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An algorithm based on molecular protocols to improve the detection of *Plasmodium* in autochthonous malarial areas in the Atlantic Forest biome

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ABSTRACT

Malaria is the most important vector-borne disease in the world and a challenge for control programs. In Brazil, 99% of cases occur in the Amazon region. In the extra-Amazonian region, a non-endemic area, epidemiological surveillance focuses on imported malaria and on autochthonous outbreaks, including cases with mild symptoms and low parasitemia acquired in the Atlantic Forest biome. In this scenario, cases are likely to be underreported, since submicroscopic parasitemias are not detected by thick blood smear, considered the reference test. Molecular tests are more sensitive, detecting asymptomatic individuals and mixed infections. The aim of this study was to propose a more efficient alternative to detect asymptomatic individuals living in areas of low malaria endemicity, as they are reservoirs of *Plasmodium* that maintain transmission locally. In total, 955 blood samples from residents of 16 municipalities with autochthonous malaria outbreaks in the Sao Paulo State were analyzed; 371 samples were collected in EDTA tubes and 584 in filter paper. All samples were initially screened by a genus-specific qPCR targeting ssrRNA genes (limit of detection of 1 parasite/μL). Then, positive samples were subjected to a nested PCR targeting ssrRNA and dihydrofolate reductase-thymidylate synthase genes (limit of detection of 10 parasites/μL) to determine *Plasmodium* species. The results showed a statistically significant difference (K = 0.049; $p < 0.0001$) between microscopy positivity (6.9%) and qPCR (22.9%) for EDTA-blood samples. Conversely, for samples collected in filter paper, no statistical difference was observed, with 2.6% positivity by thick blood smear and 3.1% for qPCR (K = 0.036; $p = 0.7$). Samples positive by qPCR were assayed by a species-specific nested PCR that was in turn positive in 26% of samples (16 *P. vivax* and 4 *P. malariae*). The results showed that molecular protocols applied to blood samples from residents in areas with autochthonous transmission of malaria were useful to detect asymptomatic patients who act as a source of transmission. The results showed that the genus-specific qPCR was useful for screening positives, with the subsequent identification of species by nested PCR. Additional improvements, such as standardization of blood plotting on filter paper and a more sensitive protocol for species determination, are essential. The qPCR-based algorithm for screening positives followed by nested PCR will contribute to more efficient control of malaria transmission, offering faster and more sensitive tools to detect asymptomatic *Plasmodium* reservoirs.

KEYWORDS: Malaria. Polymerase chain reaction. *Plasmodium vivax*. *Plasmodium malariae*. Asymptomatic infections.

INTRODUCTION

Malaria is an infectious disease transmitted to humans by the bite of the

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female *Anopheles* mosquito infected with *Plasmodium*. Transmission can also occur through blood transfusion, organ transplantation or from mother to fetus. Five *Plasmodium* species cause human malaria, namely *P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale* and *P. knowlesi*[1]. According to the World Health Organization[2], 229 million cases were notified in the world in 2019, with 409,000 deaths. *P. falciparum* is the most prevalent species in the African region accounting for 99.7% of cases, 62.8% in the Southeast Asian region, 69% in the Eastern Mediterranean and 71.9% in the Western Pacific. *P. vivax* is the most prevalent in the Americas, accounting for 74.1% of cases[2].

In Brazil, 99% of malaria cases occur in the Amazon region. In 2019, 157,454 cases of malaria were reported, 19.1% less than in 2018. *P. vivax* accounted for 89.3% of cases, while *P. falciparum* and mixed infections contributed with 10.7%[3]. *P. malariae* is probably underreported due to morphological similarities with *P. vivax*[4].

In 2019, 536 cases were reported in the extra-Amazonian region, with a decrease of 44% in 2020[5] probably due to travel restrictions imposed by the COVID-19 pandemic. In this region, transmission is influenced by migration to endemic areas, when individuals return infected and cause outbreaks due to the local presence of the vector[5,6]. In addition to these imported cases, there are reports of autochthonous transmission in the extra-Amazonian region, mainly in the Atlantic Forest biome. From 2018 to 2020, most autochthonous cases were reported in the States of Espirito Santo (148 cases), Bahia (77 cases) and Sao Paulo (36 cases), according to data from the Ministry of Health[5].The extra-Amazonian region comprises 18 States and covers about 40.25% of the Brazilian territory, with 86.6% of the national population living in this area. In this region, malaria can occur as a zoonosis, involving non-human reservoirs. Some characteristics of the Atlantic Forest biome, such as high temperature and humidity, as well as the widespread occurrence of bromeliads, are essential for the proliferation of *Anopheles*. In addition, deforestation impacts the population dynamics of mosquitoes, with negative consequences for the population adjacent to these areas[6].

