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Research brief

Plasmodium vivax: allele variants of the *mdr1* gene do not associate with chloroquine resistance among isolates from Brazil, Papua, and monkey-adapted strains

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Abstract

We describe here the sequence of the *Plasmodium vivax mdr1* gene from 10 different isolates differing in chloroquine sensitivity. The deduced amino acid sequence of PvMDR1 shares more than 70% similarity with other malarial MDR proteins and it displays consensus motifs of an ABC family transporter including two transmembrane domains and two ATP binding cassettes. Similarity and dendrogram analyses revealed that sequences could be grouped according to their geographical origin. Within each geographical group however, no correlation was found between chloroquine resistance and specific mutations.

Keywords: *Plasmodium vivax*; *mdr1* genes; ABC family transporters; Chloroquine resistance; Allele variants

Plasmodium vivax is the most widely distributed human malarial parasite outside sub-Saharan Africa and responsible for 70–80 million clinical cases annually (Mendis et al., 2001). Despite its major socio-economical burden, research in *P. vivax* remains largely neglected due to the lack of a continuous in vitro culture and the low parasitemias associated with natural infections.

Drug resistance is a major factor in the present resurgence of malaria worldwide. Several genes have now been related to drug resistance in *Plasmodium falciparum*, which causes the most lethal form of human malaria: *pfdhfr* and *pfdhps*, where point mutations confer resistance to dihydrofolate reductase inhibitors (Cowman et al., 1988; Peterson et al., 1988) and sulfadoxine (Brooks et al., 1994; Triglia and Cowman, 1994), respectively; cytochrome *b* gene in which the point mutation Y268S can be rapidly selected by atovaquone monotherapy (Korsinczky et al., 2000; Srivastava and Vaidya, 1999); *pfcrt*, which contains specific mutations associated with chloroquine resistance (CQR) (Fidock et al., 2000); *pfmdr1* and *pfmdr2* which encode P-glycoproteins from the ABC (ATP binding cassette) transporters family (Foote et al., 1989; Wilson et al., 1989; Zalis et al., 1993), and some other transporters that may be involved in chloroquine and quinine resistance (Mu et al., 2003).

Orthologous genes of *pfdhfr* and *pfcrt* have been described in *P. vivax*, namely *pvdhfr* (Eldin de Pecoulas et al., 1998), where point mutations are also associated with resistance against dihydrofolate reductase inhibitors, and *pvcg10* (Nomura et al., 2001), which in contrast to *pfcrt* contains no specific mutations associated with chloroquine resistance (CQR). The purpose of this work was to identify the ortholog of *pfmdr1* in *P. vivax*, to establish its primary structure and to verify whether amino acid substitutions were associated with CQR among isolates from different regions of the world displaying different phenotypes of chloroquine sensitivity.

To identify *P. vivax mdr* genes, the oligonucleotides 5′-ATGA AAAAGGATCAAAGGCAAC-3′ (forward) and 5′-CTACTTAGC CAGCTTGACGTAC-3′ (reverse) were used in PCR amplifications of *P. vivax* genomic DNA from 10 different chloroquine sensitive (CQS) and chloroquine resistant (CQR) isolates or strains from differ-

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ent geographical regions. Thus, three samples were from the Brazilian Amazon State of Rondônia (RO14, RO19, ROB2, and all CQS), one sample was from El Salvador in Central America (*Sal*I, CQS), one from Papua New Guinea (NGIV, CQS), one from Indonesia (IndoF, CQR), and four from Papua (CL001, CQS; CL004, CQR; CL007, CQS; and CL014, CQR). Thirty-five PCR cycles were performed, each consisting of denaturation for 30 s at 94 °C, renaturation for 30 s at 45 °C, and elongation for 5 min at 68 °C using elongase (Invitrogen). Unique 4.5 kb DNA fragments were amplified from all samples, cloned in pGEM T-easy plasmids (Promega) and sequenced using an ABI-3100 automatic sequencer (Applied Biosystems). Chromatograms and contigs were evaluated and assembled using the Phred-Phrap-Consed program (http://www.phrap.org/). To guarantee the authenticity of these sequences, all sequences were assigned a Phred value above 40. Moreover, polymorphisms were confirmed by sequencing independent amplifications to exclude PCR artifacts. Synteny was examined using the ACT tool (http://www.sanger.ac.uk/Software/ACT/), and comparing a *P. vivax* contig containing the *P. vivax* mdr sequences obtained from the *P. vivax* genome project at TIGR (http://www.tigr.org/tdb/e2k1/pva1/) with the *P. falciparum* genome as annotated in PlasmoDB (http://plasmodb.org/). GenBank Accession Nos: CL001 AY571975, CL004 AY571976, CL007 AY571977, CL014 AY571978, IndoF_H4 AY571979, NGIV_H7 AY571980, RO14 AY571981, RO19 AY571982, ROB2 AY571983, and SalI AY571984.

