Original Article

Single-Nucleotide Polymorphisms in Genes Associated with Drug Resistance of *Plasmodium vivax* in Two Regions of South India

G. Mukthayakka, Annapurna G. Sajjan, Ragini Ananth Kashid[1]

Department of Microbiology, Shri B M Medical College Hospital and Research Centre, Vijayapura
[1]Department of Microbiology, BGS Global Institute of Medical Sciences, Bengaluru, Karnataka

Abstract

Background: *Plasmodium vivax* is reported to be the predominant cause of malaria in India. Recent emergence of resistance to chloroquine (CQ) and sulfadoxine-pyrimethamine (SP) drugs has been attributed to multiple mutations in *pvmdr*-1 and *pvdhfr* regions of *P. vivax,* respectively. Hence, in this study, we have assessed the single-nucleotide polymorphisms (SNPs) in *pvmdr*-1 and *pvdhfr* genes for CQ and SP drug resistance, respectively, in cases of malaria from two regions of South India. **Materials and Methods:** A total of 36 *P. vivax* isolates from Bengaluru and Vijayapura were collected and sequenced for *pvmdr*-1 and *pvdhfr* genes, and the SNPs were analyzed. **Results:** Out of the total 45 positive *P. vivax* samples assessed in this study, 36 samples were mono infection cases. No wild-type *pvmdr*-1 gene was observed in any of the samples analyzed. Predominant presence (83.3%) of double mutations (T958M and F1076L) was observed. In the *pvdhfr* gene, wild-type (36.1%) and two mutations (41.6%; S58R and S117N) were observed. New mutations were not detected in *pvmdr*-1 or *pvdhfr* gene in this study. **Conclusion:** The present study reports the presence of SNPs in both *pvmdr*-1 and *pvdhfr* genes in *P. vivax* isolates from Bengaluru and Vijayapura. Continuous monitoring of drug resistance to antimalarial drugs is essential for undertaking optimal public health measures tailored to selected regions.

Keywords: Chloroquine resistance markers, *Plasmodium vivax*, pvdhfr, pvmdr-1, single-nucleotide polymorphisms, sulfadoxine/pyrimethamine

Introduction

Majority of the cases of malaria in India are caused by *Plasmodium vivax* infections.[1] Chloroquine (CQ) and primaquine are first line of drugs used to treat malarial infection. The emergence of resistance to the antimalarial drugs can significantly compromise the strategies used in controlling the infection, especially in the endemic regions. In a major setback, reduced susceptibility to all the frontline antimalarial drugs (amodiaquine, CQ, mefloquine, quinine, and sulfadoxine-pyrimethamine [SP]) used for controlling *P. falciparum* infection is reported.[1-3] More recently, the reports of resistance to artemisinin derivatives are of major concern.[2,3] The first case of resistance to CQ in *P. vivax* was reported from Papua New guinea.[4] Understanding the resistance mechanisms to antimalarial drugs in *P. vivax* is limited due to lack of continuous *in vitro* culture method. However, the genotypic analysis of *P. vivax* species isolated from clinical cases with treatment failure has helped to analyze the single-nucleotide polymorphisms (SNPs) in the genes likely to be associated with drug resistance.[5] Several SNPs are reported in *pvmdr*-1 gene for CQ drug resistance in *P. falciparum* and *P. vivax*. Among several SNPs reported, mutations in Y976F and F1076 region of *pvmdr*-1 gene are associated with CQ resistance.[6-8] However, few studies

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Address for correspondence: Mrs. G. Mukthayakka
Department of Microbiology, Shri B M Medical College Hospital and Research Centre, Vijayapur - 586 103, Karnataka, India.
E-mail: muktha.micrompl@gmail.com

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have failed to observe any correlation between mutations in *pvmdr*-1 gene and the clinical outcome of *P. vivax* infections to treatment with CQ.[9-11] Few studies have also reported the association of variations in *pvmdr*-1 gene copy numbers with CQ drug resistance.[12,13]

