Distribution of Plasmodium vivax variants (VK210, VK247 and P. vivax-like) in three endemic areas of the Amazon region of Brazil and their correlation with chloroquine treatment

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Abstract
The present study evaluated the glass fibre membrane (GFM) polymerase chain reaction (PCR)–enzyme-linked immunosorbent assay (ELISA) technique for genotyping the Plasmodium vivax variants, to verify the distribution of P. vivax variants (VK210, VK247 and P. vivax-like) in parts of Brazil and their correlation with levels of parasitaemia, previous malaria experience and clearance of parasitaemia linked to different treatment schedules. The samples were taken from individuals living in Macapá, Porto Velho and Belém, all of which are endemic areas of vivax malaria in the Amazon region of Brazil. Blood samples were collected on GFMs. The gene that codes for the circumsporozoite proteins of P. vivax variants was amplified by PCR and the amplified fragments were hybridized to variant-specific, digoxigenin-labelled oligonucleotide probes by ELISA. The GFM–PCR–ELISA technique was shown to be accurate for epidemiological surveys of the vivax complex. All variants were detected in all 3 areas, but only P. vivax VK210 was found as a single agent of infection, while the other 2 occurred as mixed infections. The P. vivax-like variant was found to be associated with low parasitaemia and VK210 with the highest parasitaemia levels; none of the P. vivax variants was linked with a previous malaria experience. In all cases parasitaemia clearance was identical regardless of the type of treatment and consequently it is not possible to confirm the previously reported correlation between P. vivax genotype and response to chloroquine.

Keywords: malaria, Plasmodium vivax, variants, VK210, VK247, Plasmodium vivax-like, epidemiology, chloroquine, glass fibre membranes. polymerase chain reaction. ELISA. Brazilian Amazon

Introduction
Of the 4 known human malaria parasites, Plasmodium vivax, P. falciparum, P. malariae, and P. ovale, only the first 3 species have been detected in Brazil (QARI et al., 1993a). During the past 5 years P. vivax has been responsible for 77.2% of all the reported malaria cases (BRASILIAN MINISTRY OF HEALTH, 1999).

Although the circumsporozoite protein (CSP) of the infective sporozoite has been a major target in the development of recombinant malaria vaccines, this approach has had to be re-evaluated because of the discovery of sequence variation in the CSP gene (QARI et al., 1993a; GODWIN et al., 1994). Based on the CSP gene, ROSENBERG et al. (1989) described a P. vivax variant form (VK247) in Thailand, and QARI et al. (1993a) reported the presence in Papua New Guinea of a human malaria parasite, referred to as ‘P. vivax-like’, morphologically resembling P. vivax but with the repetitive elements of the CSP differ from the 2 described types of P. vivax. Several studies have been conducted to evaluate the global distribution of variant VK247: It was detected in indigenous populations of Brazil (COCHRANE et al., 1990), in endemic areas of Thailand (WIRTH et al., 1991; KAIN et al., 1992, 1993a, 1993b), and in South America, Africa (KAINE et al., 1991), Mexico, Afghanistan and Papua New Guinea (KAIN et al., 1992). In addition, blood samples from Papua New Guinea, Indonesia, Brazil and Madagascar were positive for P. vivax-like DNA (QARI et al., 1993b). Serological tests have detected all 3 P. vivax variants in samples from São Paulo State (CURADO et al., 1995) and indigenous communities of the Amazon region of Brazil (ARRUDA et al., 1996, 1998).

