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Polymorphisms in genes associated with drug resistance of *Plasmodium vivax* in India

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ABSTRACT

Malaria is a sterning public health concern in India and contribute to a major part of malaria burden in Southeast Asia. Being more populated and diverse geographic conditions makes more suitable place for sustaining malaria parasite in India. Anti-malarial resistance is a major concern in the battle against malaria, and the identified molecular markers will aid us to monitor the drug resistance in endemic areas. The aim of the current study is to determine the genotype of drug resistance associated genes *pvmdr-1* and *pvcrt-o* from four different regions of India. Especially from Puducherry and Jodhpur, there were no prior studies focused on screening of drug resistance genes in *P. vivax* parasite. A total of 240 positive *P. vivax* infected patient samples were collected from four tertiary care hospitals from four different regions of India, namely, Puducherry (PDY), Mangaluru (MAQ), Cuttack (CTC), Jodhpur (JDH). All samples were screened by microscopy, RDT, QBC, and further DNA was extracted and vivax mono-infection was confirmed by nested PCR. Randomly selected amplicons were further subjected to nucleotide sequencing. The prevalence of K10 insertion in *pvcrt-o* gene was detected with 18.8% in PDY, 12.5% in MAQ and 6.3% in CTC *P. vivax* isolates, whereas no change in nucleotide was identified in *P. vivax* isolates collected from JDH region. Based on the F1076L mutation in *pvmdr-1* gene, resistant *P. vivax* isolates was highly predominant in both the regions, JDH and CTC, with 100%, followed by MAQ with 93.3% and PDY with 73.3%. This study showed less frequency of *pvcrt-o* and high frequency of *pvmdr-1* gene variants associated with CQ resistance, which act as an indicator and the onset of *P. vivax* drug resistance trend in four different regions of India. Due to the poor phenotypic studies available for *P. vivax* parasite, the present study data for CQ resistance based on *pvcrt-o* and *pvmdr-1* markers should assist by providing base-line data for future monitoring of drug resistance.

1. Background

Human malaria infection is a life-threatening disease caused by the sporozoites, which are the infected form of malaria parasite, transmitted through the bite of female *Anopheles* mosquito [1]. An estimated 219 million cases of malaria occurred in 2017 worldwide which are slightly upward in trend compared to 216 million cases of infection in 2016 [1]. Of the five *Plasmodium* species which causes human malaria infection, *P. falciparum* is the deadliest parasite in terms of its morbidity and mortality, whereas, *P. vivax* is spread geographically in the most densely populated regions [2]. In Southeast Asia, 58% of the malaria cases occurred due to *P. vivax* parasite, whereas, 82% of the estimated

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Fig. 1. Map of India illustrating the surveillance of chloroquine drug resistance reported in *P. vivax* parasite, as reproduced with the permission of the WorldWide Antimalarial Resistance Network (WWARN). The data presented was analysed using the Vivax Surveyor developed by the WorldWide Antimalarial Resistance Network (www.wwarn.org/vivax/surveyor/#0) and exhibits a range of tools and filters to access the spread of molecular markers associated with various antimalarials.

vivax malaria cases arise from five countries with notably 48% are only from India [1]. Despite the high disease severity, there is lack of understanding in the pathogenesis of *P. vivax* at molecular level and largely been neglected due to various technical adversities which has led to obstruct the control measures and even eradication of malaria in the long run [3].

Chloroquine (CQ) is the first line treatment for *P. vivax* malaria in India like many other parts of the world. Resistance to CQ was first reported from Papua New Guinea in 1989 [4], followed by different endemic regions from Southeast Asia [5]. Reports of CQ treatment failure and resistance was observed in different regions of India, viz., Mumbai [6,7], Mathura [8], South Bihar [9], Kolkata and North Odisha [10]. In 1989, clinical cases from Hardiwar have shown inadequate treatment with Primaquine drug in preventing relapses, which is the only effective drug against hypnozoite stage [11]. Fig. 1 depicts the surveillance of chloroquine drug resistance of *P. vivax* malaria reported in different regions of India, as obtained from WWARN Vivax Surveyor [12].

