

Prevalence and level of antibodies to the circumsporozoite protein of human malaria parasites in five states of the Amazon region of Brazil

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The aim of this study was to determine the prevalence of malaria infection and antibodies against the repetitive epitopes of the circumsporozoite (CS) proteins of Plasmodium falciparum, P. malariae, P. vivax VK210, P. vivax VK247, and P. vivax-like in individuals living in the states of Rondônia, Pará, Mato Grosso, Amazonas, and Acre. Active malaria transmission was occurring in all studied sites, except in Acre. P. falciparum was the predominant species in Pará and Rondônia and P. vivax in Mato Grosso. Infection by P. malariae was low but this Plasmodium species was detected in Rondônia (3.5%), Mato Grosso (2.5%), and Pará (0.8%). High prevalence and levels of serological reactivity against the CS repeat peptides of P. falciparum were detected in Rondônia (93%) and Pará (85%). Sera containing antibodies against the CS repeat of P. malariae occurred more frequently in Rondônia (79%), Pará (76%), and Amazonas (68%). Antibodies against the repeat epitope of the standard CS protein of P. vivax VK210, P. vivax VK247, and P. vivax-like were more frequent in Rondônia, Pará, and Mato Grosso. The high frequency of reactions to P. malariae in most of the areas suggests that the infection by this Plasmodium species has been underestimated in Brazil.

Key words: Plasmodium - infection - Amazon region - Brazil

Malaria remains as a serious health problem in the Amazon Basin of Brazil and approximately 99% of some 600,000 cases notified annually occur in this region (Ministério da Saúde 2003, Coura et al. 2006). Malaria is heterogeneously distributed in the nine states of the Legal Amazon and the number of annual cases fluctuates, increasing in some states where gold mining activities take place, massive migration occurs, and new settlements projects are implemented. However, the states of Pará, Amazonas, and Rondônia are always among the major malaria-endemic regions. Based on microscopic examination from the records of the Brazilian Ministry of Health, *P. falciparum* and *P. vivax* are the most prevalent *Plasmodium*. The reported prevalence of *P. malariae* is very low (< 1%). In the last 15 years the number of *P. vivax* has increased and presently accounts for 78% of all malaria cases reported in the country. Besides the classic *P. vivax* VK210, two species-specific circumsporozoite (CS) protein variants *P. vivax* VK247 (Rosenberg et al. 1989) and *P. vivax*-like (Qari et al. 1993, 1994) were described. The human *P. vivax*-like parasite has a CS repeat which corresponds to the simian parasite *P. simiovale*. Otherwise the identity of these parasites is not clear, since a line of the human isolates has not yet been obtained and the clinical manifestation of these infections is unknown.

Antibodies to sporozoites represent a serological transmission indicator and have been used in epidemiological studies to estimate the level of malaria in endemic areas. It is well known that individuals living in malaria endemic areas exposed to sporozoite infections develop antibodies directly to the repetitive epitopes of the CS protein. The prevalence and levels of anti-sporozoite antibodies to *P. falciparum* increase with age and have been shown to correlate with entomological inoculation rate assessed at the same time and place (Nardin et al. 1979, Druilhe et al. 1986, Del Giudice et al. 1987, Greenwood 1990). In malaria-endemic areas of Brazil it is difficult to appraise entomological data quantitatively and the serological transmission indicator may serve as an alternative in control programs. In these regions very few serological studies to all three human malaria species have been performed (Arruda et al. 1989, Krensmer et al. 1992, Machado et al. 2000). Most studies reported only the prevalence of antibodies to *P. vivax* and its variants. Antibodies to *P. vivax* VK247 sporozoites was first detected in the state of Pará in sera samples from indigenous populations during a survey conducted from 1986 to 1988 (Cochrane et al. 1990). A retrospective study showed that the *P. vivax* VK247 and *P. vivax*-like were present in indigenous populations since 1987 (Arruda et al. 1998). Antibodies to these variants were also detected in other regions of the Brazilian Amazon; in the states of Acre (Krensmer et al. 1992, Branquinho et al. 1993, Marelli et al. 1997, Machado et al. 2000). More recently antibodies to the CS repeats of these two variants were also detected in areas of very low endemicity in the states of Espírito Santo (Arruda, unpublished observations) and São Paulo (Curado et al. 1997).

