Asymptomatic infections in blood donors harbouring *Plasmodium*: an invisible risk detected by molecular and serological tools

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Background. Transfusion-transmitted malaria due to asymptomatic *Plasmodium* infections is a challenge for blood banks. There is a lack of data on the prevalence of asymptomatic infected blood donors and the incidence of transfusion-transmitted malaria in low endemicity areas worldwide. We estimated the frequency of blood donors harbouring *Plasmodium* in an area in which asymptomatic infections have been reported.

Material and methods. To estimate the frequency of blood donors harbouring *Plasmodium* we used microscopy and molecular tools. Serological tests were applied to measure the exposure of candidates to *Plasmodium* antigens. Venous blood was collected from 91 candidates attending the "Pró-Sangue" Blood Centre Foundation in São Paulo, who lived in the municipality of Juquitiba, São Paulo, Brazil, where sporadic autochthonous cases of malaria have been described. Blood samples were used for parasitological, molecular and serological studies.

Results. Among the 91 samples examined, rare *Plasmodium* forms were observed in two donors. Genus real-time polymerase chain reaction analysis demonstrated *Plasmodium* amplification in three candidates and species-specific nested polymerase chain reaction identified *P. malariae* in two. ELISA-IgG was reactive in 42.9% of samples for *P. vivax* (*Pv*-MSP1$_{19}$) and in 6.6% for *P. falciparum (Pf-Zw)*. ELISA-IgM was reactive in 2.2% of samples for *P. vivax* and in 4.4% for *P. falciparum.* An indirect immunofluorescence assay was reactive for *P. malariae* in 15.4% of cases.

Discussion. Reservoirs of *Plasmodium* represent a challenge for blood banks, since studies have shown that high levels of submicroscopic infections can occur in low transmission areas. The risk of transfusion-transmitted malaria presented here points to the need to conduct molecular investigations of candidate donors with any positive malarial antibody test.

Keywords: transfusion-transmitted malaria, blood donors, asymptomatic infection, molecular tools, serological tools.

Introduction

Transfusion-transmitted malaria (TTM) represents a challenge for blood bank services because of the occurrence of asymptomatic infections[1,2] and has been addressed as a risk in both endemic and non-endemic areas. Asymptomatic infections have been described worldwide[3-6] and are associated with the five malaria-causing *Plasmodium* species. Semi-immune individuals from endemic areas, with partial immunity to malaria, may have a mild febrile illness or even no symptoms at all, keeping parasites at low densities. It is generally assumed that in areas of high malaria transmission, where individuals rapidly acquire immunity, the levels of asymptomatic infections are high[7]. However, a high prevalence of asymptomatic infections has also been found in areas of low endemicity, due to the persistence of residual immunity, with parasites maintained at submicroscopic levels[8]. In Brazil, 99.6% of malaria transmission occurs in the Amazon Region, an area of moderate endemicity. However, outside this region, sporadic cases of malaria have been reported in areas covered by the Atlantic Forest, near the coast of the country, where asymptomatic infections have been described[9,10].

The most common sources of TTM are whole blood and red blood cell concentrates[11], in which *Plasmodium* can survive for up to three weeks during storage at temperatures between 2 °C and 6 °C[2]. Asymptomatic blood donors may have a low level parasitaemia, such as 1-2 parasites/μL, which is undetectable by microscopy. Nevertheless, this level of parasitaemia means that the recipient would receive about 400,000-800,000 parasites per unit of whole blood[12].

Brazilian guidelines[13] recommend blood screening for malaria, with different protocols for endemic and non-endemic areas. The endemic area is classified into low, medium or high risk, based on the Annual Parasite Index (API). The recommendation for the endemic area is to consider the API of the municipality: in regions with active transmission of malaria, regardless of the API, tests must be conducted to detect *Plasmodium* or its antigens (microscopy of a blood film and rapid diagnostic test). In endemic areas, candidate donors who have had malaria in the 12 months preceding the potential donation as well as those who present with a fever, those suspected of having had malaria in the preceding 30 days, or those who have come from an area of high malaria risk are deferred. In non-endemic areas, candidates who have come from an endemic area in the 30 days preceding the potential donation are ineligible. Tests to detect *Plasmodium* are required for potential donors who were in an endemic area between 30 days and up to 12 months prior to the donation. After 12 months, tests for *Plasmodium* detection are not required; candidates are also eligible for blood donation after 12 months of treatment. In both endemic and non-endemic areas, donors who have had *P. malariae* are permanently excluded. However, these criteria are not completely sufficient to avoid TTM because *P. malariae* infections may be misdiagnosed with infections by other *Plasmodium* species[14] and candidates may harbour submicroscopic, asymptomatic infections.

