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BRIEF COMMUNICATION

EVALUATION OF CIRCUMSPOROZOITE PROTEIN OF *Plasmodium vivax* TO ESTIMATE ITS PREVALENCE IN OIAPOQUE, AMAPÁ STATE, BRAZIL, BORDERING FRENCH GUIANA

Margarete do Socorro Mendonça GOMES(1,2), José Luiz Fernandes VIEIRA(2), Gustavo Capatti CASSIANO(3), Lise MUSSET(4), Eric LEGRAND(4), Mathieu NACHER(5), Vanja Suely Calvosa D'Almeida COUTO(6), Ricardo Luiz Dantas MACHADO(2,3,7) & Álvaro Augusto Ribeiro D'Almeida COUTO(2)

SUMMARY

Malaria is a major health problem for people who live on the border between Brazil and French Guiana. Here we discuss *Plasmodium vivax* distribution pattern in the town of Oiapoque, Amapá State using the circumsporozoite (CS) gene as a marker. Ninety-one peripheral blood samples from *P. vivax* patients have been studied. Of these, 64 individuals were from the municipality of Oiapoque (Amapá State, Brazil) and 27 patients from French Guiana (August to December 2011). DNA extraction was performed, and a fragment of the *P. vivax* CS gene was subsequently analyzed using PCR/RFLP. The VK210 genotype was the most common in both countries (48.36% in Brazil and 14.28% in French Guiana), followed by the *P. vivax*-like (1.10% in both Brazil and French Guiana) and VK247 (1.10% only in Brazil) in single infections. We were able to detect all three CS genotypes simultaneously in mixed infections. There were no statistically significant differences either regarding infection site or parasitaemia among individuals with different genotypes. These results suggest that the same genotypes circulating in French Guiana are found in the municipality of Oiapoque in Brazil. These findings suggest that there may be a dispersion of parasitic populations occurring between the two countries. Most likely, this distribution is associated with prolonged and/or more complex transmission patterns of these genotypes in Brazil, bordering French Guiana.

KEYWORDS: *Plasmodium vivax*; Circumsporozoite protein; Brazil–French Guiana border; Genetic marker.

Despite continuous efforts to control malaria, outside Africa, more malaria cases are caused by *Plasmodium vivax*, resulting in a daunting morbidity and economic burden for many countries across Asia and the Americas, especially in rural areas¹. Nowadays, *P. vivax* malaria transmission has decreased during the last decade, although its distribution persists and is heterogeneous in different areas².

Among Amazon countries, Brazil has the highest proportion of malaria cases (56%)³. In Brazil, gradual decreases from 2006 until 2012 have been observed. Nevertheless, *P. vivax* now accounts for more than 84% of clinical malaria cases annually reported in the Amazon region⁴. The circumsporozoite protein (CS) of the infective sporozoite is considered to be a major target for the development of recombinant malaria vaccine⁵ and, this protein can be evidenced in the process of sporozoite maturation and salivary invasion in the vector as well as in human liver cells^{6,7}. By serological and/or molecular approaches, different authors have evaluated the occurrence of *P. vivax* variants (VK210,

VK247 and *P. vivax*-like) in endemic areas of the Amazon region^{8–11}. Furthermore, the VK210 and VK247 were detected in *An. aquasalis* and *An. darlingi* in endemic areas of Pará State, Brazil¹².

Border areas between countries are often characterized by intense cross-border population flows¹³. The city of Oiapoque lies on the border between French Guiana and the Brazilian State of Amapá, where there is a well-documented and intense population flux with the French municipality of Saint Georges¹⁴. In French Guiana malaria is endemic and distributed along the Maroni and Oiapoque rivers, whereas the coastal area bordering the Atlantic Ocean has almost no malaria transmission¹⁴. A serological study in seven locations of the French Guiana (Cayenne, Camopi, Maripa Soula, Saint Laurent du Maroni, Gran Santi and Sinnamary) suggested that the three variant forms of *P. vivax* are circulating in these areas¹⁵.

