**Plasmodium vivax: Chloroquine Drug Resistance in Strains Isolated from Navi Mumbai, Maharashtra, India**

Gurjeet Singh\(^1\), Raksha\(^2\), Anant D Urhekar\(^3\)

**Abstract**

**Aim:** Malaria imposes a significant public health burden worldwide. Chloroquine (CQ) resistance has been shown to be associated with point mutations in *Plasmodium vivax* chloroquine resistance transporter (PvCRT) and *Plasmodium vivax* multidrug resistance transporter (Pvmrdr1). The present study was carried out to study the association of PvCRT-o K10 (lysine) insertion and Pvmrdr1 Y976 mutations with CQ resistance in Northeast Indian *Plasmodium vivax* isolates.

**Materials and methods:** The study was conducted in the Parasitology Laboratory at the Department of Microbiology, Mahatma Gandhi Mission Medical College and Hospital, Kamothe, Navi Mumbai, Maharashtra. A total of 22 *Plasmodium vivax* isolates were subjected to the in vitro CQ-sensitivity test and the polymerase chain reaction (PCR) test for the Pvmrdr1 Y976 and PvCRT-o K10 (lysine) insertion mutations.

**Result:** Five isolates of *Plasmodium vivax* were found to be resistant to CQ by the in vitro antimalarial drug-sensitivity test, while 17 were found to be CQ sensitive. All the CQ-resistant isolates showed the presence of Pvmrdr1 and PvCRT mutations. CQ-sensitive isolates were negative for these mutations. Strong linkage disequilibrium was observed between the alleles at these two loci [Pvmrdr1 Y976 and PvCRT-o K10 (lysine) insertion].

**Conclusion:** Our study supports the use of molecular methods for the detection of Pvmrdr1 Y976 and PvCRT-o K10 (lysine) insertion mutations to identify CQ drug resistance in *Plasmodium vivax* and to provide early and proper treatment to patients suffering from vivax malaria.

**Keywords:** Chloroquine, Polymerase chain reaction, Vivax malaria.

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**Materials and Methods**

**Study type:** Prospective and analytical study.

**Study period:** January 2014 to December 2014.

**Place of study:** The study was conducted in the Parasitology Laboratory at the Department of Microbiology and Central Research Laboratory, Mahatma Gandhi Mission Medical College and Hospital, Kamothe, Navi Mumbai, Maharashtra, India, and Eurofins Genomics, Bengaluru, Karnataka, India.

Ethical committee approval was obtained from MGM Institute of Health Sciences, Navi Mumbai, before conducting the study.

**Study participants:** A total of 22 patients having confirmed *Plasmodium vivax* malaria were included in this study.

**Sample size:** Twenty-two *Plasmodium vivax* malaria-positive samples.

Informed written consent was obtained from the patients. For antimalarial drug-sensitivity and molecular analyses, approximately 3 to 5 mL of the blood sample was collected from the patients who tested positive for *Plasmodium vivax*. Blood samples with proper...
identification number were stored in cryovials at −20 °C. A total of 22 blood samples positive for *Plasmodium vivax* malaria were included in the study. The DNA extraction of the samples was done by using the DNA extraction kit (Invitrogen, USA) spin column method. Primers were procured from Eurofins Genomics, Bengaluru, Karnataka, India. Primers for nested polymerase chain reaction (PCR) for the detection of a drug-resistant gene in *Plasmodium vivax* were selected from articles.15,16 For the *Plasmodium vivax* pvmdr1, the forward primer was CGCAGGACTGAAATAAGT ACTCCCTCTTA and the reverse primer was GCCTCGGCATATAAA, and for pvcrt-o, the forward primer was CGGCTTGCAAGAGCC and the reverse primer was AGTTTCCCTCTACAC CG. DNA was extracted from 200 µL of *Plasmodium vivax*-positive blood with the DNA extraction kit (Invitrogen, USA) per the instructions given in the manual and stored at 4 °C until PCR could be completed. Nested PCR amplifications were done using a standard procedure. Known *Plasmodium vivax*-positive and -negative samples were used as controls. DNA bands were visualized and documented by using the gel documentation system (BioEra, India).

The amplified products of nested PCR containing genes pvcrt-o and pvmdr1 were directly subjected to sequencing in both directions using a 3730XL DNA sequencer (Sanger method, big dye terminator chemistry, and Pop 7 polymer gel) from Eurofins IT Solutions India Pvt Ltd. (Bengaluru, India). We found that all isolates showed the mutant allele F976 of codon 976 in pvmdr1 genes and K10 (lysine) insertion in pvcrt-o genes.

**Results**

Out of 22 samples, five isolates showed resistance to CQ, whereas 17 isolates were sensitive to CQ. All 22 isolates were subjected to the nested PCR test, of these, five showed pvmdr1 and pvcrt-o genes and the rest 17 isolates showed none of the genes. The results showed the same sensitivity using both methods. All 22 amplified products of nested PCR were subjected to purification on gel to proceed for gene sequencing and confirm the mutations. Out of 22 amplified products, only five amplified products showed band on gel and rest 17 showed no band on gel that means only five isolates had pvmdr1 and pvcrt-o genes.

Sequencing analysis of 22 *Plasmodium vivax*-positive strains (five CQ resistance and 17 CQ sensitive) showed that the mutant allele F976 of codon 976 was detected in five samples, whereas the normal allele Y976 of codon 976 of Pvmdr1 was seen in 17 samples. For Pvcrt-o K10 codon, five samples showed K10 (lysine) insertion, whereas 17 samples did not show lysine insertion. Sequencing studies of pvmdr1 and pvcrt-o genes in our study revealed that the mutant F976 gene in codon 976 of pvmdr1 was found in 22.73% samples of CQ resistance (Table 1).