Measures to prevent TTM have been adopted in non-endemic areas, because of immigrants from and travellers to endemic areas[15]. Some non-endemic countries have implemented selective testing. Recommendations from European guidelines[16] defer: (i) for three years after their last visit to an endemic area, candidates who lived in a malarial area within the first five years of life, provided the person remains symptom-free; this period may be reduced to four months if an immunological or molecular test is negative; (ii) for three years individuals with a history of malaria after treatment and absence of symptoms; they are accepted thereafter if an immunological or molecular test is negative; (iii) for six months after leaving the endemic area those candidates who have visited malarial areas, unless an immunological or molecular test is negative; (iv) for three years following resolution of symptoms those individuals with undiagnosed fever within six months of a visit to an endemic area; this period may be reduced to four months if an immunological or molecular test is negative. In the USA, recommendations from the Food and Drug Administration[17] preconize deferral: (i) for three years for candidates who have a history of malaria or who lived in a malaria-endemic country; (ii) for one year after the last departure from a malaria-endemic area for those donors who are residents of a non-endemic country and who have travelled to or through any malaria-endemic area; (iii) for three years after a visit to a malaria-endemic area for donors who are prior residents of a malaria-endemic country and who lived in non-endemic countries for less than three consecutive years; (iv) for one year from the time that the donor returns to the non-endemic country, if that donor was a prior resident of a malaria-endemic country and returns to a malaria-endemic area after residence for three consecutive years in non-endemic countries.

There have been reports of TTM due to asymptomatic donors who referred displacement to the Atlantic Forest inside São Paulo State[1]. Nevertheless, there is a lack of data on the prevalence of asymptomatic infected blood donors in such areas of Brazil. In this study, we aimed to investigate blood donors coming from Juquitiba, which is a municipality covered by the Atlantic Forest in the State of São Paulo. It is considered a non-endemic area, in which sporadic malaria transmission has been reported. Ecotourism activities are common in this region, due to the preserved native biome. In order to estimate the presence of asymptomatic blood donors harbouring *Plasmodium* in this group of candidates we used microscopy and molecular tools. Serological tests were applied to measure exposure of the candidate donors to *Plasmodium* infections.

Materials and methods

Sample collection

Blood samples were collected from three groups of candidates who voluntarily presented, in different periods (2007, n=17; 2013, n=39; 2014, n=35), at the Pró-Sangue Blood Centre Foundation in São Paulo to donate blood. All candidates were inhabitants of Juquitiba, a municipality located in the Atlantic Forest biome, 71 km from São Paulo, capital of São Paulo State. Each group was analysed at the time of donation and afterwards all the samples were re-examined, with exception of thick blood smears (TBS), which were examined only at the time of donation. Candidates were accepted based on clinical and epidemiological screening, according to the Brazilian guidelines[13]. As previous studies by our group had found cases of asymptomatic malaria in this region, parasitological, serological and molecular tests were performed before transfusion.

Peripheral venous blood was collected into 5 mL test-tubes containing ethylenediamine tetraacetic acid (EDTA). The blood was used to prepare a TBS and perform polymerase chain reaction (PCR) analysis and immunoassays. After centrifugation, erythrocytes and plasma were stored at −20 °C for DNA extraction and serological assays, respectively.

Microscopy of thick blood smears

TBS were prepared in duplicate and slides stained with Giemsa. Counting was performed in 500 white blood cells, which corresponded to 25 minutes of observation[18]. In order to determine the number of parasites/µL, two independent and highly experienced microscopists carried out readings, following the criteria recommended by the Brazilian Ministry of Health, and assuming a standard of 6,000 leucocytes/µL[19]. TBS parasitaemia was calculated as numbers of parasites/µL:

$$\frac{\text{total parasite counting} \times 6{,}000}{500 \text{ leucocytes}}$$

The same laboratory staff examined the TBS of all three groups.

