Plasmodium vivax circumsporozoite variants and Duffy blood group genotypes in the Brazilian Amazon region

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Brazil

Summary
The circumsporozoite protein (CSP) of the Plasmodium vivax infective sporozoite is considered to be a major target for the development of recombinant malaria vaccines. The Duffy blood group molecule acts as the red blood cell receptor for P. vivax. We review the frequency of P. vivax CSP variants and report their association with the Duffy blood group genotypes from Brazilian Amazon patients carrying P. vivax malaria. Peripheral blood samples were collected from 155 P. vivax-infected individuals from five Brazilian malaria-endemic areas. The P. vivax CSP variants and the Duffy blood group genotypes were assessed using PCR/RFLP. In single infections, the VK210 variant was the commonest followed by the P. vivax-like variant. The typing of P. vivax indicated that the frequency of variants among the study areas was significantly different.
1. Introduction

In Brazil since 1993, the number of *Plasmodium vivax* cases has increased and they now account for more than 80% of clinical malaria cases annually reported in the Amazon region. The circumsporozoite protein (CSP) of the infective sporozoite is considered to be a major target for the development of recombinant malaria vaccines. 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.

The Duffy blood group acts as a receptor for *P. vivax* on the surface of the red blood cells and, therefore, its polymorphisms have an important impact where vivax malaria predominates. The FY gene, located on human chromosome 1, encodes the Duffy proteins by the two co-dominant alleles: FYA and FYB. The FYB allele is the result of a point mutation in the GATA box of the Duffy antigen/receptor for chemokines (DARC) promoter, which silences the gene encoding the Duffy system antigens in the red blood cells.

Previous reports from Latin America have suggested different abilities for vector infection between the VK210 and VK247 phenotypes. Ryan et al. described the possibility that the VK247 CSP variant marks a *P. vivax* population capable of using receptors other than Duffy. Furthermore, two Brazilian Duffy antigen-negative individuals infected by *P. vivax* were detected, whose CSP genotypes were VK210 and/or *P. vivax*-like. In our original study, we identified that individuals with the FYA/FYB genotype have higher susceptibility to *P. vivax* malaria in the Brazilian Amazon region.

We review the frequency of *P. vivax* CSP variants and report their association with the Duffy blood group genotypes in patients infected by *P. vivax* single (only one variant) or mixed (more than one variant) infections from the Brazilian Amazon.

2. Materials and methods

2.1. Sample collection and DNA extraction

A subset of 155 patients was analysed out of 312 individuals previously evaluated by Cavasini et al. The peripheral blood samples, which had been kept at −70°C, were from *P. vivax* carriers who lived in five Brazilian malaria-endemic areas: Novo Repartimento, Pará State; Macapá, Amapá State; Porto Velho, Rondônia State; Plácido de Castro, Acre State and Cuiabá, Mato Grosso State (Figure 1). The patients who were enrolled in this study complied with the following criteria: they sought medical assistance for clinical malaria symptoms, were over 18 years old and had a positive malaria diagnosis by thick blood film or molecular techniques, after written informed consent had been signed. The DNA was extracted from frozen pellets of infected erythrocytes using the Easy-DNA™ extraction kit (Invitrogen, Carlsbad, CA, USA).

2.2. Amplification of the *Plasmodium vivax* CSP gene fragment and RFLP analysis

The CSP *P. vivax* variants were assessed using PCR/RFLP as previously described by Alves et al. To amplify the CSP gene a set of forward (PR1: 5′- ATT TCT ATG CTA CTT TGT GTC TC-3′) and reverse (PR2: 5′- ATG GAC TCC ATG CAG TGT AAC C-3′) primers (Invitrogen, Portland, OR, USA) were designed based on the conserved central portion of the CSP gene. DNA (1.5 μl) was amplified in a total reaction volume of 25 μl consisting of 1× PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl), 1.5 mM of MgCl₂, 1.0 μM of each primer, 200 μM dNTPs, 2.5 U ampli-Taq DNA polymerase (Invitrogen, São Paulo, Brazil), 1% betaine and water (25 μl). Twenty-five cycles of amplification were performed in a thermocycler (DNA MasterCycler; Eppendorf, Madison, WI, USA) after initial denaturation of DNA at 94°C for 5 min. Each cycle consisted of a denaturation step at 93°C for 60 s, an annealing step at 41°C for 90 s and an extension step at 72°C for 2 min, with a final extension at 72°C for 10 min following the last cycle. Before typing CSP variants we progressively tested lower annealing temperatures (0.5°C lower at a time) until we reached good quality PCR products without unspecific amplification. The PCR products were analyzed by electrophoresis using 1.5% agarose gels and stained with ethidium bromide. The restriction enzymes selected were required to have at least one cleavage site in the amplification of each variant, resulting in DNA fragments that were easily visible in polyacrylamide gel. Restriction digestes were set up with 10 μl of the PCR product and 1 U of each enzyme (Alul and DpnI, Promega, Madison, WI, USA), incubated for 1 h at 37°C. Restriction fragments were separated by electrophoresis in 12.5% polyacrylamide gels. The gels were stained with ethidium bromide and analyzed with a Gel Doc 2000 illuminator (Bio-Rad Laboratories, Hercules, CA, USA).

