visualized under UV light. A sample was considered positive if a 120, 144, or 205 bp PCR product (*P. vivax*, *P. malariae,* and *P. falciparum,* respectively) was detected. A sensitivity level of 0.001% for PCR detection of parasitemia levels is appropriate for the diagnosis of subpatent infections[14]. Positive results were re-tested twice to verify the results.

Serological analysis

Serological examinations were performed using enzyme-linked immunosorbent assay (ELISA) with erythrocytic antigens for *P. falciparum* and *P. vivax* and immunofluorescence assay (IFA) for *P. malariae*. IgG antibodies were detected by ELISA using a crude blood stage *P. falciparum* antigen extracted with Zwittergent® (Calbiochem, Billerica, MA, USA)[15] and MSP1-19 recombinant antigen of *P. vivax*[16]. Reactions were assessed by measuring the absorbance at 492 nm using Titertek Multiskan MCC/340 (Labsystems Diagnostics Group, Vantaa, Finland). To determine the cut-off for the ELISA using *P. falciparum* and *P. vivax* antigens, receiver operating characteristic (ROC) curves were constructed based on the absorbance of positive and negative samples[17] (**Figure 2**). The reactivity index (RI=absorbance/cut-off) was calculated for all samples, and samples with RI ≥1.1 were considered positive. Samples with values between 0.9 and 1.1 (gray zone) were considered inconclusive. IgG antibodies against *P. malariae* were detected using the IFA protocol described by Ferreira and Sanchez[16]. As the quantity of *P. malariae* antigen was low, this serological test was performed only for the PCR-positive samples and all reactive and inconclusive serologies. The experiments were performed at the Laboratory of Seroepidemiology and Immunobiology at the Institute of Tropical Medicine of São Paulo, Brazil.

A malaria case was defined as an individual with any of the typical malaria symptoms (fever, chills, sweating, headache) at the time of the interview and with a positive thick blood smear or PCR result. An asymptomatic *Plasmodium* carrier was defined as an individual with *Plasmodium* species detected in the thick blood smear and/or the PCR but without symptoms 30 days before and after sample collection and without using antimalarial drugs.

Ethical considerations

All the procedures followed the ethical standards of the Ethics Committee of Research involving Human Subjects of the Research Institute Evandro Chagas-INI/Fiocruz, Brazil and in accordance with the Helsinki Declaration (Protocol 0229.0.000.009/10 approved).

Statistical analysis

Exploratory analyses were performed using contingency tables and chi-square and Fisher tests to verify possible relationships between the dependent and independent variables. This relationship was modeled using a series of simple binomial generalized linear models (GLM). Only variables with $p<0.2$ in the bivariate analysis were evaluated in the model. Multiple binomial GLMs were used to model the effects of all independent variables on each dependent variable. Interaction effects were tested in each model formulation, but no significant interaction was found. We employed the Firth's Bias-Reduced Logistic Model to account for numerical problems and model convergence in the *P. falciparum* and *P. malariae* models. Data were analyzed using the freely available EPI2000 statistical program (Centers for Disease Control, Atlanta, Georgia, USA),