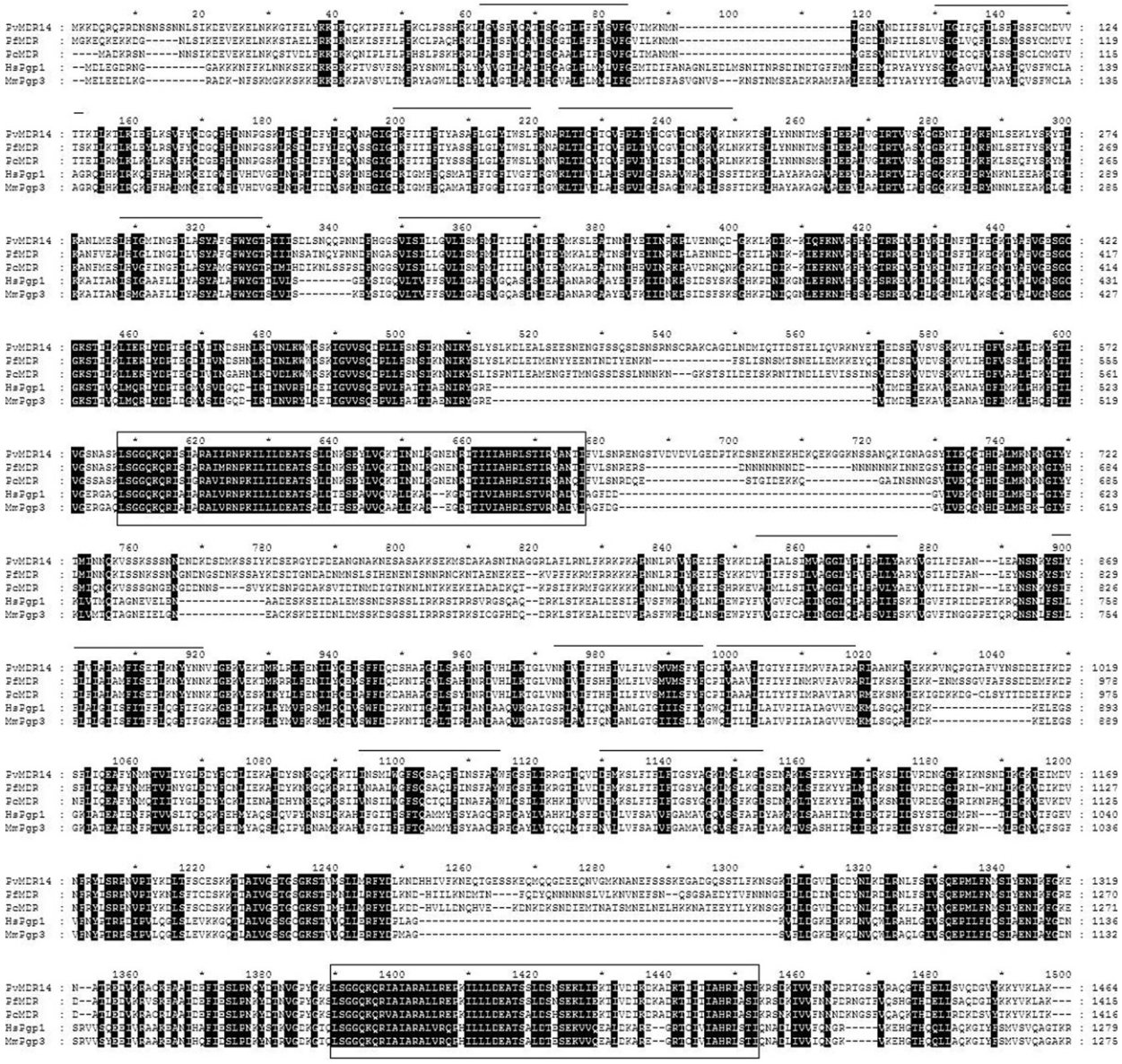

Fig. 1. Amino acid sequence comparisons of PvMDR1 and related MDR proteins. Deduced amino acid sequence of PvMDR1 from a *P. vivax* isolate *(pvmdr14*—Accession No. AY571981) compared to MDR homologues of *P. falciparum* (*pfmdr*—Accession No. NP703574), *P. chabaudi* (*pcmdr*—Accession No. AAM82617) and Pgp1, one of the human MDR proteins (Accession No. NP000918) and MDR3, of mouse (Accession No. P21447). Sequences were aligned using the ClustalX program (ftp://ftp-igbmc.ustrasbg.fr/pub/ClustalX/). Predicted Transmembrane regions were predicated with the SOUSI (Classification and Secondary Structure Prediction of Membrane Proteins) program (http://sosui.proteome.bio.tuat.ac.jp/sosuiframe0.html) and are shown with a line over the correspondent sequence. Black shading highlights both conserved residues and those where the amino acid substitutions are conservative. Boxes enclose potential nucleotide binding consensus sequences.

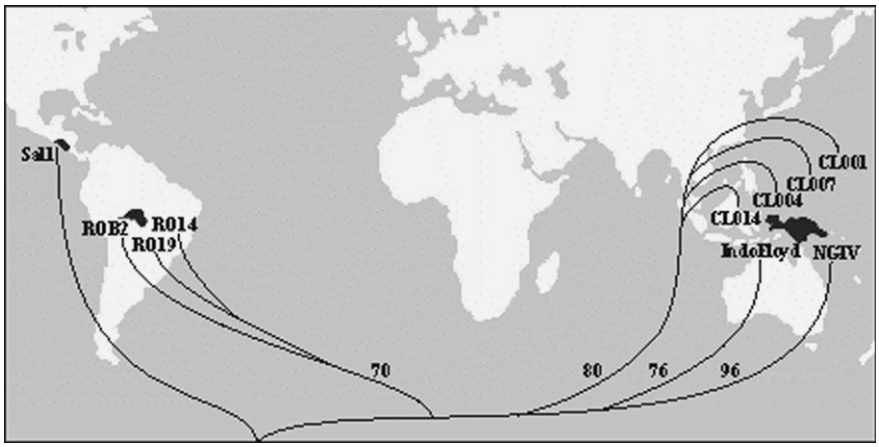

Fig. 2. Phylogenetic tree and geographic distribution of *pvmdr1* allele variants. A phylogenetic tree based on sequence alignments of deduced amino acid sequences from putative PvMDR1 of all 10 isolates was generated using Molecular Evolutionary Genetics Analysis, MEGA, software version 2.1 (http://www.megasoftware.net/text/news.sht). This tree was adapted to display the geographical distribution of the samples. The neighborjoining model was used to construct the bootstrap consensus tree (1000 replicates used to estimate the standard error) with distance option of amino acids number of differences. Shaded areas represent the geographical origin of the different samples.

