Identification of the Plasmodium vivax mdr-Like Gene (pvmdr1) and Analysis of Single-Nucleotide Polymorphisms among Isolates from Different Areas of Endemicity

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Because of the lack of methods for continuous in vitro culture of Plasmodium vivax, little is known about drug-resistance mechanisms in this malaria-causing parasite. Therefore, identification of all the genes potentially involved in drug resistance and of molecular markers related to drug resistance would provide a framework for studying the incidence and spread of drug-resistant P. vivax strains. We have identified the P. vivax orthologue of the pfmdr1 gene (pvmdr1), which was shown to have a role in the drug resistance of Plasmodium falciparum. Comparison of the alignments of both nucleotide and amino acid sequences of pvmdr1 with those of other Plasmodium multidrug-resistance genes revealed an open-reading frame of 4392 base pairs encoding a deduced protein of 1464 amino acids. Nucleotide polymorphisms at 2 codons of the pvmdr1 gene—Y976F and F1076L—were found in 14 of 23 P. vivax isolates from different areas of endemicity, including Thailand, Indonesia, Turkey, Azerbaijan, and French Guyana.

Plasmodium vivax causes more than half of all malaria infections outside Africa, with an estimated burden of ~70–80 million cases/year [1]. In most areas of endemicity, chloroquine still constitutes the first-line therapy against uncomplicated malaria caused by P. vivax, followed by primaquine for eradication of asexual stages and hypnozoites. However, since 1989 (i.e., ~30 years after chloroquine resistance [CQR] in Plasmodium falciparum was reported), the phenomenon of CQR in P. vivax has appeared in New Guinea and Sumatra [2], and there have been sporadic reports of CQR from other geographic locations [3]. Two genes in P. falciparum, pfcr [4] and pfmdr1 [5] (both coding for proteins associated to membrane transport), have been proposed to be involved in CQR. Field studies have confirmed that the pfcr mutant allele K76T generally correlates with low clinical responses to the drug in patients with malaria and with parasite CQR detected in vitro [6–10]. Other studies have suggested that the mutant allele N86Y of the pfmdr1 gene may confer higher levels of resistance to parasites bearing the pfcr mutant allele K76T [6, 11].

It has been demonstrated that CQR in P. vivax is not mediated by codon mutations in the pfcr orthologue (pvcg10) [12]. Thus, it could be supposed that CQR mechanisms of P. vivax are different from those of P. falciparum.

Of interest, the multidrug-resistance gene (mdr) shows orthologues in several Plasmodium species other than P. falciparum, including P. berghei [13], P. chabaudi [14], and P. yoelii [15]. In this report, we identify and
characterize an mdr-like gene (pymdr1) in P. vivax isolates. We were interested to look for single-nucleotide polymorphisms (SNPs) in the sequence of this gene, to be used as molecular markers in the surveillance of CQR in P. vivax. Here, a relative high frequency of 2 SNPs in the pymdr1 gene was found at codons not associated with CQR in P. falciparum.

**MATERIALS AND METHODS**

**Parasite samples.** Genomic P. chabaudi and P. yoelii DNA were a gift from Robert Menard and Paul Rick (Institut Pasteur de Paris, Paris). DNA from P. falciparum was obtained from culture-adapted strain 3D7, from The Netherlands, as described elsewhere [16]. After informed consent was obtained, 23 peripheral-blood samples were collected from patients with microscopically confirmed malaria caused by P. vivax, in different geographic locations, as follows: 9 blood samples were collected by fingerprick from adult patients visiting the Mae Sod malaria clinic, Tak province, Thailand; 3 blood-spot samples were collected during a malarialometric survey in Alor district, East Nusa Tenggara province, Indonesia; 3 blood samples were collected by venipuncture into EDTA tubes from patients consulting at the Institute of Malariology of Adana in southeastern Turkey; 4 blood samples were collected into EDTA in central Azerbaijan during an active survey performed in the framework of a malaria project supported by the European Commission (INCO COPERNICUS-2 project [VIVAXNIS]); and 4 blood samples were collected into EDTA tubes from patients hospitalized in France who presented with P. vivax malaria imported from French Guyana.

