Malaria in pregnant women living in areas of low transmission on the southeast Brazilian Coast: molecular diagnosis and humoural immunity profile

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Studies on autochthonous malaria in low-transmission areas in Brazil have acquired epidemiological relevance because they suggest continued transmission in what remains of the Atlantic Forest. In the southeastern portion of the state of São Paulo, outbreaks in the municipality of Juquitiba have been the focus of studies on the prevalence of Plasmodium*, including asymptomatic cases. Data on the occurrence of the disease or the presence of antiplasmodial antibodies in pregnant women from this region have not previously been described. Although* Plasmodium falciparum *in pregnant women has been widely addressed in the literature, the interaction of* Plasmodium vivax *and* Plasmodium malariae *with this cohort has been poorly explored to date. We monitored the circulation of* Plasmodium *in pregnant women in health facilities located in Juquitiba using thick blood film and molecular protocols, as well as immunological assays, to evaluate humoural immune parameters. Through real-time and nested polymerase chain reaction,* P. vivax *and* P. malariae *were detected for the first time in pregnant women, with a positivity of 5.6%. Immunoassays revealed the presence of IgG antibodies: 44% for ELISA*-Pv*, 38.4% for SD-Bioline*-Pv *and 18.4% for indirect immunofluorescence assay*-Pm*. The high prevalence of antibodies showed significant exposure of this population to* Plasmodium*. In regions with similar profiles, testing for a malaria diagnosis might be indicated in prenatal care.*

Key words: autochthonous malaria - pregnancy - molecular diagnostic techniques - humoural - immunity - asymptomatic infections

Pregnant women and children are the main groups at risk of acquiring malaria worldwide. Every year, 125 million women from endemic countries become pregnant. In areas of low transmission for *Plasmodium falciparum*, pregnant women have little or no immunity against the disease and usually suffer severe episodes of malaria. In areas of high or moderate transmission, there are significant levels of acquired immunity and the effects on the mother and foetus are less severe. Although malaria caused by *P. falciparum* in pregnant women has been widely investigated, the interaction of this cohort with *Plasmodium vivax* and *Plasmodium malariae* requires a more comprehensive approach. The World Health Organization (WHO) recommends four antenatal care visits, including malaria tests. However, this criterion depends on the local conditions and specific orientations for each area (Dellicour et al. 2010, WHO 2013). *P. vivax* is responsible for most cases of malaria in Asia and the Americas, with reports of morbidity in pregnant women and serious consequences, such as maternal anaemia and low birth weight in children (Nosten et al. 1999). The geographic distribution of *P. malariae* infections in pregnant women and the adverse effects on maternal and newborn health are unknown (Dellicour et al. 2010).

In Brazil, 99% of malaria infections occur in the Amazon Region and 166,864 cases were registered in 2013 (MS 2014a). Martínez-Espinosa (2003) reported a prevalence of 4.3% in pregnant women in the municipality of Coari, state of Amazonas. Non-pregnant women of the same age showed a positivity of 0.8%. Luz et al. (2013) reported that in 2007, malaria cases diagnosed in pregnant women comprised 6.7% of fertile-age women in three municipalities located in the Amazon Region. *P. vivax* was the most predominant species (80%). From the 13,308 malaria cases reported in Manaus from 2003-2006 among women aged 10-49 years, 6.1% were in pregnant women. *P. vivax* was responsible for 85% of the infections and *P. falciparum* for 14.3% (Almeida et al. 2010).

