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Mixed *Plasmodium malariae* Infections Were Underdetected in a Malaria Endemic Area in the Amazon Region, Brazil

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Abstract. *Plasmodium malariae* infections are often asymptomatic and long-lasting. Mixed infections are often underdetected in areas where *P. malariae*, *P. vivax*, and *P. falciparum* are coendemic. In this study, we described the occurrence of these species circulating as single or mixed infections in Pará state, Brazil, in the Amazon region, with the purpose of clarifying the impact of misidentification of parasite species based only on morphological description using thick blood smear. By using real-time polymerase chain reaction based on the amplification of the mitochondrial DNA, we detected a prevalence of 46% (58/126) mixed infections with 33.3% *P. malariae*/*P. vivax* which were read as *P. vivax* monoinfections by microscopy detection. Our findings confirmed the high circulation of *P. malariae* in a malaria endemic area in the Brazilian Amazon region.

Malaria is a disease caused by mono or multi-species infections of *Plasmodium* parasites.[1] In studies with patients admitted to hospitals and in epidemiological surveys, *Plasmodium* coinfections detected by microscopy are found at an estimated frequency of 2–30% in Asia.[1,2] In general, mixed infections are reported for *Plasmodium vivax* and *P. falciparum*, the two most prevalent malaria parasite species.[3] Of these, *P. vivax* has the largest geographical distribution worldwide with predominance in Brazil where it accounts for 83.7–90% of all malaria cases registered over the last two decades; additionally, two other species are also in coendemicity, *P. falciparum* (9.7–16.3%), and *P. malariae* with very low prevalence (0.01–0.3%).[4–6]

Plasmodium malariae infection is not associated with relapse but can cause chronic, low-grade infections that persist for several years, often at levels that are lower than microscopic detection limits.[2,7,8] Cases of chronic and submicroscopic *P. malariae* infections have been reported, and approximately 2% of patients develop severe complications.[9] As a case detected after a long period of infection, an asymptomatic 74-year-old Greek woman with splenomegaly was monitored, and the submicroscopic infection was confirmed by nested polymerase chain reaction (PCR) specific for *P. malariae* 18S ribosomal RNA (rRNA).[10] Furthermore, in addition to understanding the distribution of mixed infections in a *P. vivax* endemic area, it is important to evaluate how coinfection influences malaria outcome or causes different symptoms modulated by two or three different malaria parasite species.[7,11] Considering the limitations of microscopic detection of *P. malariae* parasites, which can be mistaken for *P. vivax* because of the altered shape of the parasite on thick blood smear (TBS) slides,[1,4] it may be important to investigate such infections using approaches other than morphological differences among malaria parasite species, such as a sensitive PCR technique.[12,13] Currently, technological advances are allowing early diagnosis of malaria by real-time quantitative PCR (RT-qPCR), including mitochondrial DNA (mtDNA) amplification to detect *P. malariae*.[14] Infection caused by *P. malariae* is often missed in the analysis of TBS, which are commonly used for malaria diagnosis, and confirmatory thin blood smears are rarely examined to identify and distinguish *P. vivax* from *P. malariae*, causing an inadequate capacity of the malaria vigilance system to register the actual situation of detecting *P. malariae* as single or mixed infections.[1,4,15] In this scenario, PCR techniques should be used to estimate the prevalence of this species, as well as the impact of *P. vivax* infection associated with the chronic condition caused by *P. malariae* or blood transfusion–transmitted malaria.[2,7,15]

To characterize the *Plasmodium* species associated with malaria cases in Pará, Brazil, a low malaria transmission area, we described the occurrence of *P. malariae*, *P. vivax*, and *P. falciparum* employing a molecular method. A total of 126 blood samples were collected in two cities, Belém (51) and Tucuruí (75) from 2006 to 2010. All patients were diagnosed as positive by TBS during the survey following the recommendations of the Brazilian Ministry of Health.[16] The DNA was extracted and stored at −20°C as described.[13] Next, samples were analyzed using a molecular approach to detect *P. malariae, P. vivax*, and *P. falciparum* mtDNA by RT-qPCR as previously described.[14] This study was approved by the Ethical Committee of the Evandro Chagas Institute (011/2006; 015/2007) and the Federal University of Pará (4.122.498/2020). The results obtained by RT-qPCR confirmed all positive infections detected by microscopy and revealed 58 mixed infections (46%) (Table 1). This number of cases was notable when compared with the one mixed case identified by TBS, which had *P. vivax* and *P. falciparum* blood stages. *Plasmodium vivax* mtDNA was detected in all mixed infections, with 42 cases (33.3%) of these coinfections associated with *P. malariae* and 12 cases (9.5%) associated with *P. falciparum*, and four cases were identified as triple species infections (3.2%). The remaining 68 samples (54%) were confirmed as single infections by RT-qPCR, with 26 cases of *P. vivax* (20.7%) and 42 cases of *P. falciparum* (33.3%). Among all samples, 93 samples had parasite density estimated by TBS. Of these, 48 samples were *P. vivax* (51.6%), and 44 samples were *P. falciparum* (47.3%). Only

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TABLE 1
Mixed infections detected by RT-qPCR and parasite density determined by microscopic examination of TBS detecting *Plasmodium malariae*, *P. vivax*, and *P. falciparum* as species infective in individuals living in Pará state, Amazon region, Brazil