Asymptomatic infections represent a challenge for malaria control worldwide, as these silent, human symptom-free reservoirs do not seek medical care, being a source for the maintenance of transmission[7,8,9]. The submicroscopic parasitemia observed in low transmission areas is a consequence of several factors such as previous exposure, promoting a semi-immune profile[10] leading to asymptomatic infections that impact malaria elimination goals. Furthermore, it increases the risk of transfusional malaria from asymptomatic donors, especially those with *P. malariae*[11,12].

The Sustainable Development Goals (SDGs) aim to reduce global inequalities and eliminate malaria by 2030. In 2015, WHO proposed the Global Technical Strategy for Malaria, with the aim of reducing at least 90% of cases and deaths worldwide and preventing the reintroduction of malaria in areas without transmission. In Brazil, the National Health Plan 2020-2023 proposed a 50% reduction in cases by 2023, based on the 187,756 cases reported in 2018. After reviewing this plan, the target of 93% reduction by 2030 and elimination of malaria by 2035 was established. These objectives are based on strategies to increase the detection, treatment, and surveillance of cases, in partnership with different areas of the health system, such as basic health units, specific areas of care for the indigenous population and environment autorities[3].

Elimination is defined as the interruption of transmission of all *Plasmodium* species for at least three years[13]. To achieve this goal, WHO recommends outbreaks identification based on an integrated approach aimed at identifying *Plasmodium* populations, hosts and vectors involved in the focal transmission. The Pan American Health Organization (PAHO) defines the following scenarios: I. Not receptive, without vectors; II. Receptive, without autochthonous cases; III. Receptive, without autochthonous cases, vulnerable, with imported cases; IV. Receptive, with autochthonous cases, including active and residual outbreaks[3].

Although the thick blood smear (TBS) is the gold standard for the laboratory diagnosis of malaria, it is not suitable for detecting low parasitemias[12], therefore, the number of cases in Sao Paulo State may be higher. The use of more sensitive and specific techniques such as PCR[14] allows the detection of asymptomatic infections, mapping with greater precision the transmission areas, as well as the geographical distribution of *Plasmodium* species, improving the quality and efficiency of control programs. Lima *et al.*[14] reported a sensitive genus-specific quantitative real-time PCR (qPCR) targeting ssrRNA genes, with limit of detection (LoD) of 1 parasite/µL, a suitable assay for screening a large number of samples. To determine *Plasmodium* species, the most used protocol is the nested PCR targeting ssrRNA described by Snounou *et al.*[15], with LoD of 10 parasites/µL. Similar sensitivity is reported by Tanomsing *et al.*[16], who described a protocol targeting dihydrofolate reductase-thymidylate synthase genes for the detection of five human plasmodia.

The aim of this study was to propose a more efficient alternative to detect asymptomatic individuals who live in areas of low malaria endemicity as they are reservoirs of *Plasmodium* that maintain local transmission. To detect positive individuals, an algorithm based on an in-house

genus-specific qPCR followed by a species-specific nested PCR was proposed.

MATERIALS AND METHODS

Study area and population

This is a retrospective cross-sectional observational analytical study, including blood samples from residents of autochthonous areas of malaria outbreaks in Sao Paulo State. Samples were collected during epidemiological surveillance activities and evaluated by TBS at the time of the outbreaks, in accordance with the recommendations of the National Malaria Control Program - PNCM[17]. Blood was collected in the field by finger prick using disposable devices. TBS were stained with Giemsa and examined with oil immersion and 100 X magnification by experienced microscopists from different laboratories, according to the geographic area of the outbreak. Species and parasitemia were determined in accordance with the PNCM recommendations[18]. DNA extraction, qPCR and nested PCR were performed for this survey.

This study included 16 Sao Paulo State municipalities located in an area of Atlantic Forest. For assembling the municipalities, the study considered the following Immediate Regions (IR) of Sao Paulo State, namely: Registro, Sorocaba, Santos, Sao Paulo, Caraguatatuba-Ubatuba-Sao Sebastiao and Taubate-Pindamonhangaba, as presented in Figure 1.