Over the past 30 years, widespread resistance of malaria parasites to chloroquine has, so far, been restricted to P. falciparum, and chloroquine still remains the drug of choice for both prophylaxis and treatment of P. vivax infection (RIECKMANN et al., 1989; BALDASSARE et al., 1991). The first evidence that P. vivax is developing resistance to chloroquine was reported in Papua New Guinea by RIECKMANN et al. (1989). It is difficult to ascertain how common chloroquine resistance is in P. vivax infection, particularly as resistance does not appear to be absolute (COLIGNON, 1994). Reduction in susceptibility to chloroquine was reported from Solomon Island (WHITBY et al., 1989), Papua New Guinea (SCHUURKAMP et al., 1992; MURPHY et al., 1993) and India (GARG et al., 1995). Studies conducted by KAIN et al. (1993a) showed that the response to chloroquine may vary depending on the type of P. vivax variant. However, if there is a relationship between CSP genotype and parasite clearance following treatment with chloroquine, the underlying mechanism is unknown (KAIN et al., 1993b).

The development of a sensitive and specific polymerase chain reaction (PCR) method for detection of parasite DNA in blood samples needed to consider criteria such as the selection of specific DNA, suitable DNA extraction procedure and the conditions of this extraction (WILSON et al., 1991). As the PCR technique yields amplification of only a single copy of the micro-organism's genes (FOOTE et al., 1989; WILSON et al., 1989) it is required as an excellent method to detect low levels of DNA (KAIN et al., 1993b). Nevertheless, the major difficulty in the routine use of PCR amplification for human blood samples is in obtaining and purifying the DNA. Furthermore, it is known that haemoglobin and the other proteins can inhibit the PCR and that purified DNA can contain traces of denaturing and inhibiting proteins (MERCIER et al., 1990; LONG et al., 1995). MACHADO et al. (1998) reported an adaptation of a method for blood sample collection (WARBURST et al., 1991) for the extraction and amplification of Plasmodium DNA in the diagnosis of malaria infection (OLIVEIRA et al., 1995). This new technique involving glass fibre membrane (GFM), GFM–PCR–ELISA, requires less expertise, saves time and reduces the cost for specific malarial gene sequences.

The objective of the present study was to evaluate the GFM–PCR–ELISA methodology for genotyping the P. vivax variants, to verify their distribution in 3 endemic areas of the Amazon region of Brazil and to correlate them with initial parasitaemia, previous malaria experience and the time of parasitaemia clearance linked to different treatment schedules.

Materials and Methods

Study population
Human blood was collected in Macapá (Amapá State), Belém (Pará State) and Porto Velho (Rondônia State), Belem (Pará State) and Porto Velho (Rondônia State).

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State). Macapá and Belém are in the eastern Amazon, while Porto Velho is in the western Amazon (Figure). The patients were randomly recruited to participate in this study at the National Health Foundation units in each study area, with the assistance of their physicians.

**Blood sample collection**

A questionnaire for information on malaria episodes, patient's identification, epidemiological details, and the mode of treatment was completed for each individual. The samples from Macapá (n = 42) were collected in September 1995, from Porto Velho (n = 33) in April 1996 and from Belém (n = 40) during August and September 1996, with informed consent from all individuals. The blood samples were obtained before therapy was initiated, and only from those individuals who had a thick blood film and/or Quantitative Buffy Coat (QBC™) microscopically positive for *P. vivax* parasites and who had agreed to participate in the study. Vacutainer tubes containing EDTA (Becton Dickinson, UK) were used to collect 10 mL of blood/individual for PCR typing of *P. vivax* and cryopreservation; 100 μL of whole blood were used for preparing the GFM disc (Titertek, ICN Bio medicals Limited, UK) following the protocol described by Warhurst et al. (1991).

**Treatment**

Patients from Belém were treated as follows: chloroquine 10 mg/kg in a single dose, plus primaquine 0-50 mg/kg during 7 days (scheme A). Those from Porto Velho and Macapá were treated with chloroquine 25 mg/kg over 3 days (10 mg/kg on day 1 and 75 mg/kg on days 2 and 3), plus primaquine 0-25 mg/kg for 14 days (scheme B). These were the standard treatments recommended by local physicians at each locality.