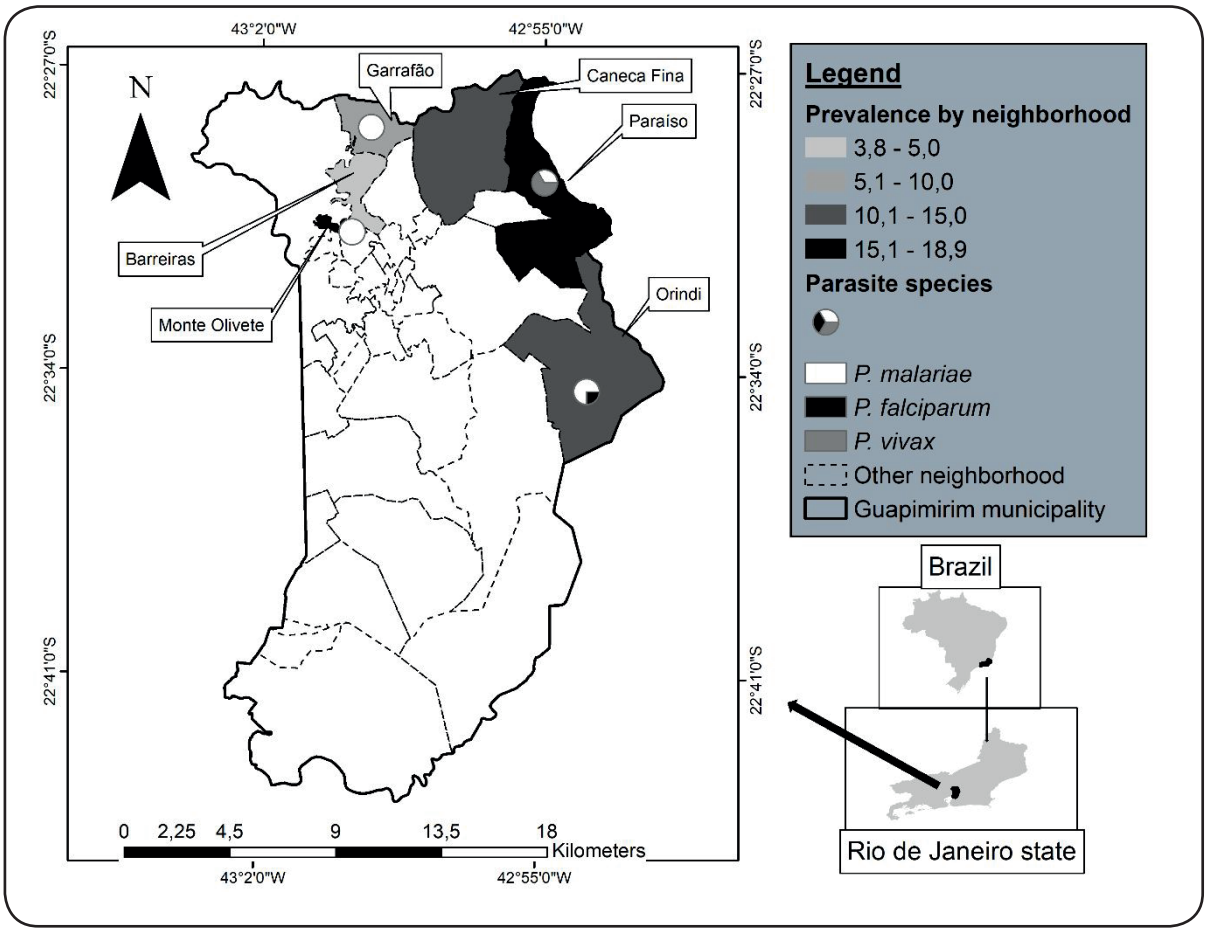

FIGURE 1: Study locations and prevalence of asymptomatic *Plasmodium* infections in the municipality of Guapimirim, Rio de Janeiro, Brazil.