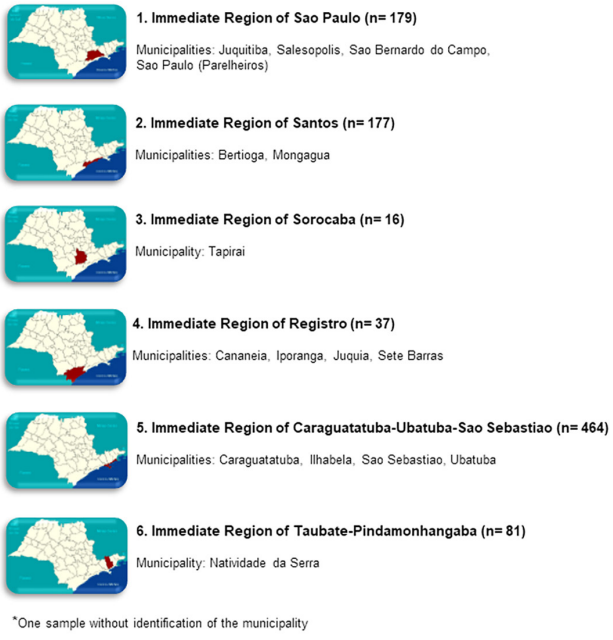

Figure 1 - Distribution of the 16 municipalities within the areas of autochthonous outbreak according to the Immediate Regions of Sao Paulo State.

In total, 955 blood samples collected in active case detection were analyzed. Among them, 371 were collected by venipuncture in EDTA tubes (Greiner Bio-One, Kremsmünster, Austria) and 584 by fingerstick plotted as a dried blood spots (DBS) on Whatman® FTA® filter paper (Merck, Darmstadt, Germany).

Genomic DNA extraction from whole blood samples

DNA from blood samples collected in EDTA tubes was extracted after lysis of red blood cells (RBC) with 1% saponin (Serva, Heidelberg, Germany), followed by two washes with ultrapure water. A 200 µL volume of the pellet was used for genomic DNA extraction with QIAamp DNA Blood Mini Kit (QiAgen®, Hilden, Germany), according to the manufacturer's instructions. Although the protocol recommends the elution of DNA in 200 µL, we eluted it in a volume of 50 µL to concentrate the DNA.

Genomic DNA extraction from DBS

Chelex®100 protocol

An area of around 1 cm^2 was removed from the DBS and transferred to a sterile 1.5 mL microtube. A 1 mL volume of 0.5% saponin in phosphate buffered saline (PBS) was added to each microtube and incubated at 37 °C for 90 min. After removing the supernatant, 1 mL of 1 x PBS was added, and incubated at 4 °C for 30 min. A solution containing 50 µL 20% Chelex®100 (Bio-Rad™, Hercules, California, USA) in 150 µL sterile distilled water was boiled at 100 °C. After removing PBS from incubated samples, the heated Chelex®100 was added to each tube, vortexed and incubated at 100 °C for 10 minutes. Microubes were centrifuged at 10,000 g for 3 min, and the supernatant was transferred to a new microtube and stored at -20 °C[19].

To evaluate the performance of qPCR, serial dilutions of *P. falciparum* parasites obtained in culture medium RPMI 1640 (Sigma-Aldrich, St. Louis, Missouri, USA), with parasitemia ranging from 2,500 parasites/µL to 5 parasites/µL were used to extract DNA using both protocols. For DBS, a volume of 50 µL was obtained.

Parasitemia was calculated as follows:

$$\frac{\text{number of parasites in 10,000 RBC} \times 100}{10{,}000} \times \frac{\text{number of RBC/µL}}{100}$$

Sample processing by qPCR

The assays were carried out according to the protocol described by Lima *et al*.[14] with primers M60 and M61 and probe M62 labeled with FAM™ and TAMRA™ (Applied

Biosystems™, USA) for the amplification of *Plasmodium* 18S rRNA gene sequences. The qPCR reaction was prepared with 2.5 µL of genomic DNA added to a 22.5 µL volume containing 12.5 µL of TaqMan® Universal PCR Master Mix 2x (Applied Biosystems™, Thermo Fisher Scientific, Waltham, Massachusetts, USA), 0.50 µM of each genus-specific (M60 and M61) primer and 0.3 µM of the M62 probe. The amplification and detection conditions were: 50 °C for 2 min, 95 °C for 10 min followed by 40 cycles of 94 °C for 30 s and 60 °C for 1 min. The samples were tested in duplicate in the 7500 Real-Time PCR System (Applied Biosystems™, Thermo Fisher Scientific, Waltham, Massachusetts, USA). Positive and negative controls were used in all tests. Positive controls were obtained from serial dilutions of *P. falciparum* cultures (0.1; 1; 10; 100; 1,000 and 10,000 parasites/µL) to obtain a standard curve for the quantification of parasitemia. Individuals without malaria who tested negative were used as negative controls. The LoD of this protocol is 1 parasite/µL, validated in a previous study[14].

