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Mixed *Plasmodium falciparum* infections and its clinical implications in four areas of the Brazilian Amazon region

Alexandre Lorenzetti[a], Patrícia Aparecida Fornazari[a], Ana Carolina Bonini-Domingos[a], Roberta de Souza Rodrigues Penhalbel[a], Érika Fugikaha[a], Cláudia Regina Bonini-Domingos[b], Valéria Daltibari Fraga[a], Luciana Moran Conceição[a], Andréa Regina Baptista Rossit[a,d], Carlos Eugênio Cavasini[a], Vanja Suely Calvosa D'Almeida Couto[c], Ricardo Luiz Dantas Machado[a,d,*]

[a] *Centro de Investigação de Microrganismos, Departamento de Doenças Dermatológicas, Infecciosas e Parasitárias, Faculdade de Medicina de São José do Rio Preto, Avenida Brigadeiro Faria Lima 5416, 15090-000 São José do Rio Preto, São Paulo, Brazil*
[b] *Departamento de Biologia, Universidade de São Paulo, Rua Cristóvão Colombo 2265, 15054-000 São José do Rio Preto, São Paulo, Brazil*
[c] *Programa de Malária, Instituto Evandro Chagas, Secretaria de Vigilância em Saúde, BR316 Km 7, 67030-000 Ananindeua, Pará, Brazil*
[d] *Fundação Faculdade de Medicina de São José do Rio Preto, Avenida Brigadeiro Faria Lima 5544, 15090-000 São José do Rio Preto, São Paulo, Brazil*

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ABSTRACT

The aim of this study was to assess the prevalence pattern of mixed-*Plasmodium falciparum* malaria infections in Brazil by molecular diagnosis and to address its clinically important features. DNA was extracted from 115 thick blood film *P. falciparum* human blood positive samples using the phenol–chloroform method, followed by a semi-nested PCR protocol with species-specific primers. Seventy-three percent of *P. falciparum* single infections and 26.95% of mixed infections were found. Amongst mixed infections, the majority was double infection (96.77%). Our results suggest that the prevalence of one species over the other can be important on weakening *P. falciparum* malaria clinical symptoms. We confirm that *P. falciparum* co-infections frequently occur in Brazilian malaria endemic areas, with underestimated diagnosis. The results point to the need of improving microscopy or changing for another accurate diagnosis technique to differentiate among human malaria species, as this is essential to choose the best treatment and control measure for malaria. More investigations are necessary in order to clarify the role of mixed-infections in the severity of *P. falciparum* disease.

1. Introduction

Malaria is an infectious disease caused by four species of intraerythrocytic protozoan parasite of the *Plasmodium* genus. Of the known human malaria parasites, only *Plasmodium falciparum*, *Plasmodium vivax*, and *Plasmodium malariae* have been detected in Brazil with 99.7% of cases occurring in the Amazon region (Póvoa et al., 2000; Ministério da Saúde, 2003).

Previous reports suggest that in mixed infections, one *Plasmodium* species may suppress the blood-stage density of another (Hill et al., 1943; Bruce-Chwatt, 1963). Over the last years, the relationships among the human malaria parasite infections have been investigated with contradictory results. Cohen's (1973) and Richie (1988) reported that mixed infections occurred less than would be expected from cross-sectional prevalences of the individual species. Molineaux et al. (1980) reviewed prevalence surveys and concluded that mixed-species infections were actually more common than expected and Richie (1988) showed no general pattern in their frequencies. In contrast, other studies found that lower than expected frequencies of dual *P. vivax–P. falciparum* infections correspond to a higher overall malaria prevalence and also that *P. malariae–P. falciparum* infections are invariably correlated with a large number of mixed infections (McKenzie and Bossert, 1997, 1999). Additionally, a mathematical model of the *P. malariae–P. falciparum* infection versus the human host dynamics suggested that a *P. malariae* infection can reduce the peak parasitemia of a subsequent *P. falciparum* infection (Mason et al., 1999).

Cohen's (1973) reports associated a decreased spleen size with mixed infections. Severe malaria was approximately four times more common in *P. falciparum* single infected patients than in those with dual *P. falciparum–P. vivax* infections (Luxemberger et al., 1997). Black et al. (1994) suggested that *P. malariae* infections reduce the severity of subsequent *P. falciparum* infections, and that

* Corresponding author at: Centro de Investigação de Microrganismos, Departamento de Doenças Dermatológicas, Infecciosas e Parasitárias, Faculdade de Medicina de São José do Rio Preto, Avenida Brigadeiro Faria Lima 5416, 15090-000 São José do Rio Preto, São Paulo, Brazil. Tel.: +55 17 3201 5736; fax: +55 17 3201 5909.
E-mail address: ricardomachado@famerp.br (R.L.D. Machado).

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individuals concurrently infected by both species have experienced significant reductions in fever. Recently, it was reported in Thailand that patients with dual *P. vivax–P. falciparum* infections have higher fevers than those with single-species infections (McKenzie et al., 2006).