Genomic DNA extraction

After plasma and buffy coat removal, 1 mL of packed red blood cells were lysed using 3 mL 1% saponin/ultrapure water (Sigma-Aldrich, St. Louis, MO, USA) and washed three times (1,200 *g*, 10 minutes) with ultrapure water. From the pellet, 200 µL were used for DNA extraction with a QIAamp® DNA Blood Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

Real-time polymerase chain reaction

The real-time quantitative PCR (qPCR) protocol 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 genomic DNA, 12.5 µL of 2× TaqMan® Universal PCR Master Mix, 500 nM of each primer and 300 nM of FAM™ and TAMRA™- labelled probe (Applied Biosystems, Foster City, CA, USA). Duplicate samples were assayed on an ABI Prism 7300 or 7500 system (Applied Biosystems) using negative (ultrapure water) and positive (*P. falciparum* DNA from a 1 parasite/µL sample) controls. The threshold cycle (Ct) cut-off value of 37.28 was set based on the receiver operating characteristics (ROC) curve using Ct values from 1 parasite/µL, as described previously[20].

Nested polymerase chain reaction

Samples with positive results in qPCR were processed by nested PCR targeting ssrRNA genes. The first reaction employed the genus-specific primers rPLU5 and rPLU6 and the second reaction used species-specific primers rFAL1 and rFAL2, rVIV1 and rVIV2, rMAL1 and rMAL2, rOVA1 and rOVA2 for *P. falciparum*, *P. vivax*, *P. malariae* and *P. ovale*, respectively[21]. 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. Resulting fragments were separated by electrophoresis in 1.5% agarose gel in Tris-borate-EDTA buffer and visualised with ethidium bromide (Bio-Rad Laboratories, Hercules, CA, USA), through ultraviolet light. Negative (ultrapure water) and positive (genomic DNA of *P. falciparum*, *P. vivax* and *P. malariae*) controls were included in all tests[21].

Enzyme-linked immunosorbent assays (ELISA)

Adapted protocols described elsewhere[22,23] were applied for antigen production and detection of IgG and IgM (immunoglobulins G and M) antibodies against *P. vivax* (*Pv*-$MSP1_{19}$) and *P. falciparum* (*Pf-Zw*).

Pv-MSP1$_{19}$

Briefly, *E. coli* BL21-CodonPlus® (DE3)-RIL (Novagen, Merck KGaA, Darmstadt, Germany) transformed with the plasmid pET14b-*Pv*$MSP1_{19}$ (kindly provided by Dr. Irene da Silva Soares, São Paulo University, São Paulo, Brazil) was used for protein expression. Protein was purified by affinity chromatography using Ni Sepharose 6 FastFlow resin (GE Healthcare, Uppsala, Sweden) and buffers containing 8 M urea. The aliquots containing the fraction of 19 kDa (pH 5.3 and 4.0) were dialysed and protein content was estimated with a BCA (bicinchoninic acid) Protein Assay Kit (Thermo Fisher Scientific, Roskilde, Denmark)[22]. For IgG antibodies[22], Costar 3590, High Binding polystyrene plates (Corning Inc., New York, USA) were coated with 200 ng/well of His_6-*Pv*$MSP1_{19}$ recombinant protein and were blocked with 5% skimmed milk. Plasma samples diluted to 1/100 and anti-human IgG (Fc specific)-peroxidase antibody produced in goats (A-0170, Sigma-Aldrich) diluted at 1/20,000 were incubated for 30 minutes at 37 °C. For colour development tetramethylbenzidine (TMB)/H_2O_2 chromogen (Life Technologies, Carlsbad, CA, USA) was incubated for 10 minutes at room temperature in the dark. The reaction was interrupted by adding 2N H_2SO_4 (Merck KGaA). The spectrophotometric reading was performed on Multiskan GO (Thermo Scientific, Vantaa, Finland) at 450 nm. For IgM antibodies, anti-human IgM-peroxidase antibody (A6907, Sigma-Aldrich) was used at a dilution of 1/5,000. The chromogen solution was incubated for 15 minutes.

Pf-Zw

Crude *P. falciparum* antigen was extracted with Zwittergent® (Calbiochem, Billerica, MA, USA) (*Pf*-Zw) and employed for the detection of antibodies against *P. falciparum* and eventually all *Plasmodium* species through cross-reactions[23]. For IgG antibodies[23], Nunc Polysorp polystyrene plates (Thermo Fisher Scientific,

Roskilde, Denmark) were coated with 500 ng/well of *Pf*-Zw. All other steps were the same as for ELISA-*Pv*-MSP1$_{19}$. For IgM antibodies, anti-human IgM-peroxidase antibody (A6907, Sigma-Aldrich) was used at a dilution of 1/5,000.