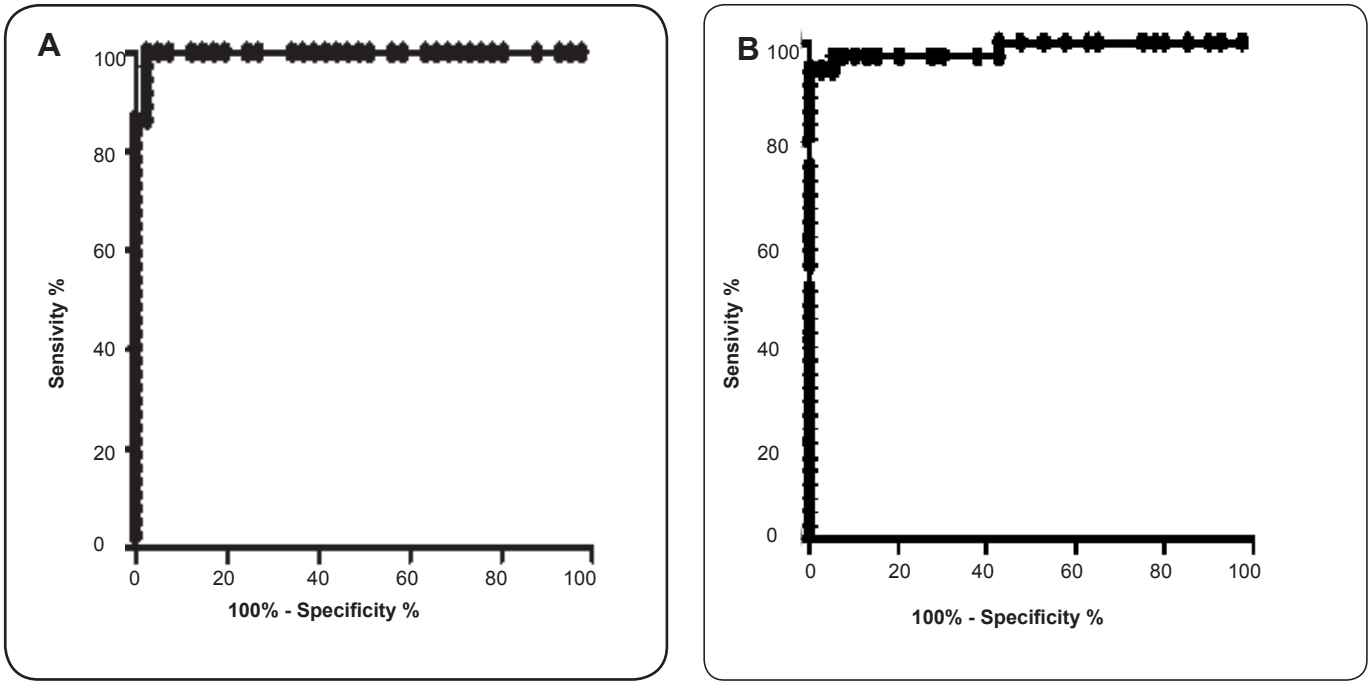

FIGURE 2: Receiver operating characteristic (ROC) curves to determine enzyme-linked immunosorbent assay (ELISA) performance for anti-PvMSP119 IgG and anti-Pf IgG. **Panel A** shows the curve obtained for samples from *P. vivax*-infected patients (n=41) and healthy subjects (n=37). **Panel B** shows the curve for samples from *P. falciparum*-infected patients (n=41) and healthy subjects (n=37). PvMSP119-and Pf-ELISAs considered a cut-off of 0.100 in the ROC curves. Anti-IgG PvMSP119 for *P. vivax* was detected in 100.0% (range: 90.5-100.0, CI: 95%) of *P. vivax* cases with a specificity of 97.6% (range: 87.1-99.9, CI: 95%). Anti-IgG for *P. falciparum* was detected in 94.4% (range: 81.3-99.3, CI: 95%) with a specificity of 100.0% (range: 91.2-100.0, CI: 95%).

the R software, and the RStudio software with the Companion to Applied Regression (CAR) package, R Foundation for Statistical Computing, Vienna, Austria.

RESULTS

Demographics aspects

We interviewed a total of 324 individuals [186 women (57.4%) and 138 men (42.6%)], with a mean patient age of 32.6± 9.8 years without differences between women and men (p=0.16). Of these, 215 participants (66.4%) lived in rural areas and 109 (33.6%) in peri-urban areas. Most individuals were born in the state of Rio de Janeiro (260/324, 81.8%). The mean educational duration of the participants was 6.9±3.6 years, and 269 people (85.4%) had attended school and could read and write. The average time of residence in the Guapimirim municipality was 18.6±13.1 years, the time of residence in the respective locality was 17.7±12.8 years, and that in the current home was 9.8±12 years. A total of 36 individuals (36/324, 11.1%) had changed housing in the last 5 years, 20 of which (6.2%) moved within the municipality.

Previous malaria episodes

Of the 324 participants, 316 (97.5%) never had malaria, 4 (1.2%) did not remember, and 4 (1.2%, 2 women and 2 men) had previously suffered a single episode of malaria. One of these women (age: 69 years) had malaria at the age of 27 years while staying in Mozambique and the other (age: 56 years) had malaria at the age of 6 while staying in the municipality of Mage (state of Rio de Janeiro). One of the previously infected men (age: 70 years) had malaria at the age of 6 years but did not remember the place, while the other man (age: 89 years) had malaria due to *P. falciparum* infection at the age of 59 years while staying in Rondônia (Amazonian region in Brazil). None of these individuals had symptoms associated with malaria in the year preceding the survey and all of them had negative results in the thick blood smear and PCR tests.