**Preparation of DNA template from blood samples.** Parasite genomic DNA from all blood samples collected into EDTA tubes was extracted by use of a QIAamp DNA blood kit (Qiagen), following the manufacturer’s instructions, with minor modifications: the incubation time with proteinase K was increased to 1 h at 56°C, to improve the yield of the extraction, and DNA was eluted from the column by use of 100 μL of polymerase chain reaction (PCR)-grade H₂O. DNA extraction of P. vivax blood-spot samples from Thailand and Indonesia was performed by use of the QIAamp DNA blood kit, following the Dried Blood Spots Protocol provided in the kit, with the same minor modifications described above.

**Identification of P. vivax mdr1 gene.** Degenerate primers were designed to amplify within an mdr-like consensus sequence conserved between P. falciparum, P. chabaudi, and P. yoelii and were identified by a basic local alignment search tool (BLAST) search of National Center for Biotechnology information databases (available at: http://www.ncbi.nlm.nih.gov/BLAST/). The degenerate oligonucleotide primers were pymdr-F2905 (forward; 5’-CGKGTTRTGAAATAATATTG-3′) and pymdr-4007 (reverse; 5’-CATATTTAGGAATAGGTC-3′), amplifying an expected fragment size of ~1100 bp. Genomic DNA from P. chabaudi, P. yoelii, P. falciparum, and a P. vivax isolate from Azerbaijan (Sab 2) were amplified in a total volume of 20 μL containing 0.20 mmol/L each dNTP, 1 μmol/L each primer, 1 U of Platinum Taq DNA Polymerase (Invitrogen), 1.5 mmol/L MgCl₂, and 5 μL of 10X PCR buffer. PCR was performed under the following conditions: 34 cycles of denaturation at 94°C (2 min in the first cycle and 30 s in subsequent cycles), annealing for 1 min at 57°C (during the first 5 cycles, the temperature between the annealing and extension phase increased to 68°C, with a slope of 1°C/4s), extension for 1.5 min at 68°C, and a final primer extension for 5 min. Before sequencing of plasmid DNA, PCR products were separated on agarose gel, and the P. vivax product of expected size was gel-purified by use of a GeneClean turbo kit and was cloned by use of a TOPO TA Cloning kit (Invitrogen). BLAST searches to the P. vivax genome sequence data were performed at http://PlasmoDB.org. The complete P. vivax sequence gene was retrieved in the chloroquine-sensitive (CQS) Sal-1 strain from El Salvador [17] and was submitted to GenBank under accession numberAY618622.

**SYBR Green PCR amplification.** Real-time PCR was performed using SYBR Green I dye binding specifically to double-stranded DNA. The sequences of PCR primers used to amplify 2 fragments (~450 and 800 bp) from the P. vivax mdr1 gene of the Sal-1 strain (GenBank accession no. AY618622), corresponding to 2 polymorphic regions of pymdr1 gene, were as follows: pymdr1-F (5’-CACCTTGCCCTTTTTCGG-3′) and pymdr1-R (5’-TCCACCTCTCTGTTGCAAAATAC-3′), and pymdr1-S (5’-ATAGTATGCCCCAGGT-3′) and pymdr1-AS (5’-ACGTTTGCTGGACAAGTATC-3′).

Five microliters of DNA template was added to the PCR mixtures (20 μL) containing 0.5 μmol/L each primer, 3 mmol/L MgCl₂, and 2 μL of LightCycler FastStart DNA Master SYBR Green 1 buffer (Roche Molecular Biochemicals). The PCR program was performed for 40 cycles, as described elsewhere [18]. PCR product identity was assessed by a specific melting temperature obtained for each genotype.