ROC curves[24] were constructed from the absorbance of 100 positive and 100 negative samples for IgG and 20 positive and 20 negative samples for IgM. Positive samples were collected from patients diagnosed with *P. vivax* or *P. falciparum* by TBS. Negative samples were collected from individuals from non-endemic regions with no history of previous malaria or displacement to areas of malaria transmission. For each sample, the reactivity index (RI: absorbance/cut-off) was calculated and samples with RI≥1 were considered positive.

Indirect immunofluorescence assay - *P. malariae*

To detect anti-*P. malariae* antibodies, the indirect immunofluorescence assay (IFA) protocol described by Ferreira and Sanchez[25] was applied. Multispot slides (Thermo Fisher Scientific, Rockford, IL, USA) coated with blood from a patient infected with *P. malariae* and no previous malaria were used, with 30,000 parasites/µL. Briefly, the blood sample was centrifuged (1,200 *g*, 10 minutes) to remove plasma and buffy coat. The pellet was washed three times with RPMI 1640 (Sigma-Aldrich), diluted in RPMI 1640 to a 50% haematocrit, dispensed on Multispot slides (15 µL/well), dried at room temperature and stored at −80 °C. Plasma samples diluted 1/40 and fluorescein isothiocyanate-conjugated goat anti-human IgG (γ chain-specific) (Fluoline G, BioMérieux, Marcy l'Etoile, France) diluted 1/200 were incubated for 30 minutes at 37 °C. The slides were mounted in alkaline glycerine (pH 9.5) and observed under a fluorescence microscope with 25× and 10× ocular water immersion objectives. The readings were scored with crosses: 1 to 4 for the positive samples.

SD Bioline Malaria *Pf/Pv* immunochromatographic test

SD Bioline Malaria *Pf*/*Pv* is a commercial immunochromatographic test for the detection of antibodies against circumsporozoite surface protein (CSP) and merozoite surface protein (MSP) recombinant antigens of *P. falciparum* and *P. vivax*. It was used according to the manufacturer's instructions, with 10 µL of plasma dispensed in the device well, followed by 110 µL of the assay diluent and test interpretation after 15 minutes. This test was applied only in 56 samples because of the unavailability of the product in Brazil since 2014.

Statistical analysis

The data were analysed with GraphPad Prism 5.0 (GraphPad Software Inc., San Diego, CA, USA) and SigmaStat Analysis System Software 3.5 (Systat Software, Richmond, CA, USA). Positivity rates (%) with 95% confidence intervals (95% CI) were calculated. Agreement between ELISA *Pv*-MSP1$_{19}$ and SD Bioline-*Pv* was assessed using the κ index and marginal association was verified using McNemar's test. Fisher's exact test was employed to measure associations between positivity in ELISA-*Pf*-IgG and IFA-*Pm*-IgG. Differences were considered statistically significant when p values were less than 0.05 (α=0.05).

Results

Positivity of candidates based on detection of *Plasmodium*

Considering microscopy, among the 91 samples examined, TBS showed rare *Plasmodium* forms (24 parasites/mm^3) in two donors (positivity 2.2%; 95% CI: 0.6-7.6) after two independent readings (Figure 1). As far