Although malaria is not considered endemic outside the Brazilian Amazon Region, autochthonous cases are registered in areas covered by the Atlantic Forest. This biome, distributed in 17 coastal states, comprises a unique ecosystem in which important mountain ranges are located. In this region, from 2012-2013, 188 autochthonous cases were reported. The states of Espírito Santo (ES), São Paulo (SP) and Piauí (PI) reported 72.3% of the infections, with 75, 43 and 27 cases, respectively (MS 2014b). In a retrospective study conducted in SP

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from 1980-2007, *P. vivax* accounted for 97.2% of 816 malaria autochthonous cases, most of which were transmitted along the Atlantic Coast. The main symptoms were fever, headache and chills and 9.6% were asymptomatic (Couto et al. 2010). In the southeastern part of SP, malaria outbreaks have been reported in the municipality of Juquitiba, located in the Atlantic Forest biome, where surveys on *Plasmodium*'s occurrence show the presence of submicroscopic parasitaemias and asymptomatic cases in inhabitants (Branquinho et al. 1997). In this region, where the transmission of *P. vivax* was established by several studies (Carvalho et al. 1988), our group detected, for the first time using molecular tools, the occurrence of *P. malariae* (Kirchgatter et al. 2005). In addition, the transmission in this region seems to involve wild primates that act as reservoirs for *Plasmodium brasilianum* and *Plasmodium simium* (Curado et al. 2006). The primary vectors involved in this scenario are *Anopheles (Kerteszia) cruzii* and *Anopheles (K.) bellator*; however, *Anopheles (N.) marajoara* may participate in the dynamics of malaria transmission. *Anopheles (K.) cruzii* is present mainly in the forested slopes of the Serra do Mar, *An. (K.) bellator* in the coastal land and *An. (N.) marajoara* in the deforested areas (Laporta et al. 2011).

Despite autochthonous malaria transmission reports having been well established outside the Amazon Region, there is a lack of data on the prevalence of autochthonous malaria in pregnant women living in low-endemicity areas of Brazil. In this study, we aimed to determine the frequency of pregnant women harbouring *Plasmodium* and the level of antiplasmodial antibodies in a cohort living in a low-endemicity area.

SUBJECTS, MATERIALS AND METHODS

Study area and population - Juquitiba is located 71 km from the capital of SP (Fig. 1) in the Atlantic Forest biome, at an altitude of 685 m. Approximately 67% of its length is covered by bushland and the preserved native forest promotes great movement of individuals due to ecotourism. Juquitiba has a population of 28,737 inhabitants, of whom 49.42% are female (Brazilian Institute of Geography and Statistics, available from: ibge.gov.br/home/estatistica/populacao/censo2010/tabelas_pdf/total_populacao_sao_paulo.pdf). Approximately 450 pregnant women are recorded annually, according to the health secretary of the municipality. These women receive prenatal care in five public healthcare units: Centro, Barnabés, Justino, Jardim das Palmeiras and Palmeiras.

Fig. 1: location of the municipality of Juquitiba. The small map on the left shows the location of the state of São Paulo, Brazil.

Study design and data collection - This is a prospective cohort study with no probabilistic consecutive sampling. The population at risk was defined as childbearing age women (10-49 years) living in Juquitiba, a definition that encompassed 9,169 women (Brazilian Institute of Geography and Statistics, available from: ibge.gov.br/home/estatistica/populacao/censo2010/tabelas_pdf/total_populacao_sao_paulo.pdf). The inclusion criterion was pregnant women of any age living in Juquitiba and the exclusion criterion was the presence of any infectious disease other than malaria. The exposure variable was defined to encompass pregnant women living in or having been displaced to areas near the forest in Juquitiba. The outcome variable was the presence of parasites in the cohort of pregnant women. The pregnant women were invited to participate in the study at the time they received prenatal care in the five health facilities in the municipality. Blood collections were held in the five healthcare units of the municipality, between October 2012-November 2013, by signing the informed consent form. At the moment of inclusion, each pregnant woman answered a questionnaire with the assistance of a health professional with regard to her address, age, civil status, race, a previous pregnancy history, any personal and/or family history of malaria, any displacement to endemic areas, a clinical description, the presence of infectious disease and other health issues.