**DNA template preparation, PCR amplification, electrophoresis and hybridization**

We used the GFM-PCR-ELISA technique (Machado et al., 1998). In brief, blood samples obtained directly from patients were spotted on GFM and prepared for PCR using the method of Warhurst et al. (1991). PGR amplification was carried out in 50 μL volume utilizing prepared DNA (5 μL), with freshly prepared master-mix [33.25 μL of double-distilled water, 0.25 μL (1.25U) of Taq polymerase (Bioline, London, UK), 1 μL of each dNTP to provide 200 μM final concentration and 5 μL of 10× reaction buffer (10 mM tris-HCl pH 8.3; 0.01% (w/v) gelatin; 1.5 mM MgCl₂; 50 mM KCl)]. The biotinylated primer and the unlabelled primer designed from specific terminal ends of the *P. vivax* CSP gene (Qari et al., 1993a) were used for amplification of the CSP gene. The reaction mixture was initially denatured at 94°C for 5 min, followed by 30 cycles of amplification (94°C for 1 min, 42°C for 50 s and 72°C for 90 s) and a final extension at 72°C for 5 min. The PCR products (6 μL) were electrophoresed on a 1% agarose gel and stained with ethidium bromide. The PCR products (5 μL) were hybridized by liquid-phase non-isotopic method (Oliveira et al., 1995) for homologous *P. vivax* variant-specific digoxigenin-labelled probes, designed from the CSP repetitive region (Qari et al., 1993a). Primer and probe sequences and concentrations were described previously by Qari et al. (1993a) and the hybridization conditions were described by Oliveira et al. (1995). For negative control we used 2 samples of non-infected human blood and human DNA. Positive controls consisted of DNA from 3 different plasmids containing the CSP repetitive region of variants.

**Statistical analysis**

The data were organized in a database manager (Dbase III) and later on processed and analysed in an epidemiology and statistical program (EpiInfo 6.0). For testing the significance of the variables we used the Kruskal-Wallis and Mann-Whitney 2-tailed tests. To obtain the independence among the proportions, the χ² test was applied by Yates' correction or Fisher's exact test (2-tailed). The adopted significance level for statistical inference was P < 0.05.

**Results**

All 115 samples collected were both microscopically and QBC™ positive for *P. vivax*. The parasitaemia in the
Macapá patients ranged from 40 to 26,500 infected red blood cells/mm³ (geometric mean (GM) 12251; in those from Porto Velho it was from 375 to 6800 (GM 1443); and from Belem 350 to 23,000 (GM 3126).

Samples with parasitaemia <500 infected red blood cells/mm³ (n = 30) did not produce a result using the PCR–ELISA technique. However, using 5 µL of the first PCR products for re-amplification, it was possible to demonstrate the same fragment (1-2 Kb) as that obtained from the samples with a parasitaemia >500 (n = 85) in agarose gel.

The typing of P. vivax indicated that the prevalence and frequency of variants among the study areas were not significantly different from one area to another (P > 0.05), since all types were present in all areas (Table 1).

Correlation of the P. vivax genotype with the highest initial parasitaemia was significant (P < 0.05) for P. vivax VK210 pure or in mixed infection with VK247, whereas the P. vivax-like parasite was associated with the lowest initial parasitaemia (Table 2).

Table 2 shows that the time required for parasitaemia clearance determined by thick smear for all P. vivax genotypes was similar, regardless of the treatment used (scheme A or B), and the differences did not reach statistical significance. Table 2 also demonstrates that there is no correlation between the mean number of previous malaria episodes and the infecting P. vivax type (P > 0.05) in all 3 study areas.

Discussion

The existence of a new species or subspecies of Plasmodium causing human malaria would have important implications for diagnosis and vaccine design. Furthermore, the P. vivax malaria variants may have different characteristics in the intensity of symptoms, the response to the drug treatment and to vector preference, which could cause drug resistance and failure of control measures (GOPINATH et al., 1994). It is important, therefore, to determine the prevalence and distribution of infection with these P. vivax genotypes.

The results of this study have indicated that P. vivax DNA can be detected and genotyped by GFM–PCR–ELISA, even when parasitaemia is very low. This technique offers advantages over microscopy for the study of the P. vivax variants because they cannot be distinguished morphologically, and the technique is also suitable for epidemiological surveys on the vivax complex.