	Number of positive samples using molecular assay and optical microscopy		Parasite density (Parasites per microliter of blood in TBS)
Plasmodium species	RT-qPCR *n* (%) [95% CI]	TBS *n* (%) [95% CI]	Mean ± SD (Range)
P. vivax (Pv)	26 (20.7) [13.6–27.7]	48 (51.6) [41.5–61.8]	8,829 ± 16,184 (10–63,500)
P. falciparum (Pf)	42 (33.3) [25.1–41.6]	44 (47.3) [37.2–57.5]	9,102 ± 10,779 (20–37,250)
*P. vivax and P. falciparum**	12 (9.5) [4.4–14.6]	1 (1.1)	na†
P. malariae (Pm)	0	0	0
P. vivax and *P. malariae*	42 (33.3) [25.1–41.6]	0	0
Three species (Pm, Pv, and Pf)	4 (3.2) na†	0	0
Total	126	93	na

A total of 126 samples were analyzed by RT-qPCR, of these 93 were positive in TBS and the number of parasites was calculated per microliter of blood; other 33 was expressed as positive using cross without count of parasites. RT-qPCR = real-time quantitative PCR; SD=: standard deviation; TBS = thick blood smear.
* One sample was double positive for Pv (15,500 parasites) and Pf (18 gametocytes) per microliter of blood.
† na = not applicable.

one sample was mixed infection (1.1%). *Plasmodium falciparum* positivity rates were similar in both methods, but the mean parasite density determined by TBS for *P. vivax* and *P. falciparum* did not show significant differences. *Plasmodium malariae* as a single infection was not detected by either method, and it only appeared as part of mixed infections when detected by RT-qPCR, which amplified the mtDNA of *P. malariae* in 36.5% of samples (46/126). Of these, 42 infections were caused by *P. malariae* and *P. vivax* and four infections were caused by three species, including *P. falciparum.*

This finding confirmed that *P. malariae* and two other *Plasmodium* species are coendemic in Brazil, where the endemicity for *P. vivax* is higher than that for *P. falciparum*.[3–5] In this low malaria transmission area, *P. malariae* has seldom been reported.[4,5] However, the official data reported for 1997 showed that the prevalence of *P. malariae* was 0.3%, and the majority of these cases (97%) were individuals living in Pará state, located in the eastern Brazilian Amazon.[6] More recently, the prevalence of this species determined by microscopy has remained low and was estimated to be lower than 0.1% among all positive cases notified in 2019 by Brazilian Malaria Surveillance System (www.saude.gov.br/sivep_malaria).

Our findings agreed with previous studies reporting the occurrence of *P. malariae* in two Amazonian states where samples were tested by molecular assays, and showed prevalence rates in Rondônia and Mato Grosso of 10% and 11.9%, respectively.[6,17] However, differences in prevalence between our results may be partly explained by both prior studies using nested PCR to detect parasite rRNA whereas we used mtDNA amplification by RT-qPCR, a more sensitive PCR technique.[13,14]

Studies on experimental or natural infections caused by *P. malariae* have been reported.[2,7,9,15] In 1973, a total of 23 human volunteers were infected with two strains of this parasite, one from the Philippines and the other from Nigeria. In infections induced by the intravenous inoculation of parasitized blood or by bites of infected mosquitoes, the peak parasitemias values ranging from 1,400 to 27,000 parasites per cubic millimeter were attained between days 13 and 28 of patent parasitemia.[18] However, in natural infections the parasite density may be very low, and it has been described as submicroscopic infection confirmed by PCR.[6–8,10,17] In our study, the presence of *P. malariae* was revealed by molecular diagnostic with sensitivity and specificity to detect species-specific sequences of mtDNA as previously described.[14]

In Colombia where malaria is also endemic a study using PCR has reported 43.8% *P. malariae* positive infections among 671 symptomatic patients. Of these, mixed *P. malariae* and *P. vivax* infections accounted for 28.3%, whereas single *P. malariae* infections were found in 15.5% of samples.[19] In contrast to that found in Colombia, we did not detect *P. malariae* single-species infections.

In Brazil, PCR is not routinely applied in public health services. Thus, this species may be underdetected. Consequently, there have been few *P. malariae* malaria cases registered by surveillance systems.[3–5,15] Mixed malaria parasite infections are a small proportion of microscopically diagnosed cases. In part, this is due to some limitations in distinguishing the young ring-form of the malaria parasite, especially when there is a need to identify *P. vivax* and *P. malariae* by TBS.[4] One of the main issues is that even expert microscopists can misdiagnose low-density infections as single infections.[1,2,4]

An accurate estimate of malaria parasite coinfection prevalence is essential for the successful implementation of malaria control and elimination programs. If such measures are undertaken, they may provide important insights for prevention, adequate disease management, and treatment, consequently reducing malaria transmission. In the present study, a molecular approach using mtDNA amplification by qPCR identified 33.3% of mixed *P. malariae* and *P. vivax* infections not detected by conventional microscopy, confirming the high circulation *P. malariae* in Brazil where such coinfections may be underestimated by the sole use of morphology-based parasite identification method.

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