Sample processing by 18S rRNA nested PCR

After screening by qPCR, *Plasmodium*-positive samples were analyzed by nested PCR targeting 18S rRNA genes, with a total volume of 25 µL in each reaction, as follows: a first round of amplification with 250 nM of each genus-specific primer (rPLU5 and rPLU6), 125 µM dNTPs, 2 mM $MgCl_2$, 50 mM KCl, 10 mM Tris pH 8.3, 0.4 U of Taq polymerase and 1 µL of gDNA as template. Amplification occurred with one cycle at 95 °C for 5 min, 58 °C for 2 min, 72 °C for 2 min and 24 cycles at 94 °C for 1 min, 58 °C for 2 min and 72 °C for 2 min, with a final cycle of 72 °C for 5 min. The second round of amplification followed the same protocol, using the species-specific primers rVIV1/rVIV2, rMAL1/rMAL2, rFAL1/rFAL2 and 1 µL of the first amplification as the DNA template. Thirty cycles were performed under the same conditions. The DNA fragments obtained were separated by electrophoresis in 1.5% agarose gels in Tris/Borate/EDTA buffer (TBE) and stained with Blue Green (LGC Biotecnologia Ltda., Cotia, Sao Paulo, Brazil). A molecular weight marker ranging from 100-2000 bp (Norgen Biotec Corp., Thorold, Ontario, Canada) was used. Positive and negative controls were included in all PCR assays. DNA from PCR-confirmed samples containing *P. vivax*, *P. falciparum* and *P. malariae* DNA were used as positive controls. Blood samples negative for *Plasmodium* collected from individuals without displacement to endemic areas or previous reports of malaria were used as negative controls[15].

Sample processing by DHFR-TS nested PCR

The protocol described by Tanomsing *et al.*[16] was used, with two cycles of amplification for the genus *Plasmodium,* followed by nested amplifications for the species *P. vivax*, *P. malariae* and *P. falciparum* identification. In the first reaction, the genus-specific primers Pla-DHFR-F and Pla-TS-R were used, producing a 1 Kb amplification fragment; in the second reaction with the genus-specific primers Pla-DHFR-NF and Pla-TS-R, a fragment of 509-587 bp was produced. The following nested PCR assays used *Plasmodium* species-specific primers (PV-Lin-Forward and PV-Lin-Reverse for *P. vivax*; PM-Lin- Forward and PM-Lin- Reverse to P. *malariae*; PF-Lin- Forward and PF-Lin- Reverse for P. *falciparum*). All reactions were performed with 25 µL of final volume, as follows: 250 nM of each genus-specific or species-specific primer (Invitrogen™, Thermo Fisher Scientific, Waltham, Massachusetts, USA), 125 µM dNTPs (Thermo Fisher Scientific, Waltham, Massachusetts, USA), 50 mM KCl, 10 mM Tris pH 8.3, 0.4 U of Taq polymerase GoTaq HotStart (Promega, Madison, Wisconsin, USA) and 1 µL of genomic DNA for the first round of amplification or 1 µL of the amplicons obtained in previous reactions. The $MgCl_2$ concentration was 3 mM in the genus-specific and *P. malariae* reactions. For *P. falciparum* and *P. vivax*, the $MgCl_2$ concentration was 2 mM. The fragments were separated by electrophoresis in a 2% agarose gel/TBE buffer, stained with Blue Green (LGC Biotecnologia Ltda., Cotia, Sao Paulo, Brazil). A molecular weight marker ranging from 100-2000 bp (Norgen Biotec Corp., Thorold, Ontario, Canada) was used. The fragments obtained were 144 bp for *P. vivax*, 177 bp for *P. malariae* and 160 bp for *P. falciparum*. The LoD of the two nested PCR protocols is 10 parasites/µL[15,16].

The results were analyzed using GraphPad Prism 5.0 and QuickCalcs GraphPad software (GraphPad Software Inc., San Diego, California, USA). The positivity of the tests was calculated with a confidence interval (CI) of 95% and for the comparison of proportions between the tests, considering the total sampling and each municipality, the Fisher's Exact Test was used. The agreement between the tests was calculated using the Kappa Index (k). The significance levels of the tests used in the statistical analysis were established accepting a type 1 error of 5% (α=0.05).

This study was approved by the Ethics Committee of the Department of Infectious and Parasitic Diseases, School of Medicine, University of Sao Paulo and by its Ethics Committee (process Nº 2.728.246).