As compared to *P. falciparum*, the molecular mechanisms underlying the drug resistance in *P. vivax* are less studied and understood, which is probably because of its benign nature and also, its inability of continuous culture by *in vitro* method [13]. Nevertheless, the impact of its negligence might have shown its effects on the socio-economic development and the quality of life [13]. Drug resistance in *P. vivax* needs to get addressed as quickly as possible with the evaluation of molecular mechanisms involved in drug resistance and its associated genes across the different endemic areas of the globe [14]. Also, studies have shown the association of *P. vivax* CQ resistance and severe *P. vivax* malaria infection with the increased expression levels of resistant markers (*pvcrt-o* and *pvmdr-1*) when compared to the susceptible *P. vivax* infected patients in the Brazilian Amazon [15]. Drug resistance in malaria have several consequences, such as, greater economic impact, slow down of initial therapeutic response, increased gametocyte transmission and carriage, increased mortality, etc [16].

Studies on the identification of single nucleotide polymorphisms (SNPs) in drug-resistant genes have been showed important which aid in monitoring the therapeutic efficacies and the prevalence of drug-resistant circulating parasites in different malaria-endemic settings [14]. Mutation in both *P. vivax* chloroquine resistance transporter (*pvcrt-o*) and multidrug resistance 1 (*pvmdr-1*) genes have been

identified as possible molecular markers to detect CQ resistance in *P. vivax* [13,17]. Studies have proven strong correlation of lysine aminoacid residue insertion at the 10th position in the *pvcrt-o* gene which contains other five SNPs [18]. Similarly, two non-synonymous mutations, Y976F and F1076L, in *pvmdr-1* have been studied and reported to be associated with CQ resistance [19]. Scanty information is available regarding the potential relationship between the CQ resistance and its associated genes (*pvcrt-o* and *pvmdr-1*) [20].

The data on the prevalence of *pvcrt-o* and *pvmdr-1* genes in India is very limited and confined to a particular region, and therefore, the present study is mainly focused to determine any mutations or SNPs in the drug-resistant molecular markers of *P. vivax* from different regions of India with distinct endemicity. This study will aid in understanding the evolution and emergence of drug-resistant *P. vivax* parasites in the population, which may lead to therapeutic failure in the vivax malaria, and additionally, helps in continuous monitoring of the circulating drug-resistant parasites and malaria elimination. This study is the first of its kind in India which includes different endemic regions, especially, Puducherry and Jodhpur where no previous data is available till date.

2. Materials and methods

2.1. Ethics approval

This study protocols were reviewed and approved by JIPMER Scientific Advisory Committee and Institute Ethics Committee in Puducherry and also approval from other three centers i.e., Kasturba Medical College (KMC) in Mangaluru, AIIMS in Jodhpur and SCB Medical College in Cuttack has also been obtained.

2.2. Sample collection, study area

A total of 240 positive *P. vivax* infected patient samples were collected from four tertiary care hospitals from four different regions of India, namely, Puducherry (PDY), Mangaluru (MAQ), Cuttack (CTC), Jodhpur (JDH), respectively. Routine samples which have come for malaria investigations in the respective laboratory of the four hospitals were screened primarily by different diagnostic methods, viz., thick and thin smears using Giemsa stain, antigen detection by rapid diagnostic test (RDT) and examination of blood by quantitative buffy coat (QBC) technique. The samples that were found to be positive for *P. vivax* infection by the above mentioned diagnostic methods were selected randomly and included in the study. The details of sample collection from four different regions were described in Table 1.

2.3. Species differentiation and haplotype confirmation

Genomic DNA was extracted using commercially available kit (Qiagen Blood DNA Mini kit, Germany) from 200 µl of EDTA blood following manufacture's protocol and stored in −20 °C until further use. The extracted DNA was additionally confirmed for *P. vivax* malaria parasite by targeting 18S ribosomal RNA gene using nested PCR (first round for genus-specific and second round for species-specific), as described previously [21].

2.4. Amplification of pvcrt-o and pvmdr-1

Target-specific fragments of *pvcrt-o* and *pvmdr-1* genes have been amplified using primers as described [14] with minor modifications in the reaction conditions using PCR (Agilent Sure Cycler 8800, CA, USA). The PCR reaction condition of both rounds for *pvcrt-o* gene are same except for annealing temperature in outer reaction as 52 °C for 30 s and in nested reaction as 57 °C 30 s with initial denaturation of 94 °C for 2 min; 30 cycles of denaturation at 94 °C for 15 s, extension at 72 °C for 1 min; final extension of 72 °C for 10 min. The PCR reaction condition for *pvmdr-1* for both outer and nested reaction are as follows: initial

Table 1
Details of sample collection from four different study centres from four distinct geographical regions of India.