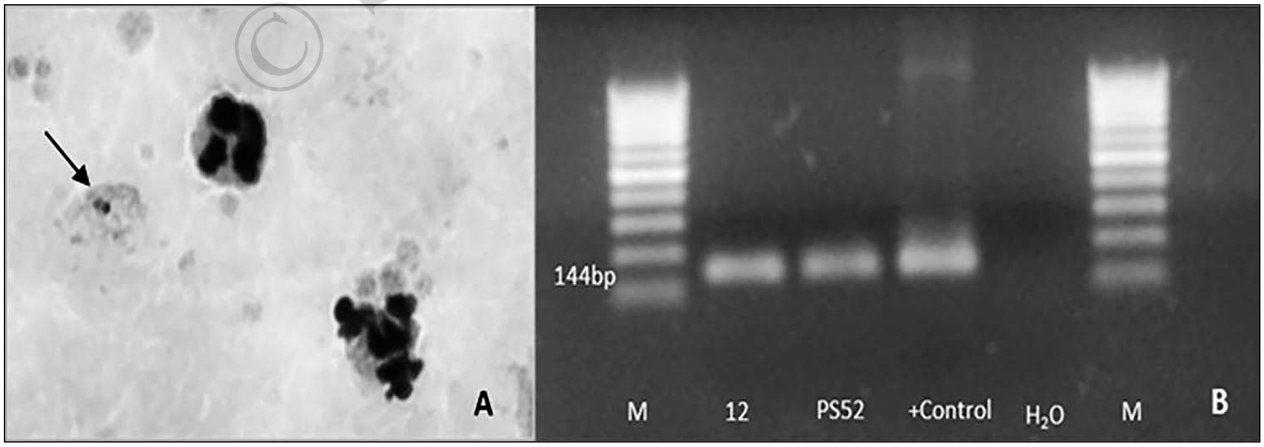

Figure 1 - (A) Microscopy showing rare parasites in a positive TBS and (B) nested PCR showing amplification of *P. malariae* in blood donor candidates from Juquitiba, SP, Brazil.
M: molecular marker 100 bp; 12 and PS52: positive blood donor candidates; +Control: *P. malariae* positive control; H_2O: negative control.

as molecular testing is concerned, genus qPCR revealed *Plasmodium* amplification in the duplicates of three candidates among the 91 assayed, with mean Ct value below the cut-off of 37.28. The rate of positivity was 3.3% (95% CI: 1.1-9.2). Species-specific nested PCR showed amplification in two candidates (positivity 2.2%; 95% CI: 0.6-7.6). Donor 6, diagnosed with very low parasitaemia by TBS, did not show amplification in two molecular protocols; positivity by TBS in donor 12 was confirmed by qPCR and nested PCR, and diagnosed as *P. malariae*; two candidates (14 and PS52) with negative TBS showed amplification in qPCR, nevertheless only PS52 had a positive reaction for *P. malariae* in nested PCR (Table I). Positive candidates were treated for malaria according to Brazilian guidelines[26].

Positivity of candidates based on the detection of anti-*Plasmodium* antibodies

ELISA-IgG was reactive in 42.9% (95% CI: 33.2-53.1) of samples for *P. vivax* (*Pv*-MSP1$_{19}$) and 6.6% (95% CI: 3.1-13.6) for *P. falciparum (Pf-Zw)*. ELISA-IgM was reactive in 2.2% (95% CI: 0.6-7.6) for *P. vivax* and 4.4% (95% CI: 1.7-10.8) for *P. falciparum.* Reactivity indices are presented in Figure 2. IFA-*Pm* was reactive for *P. malariae* in 15.4% (95% CI: 9.4-24.2) of samples, based on the independent readings of two investigators using positive and negative controls. Among 39 positive samples by ELISA-*Pv*-MSP1$_{19}$ -IgG, two were also reactive for IgM antibodies. For *Pf-Zw*, six samples were positive only for ELISA-IgG and four were positive only for ELISA-IgM. From the 14 positive samples for IgG antibodies in IFA-*Pm*, 13 were also reactive in ELISA-*Pv*-MSP1$_{19}$ -IgG and two in ELISA-IgM; six samples were positive in ELISA-*Pf-Zw*, five of which for IgG and one for IgM antibodies. One sample was reactive only for IFA-*Pm* (Table I).

ROC curves showed 100.0% sensitivity for both ELISA-*Pv*-MSP1$_{19}$-IgG and IgM, 94.0% for ELISA-*Pf-Zw*-IgG and 95.0% for ELISA-*Pf-Zw*-IgM. Specificity was 100.0% for all tests.

The SD Bioline *Pf/Pv* detected antibodies against *P. vivax* in 26/56 cases, a positivity rate of 46.4% (95% CI: 34.0-59.3). All samples tested were negative for *P. falciparum* antigens. Very good agreement was observed between ELISA-*Pv*-MSP1$_{19}$ -IgG and SD Bioline considering *Pv*-IgG detection (κ=0.893; 95% CI: 0.776-1.000; McNemar's test p=0.248).

Among the 41 samples positive by serology (IgG and/or IgM), 95.1% were detected by ELISA-*Pv*-MSP1$_{19}$, 34.1% by IFA-*Pm* and 24.4% by ELISA-*Pf-Zw*. Considering parasitological and molecular methods, 4/91 blood donors (4.4%) were probably infectious. Based on serology, 45.0% had been exposed to *Plasmodium.*

The analysis of donors who were positive in at least one malarial detection test showed that all were adults (16 to 69 years old), living in the Juquitiba municipality: 51.2% were men and 48.8% women.