An emergence of resistant to SP is specifically of concern as this may lead to treatment failure, particularly in mixed infection with *P. vivax*. This drug acts on the two enzymes involved in folate metabolism, namely dihydrofolate reductase (*dhfr*) and dihydropteroate synthase (*dhps*). Mutations in these genes are reported to be associated with the resistance to SP.[14] Pyrimethamine resistance was associated with mutations at codon regions 57, 58, 61, 117, and 113 in *dhfr* gene. Similarly, sulfadoxine resistance was associated with mutations at codon regions 382, 383, 512, 553, and 553.[15] In India, 53% of the malarial infections are due to *P. vivax,*[16] and CQ is used as a front line treatment for *P. vivax* malarial infections.Few studies have reported resistance to CQ in clinical cases from India.[17-20] Due to the prevalence of this resistance, artemisinin-based combination therapy is preferred especially in complicated mixed infection cases. Even though SP is not the treatment of choice for *P. vivax* infections in India, it is often prescribed in cases of mixed infections, exposing *P. vivax* to SP in the process.[15] Hence, regular assessment of drug resistance to both CQ and SP is essential for optimal management of *P. vivax* malarial infections. Although SNP analysis will not provide complete information about drug resistance, it is helpful as an indicator of preliminary emergence of resistance. Further, the correlation of SNPs with the gene copy numbers together with clinical outcome will offer a clear picture of drug resistance. It is also essential to look at SNPs in different geographical regions for identification of local prevalence of drug resistance. Hence, in this study, blood samples collected from patients infected with *P. vivax* from two regions of Karnataka (Vijayapura and Bengaluru), India, were assessed for SNPs in *pvmdr*-1 gene for CQ and *pfdhfr* gene for SP.

Materials and Methods

Ethics statement

The study protocol was reviewed and approved by the ethics and research committee of both the study institutions BLDEU SHRI B.M. Patil Medical College, Hospital and Research Centre Vijayapura, and BGS Global Institute of Medical Sciences, Bengaluru (BGSGIMS/GEN/296/2016-17). Before their enrollment, the study participants were orally explained about the study and informed consents were obtained. Pregnant women, immunocompromised individuals, patients on anticancer drugs, chronic alcoholics, and individuals with any underlying diseases such as diabetes and rheumatoid arthritis were excluded from the study.

Study area, sample collection, and sample storage

This study was conducted at two study centers, BLDEU SHRI B.M. Patil Medical College, Hospital and Research Centre Vijayapura, Karnataka, and BGS Global Institute of Medical Sciences, Bengaluru, Karnataka. Both the study centers are tertiary referral centers in Karnataka state. Following consent from patients, 5–10 mL of blood sample was collected into sterile EDTA tubes from June 2016 to December 2019. The blood samples were aliquoted and stored at –80°C till further analysis. All patients from 15 to 70 years age of both the genders that were presenting with symptoms of malaria were screened and cases that were smear positive or antigen positive for *P. vivax* parasite were included in the study. A total of 600 blood samples from malaria-suspected patients were screened and among them 45 malaria-positive cases were further analyzed for identification of infected parasite species and SNPs in drug resistance genes.

Malaria parasite identification by microscopy

The malarial infection was confirmed by careful microscopic examination of the Giemsa-stained peripheral blood smears. Briefly, thick and thin blood smears were made, stained by Giemsa stain (4%) and examined for the parasitological identification of malarial parasites under ×100 magnification using an oil immersion objective. In addition to microscopy, immunochromatographic principle-based malaria rapid diagnostic tests (RDTs) using Abott SD Bioline Malaria P.f/P.v test kit were performed to detect the evidence of malaria-specific antigen in the plasma of study participants.

DNA extraction and molecular confirmation of *Plasmodium vivax* species

Genomic DNA of *P. vivax* was extracted from 200 µL of each blood sample using a QIAamp DNA Blood Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions, with modification in the incubation step with proteinase K where duration of incubation time was extended to 20 min at 56°C for better DNA yield. The DNA was dissolved in TE-buffer (10 mM Tris–HCl, 0.1 M EDTA, pH 8.0) and was stored at –20°C until further analysis. The quality of total DNA was checked by running 5 µL of each DNA sample on a 1.0% agarose gel stained with ethidium bromide and visualized under ultraviolet illumination. *P. vivax* infection was further confirmed by polymerase chain reaction (PCR) analysis of 18S ribosomal RNA of parasites using the protocol described previously.[21]