Exposure to malaria

Seventy-eight participants (24.1%) had left the Guapimirim municipality in the 6 months prior to the study, most of them during the last 15 days (41 subjects, 12.7%), and 2 residents went to the Amazon region. Only 10.8% of the participants (35/324) used a mosquito net while sleeping, with 7.7% (25/324) using it always. In terms of visiting the forest region for activities such as collecting fruits, walking, working, or leisure, 75.9% (246/324) of the participants said that they did not regularly enter the forest, 3.1% (10/324) reported going occasionally (mountain climbing or walking), 10.2% (33/324) reported going often, 9.9% (32/324) reported going daily because their homes were in the forest, and 3 participants did not answer this question. Most of the participants (73%) had insufficient knowledge of malaria transmission and prevention.

Laboratory tests

None of the subjects had a positive thick blood smear result. Diagnostic PCR was performed with the samples from 320 (98.8%) individuals. Of these, 9 (2.8%) were positive for *Plasmodium* infections, 1 for *P. falciparum* (0.3%), 2 for *P. vivax* (0.6%), and 6 for *P. malariae* (1.9%). These 9 individuals did not have symptoms suggestive of malaria and all had negative smears. The individual with a positive PCR result for *P. falciparum* was a man from the Orindi municipality, and those participants with a positive PCR result for *P. vivax* were 2 women from the Paradise municipality. The 6 participants with positive PCR results for *P. malariae*, 3 women and 3 men, were residents of the Orindi, Garrafão, Paradise, and Monte Olivetti locations (**Table 1**). Although the towns of Paradise and Orindi showed a higher frequency of positive samples, there was no statistically significant association.

Serology

Serological tests were performed in 314 participants and 35 (11.1%) were reactive for any of the three *Plasmodium* species studied. The *P. falciparum* IgG antibody test was reactive in 11 participants (3.5%) and inconclusive in 5 (1.6%), while samples from 24 participants (7.7%) were reactive and 4 (1.3%) inconclusive for *P. vivax* (anti-IgG PvMSP19 antibody). Due to operational constraints, only 42 samples were tested for *P. malariae* (all positive PCR samples and/or reactive serology for *P. vivax* or *P. falciparum*). Thirteen samples were reactive for *P. malariae* antibodies (30.9%, 13/42) and 11 samples (29.7%) were reactive for more than one *Plasmodium* species. **Table 2** summarizes the presence of *Plasmodium* species in positive individuals. **Table 3** shows the association between positive PCR samples and serological reactivity for *Plasmodium* species. Cohen's kappa coefficient (κ) for agreement between molecular and serological results was considered "moderate" for *P. malariae* (κ=0.54, range: 0.27-0.82, confidence interval [CI]: 95%) and "fair" for all *Plasmodium* species (κ=0.29, range: 0.11-0.5, CI: 95%).

Table 4 shows variables associated with IgG reactivity. Individuals who entered the Atlantic Forest region (for hunting, leisure, collecting plants, or other activities) had a 2.7 times increased probability of having a reactive serology for *P. vivax* compared with individuals who did not enter the forest ($p<0.05$). On the other hand, children <15 years of age had a higher chance of reactive serology for *P. falciparum* and *P. vivax* compared with individuals ≥15 years of age ($p<0.05$). Individuals living in the Paraiso district had a higher chance of reactive serology for *P. vivax* and any *Plasmodium* species (but not specifically for *P. falciparum* and *P. malariae*) than people living in other districts ($p<0.05$). There were no associations between sex, symptoms, or past exposure to malaria and serological response to antibodies of any *Plasmodium* species. Individuals who reported previous episodes of malaria were not reactive to any species of *Plasmodium*.

DISCUSSION

The prevalence of autochthonous malaria is low in Southeastern Brazil and in the Southern states of the Atlantic Forest region[18,19,20]. The characteristics of malaria in these areas are different from those observed in the Amazon region[18]. The extra-Amazonian malaria is often described as asymptomatic,

TABLE 1: Frequency of *Plasmodium* infections diagnosed by PCR in the residents of the municipality of Guapimirim, Rio de Janeiro, Brazil, according to the different investigated variables.

