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Plasmodium vivax mdr1 genotypes in isolates from successfully cured patients living in endemic and non-endemic Brazilian areas

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Abstract

Background: *Plasmodium vivax* is the most widely distributed species causing the highest number of malaria cases in the world. In Brazil, *P. vivax* is responsible for approximately 84 % of reported cases. In the absence of a vaccine, control strategies are based on the management of cases through rapid diagnosis and adequate treatment, in addition to vector control measures. The approaches used to investigate *P. vivax* resistance to chloroquine (CQ) were exclusively in vivo studies because of the difficulty in keeping parasites in continuous in vitro culture. In view of the limitations related to follow-up of patients and to assessing the plasma dosage of CQ and its metabolites, an alternative approach to monitor chemo-resistance (QR) is to use molecular markers. Single nucleotide polymorphisms (SNPs) in the multidrug resistance gene *pvmdr1* are putative determinants of CQ resistance (CQR), but such SNPs in *P. vivax* isolates from patients with good response to treatment should be further explored. The aim of this study is to investigate the mutations in the gene, supposedly associated to QR, in *P. vivax* isolates from successfully cured patients, living in Brazilian endemic and non-endemic areas.

Methods: Blood samples were collected from 49 vivax malaria patients from endemic (Amazon Basin: 45) and non-endemic (Atlantic Forest: four) Brazilian regions and analysed for SNPs in the CQR-related *P. vivax* gene (*pvmdr1*), using PCR-based methods.

Results: Among the 49 isolates genetically characterized for the gene *pvmdr1*, 34 (70 %) presented at least one mutation. T958**M** mutant alleles were the most frequent (73 %) followed Y976**F** (15 %) and F1076**L** (12 %). Single mutation was detected in 24 (70.5 %) isolates and double mutations in ten (29.5 %). The most common single mutant genotype was the 958**M/**Y976/F1076 (79 %), followed by 976**F/**F1076 (21 %) whereas 958**M/**Y976/1076**L** (60 %) and 976**F/**1076**L** (40 %) double mutant genotypes were detected. Single mutant profile was observed only in isolates from Amazon Basin, although double mutants were found both in the Amazon and Atlantic Forest regions. Interestingly, the genotype 958**M**/Y976/1076**L** was present in all isolates from the Atlantic Forest in the Rio de Janeiro State.

Conclusions: Considering that primaquine (PQ) efficacy is highly dependent on concurrent administration of a blood schizontocidal agent and that PQ could not circumvent CQR, together with the fact that no *pvmdr1* mutation should be expected in successfully cured patients, these findings seem to indicate that the *pvmdr1* gene is not a

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reliable marker of CQR. Further investigations are needed to define a reliable molecular marker for monitoring *P. vivax* CQR in *P. vivax* populations.

Keywords: *Plasmodium vivax*, Chloroquine resistance, *pvmdr1* gene, Brazil

Background

Almost 40 % (approximately three billion) of the world's population is presently at risk of contracting malaria. The disease causes almost 200 million clinical cases and around 600,000 deaths each year [1, 2]. *Plasmodium vivax* is the most geographically widespread of the human malaria parasites, and a serious public health concern in South and Central America, Asia and Southwest Pacific [3].

In Brazil, endemic regions are restricted to the Legal Amazon (comprising Acre, Amapá, Amazonas, part of Maranhão, Mato Grosso, Pará, Rondonia, Roraima and Tocantis States), a region that presently accounts for 99.6 % of the countrywide malaria burden [4]. *Plasmodium vivax* is the predominant species, responsible for 84 % of the reported cases [5], PNCM, SVS, MS, unpublished data 2015. Extra-Amazonian autochthonous cases account for only 0.04 % of all Brazilian total registered and correspond to the autochthonous malaria existing in the Atlantic Forest, located along the southeastern Atlantic Coast [6].

Chloroquine resistance (CQR) is the main challenge for national malaria control programmes to control vivax malaria. The first cases of *P. vivax* resistant to chloroquine (CQ) were described in Papua New Guinea [7] and thereafter observed in Indonesia [8], Oceania, Asian [9, 10] and South American countries, including Brazil [11, 12]. In Brazil, CQ treatment failures, presumably related to CQR, have been reported [13]. The latest 28 day in vivo test conducted to assess the efficacy of standard supervised CQ therapy in 109 volunteers showed a proportion of 10.1 % of treatment failure (n = 11), despite an adequate absorption of CQ in these individuals on day 2 [14].

Molecular markers can represent a valuable tool for monitoring introduction and spread of drug resistance. Contrarily to *Plasmodium falciparum*, mutations at codons in the *pfcrt* orthologue (*pvcg10*) gene do not seem to mediate CQR in *P. vivax* [15]. On the other hand, the polymorphisms at codons Y976**F** and F1076**L** in the multidrug-resistant gene 1 (*pvmdr1*) has been described as molecular marker associated to CQR [16]. Indeed, in Thailand, Indonesia [17] and Myanmar [18], as well as in Mauritania [19] and Cambodia [20], it has been shown that 976**F** mutants were associated with clinical resistance to CQ. In Nepal and India, where *P. vivax* CQR has not been recorded, prevalence of the 976**F** mutation is very low (5 %) [21] or not detected [22], while in India the presence of the F1076**L** mutation was not associated to CQR. In addition, in Madagascar, despite 5 % of clinical failures more than 90 % of Y976**F** mutant parasites were detected [33].