Discussion

In this study, the risk of TTM was well defined for three candidates (12, 14, PS52) who were positive according to PCR, with *P. malariae* detected in two of them. The serology result in one candidate (PS52), who had strong reactivity by IFA-*Pm*, was consistent with this. However, candidate 12, also positive for *P. malariae*, was negative in IFA-*Pm*. Other authors have also reported the detection of *P. malariae* among seronegative donors[27], as malarial antibodies may not be present in detectable amounts at the beginning of an infection. In the blood sample of candidate 14 it was not possible to determine the *Plasmodium* species, as asymptomatic infections reported in this region covered by the Atlantic Forest are at the limit of detection, even when sensitive molecular tools are used. Samples 15, PS10, PS17 and PS50 showed amplification in qPCR above the cut-off of 37.28 and it should be considered possible that these samples, particularly PS17, which was strongly reactive in IFA-*Pm*, could harbour less than 1 parasite/µL, used to calculate the cut-off. The other three samples were positive in two serological tests, indicating contact with *Plasmodium*. Despite being negative in qPCR, the TBS of sample 6 was positive after an exhaustive reading, corresponding to 25 minutes of observation[18], and was reactive for ELISA-*Pv*-MSP1$_{19}$ -IgG. It has been reported[28] that the reproducibility of even sensitive PCR protocols (nested-PCR and qPCR) can vary between replicates, alternating positive and negative results in 38% of the samples with very low levels of parasitaemia. PCR results, therefore, do need to be interpreted carefully in submicroscopic infections, as variable results with false-negatives can occur.

The rate of positivity for anti-*Plasmodium* antibodies in candidates for blood donation found in this study (45.0%) indicates a high level of exposure of this population, consisting of semi-immune residents in an area in which asymptomatic infections have been described[10]. Of the serologically positive samples, 95.1% were detected in ELISA-IgG using *Pv*MSP1$_{19}$ recombinant antigen, which is highly immunogenic in individuals recently (<six months) exposed to *P. vivax* malaria[29]. This is consistent with the fact that two samples (10, PS45) were also reactive for IgM antibodies, which can be associated with infection; however, in asymptomatic individuals we have observed lower levels and prevalence of IgM than in patients with symptoms or severe malaria (*data not published*). As this recombinant antigen is highly specific for anti-*P. vivax* antibodies[30], one could suppose

Table I - Positive samples from blood donor candidates from Juquitiba, São Paulo State, Brazil, tested by parasitological, molecular and serological methods.

ID	TBS	SD Bioline	qPCR	Nested PCR	ELISA-IgG (RI)		ELISA-IgM (RI)		IFA-IgG
	mm³	*Pv*	*mean Ct*	*Pmal*	*Pv-MSP1$_{19}$*	*Pf-Zw*	*Pv-MSP1$_{19}$*	*Pf-Zw*	*Pm*
1	NEG	+/2	NEG	NEG	5.65	NEG	NEG	NEG	NEG
2	NEG	NEG	NEG	NEG	1.65	NEG	NEG	NEG	NEG
3	NEG	NEG	NEG	NEG	6.10	NEG	NEG	NEG	NEG
5	NEG	NEG	NEG	NEG	NEG	NEG	NEG	1.17	NEG
6	24	+/2	NEG	NEG	12.46	NEG	NEG	1.52	NEG
7	NEG	+/2	NEG	NEG	6.60	1.76	NEG	NEG	+++
8	NEG	+/2	NEG	NEG	11.41	NEG	NEG	NEG	NEG
9	NEG	+/2	NEG	NEG	10.35	3.13	NEG	NEG	+++
10	NEG	++	NEG	NEG	20.20	2.13	1.12	NEG	++
12	24	+/2	35.16	Pm	5.61	1.30	NEG	NEG	NEG
13	NEG	++	NEG	NEG	13.92	NEG	NEG	NEG	NEG
14	NEG	+/2	35.92	NEG	2.64	NEG	NEG	NEG	½ +
15	NEG	+/2	38.02	NEG	1.17	NEG	NEG	NEG	½ +
16	NEG	++	NEG	NEG	11.48	NEG	NEG	NEG	NEG
17	NEG	++	NEG	NEG	7.87	NEG	NEG	NEG	NEG
PS1	NEG	+	NEG	NEG	11.25	NEG	NEG	NEG	NEG
PS5	NEG	+/2	NEG	NEG	1.13	NEG	NEG	NEG	NEG
PS10	NEG	+	NEG	NEG	13.30	NEG	NEG	1,05	NEG
PS11	NEG	++	NEG	NEG	13.20	NEG	NEG	NEG	NEG
PS12	NEG	+	NEG	NEG	3.52	NEG	NEG	NEG	NEG
PS14	NEG	NEG	NEG	NEG	2.21	NEG	NEG	NEG	NEG
PS16	NEG	+/2	NEG	NEG	6.03	NEG	NEG	NEG	NEG
PS17	NEG	++	37.66	NEG	6.84	1.17	NEG	NEG	++++
PS18	NEG	+	NEG	NEG	8.31	NEG	NEG	NEG	+
PS22	NEG	+/2	NEG	NEG	10.33	NEG	NEG	NEG	NEG
PS27	NEG	+/2	NEG	NEG	2.22	NEG	NEG	NEG	NEG
PS30	NEG	++	NEG	NEG	8.35	NEG	NEG	NEG	NEG
PS31	NEG	+	NEG	NEG	4.76	NEG	NEG	1,05	½ +
PS32	NEG	+/2	NEG	NEG	2.46	NEG	NEG	NEG	NEG
PS37	NEG	+/2	NEG	NEG	NEG	NEG	NEG	NEG	NEG
PS39	NEG	+/2	NEG	NEG	1.73	NEG	NEG	NEG	NEG
PS43	NEG	ND	NEG	NEG	11.19	NEG	NEG	NEG	NEG
PS44	NEG	ND	NEG	NEG	20.52	NEG	NEG	NEG	+
PS45	NEG	ND	NEG	NEG	17.40	NEG	3.28	NEG	++
PS47	NEG	ND	NEG	NEG	5.83	NEG	NEG	NEG	++
PS49	NEG	ND	NEG	NEG	1.29	NEG	NEG	NEG	NEG
PS50	NEG	ND	NEG	NEG	8.12	NEG	NEG	NEG	½ +
PS52	NEG	ND	35.78	Pm	3.90	2.10	NEG	NEG	++++
PS57	NEG	ND	NEG	NEG	NEG	NEG	NEG	NEG	++
PS59	NEG	ND	NEG	NEG	6.09	NEG	NEG	NEG	NEG
PS61	NEG	ND	NEG	NEG	3.24	NEG	NEG	NEG	NEG
PS72	NEG	ND	NEG	NEG	4.94	NEG	NEG	NEG	NEG