RESULTS

The results of the 955 samples collected in EDTA tubes

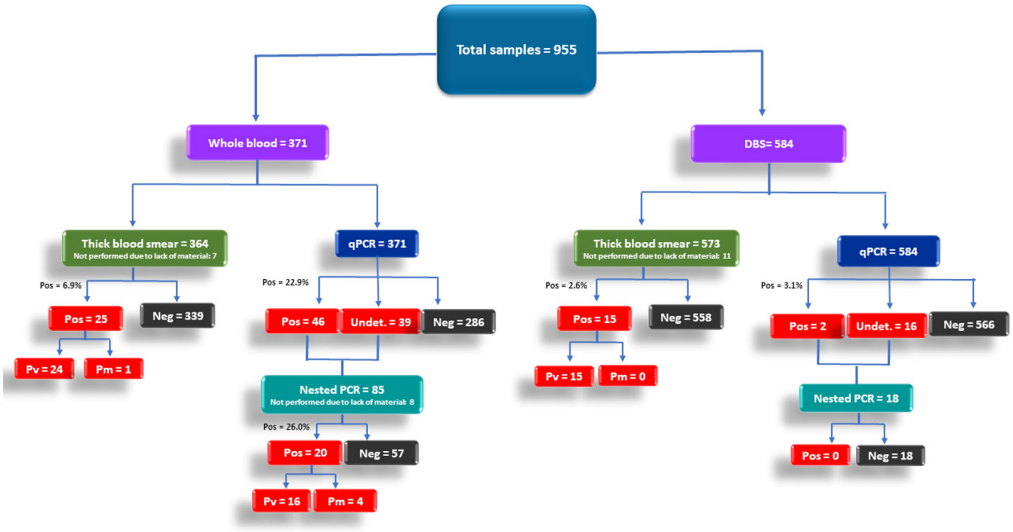

Figure 2 - Flowchart showing the number of samples analyzed according to the method of blood collection and the results obtained by each of the DNA extraction techniques used. DBS = dried blood spot; pos = positive; undet = undetermined, with amplification of only one sample of the duplicate; Pv = *P. vivax*; Pm = *P. malariae*.

or in DBS processed by TBS, qPCR and nested PCR are shown in Figure 2.

Dilutions ranging from 2,500 parasites/µL to 5 parasites/µL extracted by QIAamp DNA Blood Mini Kit and Chelex®100 showed Ct means ranging from 21.33 to 30.08 and 33.10 to 39.21, respectively. Among the dilutions of DNA extracted by Chelex®100, one dilution did not amplify and one amplified only in one well of the duplicate.

Comparison of positivity among TBS, qPCR and nested PCR in all municipalities studied

TBS was performed in 364/371 (98.1%) of blood samples collected in EDTA tubes at the time of the outbreak. Twenty-five resulted positive, with 24 *P. vivax* detections and one *P. malariae* (6.9% [CI: 4.66 - 9.98]). The qPCR detected 85 positive samples (22.9% [CI: 18.92 - 27.46]). The comparison of the two techniques (TBS and qPCR) showed statistical significance (p< 0.0001) and a weak agreement (K= 0.049). Among the 85 samples positive by qPCR, 77 (90.6%) were tested by nested PCR. As shown in Figure 2, eight samples were not analyzed by nested PCR due to lack of DNA. Twenty samples were positive, 16 detected *P. vivax* and four detected *P. malariae* (26.0% [CI: 17.42 - 36.81]), with no amplification in 57 samples. In addition, 573/584 (98.2%) samples collected in DBS were processed by TBS, with 15 detections of *P. vivax* (2.6% [CI: 1.56 - 4.31]). Among the 584 samples processed by qPCR, 18 were positive (3.1% [CI: 1.93 - 4.85]). No samples were positive by nested PCR. For the samples collected in DBS no statistical significance was observed (p= 0.7), with a weak agreement (K= 0.036) between TBS and qPCR results (Table 1).

Table 1 - Number of positive and negative samples for whole blood and dried blood spot (DBS), comparing the thick blood smear (TBS), qPCR and nested PCR (nPCR).

	WHOLE BLOOD				
	Positive	Negative	Positivity (%)	*Kappa*	*p-value*
TBS	25	339	6.9 (CI: 4.66 - 9.98)		
qPCR	85	286	22.9 (IC: 18.92 - 27.46)	K = 0.049[a]	p < 0.0001[b]
nPCR	20	57	26.0 (CI: 17.42 - 36.81)		
	DBS				
	Positive	Negative	Positivity (%)	Kappa	p-value
TBS	15	558	2.6 (CI: 1.56- 4.31)		
qPCR	18	566	3.1 (CI: 1.93- 4.85)	K = 0.036[a]	p = 0.7[c]
nPCR	0	18	0		

[a]Poor agreement; [b]Statistically significant; [c]Not statistically significant.

Comparison of positivity among TBS, qPCR and nested PCR in each municipality studied

The study included 16 municipalities (Figure 3) with 134 (14.03%) positive samples by any of the methods performed. The municipality of Sao Sebastiao showed the highest positivity (43.3%), followed by Sao Paulo (11.2%) and Bertioga (9.7%). The municipalities of Iporanga and Cananeia showed the lowest positivity (< 1%).