With regard to the distribution of P. vivax variants we have found the variant VK210 to be the most prevalent of the single variant infections. KAIN et al. (1991) had similar results when analysing samples from South America, West Africa and the Indian subcontinent. This

Table 1. Distribution of P. vivax genotypes in the three study areas in the Amazon region of Brazil

<table>
<thead>
<tr>
<th>Study area</th>
<th>Pure types</th>
<th>Mixed types</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 2 3</td>
<td>1 + 2 1 + 3 2 + 3 1 + 2 + 3</td>
</tr>
<tr>
<td>Macapá</td>
<td>11 (26.2%)</td>
<td>1 (2.4%) 1 (2.4%) 6 (14.3%) 23 (54.8%)</td>
</tr>
<tr>
<td>Porto Velho</td>
<td>9 (27.3%) 4 (12.1%) 1 (3.0%) 5 (15.2%) 14 (42.4%)</td>
<td></td>
</tr>
<tr>
<td>Belem</td>
<td>15 (37.5%)</td>
<td>8 (20.0%) 1 (2.5%) 5 (12.5%) 11 (27.5%)</td>
</tr>
</tbody>
</table>

Values are numbers (%) in each category.

Table 2. P. vivax genotypes and their correlation with initial parasitaemia, past history of malaria and the number of days for the parasitaemia clearance

<table>
<thead>
<tr>
<th>P. vivax genotype</th>
<th>Parasitaemia (parasites/mm³)</th>
<th>Parasitaemia clearance after chloroquine and primaquine treatment (days)</th>
<th>Previous malaria experience (number of episodes)</th>
<th>Total number of P. vivax genotypes found</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6122 ± 5999</td>
<td>2.2 ± 1.35</td>
<td>1.4 ± 1.98</td>
<td>35</td>
</tr>
<tr>
<td>1 + 2</td>
<td>5880 ± 5740</td>
<td>2.2 ± 1.09</td>
<td>0.8 ± 1.42</td>
<td>13</td>
</tr>
<tr>
<td>1 + 2 + 3</td>
<td>1840 ± 1597</td>
<td>2.0 ± 1.30</td>
<td>1.9 ± 1.95</td>
<td>48</td>
</tr>
<tr>
<td>1 + 3</td>
<td>900 ± 540</td>
<td>2.7 ± 1.15</td>
<td>1.7 ± 2.88</td>
<td>16</td>
</tr>
<tr>
<td>2 + 3</td>
<td>1506 ± 1708</td>
<td>2.0 ± 1.15</td>
<td>1.7 ± 2.88</td>
<td></td>
</tr>
<tr>
<td>Pd</td>
<td>0.01</td>
<td>0.86</td>
<td>0.39</td>
<td></td>
</tr>
</tbody>
</table>

Values are geometric means ± standard deviation (SD), or means ± SD.

P-values for within genotype comparisons are shown in Table 1. The P-values for between genotype comparisons are shown in Table 2.

Mann–Whitney 2-tailed test comparing P. vivax genotype with time for parasite clearance after treatment

<table>
<thead>
<tr>
<th>P. vivax genotype</th>
<th>Time for clearance (days)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type 1 vs type 2</td>
<td>0.56</td>
<td></td>
</tr>
<tr>
<td>Type 1 vs type 3</td>
<td>0.25</td>
<td></td>
</tr>
<tr>
<td>Type 1 vs type 4</td>
<td>0.82</td>
<td></td>
</tr>
<tr>
<td>Type 1 vs type 5</td>
<td>0.95</td>
<td></td>
</tr>
<tr>
<td>Type 1 vs type 6</td>
<td>0.48</td>
<td></td>
</tr>
<tr>
<td>Type 2 vs type 3</td>
<td>0.81</td>
<td></td>
</tr>
<tr>
<td>Type 2 vs type 4</td>
<td>0.93</td>
<td></td>
</tr>
<tr>
<td>Type 2 vs type 5</td>
<td>0.95</td>
<td></td>
</tr>
<tr>
<td>Type 2 vs type 6</td>
<td>0.81</td>
<td></td>
</tr>
</tbody>
</table>
fact suggests that this variant is well adapted worldwide. The VK247 variant has a worldwide distribution and is frequently found as a mixed infection with the VK210 genotype (COCHRANE et al., 1990; WIRTZ et al., 1990; CURADO et al., 1995; ARRUDA et al., 1996, 1998). While this genotype has also been reported as the keystone genotype in the Americas, Africa, and Asia than in South America, suggesting a better adaptation of this genotype in this continent (KAIN et al., 1992, 1993a, 1993b; QARI et al., 1992).