	P. falciparum		*P. vivax*		*P. malariae*		Total				
	Pos	Neg	Pos	Neg	Pos	Neg	Pos	Neg	Total	%	p-value
Age											
<15 years	0	80	0	80	1	79	1	79	80	1,3	0.558
≥15 years	1	239	2	238	5	235	8	232	240	3.3	
Total	1	319	2	318	6	314	9	311	320	2.8	
Gender											
M	1	137	0	138	3	135	4	134	138	2.9	0.794
F	0	182	2	180	3	179	5	177	182	2.7	
Total	1	319	2	318	6	314	9	311	320	2.8	
Localities											
Caneca Fina	0	39	0	39	0	39	0	39	39	0	0.08
Garrafão	0	58	0	58	1	57	1	57	58	1.7	
Barreira	0	51	0	51	0	51	0	51	51	0	
Monte Olivete	0	16	0	16	1	15	1	15	16	6.3	
Paraíso	0	54	2	52	1	53	3	51	54	5.6	
Orindi	1	101	0	102	3	99	4	98	102	3.9	
Total	1	319	2	318	6	314	9	311	320	2.8	
Entry to the Atlantic Forest region for hunting, leisure, or collecting plants											
Yes	0	74	0	74	2	72	2	70	72	2.8	0.7005
No	1	244	2	244	4	242	7	241	248	2.8	
Total	1	318	2	318	6	314	9	311	320	2.8	
Symptoms in the month preceding the study											
Yes	0	29	0	29	0	29	0	29	29	0	-
No	1	290	2	289	6	285	9	282	291	3.1	
Total	1	319	2	318	6	314	9	311	320	2.8	
Previous malaria											
Yes	0	4	0	4	0	4	0	4	4	0	-
No	1	315	2	314	6	310	9	307	316	2.8	
Total	1	319	2	318	6	314	9	311	320	2.8	

Pos: positive; **Neg:** negative.

with low parasitemia and predominance of the species *P. vivax*[20,21,22,23]. The municipality of Guapimirim is very near to the mountain ecosystem of the Atlantic Forest region, and our study was carried out in areas where malaria cases had been reported. Our results showed that the majority of the study population had lived in the municipality for the last 18 years and only 2 participants (0.6%) had a history of travel to an endemic area but without acquiring malaria. Only 1.2% had a single previous malaria episode in the past, but all of them showed negative PCR, thick blood smear, and serology results. In contrast to our expectations only one of these people had contracted malaria in the Amazon region Rondônia.

We observed no human malaria symptomatic cases and all thick and thin blood smears were negative for *Plasmodium* species. These results are not unexpected because data from the governmental surveillance system have demonstrated that cases

TABLE 2: Positive serological reactions for IgG anti-*Plasmodium* species antibodies in the population of the municipality of Guapimirim.

Parasite	Number	%
P. vivax (Pv)	14	40.0
P. falciparum (Pf)	3	8.6
*P. malariae (Pm)**	7	20.0
Pv+Pf	5	14.3
*Pv+Pm**	3	8.6
*Pm+Pf**	1	2.8
*Pv+Pf+Pm**	2	5.7
Total	**35**	**100.0**

*IgG for *P. malariae* was assessed only for samples positive in the PCR or serological tests (42 samples).

TABLE 3: Relationship between PCR and serology results of individuals in the municipality of Guapimirim, Rio de Janeiro, Brazil.

PCR	IgG anti-PvMSP119 (n=312)			IgG anti-*P falciparum* (n=313)			IgG anti-*P malariae* (n=42)			Any *Plasmodium* species (n=314)		
	Positive	**Negative**	**Total**	**Positive**	**Negative**	**Total**	**Positive**	**Negative**	**Total**	**Positive**	**Negative**	**Total**
P. vivax												
Positive	1	1	2	1	1	2	1	1	2	1	1	2
Negative	23	287	310	10	301	311	12	28	40	34	278	312
Total	24	288	312	11	302	313	13	29	42	35	279	314
P. falciparum												
Positive	0	1	1	0	1	1	0	1	1	0	1	1
Negative	24	287	311	11	301	312	13	28	41	35	278	313
Total	24	288	312	11	302	313	13	29	42	35	279	314
P. malariae												
Positive	1	5	6	0	6	6	6	0	6	6	0	6
Negative	23	283	306	11	296	307	7	29	36	29	279	308
Total	24	288	312	11	302	313	13	29	42	35	279	314