Collection and samples processing - Peripheral venous blood was collected in a 5-mL ethylenediamine tetraacetic acid (EDTA) tube in the first, second and third trimesters of pregnancy, according to the time of inclusion in the study. The blood was used to perform a thick blood smear (TBS), polymerase chain reaction (PCR), haemoglobin measurement using HemoCue® and immunoassays. After centrifugation, the pellets containing the erythrocytes and the plasma were stored at -20ºC. Blood samples from newborns whose mothers tested positive for malaria were collected by heel punctures. The samples were used to perform TBSs and were plotted on Whatman 3® filter paper (Sigma-Aldrich®, USA) for DNA extraction and real-time PCR.

Haemoscopy by TBS - All slides were air dried and stained following the Giemsa standard protocol immediately after arriving in the laboratory. Two slides were analysed for each individual. The reading was performed using an immersion lens (1,000X) until the count of 500 white blood cells (WBC) was reached, which corresponded to 25 min of observation (WHO 2009). Two investigators without knowledge of the molecular and serological results examined the slides independently.

Genomic DNA extraction - The pellet containing erythrocytes was lysed with 1% saponin (Sigma-Aldrich)

and washed and 200 µL were used for DNA extraction with a QIAamp® DNA Blood Mini Kit (Qiagen Hilden, Germany). Samples plotted on Whatman 3® filter paper (Sigma-Aldrich) were extracted with Chelex 100® (Bio-Rad™, USA) according to a protocol described elsewhere (Plowe et al. 1995).

Real-time PCR - The protocol described by Lima et al. (2011) was applied for genus-specific amplification targeting the ssrRNA gene of *Plasmodium*. The M60 and M61 primers and the M62 probe were used and the reactions were performed with 2.5 µL of gDNA, 12.5 µL of 2x TaqMan® Universal PCR Master Mix, 500 nM of each primer and 300 nM of FAM™ and TAMRA™-labelled probe (Applied Biosystems, USA). Amplification and detection were carried out according the following procedure: 50ºC for 2 min and 95ºC for 15 min, followed by 40 cycles at 94ºC for 30 s and a final cycle at 60ºC for 1 min. Duplicate samples were assayed on the ABI Prism 7300 system (Applied Biosystems) using negative (ultrapure water) and positive controls (*Plasmodium* DNA from a 1 parasite/µL culture). To monitor the performance of real-time PCR, DNA from *P. falciparum* in vitro cultures was included in all assays to obtain a standard curve, with parasitaemias ranging from 350-0.35 parasites/µL. A cut-off was established based on the average plus 2 standard deviations (SD) of the threshold cycle (Ct) values from the *P. falciparum* samples with 1 parasite/µL, included in all plates.

Nested PCR - The samples with positive results in the real-time PCR were processed by nested PCR using ssrRNA genes as the targets (Snounou et al. 1993). The first reaction employed the genus-specific primers rPLU5 and rPLU6 and the second reaction used species-specific primers for *P. falciparum*, *P. malariae* and *P. vivax*. The reaction was prepared with 25 µL, consisting of 250 nM of each primer, 125 µM of deoxynucleoside triphosphates, 2 mM $MgCl_2$, 50 mM KCl, 10 mM Tris pH 8.3, 0.4 U *Taq* polymerase and 2 µL of genomic DNA. The cycling conditions were as follows: 95ºC for 5 min, 58ºC for 2 min and 72ºC for 2 min, followed by 24 cycles at 94ºC for 1 min, 58ºC for 2 min and 72ºC for 2 min and ending with 72ºC for 5 min. In the second reaction, 30 cycles of amplification were performed under the same conditions, adding 1 µL of the first reaction product. The fragments were separated by electrophoresis in 1.5% agarose/tris-borate-EDTA gel and visualised with ethidium bromide through ultraviolet light. Negative (ultrapure water) and positive controls (gDNA of *P. falciparum*, *P. vivax* and *P. malariae*) were included in all tests.