BLASTn analysis against PlasmoDB revealed that the sequences from all isolates shared more than 70% similarities with the *P. falciparum mdr1* gene located on chromosome 5. Furthermore, deduced amino acid sequences indicated an open reading frame of 4395 nucleotides encoding a protein with predicted molecular mass of approximated 165,000 Da. BLASTP analysis showed 70, 63, and 64% similarities with predicted proteins from *P. falciparum* (Accession No. AAA29646.1), *P. chabaudi* (Accession No. AAM82617.1) and *P. yoelii* MDR1 (Accession No. EAA22011), respectively. This analysis also revealed the presence of conserved nucleotide binding sites (NBS) of the ATP binding cassette (ABC) protein family in two halves of the molecule each of which also contained 12 predicted membrane-spanning domains (Fig. 1). Multiple sequence alignments including MDR protein sequences from *P. falciparum*, *P. chabaudi*, and ABC transporters from *Drosophila melanogaster* (CG10226-PA) and human (NP 061337.1), further confirmed that the *P. vivax* sequences pertain to the ABC protein family (Fig. 1). Moreover, using the last released version of the TIGR *P. vivax* genome project, a 307,346 bp contig containing the *P. vivax mdr* sequences was identified and compared to the homologous region of the *P. falciparum* genome using the ACT tool. The results demonstrated a large degree of synteny of this genome region between these two human malaria parasite species (not shown). Together these data strongly suggest that this *P. vivax* gene encodes a P-glycoprotein orthologue of *pfmdr1* and accordingly we have termed it *pvmdr1*.