Previous studies have pointed to highly relevant limitations of traditional microscopy-based detection techniques (Snounou et al., 1993; Postigo et al., 1998). Indeed, the deficiency to detect mixed infections by the thin and thick blood film methods make treatment difficult as it is species-specific. Polymerase chain reaction (PCR) has been shown to be efficient in the diagnosis of the four human malaria parasites and, therefore, also on identifying high prevalence of mixed infections (Roper et al., 1996; May et al., 1999). The aim of this study was to assess the prevalence pattern of mixed-*P. falciparum* malaria infections in Brazil by molecular diagnosis and to address its clinically important features.

2. Materials and methods

2.1. Study population

Sample collection took place from May 2003 to August 2005. One hundred and fifteen male and female malaria patients from four regions of the Brazilian Amazon: Macapá, state of Amapá (00°02′20″S; 51°03′59″W); Novo Repartimento, state of Pará (04°19′50″S; 49°47′47″W), Porto Velho, state of Rondônia (−08°45′43″S; 63°54′14″W); and Plácido de Castro, state of Acre (10°16′33″S; 67°09′00″W) were enrolled in this study. These individuals presented on their own initiative, and were invited to participate in this study at the public healthcare clinics in each study area. They were all over the age of 18 and had positive thick blood film (TBF) results for *P. falciparum* single infection. We excluded from the study pregnant women, patients under the age of 18 years and no other concomitant illness. Participants were asked to sign a written consent form before blood samples were drawn. The consent form was co-signed by a staff member of the clinic. Clinical and epidemiological data such as age, gender, past history of malaria, and current infection information were obtained from a specific interview conducted by the physicians and also from medical records. The protocol for this study was reviewed and approved by the Research Board of the Faculty of Medicine from São José do Rio Preto.

2.2. Clinical evaluation

All patients voluntarily sought medical assistance presenting with uncomplicated clinical malaria symptoms as evaluated by the physicians and/or nurses enrolled in the malaria diagnosis and treatment routine of the Brazilian government national program. Individuals who presented at least one of the following symptoms: fever, headache, and shiver, in addition to microscopic positivity, were included in the post-diagnostic medical evaluation. Likewise, symptoms were defined as "present" or "absent" by the medical staff accordingly to the temperature measurements performed by the nurses and also by a detailed, specific interview, regarding unusual and/or previously experienced clinical malaria manifestation.

2.3. Laboratory analysis

Thick blood films (TBFs) were confirmed by independent experienced microscopists who were unaware of each result according to the World Health Organization recommended procedures. Blood samples were stored at −20 °C until laboratory analyses. Samples were treated with Proteinase K, and nucleic acids were extracted by using two rounds of phenol:chloroform:isoamyl alcohol (25:24:1), one round of chloroform and one of ether, followed by ethanol precipitation. The extracted nucleic acid samples were dissolved in sterile pure deionised water, and stored at −20 °C prior to use. The semi-nested PCR was based on the protocol accordingly to Kimura et al. (1997). The target was the SSU rDNA gene, and species-specific primers were used in the assay. Briefly, the first PCR rDNA amplification was performed with *Plasmodium* genus-specific primers. Positive samples served as template for the nested reaction. The nested PCR amplifications were performed using *P. falciparum*, *P. vivax*, and *P. malariae* SSU rDNA primers plus universal primer from the first reaction. The fragments obtained were seen at about 110-bp. As a positive control we used blood samples with *P. falciparum*, *P. vivax*, and *P. malariae* TBF plus molecular results to *Plasmodium*. As a negative control we used blood samples from blood donors living in the same areas with negative microscopy and molecular results to *Plasmodium*. The products were visualized in 2% agarose gel stained with ethidium bromide.

2.4. Data analysis

Epi Info version 6.04b (CDC, Atlanta, US) was used for data storage and statistical analyses. Proportions and categorical data were compared by the Chi-square test, with Yate's correction, in cases of 2×2 contingency tables, or Fisher exact test (two-tailed). The adopted significance level for statistical inference was $p < 0.05$.

3. Results

The parasitaemia on the thick blood films ranged from 25 to 6500 parasites/mm^3. *P. falciparum* parasitaemia was lower among patients with mixed infections than among patients with single-species infections, but this difference was insignificant (Chi-square 5403, $p > 0.7137$). In Macapá patients, the previous malaria experience (in number of episodes) was 1.5 (±2.01); in those from Porto Velho was 0.9 (±1.57); in those from Novo Repartimento was 1.7 (±2.62) and from Plácido de Castro was 1.6 (±2.57). As for their ages, the geometric means in each area were 28 (±1.35), 25 (±2.35), 32 (±1.15), and 30 (±1.02) years old, respectively, ranging from 18

Table 1
Identification of *Plasmodium falciparum* mixed-infections as determined by malaria genotypic test among 115 patients from four Brazilian Amazon areas

	Molecular diagnosis			
	P. falciparum	*P. falciparum* + *P. malariae*	*P. falciparum* + *P. vivax*	*P. falciparum* + *P. malariae* + *P. vivax*
Novo Repartimento/PA ($n = 16$)	14 (16.67%)	–	2 (7.14%)	–
Macapá/AP ($n = 37$)	26 (30.95%)	1 (50%)	10 (35.71%)	–
Porto Velho/RO ($n = 50$)	35 (41.67%)	–	14 (50%)	1 (100%)
Plácido de Castro/AC ($n = 12$)	9 (10.71%)	1 (50%)	2 (7.14%)	–
Total	84	2	28	1

PA: Pará; AP: Amapá; RO: Rondônia; AC: Acre.