ELISA - An adapted protocol described elsewhere was applied for the detection of IgG antibodies against *P. falciparum* and *P. vivax*. Crude *P. falciparum* antigen was extracted with Zwittergent® (Calbiochem®, Germany) and employed at 0.5 µg/mL for the detection of IgG antibodies against all *Plasmodium* species through cross-reactions (Sanchez et al. 1993, Coelho et al. 2007). To detect *P. vivax* antibodies, the recombinant antigen His_6*Pv*$MSP1_{19}$ was used at 4 µg/mL (Coelho et al. 2007). The plasmid pET14b-*Pv*$MSP1_{19}$ (kindly provided by Dr Irene Silva Soares) was expressed in *Escherichia coli* BL21-CodonPlus® (DE3)-RIL (Stratagene, USA) and purified by affinity chromatography using the Pro-Bond™ Purification System (Life Technologies, USA) under denaturing conditions, according to the manufacturer's instructions. Plates were coated with the antigen for 2 h at 37ºC and overnight at 4ºC and blocking was performed with 5% skim milk at 37ºC for 2 h. The plasma samples were diluted to 1/100 for the assays. Anti-human IgG (Fc specific)-peroxidase antibody produced in goats (Sigma-Aldrich) was used at a dilution of 1: 20.000. The enzymatic reaction was conducted using tetramethylbenzidin/H_2O_2 (Life Technologies) at room temperature in dark conditions. The reaction was interrupted with 2N H_2SO_4 (Merck, Germany). A spectrophotometric reading at 450 nm was performed on a Titertek Multiskan MCC/340 (Labsystems Diagnostics Group, Finland). The cut-off of each reaction was determined using the receiver operating characteristic (ROC) curve from the absorbances of the positive and negative samples (Greiner et al. 2000). The reactivity index (RI) (RI = absorbance/cut-off) was calculated and samples with RI $\geq$ 1 were considered positive.

Indirect immunofluorescence assay (IFA) - The protocol by Ferreira and Sanchez (1988) was applied to detect IgG antibodies against *P. malariae*. Multi-spot slides (Thermo Fisher Scientific, USA) were coated with blood collected from a patient infected with *P. malariae* for the first time. Plasma samples diluted to 1/40 were incubated for 30 min at 37ºC in a moist chamber and washed three times with phosphate buffered saline for 10 min. Subsequently, the antibody-antigen complex was overlaid with fluorescein isothiocyanate-conjugated goat anti-human IgG (γ chain-specific) at 1/200 (Fluoline G; BioMérieux, France) and incubated according to the same conditions. The slides were mounted in alkaline glycerin and observed under a fluorescence microscope. Positive and negative controls were used for each slide.

SD Bioline Malaria Pf/Pv immunochromatographic test - An immunochromatographic test for the detection of antibodies against *P. falciparum* and *P. vivax* was used according to the manufacturer's instructions. Briefly, 10 µL of plasma were dispensed in the device well, followed by 110 µL of the assay diluent and the test was interpreted after 15 min. The SD Bioline Malaria *Pf*/*Pv* test detects antibodies against circumsporozoite surface protein (CSP) and merozoite surface protein (MSP) recombinant antigens. The control colour band indicated that the test worked properly.

Statistical analysis - The results were analysed using Microsoft Excel 2013, Sigma Stat 3.5, GraphPad Prism 5.0 and GraphPad Software. The levels of significance were set to accept a type-1 error of 5% ($\alpha = 0.05$). The sensitivity and specificity of the ELISA tests and the 95% confidence intervals (CI) were calculated using the ROC curve. The proportions were compared using Fisher's exact test. The Mann-Whitney *U* rank sum test was used to compare ranks. The techniques were compared using McNemar's test. The agreement between the results of the techniques was assessed using the Cohen kappa (K) index and its 95% CI.

Ethics - This study was approved by the Ethical Committee of the Faculty of Medicine, University of São Paulo, and by the National Committee for Ethics on Research.