The aim of the present study was to conduct a sero-epidemiological survey of malaria in human populations

Financial support: OMS/TDR (grant 900550), FNS, CNPq, Fundep/MG, Fiocruz

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Received 19 February 2007

Accepted 24 April 2007

living in endemic areas of the Brazilian Amazon and determine the prevalence of malaria infections and antibody recognition of peptides representing the repetitive epitope of the CS protein of *P. falciparum*, *P. malariae*, *P. vivax* VK210, *P. vivax* VK247, and *P. vivax*-like human malaria parasites.

Serological studies may shed light on the dynamics of malaria in area where local health systems are lacking and baseline data are needed to better understand and develop interventions.

MATERIALS AND METHODS

Study area - Seven randomized surveys were conducted from 1993 to 2003 in five states of the Amazon region of Brazil (Fig. 1). A total of 1206 serum samples and blood films were collected from different populations in the states of Pará, Rondônia, Mato Grosso, Acre, and Amazonas. All participants were interviewed directly or through a respondent inhabitant and allocated a unique identification number. Informed consent was obtained from all individuals before admission into the study. The information collected included name, sex, age, and time remained in the area.



Fig. 1: location of the collection sites. AC, Acre; AM, Amazonas; MT, Mato Grosso; PA, Pará; RO, Rondônia.

Results discussed in this article are related to samples of blood randomized collected during the development of the following projects: "A longitudinal study to determine whether there is seasonal fluctuation in the incidence of *Plasmodium falciparum*, *P. vivax* and *P. malariae* and in the species of mosquitoes responsible for their transmission in the State of Pará, Amazon region of Brazil", "Identificação dos anofelinos responsáveis pela transmissão das diferentes espécies de malárias existentes nas áreas de garimpo dos Municípios de Peixoto de Azevedo e Matupá, Estado de Mato Grosso" and "Revisitando a Amazônia de Carlos Chagas: da borracha à biodiversidade". The ethics committee of the Fundação Oswaldo Cruz approved the first two projects.

The first survey was conducted in 1993 in Pará, where 119 serum samples and blood films were taken from natives living in four locations (Asa da Arara and Apinajes in the municipality of São João do Araguaia, and Veadas and Televisão in the municipality of Itupiranga). These locations were chosen based on a previous survey conducted during 1983-1985 that showed year-round transmission of *P. vivax*, *P. falciparum* and *P. malariae* (Arruda et al. 1986). In 1994 and 1995, serum samples and blood films were collected in Mato Grosso from 471 gold miners in 14 different mining sites in the municipalities of Peixoto de Azevedo and Matupá. In 1995, a total of 142 serum samples and blood films were collected in Rondônia from farmers living in Machadinho, who had migrated from southern Brazil to colonize this part of the Amazon in mid-1980. In 1997 a total of 180 serum samples and blood films were collected from stable riverine populations living along the Acre River. In the same survey 170 serum samples and blood films were also collected in the state of Amazonas along the Purus River, from native descendents of families that worked in the rubber plantations at the beginning of last century. In 2002, in the same state, 124 serum samples and blood films were collected from individuals that over the last several years invaded the suburban areas of the capital Manaus. The populations tested during the surveys were representative of the major malaria-endemic areas of the Amazon region of Brazil.

Blood collection - Approximately 5 ml of venous blood was collected from consenting individuals and allowed to clot for 5-6 hours at room temperature. Serum samples were stored in -20°C . Thick and thin blood films were prepared soon after the blood samples were drawn, stained with Giemsa and examined under oil immersion ($\times 100$) with a conventional light microscope. A minimum of 250 fields was examined before a slide was declared negative. The parasitological examination of the blood slides was done in the local areas and individuals found positive for malaria parasites were treated.

ELISA test for the detection of anti-CS antibodies - The ELISA for detection of antibodies to the CS protein was conducted as described by Wirtz et al. (1990). All serum samples were tested in duplicate at a dilution of 1:100. Synthetic peptides used as antigen were the repeats of the CS proteins of *P. vivax* VK210 (DGQ PAGDRAA-(GQPAGDRAD)2-GQPAG, *P. vivax* VK247 (ANGAGNQP)3-ANGAGN, *P. vivax*-like (APGAN QEGGAA)3, *P. falciparum* (NANP)8, and *P. malariae* (GNAA)2-GNDA-(GNAA)5. The mouse monoclonal antibodies Pf2A10, PvNSV3, Pv247, and Pm were used as positive controls for Pf, PVK210, PVK247, and Pm synthetic peptides. Polyclonal serum used as positive control for *P. vivax*-like was obtained from a wild monkey infected with *P. simium* and reacted strongly against the synthetic peptide. Since a level of antibody equal to 1:40 or less may be questionable as an indication of past or present malaria in endemic areas we elected to consider positive only sera with ELISA endpoints of 1:100 or higher. We calculated the cutoff value for each peptide using twenty serum samples from healthy malaria naive control individuals who never had a past

history of malaria and who had never been visited malaria transmission areas. Samples were considered positive when the mean OD values were above the mean plus 3 standard deviations of healthy control group.