P. vivax CS genotypes VK210 and VK247 have a worldwide

(1) Laboratório Central de Saúde Pública do Amapá. Amapá State, Brazil. E-mail: margarete.m.gomes@gmail.com

(2) Universidade Federal do Pará. Belém, Pará State, Brazil. E-mails: margarete.m.gomes@gmail.com; jvieira@ufpa.br; ricardomachado@iec.pa.gov.br; alvaro.couto6@hotmail.com

(3) Universidade Estadual Paulista, Instituto de Biociências, Letras e Ciências Exatas. São José do Rio Preto, São Paulo State, Brazil. E-mails: gcapatti@hotmail.com; ricardomachado@iec.pa.gov.br

(4) Laboratoire de parasitologie, Centre National de référence du Paludisme, Institut Pasteur de la Guyane. Cayenne, French Guiana. E-mails: lisemusset@gmail.com; elegrand@pasteur-cayenne.fr

(5) Université des Antilles et de la Guyane. Cayenne, French Guiana. E-mail: mathieu.nacher@ch-cayenne.fr

(6) Secretaria de Estado da Saúde do Amapá. Macapá, Amapá State, Brazil. E-mail: vs.pc@hotmail.com

(7) Instituto Evandro Chagas. Ananindeua, Pará State, Brazil. E-mail: ricardomachado@iec.pa.gov.br

Correspondence to: M.S.M. Gomes. E-mail: margarete.m.gomes@gmail.com

distribution and have been identified in several studies. However, the *P. vivax*-like genotype has been only detected in Papua New Guinea, Brazil, Indonesia and Madagascar^{16,17}. In Brazil, the presence of three variant genotypes was detected in samples obtained from indigenous populations and other communities of the Amazon region. Furthermore, there is evidence from Mexico² and Brazil¹² that VK210 and VK247 have differential infectivity rates in local vectors. Here we discuss *P. vivax* distribution pattern in the town of *Oiapoque*, Amapá State using the CS gene as a marker.

A subset of 91 patients was analyzed out of 103 individuals previously evaluated by Gomes *et al.*¹⁸. The peripheral blood samples, which had been kept at -70 °C, were from *P. vivax* carriers who lived in *Oiapoque*, Amapá State, a Brazilian malaria-endemic area (Fig. 1). The study took place from August to December of 2011 and was conducted by the staff of the Central Public Health Laboratory (LACEN) of the Amapá State. The patients who were enrolled in this study signed the written informed consent and fulfilled the following criteria: both genders, aged 16-60 years, they sought medical assistance due to clinical malaria symptoms, and had a positive malaria diagnosis by thick blood film or molecular techniques. Of the evaluated patients, for 64 individuals (70.33%) the infection site was the municipality of *Oiapoque* and for 27 patients (29.67%) it was French Guiana.

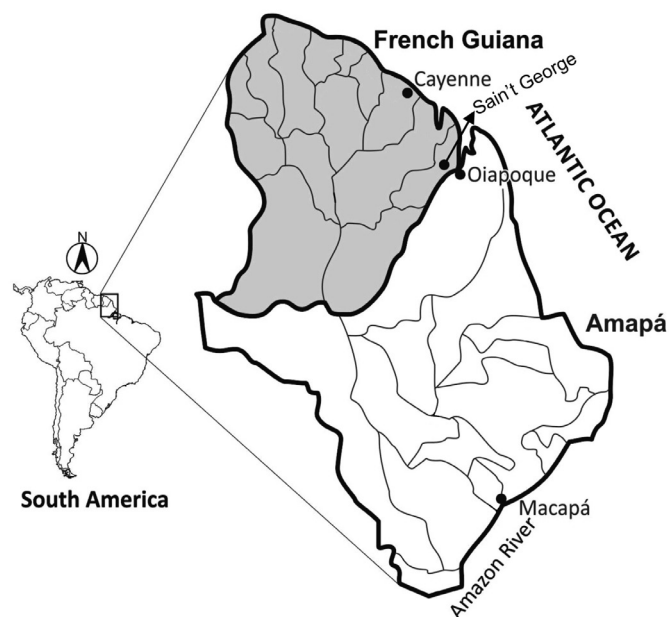


Fig. 1 - Map of the Brazilian Amazon region, showing the locations of the two populations from which isolates of *Plasmodium vivax* studied here were obtained: French Guiana and *Oiapoque*, Brazil.

