

How prevalent is *Plasmodium malariae* in Rondônia, Western Brazilian Amazon?

Qual é a prevalência de *Plasmodium malariae* em Rondônia,
Amazônia Ocidental Brasileira?

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Abstract We have compared results of *Plasmodium* species identification obtained with conventional on-site microscopy of Giemsa-stained thick smears (GTS) and a semi-nested polymerase chain reaction (PCR) in 96 malaria patients from Rondônia, Western Brazilian Amazon. Mixed-species infections were detected by PCR in 30% patients, but no such case had been found on GTS. Moreover, *P. malariae* infections were detected in 9 of 96 patients (10%) by PCR, but were not identified by local microscopists. The potential impact of species misidentification on malaria treatment and control is discussed.

Key-words: Malaria diagnosis. PCR. Brazilian Amazon. *Plasmodium malariae*.

Resumo Comparam-se neste trabalho resultados de identificação de espécie de *Plasmodium* obtidos com a microscopia convencional de gotas espessas coradas pelo Giemsa, realizada no local, e um protocolo semi-aninhado de reação em cadeia da polimerase (PCR) em 96 pacientes maláricos de Rondônia, Amazônia Ocidental Brasileira. Infecções envolvendo mais de uma espécie foram detectadas através de PCR em 30% dos pacientes, mas nenhum caso havia sido encontrado pelo exame de gotas espessas. Além disso, encontraram-se infecções por *P. malariae* por PCR em 9 dos 96 pacientes (10%), mas nenhuma havia sido encontrada pelos microscopistas locais. Discute-se o potencial impacto de erros de identificação de espécie sobre o tratamento e o controle da malária.

Palavras-chaves: Diagnóstico da malária. PCR. Amazônia Brasileira. *Plasmodium malariae*.

The incidence of malaria in Brazil has increased from 50,000 cases in 1970 to more than 400,000 cases in 1997. Transmission occurs almost exclusively in the Amazon Region, and is mainly associated with frontier agricultural settlements and professional activities such as clearing the forest for ranching projects, construction of roads, wood

extraction, and mining along the forest borders³. Most febrile patients have access to a widespread network of malaria clinics, which provide free diagnosis and treatment. Laboratory diagnosis in malaria clinics relies on microscopic examination of Giemsa-stained thick smears (GTS); confirmatory thin blood smears are rarely examined.

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Each year, 2-3 million GTS are examined in Brazil, and 10-20% of them are positive. During 1997, *Plasmodium falciparum* was detected by GTS microscopy in 95,400 patients (24%), and *P. vivax* in 305,500 patients (75%). Mixed-species (*P. falciparum* plus *P. vivax*) infections were found in only 3,042 (0.7%) patients. *P. malariae* was recorded in 1,077 patients in 1997 (0.3%), almost all of them (97%) living in the eastern Amazonian State of Pará. In contrast, no case of *P. malariae* infection was detected in the 165,000 malaria patients living in the western Amazonian State of Rondônia who were examined between 1996 and 1997 (Fundação Nacional de Saúde, unpublished data). However, morphological changes induced by hemolysis during thick smear staining may hamper the identification of *P. malariae*, and thus GTS-based prevalence data of this species in Brazil may be inaccurate. Accordingly, antibodies to synthetic peptides derived from *P. malariae* circumsporozoite protein repeats, which are thought to be narrowly species-specific⁴, have been frequently found in Amerindians living in Mato Grosso^{5,6,14}, and also among inhabitants of an area of very low malaria endemicity in São Paulo, southeastern Brazil⁴. However, *P. malariae* has rarely been detected in autochthonous infections in Mato Grosso or São Paulo in recent years (Fundação Nacional de Saúde, unpublished data).

Alternative diagnostic methods, such as the polymerase chain reaction (PCR), have recently revealed high prevalences of mixed-species infections, many of them involving *P. malariae*, in Africa^{13,17,18}, Southeast Asia^{9,11,19,20}, South America¹⁶ and Oceania¹. Most *P. malariae* infections had been missed by the local microscopists, who examined standard GTS on-site. However, no comparison between conventional microscopy and PCR-based species identification has been performed in Brazil. The present communication describes results of such a comparison, performed in the western Amazonian State of Rondônia.

Venous blood was collected, after informed consent, from 96 patients with malaria diagnosis confirmed microscopically on GTS. Blood samples were stored at -20°C until DNA extraction. Thick smears were prepared and examined by local National Health Foundation personnel using standard criteria for species identification⁸. No attempt was made to change diagnostic routines during the study period. According to on-site GTS-based species identification, 43 patients had *P. falciparum* and 53 had *P. vivax* infection; no

mixed-species or *P. malariae* infection was detected by conventional microscopy. Template parasite DNA was extracted from 200-ml aliquots of clotted blood as described⁷. All patients had a febrile illness and were clinically examined by one of the authors at outpatient clinics in Porto Velho, Rondônia, between 1995 and 1998. Antimalarial treatment was given, following the recommendations of the Fundação Nacional de Saúde⁸, based on on-site species identification, since PCR results were not available.