RESULTS

Blood film results demonstrated that malaria transmission was occurring in all populations studied, except in Acre (Table). The level of parasitemia was low in most individuals, usually lower than 0.02%. Mature asexual parasites were detected in only a few smears and three individuals showed mature gametocytes in the peripheral blood. Thick and thin blood films evaluation showed that the prevalence of malaria infection was significantly higher in Pará (40%) followed by Rondônia (17.6%) and Mato Grosso (15%). *P. falciparum* was more prevalent in Pará (29%) and Rondônia (9.5%) and *P. vivax* in Pará (10.1%) and Mato Grosso (7.6%). Infection by *P. malariae* was low but detected in Rondônia (3.5%), Mato Grosso (2.5%), and Pará (0.8%). The overall prevalence of malaria infection was highest in Pará followed by Rondônia and Mato Grosso (Table).

The prevalence of antibodies to the CS repeats of *P. falciparum*, *P. malariae*, and *P. vivax* was greater in migrants from Rondônia and natives from Pará compared to the other populations surveyed (Fig. 2). In Mato Grosso the prevalence of antibodies to all the CS repeats was lower but the frequency distribution of positive to all CS repeats was similar to Rondônia and Pará. In Amazonas the prevalence of antibodies was higher for the CS repeats of *P. falciparum* and *P. malariae* although infection by *P. malariae* was not detected by thick and thin blood smear examination. The high prevalence of antibodies to *P. malariae* contrasting with low prevalence to *P. vivax* suggests that *P. malariae* could be misdiagnosed as *P. vivax* in this area. In Acre plasmodial infection was not detected and the prevalence of antibodies to all three CS repeats was very low. This may reflect a low level of exposure of the stable riverine populations to *Plasmodium*-infected *Anopheles* mosquitoes.

Antibodies against the repeat epitope of the CS protein of *P. vivax* VK210, the variant VK247 and *P. vivax*-like were detected in all of the states surveyed. High levels of anti-sporozoite antibodies against *P. vivax* VK210 and *P. vivax* VK247 were detected, especially in migrants from Rondônia and natives from Pará while *P. vivax*-like was more prevalent in Rondônia. In

Amazonas and Acre the prevalence of antibodies to the CS repeats of *P. vivax* VK210 and both variants was very low.

The geometric mean titer (GMT) of antibodies to the CS repeats varied quite appreciated among the studied sites (Fig. 3). In Rondônia the GMT were higher for all CS repeats when compared to the other states. In Pará the highest GMT was to the CS repeat of *P. vivax* VK210 followed by *P. falciparum*. In Mato Grosso, Amazonas, and Acre the GMT for all species was low.

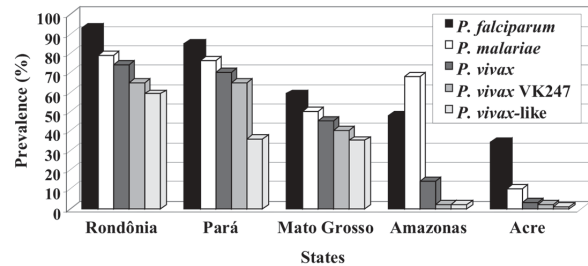


Fig. 2: prevalence of antibodies to the circumsporozoite repeats of *Plasmodium falciparum* (Pf), *P. malariae* (Pm), *P. vivax* (Pv), *P. vivax* VK247 (Pv247), and *P. vivax*-like (Pv-like) in endemic areas of the Amazon Region of Brazil. Differences in proportion were evaluated by Chi square test. Rondônia: Pf vs Pm (Chi = 9.236 p = 0.002), Pf vs Pv (Chi = 15.669 p < 0.0001), Pf vs PvK247 (Chi = 30.147 p < 0.0001), Pf vs Pv-like (Chi = 41.880 p < 0.0001); Pará: Pf vs Pm (Chi = 5.537 p < 0.018), Pf vs Pv (Chi = 6.923 p < 0.008), Pf vs Pv247 (Chi = 11.789 p < 0.0006), Pf vs Pv-like (Chi = 57.126 p < 0.0001); Acre: Pv247 vs Pm (Chi = 51.002 p < 0.0001), Pv247 vs Pv-like (Chi = 10.418 p < 0.0012).

