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

Larissa Rodrigues Gomes1,2, Natália Ketrin Almeida-de-Oliveira1,2, Aline Rosa de Lavigne1,2, Suelen Rezende Félix de Lima1,2, Anielle de Pina-Costa2,3, Patrícia Brasil1,3, Cláudio Tadeu Daniel-Ribeiro1,2, Didier Ménard4 and Maria de Fatima Ferreira-da-Cruz1,2*

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. T958M mutant alleles were the most frequent (73%) followed by Y976F (15%) and F1076L (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 958M/976F genotype (79%), followed by 976F/F1076L (60%) and 958M/976/1076L (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 958M/Y976/1076L 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
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 Tocantins 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 Y976F and F1076L in the multixcid-drug-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 976F mutants were associated with clinical resistance to CQ. In Nepal and India, where P. vivax CQR has not been recorded, prevalence of the 976F mutation is very low (5%) [21] or not detected [22], while in India the presence of the F1076L mutation was not associated to CQR. In addition, in Madagascar, despite 5% of clinical failures more than 90% of Y976F 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: T958M, Y976F and F1076L.
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 (958M, 976F and 1076L) mutations. 958M mutant alleles were the more frequent (25/34; 73 %) while 976F (5/34; 15 %) and 1076L (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 MYF profile was predominant (19/55 %) contrasting with the FF, 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 MFY allele was prevalent in Rondonia (77 %), followed by Amazonas (62 %) and Acre (67 %) (p > 0.05), although in Pará the FF single mutant was more frequent (66 %) than the MYF (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.

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 T958M 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 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) |
| 958M | 25 (51) |
| 976F | 5 (10) |
| 1076L | 4 (8) |

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

| Genotypes | Pv isolates N (%) | 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 FF | 5 (15) | 1 | 4 | 0 | 0 | 0 |
| Double FL | 4 (12) | 2 | 0 | 2 | 0 | 0 |
| Single MYF | 19 (55) | 10 | 2 | 5 | 2 | 0 |
| Double MYL | 6 (18) | 0 | 0 | 1 | 1 | 4 |

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 958M (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 958M 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 Y976F 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 976F mutant was not very common worldwide (7.4 %) [22], and the FF 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 976F/1076L mutant (12 %) and single 976F mutant (15 %) and no single 1076L 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, 976F and 1076L 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]. Probable, these mutations might have been introduced in these countries from Asia where these mutations are prevalent [23]. Interestingly, the 976F 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 976F mutations are more associated to geographical characteristics than to CQR.

Concerning codon 958, only samples from the Amazon Basin showed the MYF single mutant genotype, and double MYL 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 (MYL) 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 analysed DNA sequencing and statistical analysis and drafted the manuscript. DM and CTDPR participated in the discussions and reviewed the final manuscript. NKAO, SFRL and APL 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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