A semi-nested PCR for species identification was performed as described by Kimura and colleagues¹⁰. Briefly, two rounds of amplification were performed with oligonucleotide primers that target small sub-unit ribosomal RNA (rRNA) genes of *Plasmodium*. The first round (35 cycles) used a pair of universal (i.e. genus-specific) primers, P1 and P2, while the second round (18 cycles) used the species-specific reverse primers F2, V1 and M1 combined with the universal forward primer P1. Oligonucleotide primer sequences and detailed PCR protocols are given elsewhere¹⁰. Amplification reagents and PCR products were handled in different laboratories using pipettes equipped with aerosol-resistant filter tips, in order to prevent carry-over contamination. Laboratory personnel were unaware of GTS-based species diagnosis at the time when the first PCR experiments were performed, but later all samples showing discordant results between microscopy and PCR were re-tested.

Mixed-species infections, one of them involving three *Plasmodium* species, were frequently found by PCR among patients thought to have single-species infection based on GTS examination (Table 1). As a result, some patients were given inadequate antimalarial treatment, based on incorrect or incomplete species diagnosis. These findings are in agreement with previous comparisons of conventional microscopy and PCR in Africa^{13,17,18} and East Asia^{9,11,19,20}. As a rule, local microscopists are able to identify correctly the predominant species, but concurrent infections are frequently missed²⁰. Despite the low malaria endemicity in Rondônia, local patterns of transmission and interaction between different species and clones seem to be rather complex. About 40% of patients harboring *P. falciparum* and *P. vivax* have multiple-clone infections (i.e., a single isolate harbors more than one genetically distinct parasite clone), as detected by PCR amplification of single-copy polymorphic genes^{7,15}. Here we have shown that 30% of infections in

Table 1 - Comparison of species identification, based upon examination of Giemsa-stained thick smears and semi-nested PCR, in 96 patients from Rondônia, Brazilian Amazon.

Diagnosis by Giemsa-stained thick smears (no. of patients)	Diagnosis by nested PCR (no. of patients)							Total
	F	V	M	F+V	F+M	V+M	F+V+M	
F	35	0	0	7	1	0	0	43
V	2	30	1	13	1	5	1	53
Total	37	30	1	20	2	5	1	96

F = *P. falciparum*; V = *P. vivax*; M = *P. malariae*. Note that many of the patients with a single-species infection diagnosed on Giemsa-stained thick smears (8 of 43 with diagnosis of *P. falciparum* and 20 of 53 with diagnosis of *P. vivax*) had mixed-species infection diagnosed by PCR, and 3 patients with *P. vivax* identified on thick smears had a single but different species (either *P. falciparum* or *P. malariae*) detected by PCR.

Rondônia involve two or more *Plasmodium* species, and similar results were recently reported in an area of comparable malaria endemicity in Venezuela¹⁶. Therefore, a large proportion of local patients harbor more than one malaria species and/or genetically distinct parasite clones. The clinical and immunological consequences of such mixed-species and mixed-clone infections have been a matter of discussion in recent years¹².

Specific amplification of *P. falciparum*, *P. vivax* and *P. malariae* DNA was obtained respectively in 60 (62%), 57 (59%) and 9 (10%) of the 96 examined malaria patients. This relatively high prevalence of *P. malariae* contrasts with the reported absence of this species in Rondônia in recent years. All but one *P. malariae* infection were detected in mixed-species infections, and in all cases, the PCR-amplified fragment was 115-bp long. In contrast, *P. malariae* isolates with a 19-bp deletion in the rRNA gene fragment targeted by PCR have been commonly found in East Asia^{9,11}. Interestingly, most *P. malariae* infections reported in Rondônia in the 1980s were detected in the small town of Costa Marques (National Health Foundation, unpublished data), where an international malaria research team maintained a field station. The clustering of *P. malariae* infections in the State of Pará in recent years may also be a by-product of the extensive malaria research activities in that region, with a positive impact on local diagnostic practices.

In conclusion, a high proportion of mixed-species infections, some of them including the frequently overlooked species *P. malariae*, may be detected in the western Amazonian State of Rondônia using a sensitive semi-nested PCR protocol. As indicated by the frequent occurrence of anti-*P. malariae* circumsporozoite protein antibodies among subjects living in Mato Grosso^{5,6,14} and even in southeastern Brazil⁴, this species may be much more widespread in Brazil than suggested by official data based on GTS microscopy. The possibility exists that *P. malariae* infection occurs as a zoonosis in some areas in the Amazon^{5,6}, with predictable implications for malaria control. The long-lasting nature of low-level, symptomless *P. malariae* infections in semi-immune subjects increases the risk of transfusion malaria in both endemic and non-endemic regions². Moreover, different drug regimens are used in the treatment of the three malaria species found in Brazil, and misidentification of these species has obvious implications for patients' care. Therefore, improved diagnostic techniques such as the molecular approach applied here may be useful to investigate the distribution of human malaria species across the Brazilian Amazon and to develop adequate strategies for treatment and control of malaria in each epidemiological setting.

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