Positivity by municipality according to TBS and qPCR

The municipality of Caraguatatuba showed a positivity of 1.3% by TBS and of 0.4% by qPCR, with no statistical significant difference (p=0.62). Similarly, most municipalities studied did not present any statistically significant difference The municipality of Sao Sebastiao showed only three samples positive for TBS (1.4%) and 56 positives for qPCR (25.7%), with a significant statistical difference (p<0.0001). In the case of Mongagua, the positivity for TBS was 8.1%, with no sample positive by qPCR (p=0.01). Samples from Tapirai were positive only by qPCR (68.8%), with a significant statistical difference (p<0.0001), as shown in Table 2.

Regarding the results of nested PCR performed on qPCR-positive samples, the species distribution showed 80% of *P. vivax* and 20% of *P. malariae*. The results of each analyzed municipality are shown in Table 3.

DISCUSSION

This study revealed asymptomatic *Plasmodium* infections in residents of 14 of the 16 municipalities considered. Although asymptomatic infections have been described in the Brazilian Atlantic Forest biome, for the first time a study was carried out including so many municipalities in areas classified as Scenario IV according to PAHO, defined as receptive, with autochthonous cases, including active and residual outbreaks[3]. Identifying these silent reservoirs will improve control and elimination strategies based on timely diagnosis and treatment.

In Sao Paulo State, Hristov *et al.*[20] reported 1.6% of positivity by TBS and 5.6% by PCR, in blood samples from asymptomatic pregnant women living in Juquitiba, a municipality located in the Atlantic Forest biome. Among the seven positive samples by qPCR and nested PCR, *P. vivax* was detected in three pregnant women and *P. malariae* in four[20]. In Rio de Janeiro State, a survey carried out with 324 residents of the Guapimirim municipality revealed 2.8% of asymptomatic infections by nested PCR, without positive TBS[21]. Another study conducted in Espirito Santo State, with 92 samples from residents of an autochthonous area of malaria, showed a 3.4% positivity by PCR, with presence of *P. vivax* and *P. malariae*. All subjects were asymptomatic and negative by TBS[22].

Although most malaria cases in Brazil occur in the Amazon region[3], the autochthonous transmission in the Atlantic Forest biome of Southern and Southeastern Brazil is a challenge for the elimination of the disease. In this region, the landscape favors proliferation of *Anopheles (*subgenus Kerteszia), due to the large number of bromeliads, an ideal site for mosquito oviposition[23]. In addition, the presence of primates from the Atelidae and Cebidae families, mainly

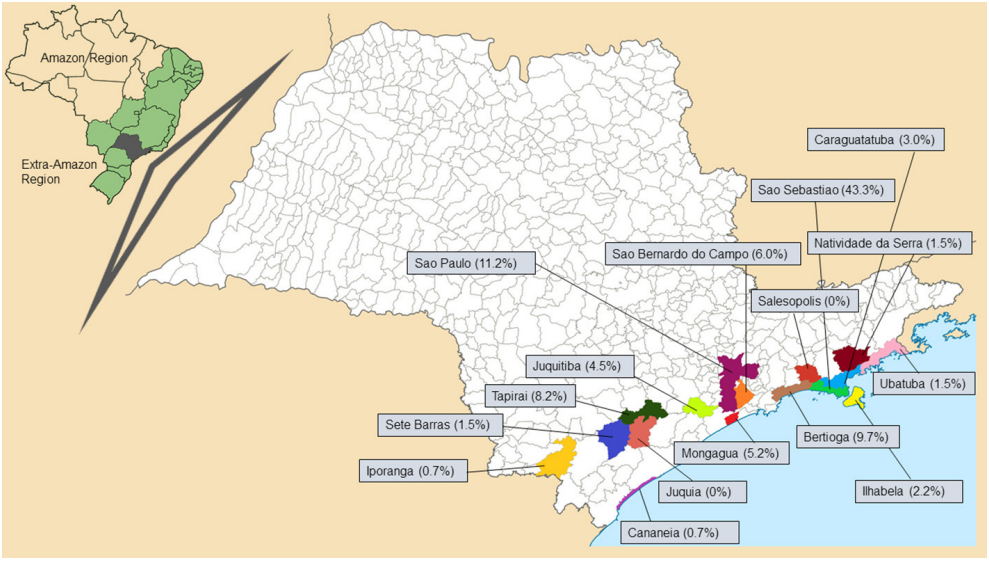

Figure 3 - Positivity among the 134 samples that were positive by TBS and/or qPCR. Distribution of positive samples in each municipality, in the studied area. *For a sample (0.7%), it was not possible to determine the origin of the infection.

Table 2 - Number of samples and positivity between the techniques performed (TBS and qPCR), by municipality.