Cohen's kappa index (κ) for associations between molecular and serological results was 0.07 (range: -0.07-0.20, CI: 95%, p>0.05) for *P. vivax*, -0.07 (range: -0.02-0.01, CI: 95%, p>0.05) for *P. falciparum*, and 0.542 (range: 0.27-0.82, CI: 95%, p<0.05, "moderate") for *P. malariae*. The same calculations were performed for all *Plasmodium* species and identified a kappa index of 0.29 (range: 0.11-0.46, CI: 95%, p<0.05, "fair"). **IgG:** Immunoglobulin G: **PvMSP1-19 :** 19 kDa C-terminal region of the Merozoite Surface Protein 1 de *P. vivax*.

occur throughout the year, with an annual average of 4 or fewer cases across the state particularly during the warmer season[7,23]. Interestingly, malaria outbreaks associated with *P. simium* occurred in areas close to the municipality of Guapimirim in the summers of 2015 and 2016[9]. In our study, 9 individuals (2.8%) with asymptomatic *Plasmodium* infection were diagnosed using PCR analysis, 2 of which were positive for *P. vivax* (0.6%), 1 (0.3%) for *P. falciparum*, and 6 for *P. malariae* (1.9%). None of these individuals developed clinical malaria within 30 days of follow-up. Although most of the infected individuals live in the towns of Orindi and Paradise, there were no significant differences between the assessed locations, probably due to the small number of positive samples. It is important to note the presence of asymptomatic infection in individuals with no previous history of malaria, travel to endemic areas, or contact with a previous malaria-infected person. We speculated that these subclinical infections were probably autochthonous cases. Only a few studies on asymptomatic infection by *Plasmodium* species in the extra-Amazon region have been carried out to date. Curado et al.[24] demonstrated the presence of asymptomatic infections with *P. falciparum* and mixed malaria caused by *P. falciparum* and *P. vivax* in the Atlantic Forest region in São Paulo state. Cerutti et al.[20] conducted a population-based study in the Espírito Santo state and obtained similar results. No parasites were detected by microscopy in 1,777 blood samples from residents of the area with reported malaria cases, but asymptomatic infections with *P. vivax* (1.5%), *P. malariae* (0.9%), and *P. falciparum* (0.5%) were diagnosed by PCR. De Alencar et al.[25] in Espírito Santo state found a prevalence of 3.4% of *P. malariae* and *P. vivax* infections. Maselli et al[26] revealed a prevalence of *P. falciparum* (5.14%) and *P. vivax* (2.26%) infections in healthy blood donors in São Paulo state. Donors with asymptomatic *Plasmodium* infection could be reservoirs of transfusion-transmitted malaria (TTM). As these individuals have low parasitic infections with negative thick blood smear results, detection can be difficult using routine laboratory tests[26]. In our study, we identified 2.8% of participants with asymptomatic infections, 6 of which were infected with *P. malaria*, the most frequent parasite associated with TTM in the Americas. Thus, further studies should be conducted to identify risk factors in these asymptomatic individuals, to establish diagnostic methods, and to prevent TTM.

In contrast to asymptomatic *Plasmodium* infections that can only be detected by PCR, the presence of anti-*Plasmodium* antibodies can reveal recent or past infections[27]. In our study, 11.8% of specimens were reactive to any of the tested antigens, with 3.5% reactive to *P. falciparum*, 7.7% to *P. vivax,* and 30.9% of a subsample of 42 individuals reactive to *P. malariae.* The frequency of reactivity was low in relation to the study of Azevedo (1997, unpublished data) in which 47.8% of samples from the outbreak of 1993 in the Rio Bonito district showed reactivity of IgG antibodies and 17.4% of IgM antibodies (indirect immunofluorescence). These studies were repeated in the area in 1996 with a higher number of subjects and revealed a frequency of 35.4% of IgG antibodies against the asexual blood forms of *P. vivax*, while no IgM antibodies were found. These findings were corroborated by Mattos et al.[6] Cerutti et al.[20] performed a cross-sectional study of 65 patients and all specimens showed a positive reaction to all the variants of antibodies against the circumsporozoite protein of *P. vivax*, with positive reactions to *P. vivax* classic (VK210) in 25.4%, to VK247 in 6.3%, to *P. vivax*-like in 10%, and to *P. malariae* in 15.1% specimens. The same authors assessed healthy individuals that had been in contact with malaria patients and observed a high percentage of antibodies to *P. vivax* (37.7% IgG and 6.2% IgM) and *P. malariae* (44.6% IgG and 15.8% IgM) and a prevalence of *P. falciparum* reactivity of 13.5% for IgM and 13% for IgG antibodies against asexual forms of the parasite. With our results, we cannot conclude that serology can predict infection.