2.3. Duffy blood group genotyping

Duffy blood group genotypes were assessed using PCR/RFLP as described previously, with modifications. Briefly, PCR was performed with 100 ng of DNA, 50 pmol of each primer
Figure 1  Study area for the *Plasmodium vivax* variants in the Amazon region of Brazil. Plácido de Castro, Acre State (AC; 10°16′33″S; 67°09′00″W); Porto Velho, Rondônia State (RO; 08°45′43″S; 63°54′14″W); Cuiabá, Mato Grosso State (MT; 15°17′05″S; 56°56′36″W); Novo Repartimento, Pará State (PA; 04°19′50″S; 49°47′47″W); Macapá, Amapá State (AP; 00°02′20″S; 51°03′59″W).

(Invitrogen, Portland, OR, USA), 2 nmol each dNTP, 1.0 U Taq DNA polymerase (Invitrogen, São Paulo, Brazil), and buffer, in a total volume of 50 μl. The promoter region was amplified using the FYN1 and FYN2 primers that flank the GATA box motif. To determine the Duffy red blood cell polymorphism, FYAB1 sense and FYAB2 reverse sense primers were used.19 The amplification conditions were performed as described by Castilho et al.18 PCR products were run on 1.5% agarose gel, followed by ethidium bromide staining and photo-documentation using a Gel Doc 1000 (Bio-Rad Laboratories). The RFLP analysis was performed through 3 h of digestion with *Ban*I, *Msp*AI and *Sty*I restriction enzymes (Promega).

2.4. Statistical analysis

Analyses were performed using R version 2.4.1 statistical software (The R Foundation for Statistical Computing, Vienna, Austria [http://www.r-project.org]). The analysis of dependency was applied to evaluate the distribution of *P. vivax* CSP variants within the five studied areas.20 To obtain the independence among the proportions, Fisher's exact test was applied with a significance level of *P* < 0.05.

3. Results

The distribution of *P. vivax* CSP genotypes and the genotypic frequencies of the Duffy blood group in the 155 blood samples obtained from malaria patients are summarized in Tables 1 and 2, respectively. The VK210 genotype was the commonest (58.7%), followed by *P. vivax*-like (9.7%) in single infections. The typing of *P. vivax* indicated that the prevalence and frequency of variants were significantly different from one area to another (Fisher's exact test; *P* = 0.046). The VK210 and *P. vivax*-like genotypes were detected as single infections in all studied areas. Only in samples from Novo Repartimento were we able to detect all three CSP variants presenting as single and/or mixed infections (Table 1). However, the distribution of the *P. vivax*-like genotype seemed to be more homogeneous than VK247 in the studied areas (analysis of dependency; *P* = 0.030). The VK247 variant was more frequently found in association with one of the two other variants (mixed infections) in Novo Repartimento and Macapá than in other studied areas. Additionally, this variant was found as a single infection exclusively in Pará State. Meanwhile, the VK210 variant as a single infection was more frequent in Porto Velho, Plácido de Castro and Cuiabá (Fisher's exact test; *P* = 0.033).

