Characterisation of *pvmdr1* and *pvdhfr* genes associated with chemoresistance in Brazilian *Plasmodium vivax* isolates

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Plasmodium vivax *control is now being hampered by drug resistance. Orthologous* Plasmodium falciparum *genes linked to chloroquine or sulfadoxine-pyrimethamine chemoresistance have been identified in* P. vivax *parasites, but few studies have been performed. The goal of the present work is to characterise* pvmdr1 *and* pvdhfr *genes in parasite isolates from a Brazilian endemic area where no molecular investigation had been previously conducted. The* pvmdr1 *analysis revealed the existence of single (85.7%) and double (14.3%) mutant haplotypes, while the* pvdhfr *examination showed the presence of double (57.2%) and triple (42.8%) mutant haplotypes. The implications of these findings are discussed.*

Key words: *Plasmodium vivax* - malaria - chemoresistance - *pvmdr1* - *pvdhfr*

Although *Plasmodium vivax* has long been neglected, it is the most ubiquitous human malaria parasite, causing up to 50% of malaria cases outside Africa (Mendis et al. 2001). In effect, more than 2.5 billion inhabitants of the Middle East, Asia, Eastern and South Africa, Central and South America and Oceania are exposed to *P. vivax* infection, leading to 71-391 million cases of malaria vivax per year (Guerra et al. 2006, Price et al. 2007). In Brazil, this parasite is responsible for around 85% of cases (MS 2008). Control measures include prompt diagnosis followed by adequate treatment with chloroquine (CQ), a blood schizonticide that remains the first-line treatment in most of the world; thus, the emergence of CQ *P. vivax* chemoresistance represents a hurdle to malaria control.

The first case of *P. vivax* resistance to CQ was described in 1989 in Papua New Guinea (Rieckmann et al. 1989). Since then periodic reports have been made in Oceanian and in Asian and South American countries (Baird 2004, Lee et al. 2009), including Brazil (Alecrim et al. 1999, de Santana Filho et al. 2007). In contrast to *P. falciparum* parasites, effective in vitro culture systems for *P. vivax* parasites are not available, a fact that makes the biological identification of CQ resistance of *P. vivax* parasites difficult. In addition, clinical recognition of CQ-resistant *P. vivax* parasites can be confusing in endemic areas in view of the difficulties in distinguishing between recrudescence due to true treatment failure, relapse from the hypnozoite stage and new infection. Thus, a database of the mutations possibly associated with in vivo drug resistance could help our understanding of the molecular mechanisms underlying CQ resistance in *P. vivax* infections.

Orthologous *P. falciparum* genes linked to CQ chemoresistance, such as *pvmdr1*, have also been identified in *P. vivax* parasites (Brega et al. 2005). However, few studies concerning these genes were conducted so far (Nomura et al. 2001, Brega et al. 2005, Sá et al. 2005, Suwanarusk et al. 2007, 2008, Barnadas et al. 2008a, Imwong et al. 2008, Marfurt et al. 2008, Fernandez-Becerra et al. 2009, Orjuela-Sánchez et al. 2009).

Another orthologous *P. falciparum* gene, in this case associated with pyrimethamine and, by extension, with sulfadoxine-pyrimethamine (SP) chemoresistance, was also identified in *P. vivax* (De Pécoulas et al. 1998a). Molecular, clinical and epidemiological studies have also shown that, as for *P. falciparum* parasites, resistance is a consequence of cumulative point mutations in the *pvdhfr* gene (De Pécoulas et al. 1998b).

Nowadays, neither *P. vivax* nor *P. falciparum* malaria cases are treated with SP in Brazil. However, in view of the increasing prevalence of *P. falciparum* CQ resistance, SP was largely employed in Brazil during the 70's and 80's to replace CQ in falciparum malaria treatment. Therefore, considering that co-infections of *P. falciparum* and *P. vivax* parasites were common in Brazil and that such co-infections could be misdiagnosed or even undiagnosed, *P. vivax* parasites must have been often exposed to this drug combination and the selection of SP-resistant *P. vivax* parasites might have occurred.

The goal of the present study was to characterise *pvmdr1* and *pvdhfr* gene single nucleotide polymorphisms (SNPs) in *P. vivax* patients, living in Paragominas, state of Pará, where no molecular investigation of these genes has been previously conducted.

PATIENTS, MATERIAL AND METHODS

Study site, blood samples and DNA extraction - P. vivax parasites were genotyped in an initial set of 28 blood samples collected in 2004 at the time of diagnosis.

The inclusion criterion comprised monoinfected *P.vivax* patients with uncomplicated malaria. Patients who were pregnant as well as individuals less than 18 years of age and indigenous populations were excluded from the study. After obtaining informed consent, venous blood collection was performed according to protocols previously approved by the Ethical Research Committees of Fiocruz and of local study sites. DNA was extracted from 1 mL of cryopreserved blood using QIAamp midi columns, as described by the manufacturer (Qiagen).