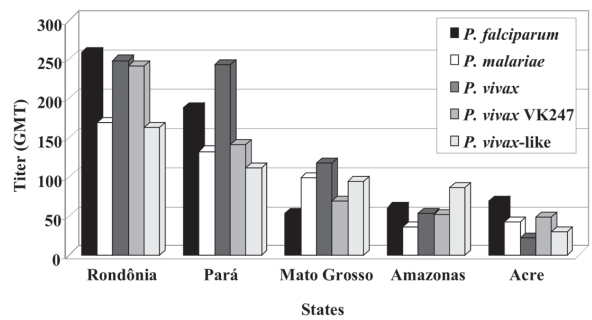


Fig. 3: geometric mean titer (GMT) of antibodies to the circumsporozoite repeats of *Plasmodium falciparum*, *P. malariae*, *P. vivax*, *P. vivax* VK247, and *P. vivax*-like in endemic areas of the Amazon region of Brazil.

TABLE

Frequency of patent malaria infections as determined by blood smears collected in five states of the Amazon region of Brazil

State	No. tested (% positive)	<i>Plasmodium falciparum</i> (Pf)	<i>P. malariae</i> (Pm)	<i>P. vivax</i> (Pv)
Rondônia	142 (17.6)	13 (9.2)	5 (3.5)	7 (4.9)
Mato Grosso	471 (15.1)	23 (4.9)	12 (2.6)	36 (7.6)
Pará	119 (40.3)	35 (29.4)	1 (0.8)	12 (10.1)
Acre	180 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Amazonas	294 (7.1)	12 (4.1)	0 (0.0)	9 (3.1)
Total	1206 (13.7)	83 (6.8)	18 (1.5)	64 (5.3)

Differences in proportion were evaluated by Chi square test. Mato Grosso: Pf vs Pv (Chi = 4.176 p = 0.041), Pv vs Pm (Chi = 16.648 p < 0.0001); Pará: Pf vs Pm (Chi = 48.400 p < 0.0001), Pf vs Pv (Chi = 20.870 p < 0.0001), Pv vs Pm (Chi = 0.012 p = 6.193).

DISCUSSION

In the Brazilian Amazon despite the high prevalence of human malaria parasites there are still states where little information about the distribution of cases caused by *P. vivax*, *P. falciparum*, and *P. malariae* is available.

Data for this seroprevalence study was collected in the course of seven random surveys conducted from 1993 to 2003 in order to evaluate the *Plasmodium* species and determine the prevalence of malaria infection and antibody recognition of peptides representing the repetitive epitope of the CS protein of *P. falciparum*, *P. malariae*, *P. vivax* VK210, *P. vivax* VK247, and *P. vivax*-like human malaria parasites. The individuals included in these surveys were living in the states of Rondônia, Pará, Mato Grosso, Amazonas, and Acre.

Blood smear results demonstrated that active malaria transmission was occurring in all studied sites, except in Acre. The level of parasitemia was low in most individuals, usually lower than 0.02%. Mature asexual parasites were detected in only few smears and three individuals showed mature gametocytes in the peripheral blood. There was a high prevalence of antibodies to the CS repeat of *P. malariae* in the states of Rondônia, Pará, and Amazonas. The high prevalence of antibodies against *P. malariae* shows the discrepancy between our data and the official cases reported by SIVEP suggesting that this *Plasmodium* species seems to be more common than suspected. The failure to report *P. malariae* in endemic areas is not surprising since the only official method for malaria diagnosis in Brazil is the microscopic examination of thick blood smear stained with Giemsa. In this procedure it is not possible to assess the morphology of infected red blood cells and the parasite-altered shape can lead to a mistaken identification of *P. malariae* as *P. vivax*. However, the antibody response to *P. malariae* antigens suggests that this parasite could possibly be more prevalent than *P. vivax* in human populations in Rondônia, Amazonas, and Pará. Recently, Cavasini et al. (2000) and Scopel et al. (2004) found significantly more cases of *P. malariae* infection in patients from Rondônia and Mato Grosso, when using nested polymerase chain reaction and comparing the results of *Plasmodium* species with conventional on-site microscopy of Giemsa-stained thick smears. These results point to the need for the development or use of a more accurate diagnosis method to distinguish between *P. malariae* and *P. vivax*. This is particularly important in view of the fact that the drug of choice for antimalarial therapy depends on the parasite species (Scopel et al. 2004).