The data show a high frequency of the FYA/FYB genotype, which was present in 55 individuals (35.5%) with all *P. vivax* CSP variants. However, the association of the *P. vivax* variants with the FYA/FYB genotype was significant only for the VK210 single infection (Fisher's exact test; *P* = 0.042). As for the VK247 or *P. vivax*-like variants, either in single or mixed infections, the Duffy blood group system genotype distribution was homogeneous. There were no differences comparing the populations from the study areas for the
Table 1  Distribution of *Plasmodium vivax* variants in five areas of the Amazon region of Brazil (2003–2005)

<table>
<thead>
<tr>
<th>Study area</th>
<th>Single types, n (%)</th>
<th>Mixed types, n (%)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>VK210</td>
<td>VK247</td>
<td>VK210 + VK247</td>
</tr>
<tr>
<td>Cuiabá (MT)*</td>
<td>33 (70.2)</td>
<td>0</td>
<td>8 (17.0)</td>
</tr>
<tr>
<td>Macapá (AP)*</td>
<td>10 (35.7)</td>
<td>0</td>
<td>11 (39.3)</td>
</tr>
<tr>
<td>Novo Repartimento (PA)*</td>
<td>10 (29.4)</td>
<td>4 (11.8)</td>
<td>12 (35.3)</td>
</tr>
<tr>
<td>Porto Velho (RO)</td>
<td>25 (96.2)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Plácido de Castro (AC)*</td>
<td>13 (65.0)</td>
<td>6 (30.0)</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>91 (58.7)</td>
<td>4 (2.6)</td>
<td>15 (9.7)</td>
</tr>
</tbody>
</table>

MT: Mato Grosso State; AP: Amapá State; PA: Pará State; RO: Rondônia State; AC: Acre State.
* Significant at *P* < 0.05; Fisher’s exact test.

Table 2  *Plasmodium vivax* circumsporozoite variants and their correlation with Duffy blood group system genotypes

<table>
<thead>
<tr>
<th>Duffy blood group system genotypes</th>
<th><em>P. vivax</em> circumsporozoite variants, n (%)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>VK210</td>
<td>VK247</td>
</tr>
<tr>
<td>FYA/FYA</td>
<td>17 (11.0)</td>
<td>0</td>
</tr>
<tr>
<td>FYB/FYB</td>
<td>17 (11.0)</td>
<td>0</td>
</tr>
<tr>
<td>FYA/FYB</td>
<td>35* (22.6)</td>
<td>2 (1.3)</td>
</tr>
<tr>
<td>FYA/FYB-33</td>
<td>10 (6.5)</td>
<td>1 (0.6)</td>
</tr>
<tr>
<td>FYB/FYB-33</td>
<td>10 (6.5)</td>
<td>1 (0.6)</td>
</tr>
<tr>
<td>FYA/FYX</td>
<td>2 (1.3)</td>
<td>0</td>
</tr>
<tr>
<td>FYB/FYX</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>91 (58.7)</td>
<td>4 (2.6)</td>
</tr>
</tbody>
</table>

* Significant at *P* < 0.05; Fisher’s exact test.
Duffy blood groups (Fischer's exact test; \( P = 0.221 \); data not shown).

4. Discussion

In the Brazilian Amazon region, two previous studies evaluated the molecular epidemiology of the CSP variants and detected the VK210 genotype in single infections, whereas the VK247 and \( P. \) vivax-like genotypes were only detected as mixed infections in three malaria-endemic areas. Interestingly, after 10 years, a new scenario is observed. VK210 continues to be the most prevalent variant in all endemic areas of the Brazilian Amazon. However, regarding the VK247 and \( P. \) vivax-like variants, the present results suggest a change in \( P. \) vivax distribution dynamics since, to our knowledge, this is the first detection of both parasite forms as single infections in Brazil. These results may indicate that VK247 is not yet totally adapted in Brazilian malaria-endemic areas, unlike earlier observations in areas of Colombia, Mexico and Asia. The exclusive detection of VK247 in single infections only in blood samples from Novo Repartimento, Pará State, might suggest a later introduction of this variant in Rondônia, Amapá, Acre and Mato Grosso States. The results of this study demonstrate that the \( P. \) vivax-like variant is more widely distributed in all five studied areas than VK247. In fact, they show a more homogeneous distribution of the \( P. \) vivax-like variant, as a single or mixed infection, suggesting that its adaptation is happening faster than that of VK247. Another hypothesis is that the introduction of VK247 occurred more recently in Brazilian areas. These findings will allow us to continue expanding our understanding of \( P. \) vivax variant epidemiology in the Brazilian Amazon region.