These polymorphisms also seem to be relatively uncommon in Latin America, where *P. vivax* CQR remains relatively infrequent [23]. In Brazil, different conclusions were drawn: either mutations in *pvmdr1* were reported in CQ-sensitive *P. vivax* parasites [24–26] or not detected in resistant *P. vivax* isolates [25, 26], as well as *P. vivax* CQR being associated with *pvmdr1* mutants only in patients with severe malaria [27].

In view of these different epidemiological data, the nucleotide polymorphisms (SNPs) of *pvmdr1* gene in successfully cured vivax malaria patients living in endemic (Amazonian) and non-endemic (Extra-Amazonian) Brazilian areas, were investigated in the present study.

Methods

Study site, blood samples and DNA extraction

Blood samples were collected between 2010 and 2014 in patients presenting vivax malaria (n = 49) at the Laboratório de Doenças Febris Agudas, INI-IPEC, Fiocruz, the Reference Laboratory for Malaria in the Extra-Amazon to the Brazilian Ministry of Health. The inclusion criterion was patients with uncomplicated vivax malaria. After obtaining informed consent, venous blood collection was performed according to protocols previously approved by the Ethical Research Committees of Fiocruz (32839013.6.00005248). Genomic DNA was extracted from 1 mL whole blood using QIAamp midi columns, as described by the manufacturer (Qiagen). *Plasmodium vivax* samples were diagnosed by microscopic examination and by polymerase chain reaction (PCR) [28]. All patients were treated with CQ plus primaquine (PQ) and followed up for 42 days and no treatment failure was detected during this period.

PCR and electrophoresis

The *pvmdr1* gene was amplified by PCR using gene-specific primers. The PCR was performed as described elsewhere [16, 29] to amplify a partial DNA sequence containing three SNPs for *pvmdr1* gene including: T958**M**, Y976**F** and F1076**L**.

DNA sequencing and SNP polymorphisms detection

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) [30] at the Genomic Platform/PDTIS/Fiocruz. The direct DNA sequencing from PCR products was compared with the reference wild type Sal I GenBank accession nº AY571984 [24, 25]. Forward and reverse sequences were analysed using the free software Bioedit Sequence Alignment Editor version 7.2.5. Statistical significance of differences of *pvmdr1* genotypes frequencies among Brazilian localities was assessed using Fisher's tests.

Results

The *pvmdr1* gene was successfully amplified and DNA sequenced in 49 isolates from the Amazon Region (Acre, Amazonas, Pará, and Rondonia) and the Extra-Amazonian State of Rio de Janeiro.

Globally, 34 (69 %) showed non-synonymous (958**M**, 976**F** and 1076**L**) mutations. 958**M** mutant alleles were the more frequent (25/34; 73 %) while 976**F** (5/34; 15 %) and 1076**L** (10/34; 12 %) were detected at lower frequencies (Table 1). Single mutation was observed in 24 isolates (70.5 %, 24/34), while double mutations were recorded in ten (29.5 %, 10/34) *P. vivax* samples. In the isolates presenting single mutant genotype, the **M**YF profile was predominant (19/55 %) contrasting with the **F**F, which was found in only five isolates (Table 2).

The *pvmdr1* wild-type allele was prevalent in Pará (54 %) followed by Acre (40 %), Amazonas (33 %), and Rondonia (24 %) states without statistically significant difference in proportion ($p > 0.05$). Single mutants were observed only in isolates from the Brazilian Amazon: the **M**FY allele was prevalent in Rondonia (77 %), followed by Amazonas (62 %) and Acre (67 %) ($p > 0.05$), although in Pará the **F**F single mutant was more frequent (66 %) than the **M**YF ($p > 0.05$). However, when double mutants were investigated, samples presenting **FL** (12 %) and **MYL** (20 %) in both Amazonian and Extra Amazonian States, were identified. Irrespective to the Brazilian State **FL** double mutant was the less frequent (Table 2) ($p > 0.05$). Interestingly, all isolates from the Extra Amazon (Rio de Janeiro State) showed double mutant genotype (**MYL**) contrasting with those from the Amazon Basin (6 %) ($p = 0.01$), where most of the isolates came from.

Table 1 Frequency of 958M, 976F and 1076L mutants in *pvmdr1* gene among 49 Brazilian *P. vivax* isolates

SNPs	Number of isolates (%)
No mutation	15 (31)
958**M**	25 (51)
976**F**	5 (10)
1076**L**	4 (8)

SNPs single nucleotide polymorphisms

Discussion

CQ and PQ remain the drugs of choice to treat vivax malaria, but recent studies have reported *P. vivax* cases of resistance to CQ in different regions of the world [9, 31], including Brazil [11–13]. Therefore, monitoring the efficacy of CQ in the treatment of vivax malaria is essential for early warning systems to promote drug policies.