ID: identification; TBS: thick blood smear; qPCR: real-time quantitative polymerase chain reaction; IgG: immunoglobulin G; RI: reactivity index; IgM: immunoglobulin M; Pv: *Plasmodium vivax*; Ct: cycle threshold; Pmal: *Plasmodium malariae*; Pm: *Plasmodium malariae*; Pf: *Plasmodium falciparum*; NEG: negative; ND: not done.

a recent infection by this *Plasmodium* species and a cross-reaction of *P. vivax* antibodies with *P. falciparum* and/or *P. malariae* total antigens. Eight samples were reactive with *P. vivax* and *P. malariae* antigens, but negative by TBS and PCR, suggesting either donor exposure to the two species or cross-reactivity between *P. malariae* antigen and anti-*P. vivax* antibodies. Additionally, anti-*P. falciparum* antibodies are supposed to be due to cross-reactivity, since in this region only *P. vivax* and *P. malariae* have been described[10]. This is corroborated by the association between positivity in ELISA-P*f-Zw*-IgG and high reactivity of IFA-*Pm* (Fisher's exact test, p=0.031).

Despite the small number of clinically diagnosed cases, there is evidence of malaria parasites circulating in the studied area. Serological surveys showed between 21% and 52% positivity for IgG antibodies against *P. vivax* in two localities of Juquitiba. A significant difference was found between the prevalence of positive sera among home caretakers resident in the area (73%) and positive sera among homeowners who visit the area for leisure and vacation (18%)[31]. In the State of Espirito Santo, Brazil, the serological profile of the population suggests exposure to *Plasmodium*, with a positivity rate of 37.7% for *P. vivax* and 7.9% for *P. malariae* antibodies. In this area, asymptomatic cases were detected using PCR[32]. This scenario has implications for the risk of TTM in other areas.

Cases of TTM have been reported in the State of São Paulo during the last decade. In 2005, *P. malariae* was responsible for the death of one transfusion recipient, an immunocompromised patient who received blood from a donor who had displaced to the coast of São Paulo State. As the recipient had undergone a splenectomy, the number of old circulating erythrocytes was very high. As *P. malariae* invades only old erythrocytes, the recipient had a very high parasitaemia, leading to death[33]. Persistence of *P. malariae* long after cessation of exposure is well documented. Vinetz *et al.*[34] described a case of asymptomatic infection lasting for at least 40 years. In our experience all cases of TTM were due to *P. malariae*[1,33].