RESULTS

Sampling - Blood samples from 125 pregnant women were collected from October 2012-November 2013 in five healthcare units: Centro (11/125), Barnabés (75/125), Justino (31/125), Palmeiras (2/125) and Jardim das Palmeiras (6/125). The mean age was 24.2 (± 6.02; range, 13-40). None of the pregnant women referenced a previous history of malaria or displacement to an endemic area. The number of subjects admitted to the study represented 27.78% of the 450 pregnant women, which was equal to the average registered in the municipality per year. At the follow-up, 78 pregnant women underwent second blood collections and 18 underwent second and third collections. Five newborns were tested using TBS and real-time PCR.

Haemoscopy by TBS - Among the 125 samples from the first collection, two tested positive by microscopy, showing positivity (95% CI) of 1.6% (0.4-5.7) after two independent diagnoses. One pregnant woman was positive for *P. vivax* and one for *P. malariae*. Parasitaemias of the TBSs were calculated as numbers of parasites/µL:

$$P = \frac{\text{WBC count/µL x number of parasites in 500 WBC}}{500}$$

Both pregnant women showed parasitaemias of 24 parasites/µL and no newborn of the positive mothers showed *Plasmodium* infection when tested by TBS.

Real-time PCR and nested PCR - Samples with Cts under 37.28 were considered to be positive in real-time PCR. This value was based on the average of the 1 parasites/µL controls ± 2 SD. Of the 125 pregnant women admitted into the study, the samples of seven were amplified by real-time and nested PCR in at least one blood collection (Table I). Among them, six were followed in the Barnabés healthcare unit and one in the Centro healthcare unit. The positivity (95% CI) in both PCR assays was 5.6% (1.7-9.0). *P. vivax* was detected in three pregnant women and *P. malariae* in four. Positive pregnant women were treated according to Brazilian guidelines: chloroquine at 25 mg/kg total doses over three days (MS 2010). None of the newborns from positive mothers showed *Plasmodium* infection when tested using real-time PCR.

Immunoassays - Immunoassays with plasma from all pregnant women included in the study (n = 125) showed positivities (95% CI) of 44% (35.6-52.7) for ELISA-*Pv*, 6.4% (3.3-12.1) for ELISA-*Pf*, 38.4% (30.3-47.1) for SD Bioline *Pv*, 2.4% (0.8-6.8) for SD Bioline *Pf* and 18.4% (12.6-26.1) for IFA-*Pm*.

The IFA-*Pm* was considered positive based on the independent readings of two investigators using positive and negative controls. The SD Bioline *Pf*/*Pv* devices were read according to the manufacturer's instructions. Cut-offs for ELISA assays were calculated using ROC curves with positive and negative controls, generating a value of 0.090 for *P. vivax* and 0.200 for *P. falciparum*.

Table II shows the immunoassays' positivities during the quarterly follow-up. Considering all samples collected from the pregnant women (n = 220), positivities (95% CI) of 34.6% (28.3-41.2) for SD Bioline *Pv* and 38.2% (31.7-44.9) for ELISA-*Pv* were obtained (McNemar's test, $p < 0.099$). The strength of agreement between the two assays detecting anti-*P. vivax* IgG antibodies was considered to be "very good", k = 0.823 (95% CI: 0.746-0.901).

The positivities of the immunoassays varied across the different neighbourhoods. Fig. 2 presents the RI of ELISA-*Pv*, showing higher IgG antibodies levels in pregnant women living in Barnabés, Juquitiba. In the first and second collections, comparing Barnabés with all other neighbourhoods studied, a significant difference existed not only in the ranks of the RI (Mann-Whitney *U* rank sum test, $p < 0.0001$), but also in positivity (Fisher's exact test, $p < 0.0001$). Due to the small number of samples, no difference was observed in the third collection (Mann-Whitney *U* rank sum test, $p = 0.9549$; Fisher's exact test, $p = 0.6029$).