TABLE 4: Reactivity associated with IgG anti-PvMSP119, anti-Pf IgG, and anti-Pm IgG in specimens from residents of the Guapimirim municipality, Rio de Janeiro, Brazil.

	***P. vivax* (n=312)**			***P. falciparum* (n=313)**			***P. malariae* (n=42)[1]**			***Any species* (n=314)**		
	%Pv	uOR (CI: 95%)	aOR (CI: 95%)	%Pf	uOR (CI: 95%)	aOR (CI: 95%)	%Pm	uOR (CI: 95%)	aOR (CI: 95%)	%	uOR (CI: 95%)	aOR (CI: 95%)
Age												
≥15 years	6.3	0.49(0.20-0.18)	**0.36(0.14-.96)***	2.1	**0.25(0.07-0.8)***	**0.03(0.00-0.09)***	29.0	0.71(0.16-3.06)	0.93(0.73-1.16)	9.7	0.57(0.27-1.21)	0.52(0.23-1.19)
<15 years	12	-	-	7.9	-	-	36.9	-	-	15.8	-	-
Total	7.7	-	-	3.5	-	-	30.9	-	-	11.1	-	-
Sex												
Male	7.5	0.96(0.41-.23)	-	3.8	1.13(0.34-3.8)	-	21.0	0.38(0.09-1.56)	-	11.2	1.01(0.49-2.06)	-
Female	7.8	-	-	3.3	-	-	39.1	-	-	11.1	-	-
Total	7.7	-	-	3.5	-	-	30.9	-	-	11.1	-	-
Entry to the forest for hunting, leisure, or collecting plants												
Yes	14.3	**2.71(1.15-6.4)***	**2.73(1.01-7.38)***	4.3	1.31(0.34-5.2)	1.02(0.54-2.20)	44.4	0.98(0.24-4.07)	0.69(0.54-0.86)	17	1.99(0.93-4.23)	2.09(0.87-4.84)
No	5.8	-	-	3.3	-	-	45.0	-	-	9.4	-	-
Total	7.7	-	-	3.5	-	-		-	-	11.1	-	-
Housing location												
Barrera	2.0	1	1	0	1	1	33.3	1	1	3.8	1	1
Monte Oliveti	13.3	1.29(0.41-9.1)	12.62(1.06-297)	0	**	-	100.0	**	-	13.3	1.24(0.27-5.74)	5.01(0.55-46.52)
Garrafão	3.7	0.41(0.09-1.8)	2.53(0.23-56.42)	0	**	-	20.0	0.52(0.05-5.20)	-	5.5	0.41(0.12-1.38)	1.55(0.24-12.25)
Caneca Fina	15.8	2.67(0.98-7.2)	8.71(1.31-173.74	7.9	2.86(0.72-11.3)	-	42.9	1.87(0.35-9.93)	-	18.4	2.00(0.81-4.21)	5.17(1.11-37.29)
Orindi	5.9	0.68(0.26-1.7)	3.48(0.55-67.97)	4	1.21(0.35-4.22)	-	23.1	0.57(0.127-2.55)	-	11.9	1.11(0.53-2.34)	3.69(0.93-24.73)
Paraiso	13.2	2.16(0.85-51)	**10(1.62-94.85)***	7.5	2.95(0.83-10.5)	-	25.0	0.67(0.147-3.02)	-	17	1.83(0.81-4.21)	**5.98(1.42- 41.21)***
Total	7.7	-	-	3.5	-	-	31.0	-	-	11.1	-	-
Symptoms in the month preceding the study												
Yes	3.8	0.46(0.06-.53)	-	3.8	1.10(0.13-9.01)		0	**	-	3.8	0.29(0.04-2.27)	-
No	8	-	-	3.5	-	-	30.9	-	-	11.8	-	-
Total	7.7	-	-	3.5	-	-	30.9	-	-	11.1	-	-
Previous malaria												
Yes	0	**	-	0	**	-	0	**	-	0	**	-
No	7.8	-	-	3.6	-	-	30.9	-	-	11.3	-	-
Total	7.7	-	-	3.5	-	-	30.9	-	-	11.1	-	-

*p<0.05, **Undefined. **CI:** confidence interval. **aOR:** adjusted odds ratio, **uOR:** unadjusted odds ratio. [1]Only 42 samples.