QARI et al. (1993a, 1993b) drew attention to the *P. vivax*-like parasite and demonstrated the global occurrence of this variant in mixed infections, including in the Brazilian Amazon Region. ARRUDA et al. (1996, 1998) and CURADO et al. (1997) had demonstrated by serological methods the occurrence of this variant co-circulating with other types, but do not indicate whether the infections were mixed or the antibodies were raised from separate single infections. Similar results were obtained in the present study. If our results are compared with those of D. A. Oliveira (personal communication) (39% of the *P. vivax*-like variant in mixed infections), we note that the *P. vivax*-like variant has a significantly wider distribution worldwide than it did in the study (2' tests: P < 0.05). The reasons for this wide distribution are still unknown, but the *P. vivax* variants can reflect differences in the transmission intensity in these areas, variability of the vectorial competence and of the different genotypes of *P. vivax*, and/or the variation, in time, in which these genotypes appear or emerge in new areas (COX, 1991).

The mean parasitaemia indexes were significantly lower for mixed infection, including *P. vivax*-like, than for pure infections, and VK210 was correlated with the highest parasitaemia levels, an observation also reported by KAIN et al. (1993a). These authors also showed that when VK210 was mixed with VK247 the parasitaemia was enhanced, which was not observed in our study. The high prevalence of genotype VK210, the oldest type of *P. vivax*, could be related to a wide distribution over a long period of time, while the *P. vivax*-like parasite, which is phylogenetically linked to *P. simiae* of Old World monkeys (ESCALANTE et al., 1995), probably causes the lowest parasitaemia because this parasite is undergoing adaptation to humans.

KAIN et al. (1993a) suggested that the response to chloroquine may vary depending on the type of *P. vivax* variant, since VK210 genotype and mixed infections with VK247 took longer to clear parasitaemia while VK247 pure infection tended to be shorter. However, if there is a relationship between CSP genotype and parasite clearance following treatment with chloroquine, the underlying mechanism is unknown (KAIN et al., 1993b). The results observed in the present study did not show any significant difference in the time of parasite clearance (P > 0.05) for the 2 treatment schedules and the small variation was independent to the infective genotype(s). The difference between our results and those from KAIN et al. (1993a) can be explained by: i) in the study by KAIN et al. parasitaemia clearance was followed over shorter intervals of time (hours) than in ours (days); ii) our patients were treated with chloroquine and primaquine which also has schizonticide action; and iii) we included an analysis of a third genotype (*P. falciparum*) that is co-circulating with the other 2. However, our results support the hypothesis that strain-variable responses to chloroquine may exist.

In our study, the mean number of previous episodes of malaria was low (1-7) and most patients were non-immune. This indicates that no correlation exists between experience and the *P. vivax* genotype. KAIN et al. (1993a) had found a higher number of previous malaria experiences (7-0-7-6) in their patients, but also found no correlation with the genotypes of *P. vivax*. As immunity in malaria is directly related to the number of infections, it is likely that our patients had not developed immunity and consequently there was no selection of genotypes.

In order to determine better the 3 *P. vivax* genotypes, studies focusing on other genes might provide further genetic information, enabling a better understanding of their phylogeny and relationship with other human and non-human malaria parasites.

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References


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