Chloroquine is still the primary chemotherapeutic drug for treatment of *P. vivax* but unfortunately resistance is emerging in several malaria regions of the world (Wellems and Plowe, 2001). To determine whether an association exists between point mutations of the *pvmdr1* gene and CQR, similarity and dendrogram analyses were performed using *pvmdr1* sequences from isolates of different geographical regions and with different chloroquine response (Fig. 2). Interestingly, these analyses revealed that sequences could be grouped according to their geographical origin and that within each geographical group resistant isolates branched independently; yet, there was no association of amino acid substitutions with CQR.

Knowledge about genetic diversity and population structure of *P. vivax* remains limited (Cui et al., 2003). Studies on gene diversity and population structure will help in predicting and monitoring the effectiveness of intervention strategies such as the usefulness of therapeutic regimens, the dispersion of drug resistance, and the emergence of multidrug-resistant parasites. For example, a direct correlation of *pvdhfr* point mutations from four different allelic variants with pyrimethamine resistance and geographic distribution of *P. vivax*, has been reported (Imwong et al., 2001). In contrast, no association has been found between point mutations of the *P. vivax* homolog gene of the *P. falciparum* CRT and the chloroquine response of different *P. vivax* isolates, including some of the samples used in the present work (Nomura et al., 2001). Similarly, our results failed to detect a correlation of *pvmdr1* allele variants and CQR and are thus in agreement with results that have not linked polymorphism of the *P. falciparum mdr1* gene with chloroquine resistance (Wellems et al., 1990). The mechanism of CQR in *P. vivax* remains to be determined.

There is presently no consensus definition of drug resistance in *P. vivax;* yet, it is clear that chloroquine resistance and multiple drug resistance will spread. Genes related to pyrimethamine resistance, *pvdhfr* (Eldin de Pecoulas et al., 1998; Imwong et al., 2001), chloroquine resistance, *pvcg10* (Nomura et al., 2001) and multiple drug resistance, *pvmdr1* (the present work) have been characterized in *P. vivax*. With the imminent release of the *P. vivax* genome (http://www.tigr.org/tdb/e2k1/pva1/), and annotations facilitated through ESTs gene discovery (Merino et al., 2003), it is likely that in silico analysis will predict additional transporter and drug resistance genes as has been recently reported for *P. falciparum* (Mu et al., 2003). Along with the development of new technologies for *P. vivax* such as the short-term in vitro cultures to monitor drug-resistance (Chotivanich et al., 2004) it is hoped that monitoring and control strategies will help control worldwide spread of multiple drug resistant *P. vivax* parasites.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.exppara.2004.12.005.

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Table 1S. Pv MDR1 amino acid substitutions and chloroquine phenotypes of the* Plasmodium vivax *isolates. CQ, chloroquine; CQS, chloroquine sensitive; CQR, chloroquine resistant.

Isolate	196	237	443	500	511	556	698	783	823	908	958	976	1076	1104	1440	1447	CQ phenotype
RO14	N	S	I	N	R	V	G	A	K	L	M	Y	F	L	R	E	CQS
RO19	N	S	I	N	R	V	G	A	K	L	M	Y	F	L	Q	E	CQS
ROB2	N	S	I	D	R	V	G	A	K	L	M	Y	F	L	Q	E	CQS
Sal1	N	S	I	D	R	V	G	A	K	M	T	Y	F	L	Q	E	CQS
NGIV	N	S	I	D	R	V	S	V	K	L	M	F	L	L	Q	E	CQS
IndoF	N	S	I	D	H	V	S	A	K	L	M	F	L	L	Q	E	CQR
CL004	N	S	I	D	H	V	S	A	E	L	M	F	L	F	Q	E	CQR
CL014	N	S	I	D	H	V	S	A	E	L	M	F	L	F	Q	E	CQR
CL001	S	P	T	D	H	V	S	A	E	L	M	F	L	F	Q	G	CQS
CL007	N	S	I	D	H	A	S	A	E	L	M	F	L	F	Q	E	CQS