The DNA was extracted from frozen pellets of infected erythrocytes using the Easy-DNA™ Kit (Invitrogen, Carlsbad, CA, USA), according to the manufacturer recommendations. The CS *P. vivax* genotypes were assessed using PCR/RFLP as previously described by Cassiano *et al.*¹⁹. Briefly, a reaction mix with a final volume of 25 µL containing *P. vivax* DNA (1.5 µL), 1 X PCR buffer (20 mM Tris-HCl pH 8.4, 50 mM KCl), 1.6 mM MgCl₂, 0.2 mM of each dNTP, 0.2 µM of each primer (5'-AGGCAGAGGACTTGGTGAGA-3' and

5'-CCACAGGTTACTACTGCATGG-3') and 1 U of Taq Platinum. The reaction was performed in a thermocycler (DNA MasterCycler, Eppendorf, Madison, WI, USA) as follows: an initial cycle of 94 °C for 15 min, followed by 30 cycles of 94 °C for 1 min, 58 °C for 1 min and 72 °C for 1 min, with a final extension at 72 °C for 10 min. As a positive control, three plasmids were used, containing a gene insert of the repeated portion of the CSP amplification product from VK210, VK247 and *P. vivax*-like variants (BlueScript, Stratagene, La Jolla, USA). As the negative control of the reaction, sterile water was used. The RFLP (Restriction Fragment Length Polymorphism) reaction was performed in a final volume of 20 µL: 10 U of the restriction enzyme AluI (Invitrogen, USA), 2 µL of the reaction buffer, 10 µL of the PCR product and 7 µL of sterile DNase-free water. Reactions were performed in a water bath at 37 °C overnight.

Analyses were performed using the R statistical software, version 2.4.1 (The R Foundation for Statistical Computing, Vienna, Austria [http://www.r-project.org]). The distribution of *P. vivax* CSP variants between the two studied areas was evaluated by the Chi-square test or the Fisher exact test, and the level of significance was set at $p < 0.05$.

The distribution of *P. vivax* CS genotypes and the genotypic frequencies of the 91 blood samples obtained from malaria patients are summarized in Table 1. The VK210 genotype was the commonest (62.64%), followed by *P. vivax*-like (2.20%) and VK247 (1.10%) in single infections. We were unable to detect all three CS genotypes simultaneously in mixed infections. However, double detections of VK210 plus VK247 (26.37%) and VK210 plus *P. vivax*-like (7.69%) were recorded. There were no statistically significant differences for *P. vivax* CS genotypes and the site of infection (Chi-square, p value = 0.1963). The parasitaemia on the thick blood films ranged from 200 to 36,000 parasites/mm³ (geometric mean + SD: 1,167.86 ± 3.32 parasites/mm³). The geometric means of parasitaemias were 1,323 parasites/mm³ (1,001- 1,750) for VK210; 1,205 parasites/mm³ (744- 1,951) for the VK210 + VK247 and 750 parasites/mm³ (295- 1,890) for VK210 + *P. vivax*-like infections. There were no statistically significant differences of the geometric mean of parasite density among the different genotypes detected (Table 1).

Epidemiological and genetic studies performed in malaria endemic areas of frontier malaria in Brazil could provide valuable information on parasite transmission and dispersion. The genetic diversity of the CS gene has been useful in molecular epidemiological studies, understanding transmission, dynamics and evolutionary relationships²⁰. Moreover, biological and genetic characteristics of the parasite, the host immunity and local vectors may influence the different patterns of demographic expansion, which are also modulated by eco-epidemiological conditions. However, the human effect can become a factor to reduce the risk of malaria, without necessarily modifying the environment^{3,21}.