It has been demonstrated that Duffy-negative individuals are protected against \( P. \) vivax infection, although in Brazil and in Africa \( P. \) vivax malaria has been shown to affect Duffy-negative individuals. Previous results have indicated that the heterozygous genotype for Duffy antigen favours infection by \( P. \) vivax in the Brazilian Amazon region, reinforcing the hypothesis that the presence of both functional Duffy alleles increases the risk of infection by this parasite. Indeed, the \( FYA/FYB \) genotype was the most prevalent in this study, which included only malaria patients. Possibly, variations in \( FYA \) and \( FYB \) allelic frequencies in populations exposed to \( P. \) vivax may determine differential susceptibility. Cavasini et al. showed that the difference in the \( FYB \) allelic proportion between malaria patients and controls (blood donors) was statistically significant (Fisher's exact test; \( P = 0.039 \)), and although no qualitative or quantitative measurements of the Duffy glycoprotein expression were performed, there was a higher number of malaria episodes among \( FYA/FYB \) patients. It is possible that the \( FYA/FYB \) genotype modulates an individual’s susceptibility to infection by \( P. \) vivax by means of quantitative and/or qualitative variations that affect the Duffy antigen expression on erythrocytes. On the other hand, the mechanism by which \( P. \) vivax uses the Duffy determinants to invade erythrocytes is mediated by the Duffy binding protein (DBP). Previous reports in Papua New Guinea and Colombia have shown that the sequence of the DBP is highly polymorphic, suggesting that the merozoite may have the capacity for rapid adaptation. Recently, Van Buskirk et al. suggested that polymorphism in the ligand domain of DBP can alter immune recognition. In Brazil, Cerávolo et al. showed that a recombinant DBP was immunogenic in Brazilian Amazon populations and that the immune response increased with exposure to malaria, reaching a peak in those subjects with long-term exposure. Moreover, Sousa et al. analyzed DBP variability and identified that other polymorphic residues seem to be unique among isolates from the Brazilian Amazon region.

In the present study a higher frequency of VK210 single infections was detected among \( FYA/FYB \) subjects. Such an association between the parameters occurring with the highest frequency: \( FYA/FYB \) genotype and single infections by the VK210 variant would be expected to be found, a limitation we acknowledge. Nevertheless, other explanations are possible based on biological differences not necessarily related to the CSP protein function, as postulated before. DARC is the receptor for \( P. \) vivax DBP and not for CSP, which is not expressed during the merozoite blood stage. Therefore, one possible explanation for the current results is that CSP and DBP variations are in linkage disequilibrium, a perspective to be explored. Likewise, \( P. \) vivax CSP variants could be tagging functional subset(s) of genetic diversity that modulate the efficiency of erythrocyte invasion towards a specific group of erythrocyte receptors.

The negative findings for single and mixed VK247 and/or \( P. \) vivax-like infections in relation to the studied Duffy genotypes point to the hypothesis that these variants characterize a \( P. \) vivax population capable of using receptors other than Duffy, as suggested by Ryan et al. In the previous report of Cavasini et al. the authors could not make this hypothesis since the presented Duffy-negative individuals were also VK247-negative.

Additional studies will be necessary to enable a better understanding of whether individuals in endemic areas of Brazil acquire \( P. \) vivax CSP variants that have preferential ability to bind the parasite ligand. Finally, the question remains whether the \( P. \) vivax CSP repeated region is a limited, mostly silent base variation or if these variants represent the existence of a new species or subspecies of \( Plasmodium \) causing human malaria, with major biological consequences. These observations provide additional data on the \( Plasmodium \)-host interactions mediated by the Duffy blood group and how these affect the capability of \( P. \) vivax to cause human malaria. Molecular and serological investigations are currently being conducted at the Center for Microorganisms Investigation, Faculty of Medicine of São José do Rio Preto, to improve the knowledge on the role of \( P. \) vivax variants in malaria epidemiology.

In conclusion, the current results highlight the possibility that \( P. \) vivax CSP variants mark biological differences towards the preferential invasion of specific erythrocyte antigen receptors, such as the Duffy antigen receptor for chemokines.

Authors’ contributions: RLDM and ARBR conceived and designed the study; AADC, MMP and CJF collected samples from individuals with \( P. \) vivax malaria; LMSM, CEC, WSN, GCC and ACPJ carried out all the genotype assays; RLDM, ARBR, CRBD and LCM analysed and interpreted the data.
and drafted the manuscript; LMSM, CEC, WCSN, GCC, ACPJ, AADC, MMP and CJF critically revised the manuscript. All authors read and approved the final manuscript. LMSM and WCSN are guarantors of the paper.

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Conflicts of interest: None declared.

Ethical approval: Research Board of the Faculty of Medicine of São José do Rio Preto, São Paulo State, Brazil.

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