ORIGINAL ARTICLE

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

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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 (Pvmdr1). The present study was carried out to study the association of Pvcrt-o K10 (lysine) insertion and Pvmdr1 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 Pvmdr1 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 Pvmdr1 and Pvcrt mutations. CQ-sensitive isolates were negative for these mutations. Strong linkage disequilibrium was observed between the alleles at these two loci [Pvmdr1 Y976 and Pvcrt-o K10 (lysine) insertion].

Conclusion: Our study supports the use of molecular methods for the detection of Pvmdr1 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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Introduction

Malaria remains one of the leading causes of morbidity and mortality in the world. About 2 million deaths are attributed to malaria each year globally. Approximately 2.48 million malarial cases are reported annually from South Asia, of which 75% of cases are from India alone. Drug resistance is one of the major factors contributing to the resurgence of malaria, especially resistance to the most affordable drugs, such as chloroquine (CQ). Prior to the emergence of resistance, CQ was considered a very effective, safe, and inexpensive antimalarial drug.[1] However, resistance to CQ developed in the early 1960s at two loci, one in Southeast Asia and another in Latin America, and has spread to all areas where malaria is present. It has been estimated that mortality from falciparum malaria increases up to five-fold in areas where resistance to the antimalarial CQ is established.[1]

To reduce the mortality rate, understanding the mechanisms of such resistance and the development of new treatments, including new drugs, are urgently required. Great progress has been made recently in studying the mechanisms of drug action and drug resistance in malaria parasites. These efforts are highlighted by the demonstration of mutations in multiple drug-resistant genes.[2]

Malaria transmission depends on two primary factors, i.e., location of mosquito breeding sites and clustering of human habitations where people serve as reservoirs of parasites for mosquito infection.[3] The resistance to CQ antimalarial drug in *Plasmodium falciparum* is one of the main issues contributing to the global increase in morbidity and mortality. Various methods have been developed to detect the antimalarial drug-resistant pattern of malarial parasites. CQ resistance in *Plasmodium vivax* has also been observed in the Pacific and developing countries including India and nearby the countries but has not yet been reported in Pakistan and Afghanistan. CQ remains effective against *Plasmodium vivax* in India.[4–14] Therefore, the present study was undertaken to detect the CQ antimalarial drug sensitivity in *Plasmodium vivax*.

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Conflict of interest: None

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 GCGAACTCGAATAAGT ACTCCCTCTA and the reverse primer was GGCGTA GCTTCCCGTAAATAAA, and for pvcrt-o, the forward primer was CGCTGTCGAAGAGCC and the reverse primer was AGTTTCCCTCTACAC CCG. 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.

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

In vitro sensitivity status CQ resistance (5) and CQ sensitive (17)		
Codon 976 of Pvmdr1 (*n* = 22)	Allele Y976	17
	Allele F976	05
Codon K10 of Pvcrt-o (*n* = 22)	Without K10 insertion	17
	K10 insertion	05

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 recrudescent parasitemia (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.[17,18,20] 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] Out of 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. 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 (Table 1 and Fig. 1). For the pvcrt-o K10 codon, five samples showed K10 (lysine) insertion, whereas 17 samples did not show lysine insertion (Table 1 and Fig. 2). Sequencing studies of pvmdr1 and pvcrt-o genes in our study revealed

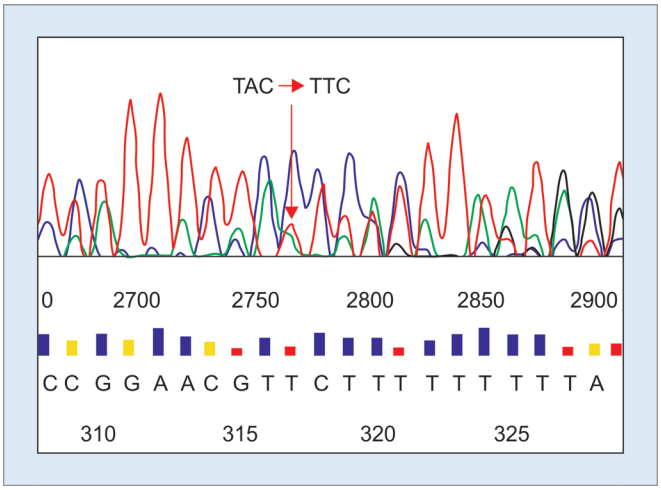

Fig. 1: Mutant allele F976 mutation in pvmdr1 gene

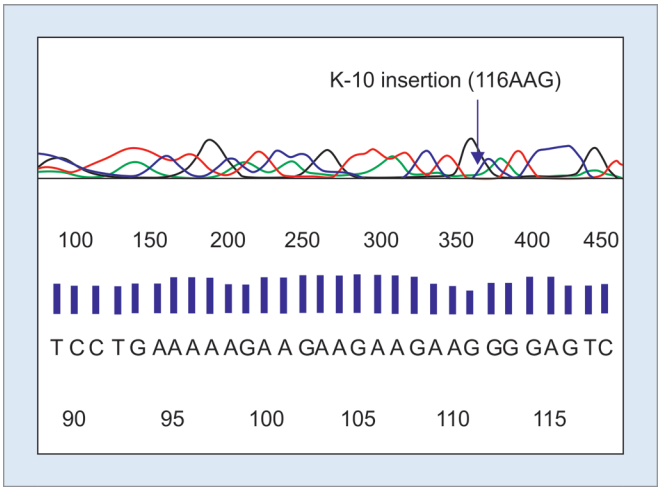

Fig. 2: Mutant allele K10 insertion in pvcrt-o gene

that the mutant F976 gene in the codon 976 of pvmdr1 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 pvdhfr genotype, while the remaining 10 isolates carried the S58R and S117N double mutations. In their study, all isolates had the wild-type pvdhps genotype SAKAV. For the pvmdr1, 75 of 103 (73%) had the wild-type Y976, and 28 (27%) carried the mutant F976. Most (98%) carried the mutant L1076 codon. Of 105 isolates, 102 (97%) had one copy and 3 (3%) had two copies of the pvmdr1 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; $p = 0.045$). For the pvdhps gene, a non-synonymous mutation was found at the codon 382 (S C) in 5/8 CQ-sensitive samples and 1/9 CQ-resistant samples ($p = 0.027$). The other molecular markers were not associated with CQ susceptibility.[16]

Conclusion

The gene sequencing study in our work revealed the presence of the mutant allele F976 of the codon 976 of pvmdr1 (*Plasmodium vivax* multidrug-resistant gene) in 5/22 (22.73%) samples and K10 (lysine) insertion in the codon K10 of pvcrt-o (*Plasmodium vivax* CQ-resistant transporter gene) in 5/22 (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.

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