Study state	Puducherry	Karnataka	Odisha	Rajasthan
Study location	 JIPMER, Puducherry	 KMC, Mangaluru	 SCB Medical College, Cuttack	 AIIMS, Jodhpur
Study period	Aug, 2015 – July 2017	Dec, 2015 – Nov, 2016	Nov, 2015 – Oct, 2016	Dec, 2015 – Nov, 2016
Total samples screened[a]	2470[b]	15,147[b]	2650	670
Total positive malaria cases	150	995	540	90
P. vivax cases randomly selected for the study	60	60	60	60
All the selected positive *P. vivax* samples from three study centres have been transported to JIPMER for molecular diagnosis and genotyping of drug resistance				
P. vivax monoinfection diagnosed by nested PCR	60	60	60	60

[a] Screening of samples were performed by routine malaria investigation techniques, like, microscopy and RDT.
[b] In JIPMER and KMC, the samples were screened additionally using QBC technique, as routine diagnostic procedure.

denaturation at 94 °C for 2 min, 33 cycles of denaturation at 94 °C for 15 s, 56 °C for 30 s, and extension at 72 °C for 1 min followed by final extension of 72 °C for 10 min.

2.5. Genotyping of pvcrt-o and pvmdr-1

To identify appropriate polymorphisms in *pvcrt-o* and *pvmdr-1* genes in our study population, 72 isolates of *P. vivax* were sequenced [14]. All these isolates are single species and monoclonal infections of *P. vivax* parasite. The PCR products were purified using MN nucleospin gel and PCR clean up kit according to manufacturer's protocol. The purified products were sequenced using ABI 3130 in both forward and reverse directions using gene-specific primers at Bioserve Biotechnologies, Hyderabad, India. DNA sequences were aligned using BioEdit 7.2 software [22], and analyzed using MEGA version 6 program [23]. Partial sequences of drug-resistant markers studied were submitted in NCBI database with the accession numbers MK051471 – MK051530 (*pvmdr-1*) and MK051531 – MK051594 (*pvcrt-o*).

2.6. Data analysis

Power calculation for sample size is not performed since the present study is a preliminary exploratory study. Chi-square test in SPSS version 16 (SPSS Inc., Chicago IL) was used for analysing the statistical association between the point mutations and the independent variable [24] and a *p*-value $\leq .05$ was considered as statistically significant. The number of haplotypes and haplotype diversity was determined by alignment of *pvcrt-o* and *pvmdr-1* coding sequences [25]. The nucleotide diversities measures, namely θw and π [26,27], were estimated distinctly for individual sample population. All the parameters were calculated using computer program DnaSP version 5.10 [28].

3. Results

Drug resistance molecular markers, *pvcrt-o* and *pvmdr-1*, were sequenced to determine the CQ resistance of *P. vivax* isolates collected from different regions of India. This study also helps to detect emerging new mutations in the molecular markers, which aids in the survival fitness of the circulating parasite. A total of 240 positive *P. vivax* samples were collected from Southeastern and Southwestern coastal regions of India (PDY and MAQ, respectively), East India (CTC), and Thar desert of Northwestern part of India (JDH), which are screened by different routine diagnostic methods, viz., microscopy, RDT, and QBC (Table 1). All the positive samples were further confirmed by nested PCR in JIPMER, Puducherry. Randomly 18 samples from each study site (total sample size, 72) were selected for CQ drug resistance markers, *pvcrt-o* and *pvmdr-1*, respectively. In this study, the sequence analysis of *pvcrt-o* is in contrast to the *pvmdr-1* sequence analysis and no association was observed between the mutation of the two genes.

3.1. Analysis of pvcrt-o gene polymorphisms

A 379 bp *pvcrt-o* gene product, targeting the first exon with K10 insertion, was successfully sequenced with the frequency of 88% (64/72). Of the samples analyzed (16 isolates from each site), the predominant of the isolates were sensitive for CQ with 90.6%, while resistant isolates were detected in only six isolates with 9.4% (Table 2). K10 insertion was detected more in PDY ($n = 3$), followed by MAQ and CTC isolates. Interestingly, all of the isolates from JDH were sensitive without the K10 insertion in the *pvcrt-o* gene. Haplotype and nucleotide diversities (Hd and π) were high in PDY and MAQ isolates, followed by CTC isolates (Table 3).