Amplification of *pvmdr*-1 and pvdhfr regions by polymerase chain reaction

After confirming infections by expert microscopic examinations of Giemsa-stained thick and thin blood smears and reconfirmed with bivalent RDT kit (Falcivax® from tulip, Goa), the genomic DNA of *P. vivax* was extracted. To amplify target-specific fragments of *pvmdr*-1 and *pvdhfr* genes, PCR amplification methods were used by employing protocols reported elsewhere with some minor modifications in the reaction conditions.[21,22] Oligonucleotide primers for amplifying *pvmdr*-1 were 200 nM concentration of forward primer 5'-GGATAGTCATGCCCCAGGATTG-3' and reverse primer 5'-CATCAACTTCCCGGCGTAGC-3. *Pvdhfr* gene was amplified by using 200 nM concentration of each forward primer, *Pvdhfr*FP 5′-ATGGAGGACCTTTCAGATGTATT-3′,

and reverse primer, *Pvdhfr* RP 5′-CCACCTTGCTGTAAACCAAAAAGTCCAGAG-3′ (expected PCR product size 715 bp). For both the genes, PCR was setup in a 20-μL reaction volume using GoTaq® Green 2X Master Mix (Cat.# M7122, PROMEGA GoTaq Green, Madison, WI, USA) containing DNA Taq Polymerase, 3 mM $MgCl_2$, 400 μM each dNTPs, and 2 μl of genomic DNA as template in a reaction. No template control was included, with each batch of PCRs serving as negative control to check for any chances of contamination in reagents or during reaction setup. The PCR cycling conditions for both the gene fragments included an initial denaturation step at 94°C for 5 min, followed by 40 cycles at 94°C for 30 s, annealing temperatures 58.2°C for 15 s in the case of *Pvmdr*-1 and 59.1°C for 15 s in the case of *Pvdhfr*, followed by 72°C for 45 s. Final extension temperature step for 8 min was also included. The amplified PCR products were resolved on 1.0% agarose gel prestained with ethidium bromide and visualized under ultraviolet light. The PCR products were stored at − 20°C until further Sanger sequencing analysis.

Sanger sequencing analysis for *pvmdr*-1 and *pvdhfr* polymerase chain reaction products

Purified PCR products were quantified by NanoDrop before proceeding for bi-directional sequencing. To identify polymorphisms in *pvmdr*-1 and *pvdhfr* genes in our study population, 36 isolates of *P. vivax* were sequenced. All these isolates were of single species and monoclonal infections with *P. vivax* parasite. Sequencing of genes from each isolate was performed on an ABI Prism 377 DNA Sequencer equipped (Wilmington, DE 19810 USA). Sequencing data were analyzed using SeqMan software (DNASTAR, Inc.,Madison,WI 53705 USA) for eliminating PCR or sequencing errors, if any. FASTA format nucleotide sequences were checked using NCBI-BLAST tool for comparing with PubMed-deposited sequences. Nucleotide change such as presence or absence of SNPs was confirmed by reading both the forward and reverse strands.

Results

A total of 45 positive *P. vivax* samples were collected after microscopic examination from the two geographical regions. Thirty-six samples were mono infection cases and the same was confirmed by immunochromatographic RDT.

Analysis of *pvmdr*-1 gene polymorphism

The *pvmdr*-1 gene was amplified and sequenced for all the 36 isolates [Table 1]. No wild-type *pvmdr*-1 gene was observed in any of the samples analyzed. Three non-synonymous mutations (T958M, F1076L, and Y976F) were detected. Predominant presence of double mutation with T958M and F1076L (83.3%) was identified. Only single mutation (Y976F or F1076 L) was observed in some samples. Any new mutations were not detected in *pvmdr*-1 gene [Table 1].

Analysis of *pvdhfr* gene polymorphism

Among the 36 *pvdhfr* gene sequenced, one wild-type and two mutations, S58R and S117N, were observed. Double mutants (S58R with S117N) were present in 41.6% of the samples, and 36.1% of the samples were of wild type. There were no new mutations detected in *pvdhfr* gene [Table 1].

Table 1: Frequency distribution of mutations in drug resistance marker genes (*pvmdr-1* and *pvdhfr*) among Plasmodium vivax isolates (*n*=36)

Molecular marker	Type of mutation	Isolates number (%)
pvmdr-1 (*n*=36)	Wild type (without any mutation)	0 (0)
	Mutant (with T958M mutation)	34 (94.4)
	Mutant (with F1076L mutation)	31 (86.1)
	Double mutant (with T958M and F1076L mutations)	30 (83.3)
	Double mutant (with F1076L and Y976F mutations)	1 (2.7)
pvdhfr (*n*=36)	Wild type (without any mutation)	13 (36.1)
	Mutant (with S58R mutation)	17 (47.2)
	Mutant (with S117N mutation)	22 (61.1)
	Double mutant (with S58R and S117N mutations)	15 (41.66)