**Discussion**

The treatment of *Plasmodium vivax* malaria has changed a little in the past 60 years. In most areas, CQ plus primaquine is the first-line treatment, but this status quo is increasingly threatened by the emergence and spread of CQ-resistant *Plasmodium vivax*.17,18 The extent of this threat is unclear because primaquine has intrinsic blood-stage activity, which could mask low-level CQ resistance, and modest reductions in therapeutic efficacy can be either masked or accentuated by various methodological issues inherent in the study designs applied.

Decreasing antimalarial efficacy is shown by the ability of malaria parasites to grow in the presence of adequate bloodstream drug concentrations. At low levels of resistance, an initial clinical response occurs, often followed by a return of illness caused by recrudescence (a late treatment failure or late parasitological failure). The length of the interval from the start of treatment to parasite recrudescence depends on the pharmacology of the initial treatment regimen, the degree of drug resistance, and the level of host immunity.19 Increasing drug resistance enables parasite growth in high drug concentrations, which slows parasite clearance and shortens the interval to the first recurrence. In studies with a greater risk of recurrence by day 28, illness tends to recur sooner (rs = −0.58). Highly resistant parasites continue to grow despite high blood concentrations of the drug, which results in early treatment failure.

The epicenter for CQ-resistant *Plasmodium vivax* studies has consistently shown high-grade resistance manifested by early clinical deterioration requiring hospitalization, by delayed parasite clearance, and by early recurrent parasitemia.17,18 Several reports of severe and fatal vivax malaria have been published in the past few years.21,22

*In vitro* antimalarial drug-sensitivity testing of CQ was done for 22 isolates of *Plasmodium vivax* using a method similar to the WHO III plate method according to Singh et al.2 All 22 isolates, only five were resistant to CQ, whereas 17 were sensitive.

All 22 isolates were subjected to the nested PCR test and there were the same numbers, i.e., five showed pvmdr1 and pvcrt-o genes and the rest 17 isolates showed no genes. The results showed the same sensitivity using both methods.

All 22 amplified products of nested PCR were subjected to purification on gel to proceed for gene sequencing and confirm the mutations. Out of 22 amplified products, only five amplified products showed band on gel and rest 17 showed no band on gel that means only five isolates had pvmdr1 and pvcrt-o genes.

### Table 1: Drug-resistant pattern of *Plasmodium vivax*

<table>
<thead>
<tr>
<th>Codon 976 of Pvmdr1 (n = 22)</th>
<th>Allele Y976</th>
<th>17</th>
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<tr>
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<td>Allele F976</td>
<td>05</td>
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<thead>
<tr>
<th>Codon K10 of Pvcrt-o (n = 22)</th>
<th>Without K10 insertion</th>
<th>17</th>
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<tbody>
<tr>
<td></td>
<td>K10 insertion</td>
<td>05</td>
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**Fig. 2:** Mutant allele K10 insertion in pvcrto gene

that the mutant F976 gene in the codon 976 of pvmrdr1 was found in 22.73% samples of CQ resistance. Mint et al.15 reported that the majority of the isolates with successful PCR amplification (76/86, i.e., 88%) were characterized to be of the wild-type pvdfhr genotype, while the remaining 10 isolates carried the SS8R and S117N double mutations. In their study, all isolates had the wild-type pvdr1 gene.15 Chehuan et al.16 reported that 12 out of 105 isolates, 102 (97%) had one copy and 3 (3%) had two copies of the pvdr1 gene.15 Chehuan et al.16 reported that 12 out of 112 isolates were considered resistant to CQ, resulting in 10.7% (IC95%, 5.0–16.4), while 3 out of 47 (6.4%; IC95%, 0.0–12.8) were resistant to mefloquine (MQ). A discrete correlation was observed between IC50s of CQ and MQ (Spearman = 0.294; P = 0.027). The occurrence of CQ drug resistance in patients in and around Navi Mumbai could call for reinforced surveillance of drug efficacy. Plasmodium vivax CQ resistance may lead to the contribution in the spread of CQ-resistant vivax malaria and the clinical severity of this disease may cause mortality of patients. Our study recommends the use of the molecular technique for early detection of drug resistance and highlights the importance of the CQ-resistant vivax malaria and antimalarial drug-resistant surveillance tests must be conducted on a regular basis to assess the efficacy of the drug.

**CONCLUSION**

The gene sequencing study in our work revealed the presence of the mutant allele F976 in the codon 976 of pvmrdr1 (Plasmodium vivax multidrug-resistant gene) in S522 (22.73%) samples and K10 (lysine) insertion in the codon K10 of pvcrto (Plasmodium vivax CQ-resistant transporter gene) in S522 (22.73%) samples.

The occurrence of CQ drug resistance in patients in and around Navi Mumbai could call for reinforced surveillance of drug efficacy. Plasmodium vivax CQ resistance may lead to the contribution in the spread of CQ-resistant vivax malaria and the clinical severity of this disease may cause mortality of patients. Our study recommends the use of the molecular technique for early detection of drug resistance and highlights the importance of the CQ-resistant vivax malaria and antimalarial drug-resistant surveillance tests must be conducted on a regular basis to assess the efficacy of the drug.

**REFERENCES**