3.2. Analysis of pvmdr-1 gene polymorphisms

The *pvmdr-1* gene product of 604 bp, targeting the Y976F and F1076L mutation, was sequenced successfully in 60 isolates with 83.3%. Out of 64 isolates sequenced for *pvmdr-1* (15 from each site), most of the isolates were resistant for CQ with 91.6% having the F1076L mutation in the *pvmdr-1* gene (Table 2). All the *P. vivax* isolates sequenced from CTC and JDH were resistant for CQ based on the *pvmdr-1* molecular marker, and the prevalence of resistance in MAQ isolates fall almost close to CTC and JDH. The prevalence of CQ resistance among the PDY isolates were 73.3%. Highest haplotype and nucleotide diversities were detected in PDY isolates, followed by MAQ, and CTC and JDH isolates, based on sequence polymorphisms observed in the *pvmdr-1* gene (Table 3). Interestingly, the T958M non-synonymous mutation, resulting due to the nucleotide change at the position 2873 from C to G, was present in all the *P. vivax* isolates collected from four different regions of India with 100% frequency.

Table 2
Detection of CQ resistance among the *P. vivax* isolates collected from different regions of India, such as, Puducherry (PDY), Mangaluru (MAQ), Cuttack (CTC), Jodhpur (JDH).

Molecular markers	PDY	MAQ	CTC	JDH	Total (%)
pvcrt-o ($n = 16$[a])					
Wild (without K10 insertion)	13	14	15	16	58 (90.6)
Mutant (with K10 insertion)	3	2	1	0	6 (9.4)
pvmdr-1 ($n = 15$[a])					
Wild (without Y976F and F1076 mutation)	4	1	0	0	5 (8.3)
Mutant (with T958M mutation)	15	15	15	15	60 (100)
Mutant (with F1076L mutation)	11	14	15	15	55 (91.6)

[a] number of samples sequenced from each study site.

4. Discussion

Drug resistance in malaria is a serious public agitating problem mainly in the tropical and sub-tropical countries of the world. The emergence of resistant parasite in *P. falciparum*, severe malaria, was first reported in the late 1950s from Southeast Asia and South America independently [29], and since then, it has evolved rapidly for different antimalarial drugs and spread globally. *P. vivax* parasite, benign and tertian malaria, causes relapse due to hypnozoites, and CQ resistance was reported first in Paupa New Guinea in 1989 [4]. In India, CQ resistance in *P. vivax* malaria was first reported in 1995 in Bombay [6], and also, Primaquine failure due to inadequate 5-day radical treatment was reported in Hardiwar [11]. Thus, unravelling the prevalence of CQ resistance in *P. vivax* malaria and detecting the emergence of new mutations will aid in understanding the phenotypic changes resulting in resistant parasite due to the change in nucleotide of genes involved in drug resistance.

In this hospital-based descriptive study, we performed DNA sequencing of the drug-resistant molecular markers, *pvcrt-o* and *pvmdr-1*, for CQ resistance in *P. vivax* isolates collected from different regions of India based on tertiary care hospital settings. India, belonging to the tropical region, has diverse climate and distinct geographical conditions across the country. This high climatic variation in the tropical environment ideally helps in the continuous survival of the mosquito vectors, which thereby aids in the distribution of vector borne diseases, like malaria, filaria, dengue, chikungunya, etc. Such climatic variation in India causes seasonal transmission of malaria infection, for example, the Southeastern (PDY) and Southwestern (MAQ) coastal region of this present study has distinct endemicity and transmission rate for vivax malaria (PDY, mesoendemic and seasonal transmission; MAQ, hyperendemic and year-round transmission). Similarly, JDH region with dry tropical climate has seasonal transmission of *P. vivax* malaria parasite with high endemicity. While the CTC region, exhibiting both tropical wet and dry climate, has high endemicity for *P. falciparum* malaria, followed by *P. vivax* with year-round transmission of malaria.

Table 3
Genetic diversity of *pvcrt-o* and *pvmdr-1* genes in *P. vivax* isolates collected from four different regions of India.

Gene name	*pvcrt-o*				*pvmdr-1*			
Study site	PDY	MAQ	CTC	JDH	PDY	MAQ	CTC	JDH
No of isolates	16	16	16	16	15	15	15	15
SNP	3	3	3	0	2	2	2	2
No of haplotypes (n)	2	2	2	1	3	3	2	2
Haplotype diversity (Hd)	0.233	0.233	0.125	0.0	0.492	0.242	0.125	0.125
Nucleotide diversity								
θw	0.301	0.301	0.301	0.0	0.00097	0.00059	0.00041	0.00041
π	0.00061	0.00061	0.00033	0.0	0.00100	0.00100	0.00100	0.00100
Tests of neutrality								
Tajima's D	−0.44832	−0.44832	−1.16221	na	−0.08238	−1.03789	−1.49796	−1.49796
Fu and Li's D*	na	na	na	na	−0.50381	−0.50381	−1.91470	−1.91470
Fu and Li's F*	na	na	na	na	−0.44901	−0.73427	−2.06018	−2.06018

* Is the way of representing that statistics according to the DnaSP program (P. Librado and J. Rozas, DnaSP v5: a software for comprehensive analysis of DNA polymorphism data, Bioinformatics 25, 2009, 1451–1452.)