The main finding of our study is the association between entering the forest and positive serology for *Plasmodium vivax*. The chance to have a positive serology was 2.7 times (range: 1.01-7.38, CI: 95%, p<0.05) higher for people who entered the forest compared with those that did not. These results differ from those obtained by Maselli et al.[26] who showed a positive association between residing in the mountain area of the Atlantic Forest region with *P. falciparum* and *P. vivax* infections and forest fragmentation. Nonetheless, the study was carried out with samples from blood donors in São Paulo state and the origin of samples could explain the divergences from our results.

The presence of asymptomatic *P. falciparum* infections detected in our study is a cause of concern, but other authors[20,24,26] made the same observation in the Espírito Santo and São Paulo states. Recently, Laporta et al.[28] showed an unexpectedly high proportion of *P. falciparum* on anophelines in the São Paulo state. These results are challenging the traditional "bromeliad-malaria" paradigm that proposes a sylvatic cycle in bromeliad areas with an interaction between an *An. kerteszia* vector (particularly *An. cruzii*), a non-human primate reservoir (*Alouatta* species, *Brachyteles* species, and *Cebinae* subfamilies), and a *P. simium* or *P. vivax* parasite[9,30,31,32,33,34,35]. As PCR used in our study cannot differentiate between a *P. vivax* and *P. simium* infection, more sophistical analyses are required. Humans could be accidentally infected when they enter the forest for recreation or work by invading the transmission cycle of the parasite in the wild[36,37,38,39,40]. On the other hand, *P. falciparum* is a virulent *Plasmodium* species in the Amazon region and any infection by this parasite should result in malaria disease if the individual is not immune. The fact that individuals do not develop symptoms in the extra-Amazonian region may be due to antigenic variability of *P. falciparum,* but this question must be further clarified in future studies involving sequencing of parasite DNA[29,41]. Another important and unexpected finding of our study was the higher

chance of obtaining positive serologies for *P. falciparum* and *P. vivax* in individuals under 15 years of age compared to older individuals. No previous study has reported a similar trend, and our findings may present a novel epidemiological scenario in this context that needs to be elucidated in future studies.

Enhancing the knowledge on malaria in residents of the extra-Amazonian areas is an important aspect of disease elimination. Although transmission in this area was interrupted 50 years ago[5], 73% of actual residents have insufficient knowledge on malaria transmission and prevention. Azevedo et al.[6] found a similar percentage in the Nova Friburgo region. It is noteworthy that 79.3% of participants associated the summer with an increase in mosquito density particularly at nightfall, but a large proportion of the population did not use any control measures for avoiding insect bites. Malaria diagnosis is a big challenge in these areas because medical doctors often do not consider the disease when a patient presents with fever[22].

According to the WHO, residual malaria occurs sporadically in places with interrupted transmission and remaining determinants of transmission[42]. These areas require assessment of favorable locations for *Anopheles* larvae habitats, food sources, and invasion of *Anopheles* by *Plasmodium*. All these conditions are present in the Atlantic Forest region, but on a much smaller scale compared to the Amazon region[36,37,38,39,40]. Albuquerque et al. [43] recently constructed a model for evaluation of territory receptivity in order to strengthen entomological surveillance of *Nyssorhynchus* mosquitoes for imported malaria cases. The study showed that pluviosity, temperature, geomorphology, and vegetation variables could be used to create a model of surveillance, but a specific model needs to be generated for *Kerteszia* anophelines responsible for bromeliad malaria in these areas.

Acknowledgments: We offer our deepest thanks to the institutions that provided technical support for our study. We thank Arianni Rondelli Sanchez and Eduardo Milton Ramos-Sanchez for preparing the *P. vivax* recombinant, Fabiana Maria de Souza Leoratti for preparing the extract, Dr. Silvia Di Santi for blade preparation of *P. malariae* and Dr. Daniel Câmara for statistical analysis. Special thanks to the research participants in the Guapimirim municipality.

Conflict of Interest: The authors declare that there are no conflicts of interest.

Financial Support: This research was funded by *Fundação Carlos Chagas Filho de Amparo à Pesquisa do Estado do Rio de Janeiro* (FAPERJ, financial support process 214879/E_06/2015) and *Coordenação de Aperfeiçoamento de Pessoal de Nível Superior* (CAPES, financial support 001).

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