TABLE I

Samples from pregnant women positive by real-time and nested polymerase chain reaction (PCR)

	First collection		Second collection		Third collection		Postpartum	
Sample	Real-time (Ct)	Nested sp.	Real-time (Ct)	Nested sp.	Real-time (Ct)	Nested sp.	Real-time (Ct)	Nested sp.
B 12	36.16	*Pv*	NEG	NEG	ND	ND	NEG	NEG
B 24	34.19	*Pm*	36.01	NEG	37.02	*Pm*	35.98	*Pm*
B 59	36.91	*Pm*	36.34	*Pm*	35.19	*Pm*	ND	ND
B 65	33.22	*Pm*	ND	ND	ND	ND	32.39	*Pm*
B 72	35.23	*Pm*	NEG	NEG	ND	ND	ND	ND
C 3	NEG	NEG	34.14	*Pv*	32.45	*Pv*	NEG	NEG
B 8	NEG	NEG	ND	ND	ND	ND	37.06	*Pv*

the threshold cycles (Ct) represent the average of the duplicates in genus specific real-time PCR assays. *Plasmodium vivax* (*Pv*) amplified a fragment of 120 base pairs (bp) and *Plasmodium malariae* (*Pm*) 144 bp. ND: not done; NEG: negative.

TABLE II

Positivity of plasma samples from pregnant women during the quarterly follow-up

Immunoassay	Sample collections n/n (%)		
	First	Second	Third
ELISA-*Pv*	50/125 (40)	27/78 (34.6)	7/17 (41.2)
ELISA-*Pf*	5/125 (4)	5/75 (6.7)	2/17 (11.7)
SD Bioline *Pv*	47/125 (37.6)	22/78 (28.2)	7/17 (41.2)
SD Bioline *Pf*	2/125 (1.6)	0/78 (0)	1/17 (5.9)
IFA-*Pm*	19/125 (15.2)	12/81 (14.8)	2/17 (11.7)

ELISA-*Plasmodium falciparum* (*Pf*) with crude extract of *P. falciparum* from culture; ELISA-*Plasmodium vivax* (*Pv*) was assayed with $His_6PvMSP1_{19}$ antigen; indirect immunofluorescence assay-*Plasmodium malariae* (IFA-*Pm*) with *P. malariae* clinical sample.

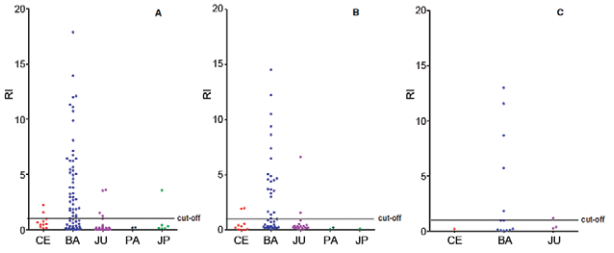

Fig. 2: reactivity index (RI) of plasma samples assayed by ELISA-*Pv* with $His_6PvMSP1_{19}$ recombinant antigen during the follow-up of pregnant women from each locality of the municipality of Juquitiba, state of São Paulo, Brazil. Cut-off = 0.090. A: first collection (n = 125); B: second collection (n = 78); C: third collection (n = 17); CE: Centro; BA: Barnabés; JU: Justino; PA: Palmeiras; JP: Jd. das Palmeiras.

Clinical aspects - Among the positive pregnant women, one reported fever and one reported chills. Other non-specific symptoms were reported, such as headache and tiredness. The haemoglobin measures were normal (≥ 12 g/dL) in all but one pregnant woman, who presented with a level of 11.4 g/dL in the first survey, when she was negative for *Plasmodium* and 9.8 g/dL after she had become infected with *P. vivax*.

DISCUSSION

For the first time, infections due to *P. vivax* and *P. malariae* were detected in pregnant women living in a low-endemicity area of the Brazilian southeastern coast. No displacements outside SP were reported by these women. In the extra-Amazon Region, 691 malaria cases diagnosed by TBS were reported in 2013, most having been imported from the Amazon Region or other countries and presenting with classical malaria symptoms. Among them, 84 were autochthonous and ES, SP and PI reported 79.8% (MS 2014b). Most cases are oligosymptomatic and present low parasitaemias, thereby decreasing the opportunity of detection by haemoscopy. The diagnosis of *Plasmodium* by TBS enables species differentiation and quantification. However, the sensitivity depends on the microscopist's experience and can range from 5-500 parasites/μL (Milne et al. 1994). In this context, molecular tools based on the detection of parasite DNA are very useful (Snounou et al. 1993, Lima et al. 2011).