**RESULTS**

**Identification of the mdr-like gene in the P. vivax genome.** In silico sequence analysis and PCR screening with codon-adjusted degenerate primers were used to identify P. vivax orthologues of P. falciparum (pfmdr1), P. chabaudi (pcmdr1), and P. yoelii (pymdr1). The ~1100-bp P. vivax fragment amplified from the Sab 2 isolate was sequenced, and data analysis gave a significant match between part of the pfmdr1-coding sequence (GenBank accession no. M29154), as well as pcmdr1 and pymdr1 sequences (GenBank accession nos. AY123625 and PY002425, respectively). Consensus prediction of the translated partial amino acid sequence confirmed the homology with PfMDR1 and showed the presence of 4 hydrophilic transmembrane domains (seg-
Figure 1. Comparison of the predicted sequences for pfmrd1 (GenBank accession no. M29154) and its homologues pvmrd1 (GenBank accession no. AY618622) and pcmdr1 (GenBank accession no. AY123625). Matching amino acids across all species are marked as follows: bold dots (.) indicate weak similarity, bold colons (:) indicate strong similarity, and bold asterisks (*) indicate identity between corresponding amino acids. Boxes with solid lines indicate putative transmembrane segments, and boxes with dashed lines enclose the 2 nucleotide-binding consensus sequences (NBS1 and NBS2). The Walker A and Walker B motifs are in shaded boxes. Bold dashes (--) representing spaces have been inserted to optimize the comparisons.
Table 1. Mutations present in the pvmdr1 gene amplified from Plasmodium vivax isolates.

<table>
<thead>
<tr>
<th>Isolate/location</th>
<th>Mutation residues in pvmdr1 amino acid</th>
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<tbody>
<tr>
<td></td>
<td>91 189 976 1076</td>
</tr>
<tr>
<td>Guy1/French Guyana</td>
<td>N Y Y F</td>
</tr>
<tr>
<td>Guy2/French Guyana</td>
<td>N Y Y F</td>
</tr>
<tr>
<td>Guy3/French Guyana</td>
<td>N Y Y F</td>
</tr>
<tr>
<td>TMPV108/Thailand</td>
<td>N Y Y F</td>
</tr>
<tr>
<td>TMPV109/Thailand</td>
<td>N Y Y F</td>
</tr>
<tr>
<td>VP1439/Thailand</td>
<td>N Y Y F</td>
</tr>
<tr>
<td>A33/Indonesia</td>
<td>N Y Y F</td>
</tr>
<tr>
<td>VP1438/Thailand</td>
<td>N Y Y F</td>
</tr>
<tr>
<td>Sal-1/El Salvador</td>
<td>N Y F L</td>
</tr>
<tr>
<td>B4/French Guyana</td>
<td>N Y Y F</td>
</tr>
<tr>
<td>VP1443/Thailand</td>
<td>N Y F L</td>
</tr>
<tr>
<td>VP1445/Thailand</td>
<td>N Y F L</td>
</tr>
<tr>
<td>F49/Indonesia</td>
<td>N Y F L</td>
</tr>
<tr>
<td>M17/Indonesia</td>
<td>N Y F L</td>
</tr>
<tr>
<td>VP1448/Thailand</td>
<td>N Y F L</td>
</tr>
<tr>
<td>VP1454/Thailand</td>
<td>N Y F L</td>
</tr>
<tr>
<td>Tur11/Turkey</td>
<td>N Y Y L</td>
</tr>
<tr>
<td>Agy6/Azerbaijan</td>
<td>N Y Y L</td>
</tr>
<tr>
<td>VP1447/Thailand</td>
<td>N Y Y L</td>
</tr>
<tr>
<td>Tur22/Turkey</td>
<td>N Y Y L</td>
</tr>
<tr>
<td>Tur3/Turkey</td>
<td>N Y Y L</td>
</tr>
<tr>
<td>Sab5/Azerbaijan</td>
<td>N Y Y L</td>
</tr>
<tr>
<td>Saa12/Azerbaijan</td>
<td>N Y Y L</td>
</tr>
<tr>
<td>Sab2/Azerbaijan</td>
<td>N Y Y L</td>
</tr>
</tbody>
</table>

NOTE. Residues that differ from the wild-type Sal-1 strain are indicated in bold type. Representative sequences were submitted to Genbank.

a GenBank accession no. AY618622 (wild-type strain).
b GenBank accession no. AY643799.
c GenBank accession no. AY351844.

The phenomenon of CQR in P. vivax has been extensively described in Papua New Guinea in 1989 [2] and in Indonesia in 1991 [20, 21]; more recently, sporadic clinical CQR has been reported in India [22, 23], Myanmar [24], and in Central and South America [25–27]. However, the mechanisms of CQR in P. vivax are still unknown.