To circumvent the limitations of in vivo and in vitro studies and to assess chemo-resistance, identification of mutations in target genes has been proposed, such as those in the *pvmdr1* gene at codons 976 and 1076, as well as the increased expression of *pvcrto* transcripts [12, 32]. Similar to previous studies performed with samples from western Brazilian Amazon [24, 25], in this work the T958**M** mutant was found to be the more frequent in the Brazilian Amazon and even in isolates from the Extra Amazonian regions. Additionally, in Madagascar [33], Nepal [21] and Thailand [34], most of the samples

Table 2 Proportion of the 4 alleles observed among 49 Brazilian *P. vivax* isolates, according to the sampling location

Genotypes	*Pv* isolates N (%)	Localities				
		Rondônia (n = 17)	Pará (n = 11)	Amazonas (n = 8)	Acre (n = 5)	Rio de Janeiro (n = 4)
Wt Sal1 type	15 (31)	4	5	4	2	0
Single **F**F	5 (15)	1	4	0	0	0
Double **FL**	4 (12)	2	0	2	0	0
Single **M**YF	19 (55)	10	2	5	2	0
Double **MYL**	6 (18)	0	0	1	1	4
		13 (76 %)	6 (54 %)	8 (67 %)	3 (60 %)	4 (100 %)

Pv, *Plasmodium vivax*

presented mutations at 958 position, although all isolates have been obtained from individuals with successful response to CQ therapy. No later than November 2015, Schousbe and colleagues [35] reported a high prevalence of 958**M** (97.6 %) among *P. vivax* samples from six different geographical sites, suggesting that this allelic variant is most likely not associated with CQR and could be an allele characteristic of Asia and Africa isolates. The present data reinforce the lack of association of 958**M** with CQR, but are not in agreement with Asian and African geographical characteristic of this allele, since this allele was present in 51 % of the Brazilian (South American) samples.

Previous studies seemed to indicate that both SNP and amplification of *pvmdr1* are associated with variation in in vitro CQ susceptibility of *P. vivax* [17, 32]. It has been shown that the geometric mean of the CQ inhibitory concentration 50 % (IC50) was significantly higher in isolates carrying the Y976**F** mutation when compared to wild-type isolates in samples from Indonesia and Thailand [17]. However, the clear association between the clinical outcome following a three-day CQ treatment and non-synonymous mutations in this gene has never been demonstrated elsewhere. In fact, the single 976**F** mutant was not very common worldwide (7.4 %) [22], and the **F**F double mutant genotype was detected only in endemic regions of three countries: Brazil [36, 37], Honduras [38] and Papua New Guinea [39]. In the samples herein analysed, no significant difference was observed between the presence of double 976**F**/1076**L** mutant (12 %) and single 976**F** mutant (15 %) and no single 1076**L** mutant was noted. These findings suggest that polymorphisms at codons 976 and 1076 may not be strong indicators of CQ resistance since all *P. vivax* isolates were obtained from patients with good response to CQ therapy. In addition, 976**F** and 1076**L** mutants were also detected in *P. vivax* isolates in several countries in Africa and in South America from patients with no history of CQ recrudescence [23]. Probably, these mutations might have been introduced in these countries from Asia where these mutations are prevalent [23]. Interestingly, the 976**F** mutation in *P. vivax* isolates from Extra-Amazonian were not detected in areas where autochthonous malaria cases from Brazilian Atlantic Forest can occur. Thus, it seems that 976**F** mutations are more associated to geographical characteristics than to CQR.

Concerning codon 958, only samples from the Amazon Basin showed the **M**YF single mutant genotype, and double **M**Y**L** mutants were observed in Amazonas and Acre State isolates. On the other hand, all isolates from the Extra Amazon State of Rio de Janeiro had the double mutant 958+1076 (**M**Y**L**) genotype and these samples were wild type for codon 976. Once again, the heterogeneity in these *P. vivax* populations could reflect the genetic diversity rather than an association with CQR in endemic areas with different endemic profiles [6].

Considering that *pvmdr1* mutations should not be expected in CQ-sensitive parasites and that PQ efficacy is highly dependent on concurrent administration of a blood schizontocidal agent [40] and thus PQ could not circumvent CQR, the present findings seem to indicate that the *pvmdr1* gene is not a reliable marker of CQR.

Conclusion

This study provides new data concerning the molecular characterization of *P. vivax* isolates from Brazilian Atlantic Forest. The SNP diversity observed in samples from New World is similar to those from Asia and Africa, and probably reflects a capacity for great functional variation, as already suggested [41]. Very little is known about the molecular mechanisms underlying drug resistance in *P. vivax* and most *loci* that have been suggested to be responsible for *P. vivax* CQR derived from orthologue *P. falciparum* drug CQR genes. Further investigations are needed to define a reliable molecular marker for monitoring CQR in the Brazilian *P. vivax* population.

Authors' contributions

MFFC carried out the study and the manuscript. LRG performed and analysis DNA sequencing and statistical analysis and drafted the manuscript. DM and CTDR participated in the discussions and reviewed the final manuscript. NKAO, SRFL and AL performed DNA extraction and PCRs. APC and PB recruited the patients. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

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