Polymerase chain reaction (PCR) and electrophoresis - Two single PCR techniques were employed to amplify a partial DNA sequence containing some SNPs for target genes including: SNPs Y976F and F1076L for *pvmdr1* and SNPs F57L/I, S58R, T61M, S117T/N and I173F/L for *pvdhfr*. The protocols for *pvmdr1* and *pvdhfr* PCRs have been described elsewhere (Brega et al. 2004, 2005). PCR products were analysed by ethidium bromide-stained agarose-gel (2%) electrophoresis.

DNA sequencing - After purification using the Wizard SV Gel and PCR Clean-Up System (Promega), the amplified fragments were sequenced using Big Dye® Terminator Cycle Sequencing Ready Reaction version 3.1 (Applied Biosystems) and ABI PRISM DNA Analyzer 3730 (Applied Biosystems) from the Genomic Platform/PDTIS/Fiocruz (Otto et al. 2008).

RESULTS AND DISCUSSION

All 28 samples were successfully amplified and the corresponding DNA was sequenced. For SNPs Y976F and F1076L, the *pvmdr1* analysis revealed the existence of two haplotypes: FF, or single-mutant (85.7%) and FL, or double-mutant (14.3%). Additionally, two other novel polymorphisms were noted in one single sample, comprising a synonymous mutation at codon 1022 (CTA to TTA, both coding for leucine) and a non-synonymous change at codon 1070, from phenylalanine to leucine. None of the recently reported SNPs from *P. vivax* parasites from another Brazilian endemic area (Manaus, Amazonia) were found in our study (Orjuela-Sánchez et al. 2009); in contrast, all of our samples presented the Y976F mutation - one of the early chemoresistance molecular marker candidates.

Surprisingly, no wild-type haplotype (YF that corresponds to CQ sensitivity) was found. This strikingly contrasts to the only 10% of CQ in vivo therapeutic failures recently reported in Brazil (De Santana Filho et al. 2007). This finding might suggest that the Y976F *pvmdr1* point mutation, i.e., the SNP associated with CQ resistance (Brega et al. 2005, Suwanarusk et al. 2007), was not valuable for monitoring CQ resistance in Brazil. The same phenomenon may be occurring in Madagascar, where this codon was mutated at a high frequency, in spite of a low prevalence of in vivo *P. vivax* CQ-resistant parasites (Barnadas et al. 2008a). In addition, the Y976F mutation was not found in isolates from three patients with recrudescent disease, a recently finding that reinforced our proposition (Orjuela-Sánchez et al. 2009).

The *pvdhfr* analysis also revealed the existence of two haplotypes, considering SNPs F57L/I, S58R, T61M,

S117T/N and I173F/L. FRTNI or double-mutant (S58R + S117N) were found in 57.2% of the samples; FRTNL or triple-mutant (S58R + S117N + I173L) were found in 42.8% of the samples. No wild-type haplotype FSTSI was found, as expected, and two samples presented an atypical exchange at codon 116, from serine to glycine, that was newly identified in French Guiana samples (Barnadas et al. 2009).

The double *pvdhfr* mutant haplotype has already been described in samples from Indonesia (Hastings et al. 2004), Thailand (Brega et al. 2004), Ethiopia (Schunk et al. 2006), China, East Timor, Philipines, Vanuatu, Vietnam (Auliff et al. 2006), Papua New Guinea (Marfurt et al. 2008), Madagascar (Barnadas et al. 2008b) and Iran (Zakeri et al. 2009). The same double and triple *pvdhfr* mutants were also found in *P. vivax* parasites from French Guiana (Brega et al. 2004, Barnadas et al. 2009), the only locality where the I173L SNP was identified before the present work. Therefore, the genetic similarity of haplotypes and this specific SNP recorded in *P. vivax* parasites from two neighbouring South-American countries could reflect the existence of a geographic subdivision of different *P. vivax* parasites in samples from the old and new worlds (Li et al. 2001, Brega et al. 2004). Although when other authors looked at the population genetics of the parasites using microsatellites, they did not find a significant differentiation between parasites from the same continent or even within the same country (Karunaweera et al. 2008).

Considering that, besides underlying immunity conditions and other host factors, in vivo *P. vivax* pyrimethamine resistance is associated with the presence of multiple mutations in the *pvdhfr* gene, we conclude that studies, such as the one reported here, can provide critical information on the potential emergence of SP resistance in endemic areas, if the increase of CQ treatment failure persuades health care policy programs to shift from CQ to this antifolate drug treatment. These findings will be further complemented by an additional analysis concerning the *pvdhps* gene, the orthologous gene to falciparum *pfdhps*, which mediates resistance to sulfadoxine, the counterpart of pyrimethamine in the SP combination.

Finally, since the molecular mechanisms of *P. vivax* CQ resistance remain elusive, more studies must be carried out on the prevalence of chemoresistance molecular markers as well as on the in vivo and in vitro sensitivity of antimalarial drugs to validate CQ molecular markers for screening and monitoring *P. vivax* CQ-resistant parasites.

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