In SP, autochthonous transmission was detected along the northeastern and southeastern coasts, which was associated with the close contact between humans and the Atlantic Forest biome, leading to exposure to *Anopheles* mosquitoes, whose breeding grounds are the bromeliads (Deane 1992, Curado et al. 2006, Marques et al. 2008). Cerutti et al. (2007) offered two hypotheses for such transmission in a similar setting. The first is a suspected greater number of undetected cases, which are self-limited or asymptomatic. The second is the presence of simian reservoirs of parasites, acting as a source of infection for humans. This latter hypothesis is corroborated by the fact that autochthonous *P. malariae* was detected in SP by our group. It is important to note that *P. malariae* from humans is undistinguishable from *P. brasilianum*, which infects non-human primates, suggesting a host transfer (Escalante et al. 1998), as well as *P. vivax* and *P. simium* (Escalante et al. 2005). In a survey of non-human primates in Brazil, the highest infection rate (35.6%) was recorded in the southeastern region, with *P. brasilianum* accounting for 46.3% and *P. simium* for 37.5% of the positive blood samples (Deane 1992).

The studied area is considered "low-endemicity". However, the asymptomatic cases detected in the region over time suggest a higher transmission rate than has previously been reported. In this scenario, our results showed a positivity of 1.6% by haemoscopy. The parasitaemias of the two positive TBSs were 24 parasites/μL, in agreement with previous reports of autochthonous cases in SP, when very low parasitaemias represented 61% of the infections (Couto et al. 2010). Because the inclusion criteria had included all pregnant women in the five public healthcare units of Juquitiba who agreed to participate, sample selection bias should not be expected. The positivity of real-time and nested PCR was 5.6%. Six out seven (4.8%) of the positive pregnant women resided in Barnabés, a district located in a rural area, very close to the forest. The other woman, despite living in a central area, probably acquired the infection during displacements to rural areas, because she is a local health worker. It is interesting to note that *P. vivax* was the species reported as the most prevalent in SP, when, based on microscopy data (Carvalho et al. 1988, Marques et al. 2008, Couto et al. 2010), *P. malariae* and *P. vivax* are frequently misdiagnosed (Kawamoto et al. 2002). Molecular tools are able to detect *P. malariae* in areas where this species is significant for the dynamics of local transmission (Kirchgatter et al. 2005). Our survey showed a prevalence of 3.2% of *P. malariae* infections in Juquitiba, 3.5 times higher than that reported in ES (Cerutti et al. 2007).

Although serology is not used for malaria diagnosis, it is useful for determining the exposure of individuals, thereby providing significant data on the parasite-host interaction. Junqueira et al. (2007) reported asymptomatic

P. vivax in the postpartum period of a woman living in the Atlantic Forest area, with IgM and IgG antibodies. Her twin newborns only presented IgG antibody titres. TBS and PCR resulted in negatives for the mother and the neonates; nevertheless, immunohistochemistry with *Plasmodium* antigens was positive. The spontaneous clearance of parasitaemia after delivery could explain the negative TBS and PCR results (Nguyen-Dinh et al. 1988). In our study, the same event occurred in a pregnant woman positive for *P. vivax* during the follow-up, for which antibodies presented only in the postpartum period. The ELISA-*Pv* positivity was 44%, in very good agreement with the SD Bioline-*Pv* (38.4%), but indicating a high exposure of pregnant women to the parasite. It is important to note that the recombinant antigens used in this study provide a specific antibody response against the MSP1 protein present in the blood stage of *Plasmodium*, indicating infection instead of merely inoculation of sporozoites, as detected by ELISA protocols based on CSP antigens. On the other hand, IFA is based on crude antigens for the detection of antibodies, thus being less sensitive and specific. This fact may explain IFA's low positivity in an area where *P. malariae* is the most prevalent species.