Discussion

The emergence of drug resistance in malaria can significantly impair the public health efforts in disease management. It is essential that identification of drug resistance in malaria is geographical region specific and continuously monitored. This study reports SNPs in both *pvmdr*-1 and *pvdhfr* genes in two regions, that is, Bengaluru and Vijayapura of Karnataka, South India. Mutations in *pvmdr1* and *pfdhfr* genes are previously reported to be associated with CQ and SP resistance. Drug resistance to CQ is previously reported in *P. falciparum*[23] and *P. vivax* species.[4,18,19] Few studies have also reported the emergence of resistance to antifolate drug SP among *P. vivax* infections in India and other countries.[24-26]

Single-nucleotide polymorphisms in *pvmdr*-1 gene

In our study, majority of the *P. vivax* isolates had mutations in T958M (94.4%) and F1076L (83.3%) regions, whereas one isolate had mutations in Y976F (2.7%) region. Similar frequency of mutations in T958 M, F1076L, Y976F was also reported previously from Mangalore region of Karnataka state, India.[2] Our observations are also consistent with those of Anantabotla *et al.* 2019 who reported similar SNPs in *P. vivax* from four different states across India (Puducherry, Mangaluru, Cuttack, and Jodhpur).[27] However, in contrast to our observations, some previous studies from India have observed mutations in Y976F.[6,28] This inconsistency perhaps reflects the emerging nature of mutations in Y96F, warrants detailed investigation in future. Although the predominant haplotypes evaluated in our study (T958M, F1076L) are commonly reported in all the previous studies from India,[2,6,27,28] an additional ten different haplotypes, including two novel mutations and K10 insertions, were reported by one study.[2] However, we did not observe any novel mutations in

our study. K10 insertions and Y96F mutations were reported to correlate with CQ resistance in studies from Thailand and Indonesia.[29] Mutations in *pvmdr*-1 and variations in gene copy numbers are commonly observed in low to high level of CQ drug resistance.[7,25,30,31] However, single-gene copy number is not significantly associated with drug resistance, whereas multiple copy number are clearly associated with treatment failure.[12,13] Consistent with this in the present study, we did observe multiple SNPs in the *pvmdr*-1 gene, indicating the prevalence of potential resistance to CQ in Bengaluru and Vijayapura regions of Karnataka, South India.

Single-nucleotide polymorphisms in *pvdhfr* gene

In this study, apart from CQ resistance, we also looked for resistance to antifolates in *P. vivax* infection by analyzing mutations in *pvdhfr* gene. Single, double, and quadruple mutants in *pvdhfr* gene have previously been reported the Indian subcontinent.[24] Consistent with these previous reports, our study also observed wild-type and single-type mutations in S58R and S117N amino acid positions of *dhfr* genes. The double-mutation frequency in our sample sets correlates with that of other studies from India.[3,24] In the case of *P. vivax* infection, resistance is due to slow clearance of SP and is often associated with mutations (S58R and S117N) in *dhfr* gene.[24,31] No novel mutations were observed in our study, which was in contrast to a study from another region of Karnataka state reporting novel mutation in K55R in combination with S58R and S117N.[3] While quadruple mutations were associated with high-level resistance,[24] in our study, only wild-type, single, and double mutations were observed, suggesting the prevalence of only milder level of resistance despite the detection of mutation in *pvdhfr* gene. The mutations observed in this study may also suggest that the development of resistance in our geographical region may be at an early evolution phase. This warrants necessary and immediate intervention to prevent the development of high-level resistance in the near future. One of the major limitations of this study is not evaluating the gene copy numbers and clinical outcome in the patients, which was due to lack of necessary resources, limiting our analysis to only one gene each associated with CQ (*pvmdr*-1) and SP (*pvdhfr*) resistance.

Conclusion

The present study detected the presence of SNPs in both *pvmdr*-1 and *pvdhfr* genes in the selected geographical area. The frequency of mutations in these genes does not indicate the development of complete resistance to CQ and SP in *P. vivax*. However, few SNPs detected in both genes suggested the probable early phase of resistance development. Hence, continuous monitoring of drug resistance to antimalarial drugs is essential for adopting optimal public health measures and avoiding development of complete resistance in future.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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