Although the action of chloroquine is probably similar in P. vivax and in P. falciparum, it appears that the development of CQR involves a different mechanism in these 2 species [12]. Codon mutations in the pfcrt gene are central to the CQR phenotype of P. falciparum, but P. vivax pvPG10, the chloroquine-resistant transporter gene (crt) orthologue of P. falciparum, has been shown to be not responsible for CQR in P. vivax [12].
The *P. vivax mdr* gene has not yet been described, probably because the role of the orthologue in *P. falciparum (pfmdr1)* is less important than that of *pfcr* in CQR. However, identification of an orthologue between the 2 major *Plasmodium* species is important for cross-species comparison of gene function and comparison of molecular mechanisms associated with a common phenotype, such as CQR [28].

In the present article, we have reported the first evidence of a *P. vivax* mdr-like gene and have described some polymorphisms in isolates from areas where CQR in *P. vivax* has been detected, such as Indonesia. The *pvmdr1* gene is characterized by a single ORF of 4392 bp, encoding a protein of 1464 aa. Two regions of the *pvmdr1* gene have been taken into consideration: one encompassing the *P. vivax* positions homologous to the polymorphic sites 86 and 184 in *P. falciparum*, and the other encompassing the *P. vivax* positions homologous to the polymorphic sites 1034 and 1042 in *P. falciparum*. Sequence analysis of the *pvmdr1* gene has shown no polymorphism at *pvmdr1* codon 91 homologous to *pfmdr1* codon 86, which is associated with drug resistance in *P. falciparum*.

These data contrast with those obtained for pyrimethamine-resistant malaria parasites, in which mutations in dihydrofolate reductase gene, the target enzyme for this folate drug, are at similar sites in the genome of both *Plasmodium* species [18]. Amplification or overexpression of the *mdr1* genes from various *Plasmodium* species have also been associated with drug resistance, but the level of expression of the *pvmdr1* gene and the copy number of this gene have not been determined for these samples.

Two *pvmdr1* mutant alleles were identified: F1076L alone and Y976F-F1076L. The locations of the 2 mutations in the MDR protein are the X and XI hydrophobic segments, respectively. The rates of distribution of *pvmdr1* genotypes found among our samples were 42% for the wild-type genotype, 33% for the single-mutant genotype, and 25% for the double-mutant genotype. Unfortunately, the role of these mutations in clinical CQR has not been evaluated in patients infected with mutant *Plasmodium* species. However, assessment of the therapeutic failures are confounded by the tendency of *P. vivax* malaria to relapse from liver hypnozoites several weeks after initial infection, and, in areas of endemicity where continuous malaria transmission occurs, it is often difficult to distinguish between reinfection, relapse, and recrudescence [29–31]. Thus, we are still unable to assume that these polymorphisms could be related to any drug resistance, and the same polymorphisms have not been described yet for *P. berghei*, *P. chabaudi*, and *P. yoelii*. Nevertheless, the high frequency of these mutations in samples from different areas of endemicity and from different continents is probably not without significance. It should be noted that all the double mutants (976–1076) were found in samples from Thailand and Indonesia. In the latter area, CQR in *P. vivax* has been reported for more than a decade. Other cases of CQR in *P. vivax* have been reported in Papua New Guinea [2], India [22, 23], and Myanmar [24], which all are Asian countries. Thus, even if most of the *P. vivax* infections detected in Thailand still seem to be CQS, we could speculate that these 2 mutations could be early molecular markers of *P. vivax* CQR and should be included in later surveys of drug resistance. A potential use of real-time PCR will be the analysis of a large number of samples in a short period of time. On the other hand, only some samples from Azerbaijan and Turkey had a single mutation at position 1076, and none had a double mutation. This result is concordant with the good efficacy rate of chloroquine against *P. vivax* in these areas [32]. Surprisingly, no mutation has been observed in samples from French Guiana, where CQR in *P. vivax* has been reported. However, the very low number of samples studied from each area led us to analyze these data with great care. Further studies are needed to explore the value of these new molecular markers in the context of increasing *P. vivax* drug resistance.

**Acknowledgments**

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**References**