Studies conducted in a similar scenario reported positivities ranging from 32-49% (Curado et al. 2006) and 37.7% (Cerutti et al. 2007) for IFA-*Pv*. With respect to the immune response to *P. malariae* in our survey, IFA-*Pm* was positive in 18.4% of the samples, very similar to the frequency of 16-19.3% reported by Curado et al. (2006). It is noteworthy that, in ES, PCR showed a positivity of 0.9% for *P. malariae*, whereas the IFA-*Pm* for IgG antibodies was positive in 7.9% of residents, suggesting that individuals could present spontaneous cures without specific treatment, because they had no symptoms. In our results, we found higher positivity in both PCR (3.2%) and IFA-*Pm* (18.4%) for the pregnant women. One can argue about the reason for such differences. In fact, our survey was conducted in an immunosuppressed cohort, because pregnant women develop an immune adaptation in order to avoid foetal rejection (Hunt 1992). The maternal immunosuppression is both specific, to maintain the foetus, and nonspecific, increasing the risk of acquiring malaria (Weinberg 1984). Mayor et al. (2013) compared the IgG humoural responses in uninfected pregnant women with those with acute, chronic or past placental infection. The group of infected women showed higher levels of IgG against all parasite and recombinant antigens tested, including $Pf\text{MSP1}_{19}$. On the other hand, during pregnancy, there is a depression of cell-mediated immunity (Riley et al. 1989) associated with the increased production of hormones essential to maintain gestation. Increased cortisol levels were detected in pregnant women who later developed parasitaemia, thereby suggesting a causal association (Vleugels et al. 1987).

P. vivax and *P. malariae*-asymptomatic infections have been described in the studied area (Couto et al. 2010). Due to the lack of symptoms, transfusional malaria cases were reported, when donors transmitted *P. malariae* to recipients, even a long period after the displacement to the Atlantic Forest areas had occurred (Kirchgatter et al. 2005, Scuracchio et al. 2011). The low parasitaemia and the non-specific symptoms observed in the positive pregnant women could be related to the high frequency of IgG antibodies, indicating constant exposure to parasites. The subclinical aspects observed could be explained by the zoonotic profile of the infections, with the transfer of non-adapted parasites between primates and human hosts. The behaviour of *An. (K.) cruzii* mosquitoes contributes to the maintenance of transmission because their vertical dispersion allows them to feed at the ground and in the canopy (Deane 1992).

The first description of pregnant women harbouring *P. vivax* or *P. malariae* in an area of low transmission in southeastern Brazil has had an impact on the surveillance of malaria transmission in that scenario. It is important to highlight that the subjects tested positive and mentioned no displacement, confirming the autochthony. Data reported here note the need for follow-ups with this special group to detect infection. Considering the low parasitaemias and the asymptomatic profile, the diagnosis should apply very sensitive tools, such as real-time and/or nested PCR, because TBS could not detect parasites in the peripheral blood in these cases. Among the molecular protocols, real-time PCR should be considered the first option for detecting *Plasmodium*. This assay is based on genus-specific primers, is very sensitive and performs rapidly. Positive samples should be assayed by nested PCR to determine *Plasmodium* species. Evaluation of exposure to *Plasmodium* ELISA should be considered due to its high sensitivity. The results obtained herein should encourage other surveys in similar areas, where transmission could be happening without knowledge of the control programs. Other aspects concerning the relationship between pregnant women and *P. vivax* and *P. malariae* should be further investigated, as well as the cell-mediated immune response and immunohistochemical approaches.

In conclusion, despite the benign appearance of malaria in the studied area, the detection of *Plasmodium* in pregnant women demonstrates the utility of testing for this parasite in routine prenatal care.

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