In this study, candidates were accepted according to the Brazilian guidelines. As far as the donors' profiles are concerned, all were adults living in Juquitiba municipality; none reported previous malaria, related symptoms or displacement to endemic areas. Nonetheless, the detection of parasites and the high prevalence of antibodies in this group points to the risk of TTM, as the current clinical-epidemiological screening does not pick up asymptomatic donors. The detection of *Plasmodium* in this scenario is challenging, because even molecular tools can fail to detect very low level parasitaemia[14] and serological techniques may not confirm active infection. The real risk of TTM, due to the occurrence of asymptomatic infections presented here, suggests the need for accurate knowledge about this silent malaria outside the Amazon Region when screening blood donors.

We must point out that donors from these areas with autochthonous malaria account for only a small fraction of the blood donor population and, in practice, the risk they pose is diluted by the majority

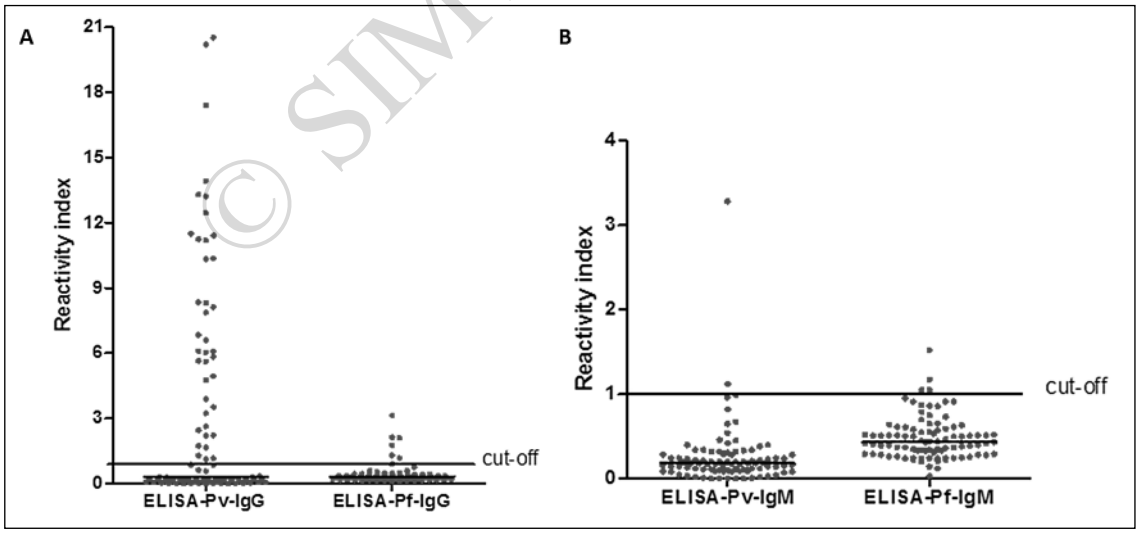

Figure 2 - Reactivity index of plasma samples assayed by ELISA-*Pv*-MSP1$_{19}$ and ELISA-*Pf-Zw* from blood donor candidates from the locality of Juquitiba, SP, Brazil.
Cut-offs: (A) *Pv*-IgG 0.100; *Pf*-IgG 0.135 (B) *Pv*-IgM 0.090; *Pf*-IgM 0.330.
ELISA: Enzyme-linked immunosorbent assay; *Pv*: *Plasmodium vivax*; *Pf*: *Plasmodium falciparum*; IgG: immunoglobulin G; IgM: immunoglobulin M.

of the donors originating from urban areas in which autochthonous malaria does not exist. The knowledge that positivity is associated with residents or individuals with close contact with the forest points to the adoption of an approach consisting of molecular screening of candidates positive for any malarial antibody test, as implemented elsewhere[35].

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Authorship contributions

GFMCL carried out the molecular assays, analysed data and wrote the manuscript; MCAS coordinated the serological assays, performed the statistical analysis and wrote the manuscript; JEL coordinated the samples collection, analysed data and wrote the manuscript; MF and LCC carried out the serological assays; ARS and EMR-S obtained the recombinant antigens; JI carried out the molecular assays; MJC-N carried out the microscopy tests and the epidemiological survey; AMJ provided data from donors and reviewed the manuscript; SMDS coordinated molecular assays, analysed data and wrote the manuscript.

The Authors declare no conflicts of interest.

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