Parasitic sampling in *Oiapoque* was performed, and followed a similar gradual pattern of *P. vivax* CS genotypes observed in others area of the Brazilian Amazon region. The three genotypes were found as single and mixed double infections, with VK210 and VK247 more frequently detected in mixed infections¹⁰. Interestingly, in French Guiana Volney *et al.*¹⁵ performed a seroepidemiological study on malaria and found positive reactions with all *P. vivax* CS peptides in malaria patients from the Maroni, *Oiapoque* and coastal areas. Antibodies against VK210,

Table 1
Distribution of *P. vivax* variants diagnosed in *Oiapoque*, Brazil, bordering French Guiana

Site of Infection*	Single types			Mixed types				Total
	VK210	VK247	<i>P. vivax</i> -like	VK210 + VK247	VK247 + <i>P. vivax</i> -like	VK210 + <i>P. vivax</i> -like	VK210 + VK247 + <i>P. vivax</i> -like	
French Guiana	13 (14.28%)	0	1 (1.10%)	10 (10.98%)	0	3 (3.30%)	0	27 (29.67%)
<i>Oiapoque</i>	44 (48.36%)	1 (1.10%)	1 (1.10%)	14 (15.38%)	0	4 (4.39%)	0	64 (70.33%)
Total	57 (62.64%)	1 (1.10%)	2 (2.20%)	24 (26.37%)	0	7 (7.69%)	0	91

*Chi-Square, $p = 0.1963$

VK247 and *P. vivax*-like were used. Our results suggest that this may be a common feature of frontier malaria. Thus, the same genotypes circulating in French Guiana were also found in the municipality of *Oiapoque* in Brazil. Moreover, the detection of VK210 subtypes circulating in the municipality of *Oiapoque* needs to be considered. In our research, some samples diagnosed as VK210 showed a nonspecific fragment in the RFLP gel. Recently, six different VK210 subtypes (VK210a, VK210b, VK210c, VK210d, VK210e the VK210f) were identified in isolates of *P. vivax* from Nicaragua and Mexico. The VK210a subtype was detected in both countries and was the most frequent. VK210f, was the less frequent, and has only been found in Nicaragua²¹. Unfortunately, the sequencing of these samples was not performed, which is a limitation that we acknowledge. Molecular investigations are currently being conducted to understand the role of VK210 subtypes in malaria epidemiology in bordering French Guiana.

This pattern of CS genotypes distribution at the border between Brazil and French Guiana, can bring serious implications to the strategies for effective malaria control. First, approximately 40% of *P. vivax* malaria cases detected in *Oiapoque* are from French Guiana⁴. From another perspective, the *P. vivax* treatment prescription used in Brazil is not the same applied in French Guiana. In Brazil the standard treatment recommended by Brazilian Ministry Health are chloroquine 25 mg/kg for three days (10 mg/kg on day 1 and 7.5 mg/kg on days 2 and 3), plus primaquine 0.50 mg/kg for 7 days. In French Guiana, when the diagnosis is confirmed, the patient has already received an unsupervised three-day treatment with chloroquine (25 mg/Kg). To receive a primaquine prescription, the individual requires a G6PD-deficiency test performed in Cayenne and a nominative temporary use authorization from the State drug authority. Thus, this procedure causes delay in the administration of primaquine. Consequently, approximately half of the population was presented *P. vivax* relapses¹⁴. Moreover, the periodicity of vivax malaria relapses may be explained by the activation of latent hypnozoites by subsequent infections with *P. falciparum*. Evidence from a simultaneous typhoid and malaria epidemic suggest that typhoid fever might activate *P. vivax* hypnozoites^{22,23}. However, previous reports suggest that in mixed infections, one *Plasmodium* species may suppress the blood-stage density of another species^{24,25}. Thus, species interactions may cause alterations in the typical clinical manifestation of infections caused by only one species, therefore additional investigations are necessary in order to clarify the role of mixed-infections on disease severity and also in the parasite transmission dynamics, considering that events taking place in French Guiana can influence the transmission and spread of the parasite on the Brazilian side.

In conclusion, a large proportion of all malaria cases in South America occur in the Brazilian Amazon region. The emergence of epidemic and endemic foci is affected by colonization of different areas by different human groups. However, it remains unclear whether parasites are commonly spread from one area to another by migrants or whether they emerge mainly from local endemic populations²⁶. The same CS genotypes circulating in French Guiana are found in the municipality of *Oiapoque* in Brazil. These findings suggest that it may occur via the dispersion of parasite populations between the two countries. Most likely, this distribution is associated with prolonged and/or more complex transmission patterns of these genotypes in Brazil, bordering French Guiana. Furthermore, studies based in CSP should consider these effects to obtain a protective vaccine.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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