The number of individuals with antibodies to the CS repeats of *P. falciparum*, *P. malariae*, and *P. vivax* were higher in migrants from Rondônia and natives from Pará, which coincided with the total of cases, reported by the Ministry of Health during the year of our survey (Rondônia 133,246 reported cases in 1995 and Pará 135,952 reported cases in 1993). However, this number could increase since repeated inoculations are needed to get a detectable level of antibodies (Mota et al. 1996). Wirtz et al. (1990) have shown that only 19% of subjects with confirmed acute *P. vivax* infection had detectable antibodies against CS protein. The age of indi-

viduals with detectable anti-sporozoite antibodies ranged from less than 5 to 64 years old.

In Mato Grosso, the prevalence of antibodies to the CS repeats of *P. falciparum* and *P. vivax* was 59 and 45%, respectively. In the surveyed area there is a continuous influx of new miners who never had malaria and others that returned after a few years from malaria free areas. This may account for the moderate prevalence in Mato Grosso when compared to Rondônia and Pará.

Antibodies against the CS protein of *P. vivax* VK247 variant occurred more frequently in Rondônia, Pará, and Mato Grosso and to a lesser extent against the repeat *P. vivax*-like. In Amazonas the prevalence of antibodies to the CS repeat of both variants was very low. This might reflect the recent introduction of these variants in the area or differences in the development of these variants in the local vector, resulting in a lower transmission rate.

The lowest prevalence of antibodies and patent infections for all *Plasmodium* species at the study sites were detected in the riverine populations of Acre. According to Funasa the number of reported cases during the year of our survey was low (6070 cases). One possible explanation for the low prevalence and titers may be related to the history and stability of these populations and the vectors species. Indeed, the participants of our study live in small-scattered communities (2-20 families) along the rivers. They practice subsistence agriculture, there is a low level of migration between communities and malaria is unstable with only occasional outbreaks. In addition, vector abundance was low, particularly for the principal vector in the Amazon Region, *An. darlingi* (Zimmerman et al. 1999) when compared to the other sites studied (Rondônia, Lourenço et al. 1989, Pará, de Arruda et al. 1986). In contrast, in Machadinho, Rondônia state, *An. darlingi* was present in high numbers and represented 98.3% of the anophelines collected (Lourenço et al. 1989).

GMT were calculated to compare differences in antibody levels between the study groups. In general, the ELISA GMT titers for *P. vivax* in Pará were considerably lower than those obtained from the examination of samples from Indians belonging to the A-Ukre and Pucanuv tribes, living in this same region of the Amazon. The overall GMT for the *P. vivax* VK247 variant in the Indians tribes living in Pará was 1073 (Arruda et al. 1998), while in natives of the same state reached only 353. The GMT to the CS repeat sequence of *P. vivax* VK210 and the variant VK247 showed similar trends in Rondônia and Pará while *P. vivax*-like were almost twice as prevalent in Rondônia.

The results of this study demonstrate that *P. malariae* infection is widely distributed in the Amazon Region of Brazil and it is more common than previously recognized. The transmission of the two variant strains of *P. vivax* VK247 and *P. vivax*-like is a common phenomenon in this region and vary from state to state even in geographically small areas.

In conclusion, seroepidemiological stratification of malaria showed to be a valuable method to recognize how variations in habits and years of contact with vectors contribute to the level of antibodies and intensity of transmission in malaria-endemic areas.

ACKNOWLEDGEMENTS

To Dr Ruth Nussensweig and Elisabeth Nardin, from the New York University, for allowing us to use their facilities. To Filipe Dantas-Torres for his critical and English review of the manuscript. To the staff of the Fundação Nacional de Saúde (Ministério da Saúde) for their technical assistance and logistic support. Also to the Ministério da Defesa and Secretaria Estadual de Saúde do Acre for logistic support.

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