Municipality	Number of samples	Number of +ve TBS	% of +ve TBS	Number of +ve qPCR	% of +ve qPCR	p- value
Bertioga	91	8	8.8	6	6.6	0.78
Cananeia	1	NR	-	1	100	-
Caraguatatuba	227	3	1.3	1	0.4	0.62
Ilhabela	12	1	8.3	2	16.7	1.0
Iporanga	24	0	0	1	4.2	1.0
Juquia	1	0	0	0	0	-
Juquitiba	80	3	3.8	4	5.0	1.0
Mongagua	86	7	8.1	0	0	0.01*
Natividade da Serra	81	2	2.5	0	0	0.49
Salesopolis	19	0	0	0	0	-
Sao Bernardo do Campo	54	5	9.3	5	9.3	1.0
Sao Paulo (Parelheiros)	26	6	23.1	12	46.2	0.14
Sao Sebastiao	218	3	1.4	56	25.7	< 0.0001*
Sete Barras	11	0	0	2	18.2	0.47
Tapirai	16	0	0	11	68.8	< 0.0001*
Ubatuba	7	2	28.6	2	28.6	1.0

+ve = positive. *Statistically significant.

Table 3 - Species-specific nested PCR results performed on previously qPCR-positive samples.

Municipality	+ve qPCR/ performed	nested PCR		
		Pv	*Pm*	**Negative**
Bertioga	6/2	2	-	-
Caraguatatuba	1/1	-	-	1
Ilhabela	2/2	-	-	2
Juquitiba	4/4	1	-	3
Sao Bernardo do Campo	5/4	2	-	2
Sao Paulo	12/11	3	-	8
Sao Sebastiao	56/56	2	3	51
Sete Barras	2/2	2	-	-
Tapirai	11/11	4	1	6
Ubatuba	2/2	-	-	2
TOTAL	101*/95	16	4	75

+ve = positive. *Two samples positive by qPCR (Cananeia and unknown origin) were not processed by nested PCR due to lack of DNA.

from the genus *Alouatta*, *Brachyteles*, *Cebus* and *Sapajus* infected with *P. simium* and *P. brasilianum*[24], hampers the usual control measures, such as those adopted by the PNCM, that focuses on human-vector transmission. The zoonotic malaria in this region was first described in 1966, with detection of *P. simium* in a human host[23]. The similarity among *P. vivax* and *P. simium*, and *P. malariae* and *P. brasilianum* supports the idea that primate hosts act as reservoirs for human infections, sharing the same epidemiological setting[25].

In Sao Paulo State, 14 Reference Units for Malaria are responsible for the diagnosis and treatment of malaria cases, which are notified to the Epidemiological Surveillance Service. Autochthonous cases are reported to the surveillance agency aiming at the immediate control of outbreaks[26]. Autochthonous transmission in well-preserved areas of the Atlantic Forest shows a continuous pattern, with a low number of cases and mild symptoms. Asymptomatic infections are only reported in active case detection activities[26]. The present study showed 134 asymptomatic residents with *Plasmodium* among the 955 evaluated and highlights the need for detecting these untreated human reservoirs who maintain the circulation of *Plasmodium*, favoring the occurrence of outbreaks[27].

Many studies have shown greater accuracy and sensitivity of molecular techniques compared to TBS[28,29]. However, according to the results presented here, TBS positivity was higher than the one of PCR in some municipalities, which may be due to the DNA extraction protocol for DBS. Although DNA extraction with Chelex®100 is widely used[30,31], in this study, samples were collected in the field by different groups, without a pattern of blood volume and DBS distribution. The lack of standardization may have affected the qPCR performance when DNA extracted from DBS samples were used. A study comparing DNA extraction methods (Chelex®-saponin, methanol and TRIS-EDTA) showed that Chelex®100 extraction performed better by qPCR when samples from 21 positive individuals were tested, with 18 asymptomatic and only four positive samples by TBS. Even with better results, the sensitivity of Chelex®100 DNA extractions

was 66.7% for *P. falciparum* and 31.6% for *P. vivax*[32]. Schwartz *et al.*[33] tested the effects of storage, extraction and amplification on DBS samples in serial dilutions of a positive control, ranging from 0.1 to 100,000 parasites/μL, concluding that sensitivity was lower for infections with low parasite density. Field sampling interferences were not considered, as the experiments were conducted under controlled conditions. Similar findings were observed in this study using qPCR-processed serial dilutions with precise parasitemia to evaluate DNA extraction protocols. When qPCR results using serial dilutions (2,500 to 5 parasites/μL) were analyzed, it was observed that the Ct range was lower for samples extracted by QIAamp DNA Blood Mini Kit than by Chelex®100. Considering that these results were processed under controlled laboratory conditions, one can conclude that the volume of blood plotted in the DBS in field conditions, and the low parasitemia of samples, in addition to the efficiency of each DNA extraction protocol, have clearly affected qPCR results. The hypothesis that Chelex®100 could yield qPCR inhibitors was ruled out, as all DNA extractions included positive controls that amplified in all assays. On the other hand, it cannot be excluded that positive TBS results from the municipalities of Mongagua and Natividade da Serra were false-positives, and some factors may have influenced these findings: the extremely low parasitemia and atypical morphology of *Plasmodium* species transmitted in this setting may require exceptional expertise from microscopists; although some of these TBS have been examined in reference centers, as in the Natividade da Serra outbreak, the unusual morphology of parasites found in these samples, circulating in the Atlantic Forest, can induce well-trained professionals to false-positive detections[34].