The prevalence of K10 insertion in *pvcrt-o* gene was detected with 18.8% in PDY, 12.5% in MAQ and 6.3% in CTC *P. vivax* isolates, whereas no change in nucleotide was identified in *P. vivax* isolates collected from JDH region. This is the second report to detect CQ resistance in Indian *P. vivax* isolates, and is the first study to report K10 insertion in *P. vivax* isolates from PDY and CTC region. Thus, the overall frequency of K10 insertion in the studied isolates was 90.6%, which is in accordance with previous reports from Mangaluru, Southwestern region of India with 94.4% [5], West Arsi district of Ethiopia with 93.9% [14], Thailand with 56% and Myanmar with 46.2% [30]. Reports from China, and border regions of Thailand conducted drug resistance molecular markers studies on the *P. vivax* field isolates were detected with only wild-type *pvcrt-o* gene (without K10 insertion) [31,32]. The sequence analysis shows that the CQ resistance in *P. vivax* isolates, based on K10 insertion in *pvcrt-o* gene, was observed more in Southern region of India as compared to North India. This spread of CQ resistance in Indian *P. vivax* isolates could be due to the ongoing malaria transmission from Papua New Guinea and Southeast Asian countries; this observation was similar to the transmission of 'SVMNT' and 'CVIKT' haplotype in CQ-resistant *P. falciparum* isolates detected from South India [33]. Although K10 insertion in *pvcrt-o* gene is detected with less frequency, this needs to be monitored continuously under regular drug resistance epidemiological surveillance for decisions on the revisal of national drug policy of India.

Based on the F1076L mutation in *pvmdr-1* gene, resistant *P. vivax* isolates was highly predominant in both the regions, JDH and CTC, with 100%, followed by MAQ with 93.3% and PDY with 73.3%. The detection of CQ resistance in *P. vivax* isolates, based on *Pvmdr-1* gene, showed that the T958M mutation was observed with 100% frequency, followed by F1076L mutation with 91.7%. However, Y976F mutation was not detected in any of the *P. vivax* isolates screened for CQ drug resistance. This finding of high predominance of T958M mutation followed by F1076L is in agreement with previous reports from Southwestern region of India with 90% T958M followed by 76% F1076L and very low prevalence of Y976F mutation with 7% [5]. Also, previous reports from India observed CQ susceptibility from clinical studies due to *P. vivax* infection [34,35] and the Y976F, possible candidate mutation for CQ resistance [36], was not observed in these two clinical studies. The major haplotype detected in the present study was T958M and F1076L which is in concordance with previous studies from India [30,35]. This suggest that *P. vivax* isolates from four different study regions of India are susceptible to CQ drug; however, the low prevalence rate of both K10 insertion in *pvcrt-o* and Y976F mutation in *pvmdr-1* gene in *P. vivax* field isolates might act as an indicator exhibiting that CQ resistance in the studied geographical regions might surface in the near future.

5. Conclusion

The surveillance of *pvcrt-o* and *pvmdr-1* gene fragments associated with CQ resistance from different regions of India (PDY, MAQ, CTC and JDH) with different endemicity and distinct geographical regions benefit us in understanding the molecular evolutionary changes in Indian *P. vivax* isolates. Few limitations of this study were the screening of less sample size for drug resistance based on genotyping of *pvcrt-o* and *pvmdr-1* genes, and it does not determine the phenotypic nature of the parasite for drug resistance based on either *in vivo* or *in vitro* studies. This study showed less frequency of *pvcrt-o* and high frequency of *pvmdr-1* gene variants associated with CQ resistance, which act as an indicator and the onset of *P. vivax* drug resistance trend in four different regions of India. Due to the insufficient phenotypic studies available for *P. vivax* parasite, the present study data for CQ resistance based on *pvcrt-o* and *pvmdr-1* markers should assist by providing base-line data for future monitoring of drug resistance. Thus, large-scale epidemiological surveillance and therapeutic efficacy studies in India that will aid in proper administration and revisal of antimalarial drugs for *P. vivax* infection.

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Competing interests

The authors declare that they have no competing interests.

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