In this study, the performance of qPCR revealed some aspects that need to be addressed. The results of molecular tests were better on blood samples collected in EDTA tubes and extracted by the QIAamp DNA Blood Mini Kit than on samples extracted by the Chelex®100. Very low parasitemia may not have been detected due to the blood volume used in DNA extraction protocols. Only five of the 39 samples collected in EDTA tubes that amplified only in one well of the duplicate by qPCR were positive by nested PCR. Regarding the 16 samples extracted by Chelex®100 that amplified only in one well of the duplicate by qPCR, none of them were positive by nested PCR. The LoDs of qPCR (1 parasite/μL)[14] and of nested PCR (10 parasites/μL)[15,16] support these results and suggest the need of high-throughput protocols to extract DNA from larger volumes of blood[35]. In a study conducted in Malaysia, microscopy was compared with species-specific nested PCR of blood samples plotted on DBS from 129 individuals living in endemic areas. This protocol included two rounds of amplification for the genus identification, followed by one cycle for the species, and the LoD was 6 parasites/μL[36], supporting the proposal of using a larger volume of blood to increase *Plasmodium* detection.

As the analyzed blood samples were expected to have very low parasitemia, an internal control was not included in the qPCR reactions. Although the qPCR validation determined a cut-off value of 1 parasite/μL in a previous report[14], in this study any amplification, even of one well of the duplicate was considered indicative of the presence of parasite DNA. In these samples, the addition of an internal control would probably increase Ct values, leading to false-negative results. Murphy *et al.*[37] showed that the LoD increases as the sample volume decreases, and for a volume of 200 μL of blood, the LoD is 5 parasites/mL or 1 parasite/200 μL. Considering a total of 50 μL sample, the LoD would be 20 parasites/mL (or 1 parasite/50 μL). Therefore, the LoD can be significantly affected by the initial volume of blood sample, the volume of extracted DNA and the DNA input in the PCR assay.

Currently, autochthonous outbreak control is performed using TBS for the detection of *Plasmodium* carriers. However, it is known that this methodology is not sensitive to detect low parasitemia[14]. The samples from this retrospective study were processed by molecular protocols after the outbreak control measures were performed according to the PNCM[17], using TBS for the diagnosis. Furthermore, the time spent reading each TBS must be considered. It is estimated that the time required to perform all TBS analyses in this study was 477 hours, whereas for the qPCR assays took only 116 hours. Considering the current cost of molecular protocols, their application is advantageous in relation to the cost attributed to the working hours of a microscopist. The results presented here point to the advantage of molecular protocols for *Plasmodium* detection and are robust enough to propose an algorithm based on the screening of positives by qPCR using a larger volume of blood, that should be applied in the active detection of cases for the control of malaria outbreaks.

CONCLUSION

Asymptomatic *Plasmodium* infections were identified in residents of malaria outbreak areas in several municipalities in Sao Paulo State. The positivity was 22.9% by qPCR and 6.9% by TBS, in whole blood samples collected in EDTA tubes, showing better results than samples collected in DBS. Blood samples were screened using qPCR targeting the genus *Plasmodium* and then positive samples were tested by nested PCR assays to identify the species of *Plasmodium*,

with a positivity of 26% among those previously positive by qPCR, revealing the presence of *P. vivax* and *P. malariae* species. These results highlight the need for more sensitive tools for the diagnosis of submicroscopic infections in areas of low malaria transmission, as these human reservoirs, undetected by microscopy, influence local transmission and jeopardize malaria elimination goals. An algorithm for the molecular detection of *Plasmodium* was proposed to identify asymptomatic carriers in low transmission areas.

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AUTHORS' CONTRIBUTIONS

MLRNF designed the study, carried out and analyzed the molecular assays and wrote the manuscript; MA contributed to the molecular assays, to the statistical analyzes and to the writing of the manuscript; MJCN contributed to the molecular assays; SMDS designed the study, coordinated and analyzed the molecular assays and wrote the manuscript. All authors read and approved the final manuscript.

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