STANDARDIZATION OF MULTIPLEX PCR FOR THE DETECTION OF PVCRT-O, PVMDR1 RESISTANT GENES IN PLASMODIUM VIVAX AND PFCRT-O RESISTANT GENE IN PLASMOIDUM FALCIPARUM

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ABSTRACT:

Malaria is considered as a neglected tropical disease because of the therapeutic failures and lack of immunoprophylactic methods. Plasmodium species develop resistant genes by selective drug pressure, detection of such resistant parasitic strains by various molecular methods is significant in improving treatment modalities. Pvcrt-o, Pvmdr1 in Plasmodium vivax and Pfcrt-o in Plasmodium falciparum are the genes responsible for antimalarial resistance. In this study
method to standardize multiplex PCR for the detection of these three resistant genes was done. From a total of 64 blood samples collected from the suspected cases of fever, 28 were positive for *P. vivax*, out of them 20 were positive for *Pvmdr1* gene and 18 were positive for *Pvcrt-o* gene, from the 10 samples positive for *P.falciparum*, 5 were positive for *Pfcrto* gene detected by PCR. Molecular diagnostic methods for the detection of resistant strains of Plasmodium species in malaria may help in better understanding of resistant pattern in various geographical areas in endemic countries.

**Key words:** *Plasmodium falciparum, Plasmodium vivax, Pvmdr1, Pvcrt-o, Pfcrto* multiplex PCR


**INTRODUCTION:**

Antimalarial drug resistance is due to mutations occurring, among various Plasmodium species which can be due to point mutations, triple mutations, double mutations, and wild type mutations. The genes like *pfcrt-o, pfmdr1, pfdhps, pfdhfr*, among *Plasmodium falciparum* and, *Pvcrt-o, Pvmdr1, Pvdhps, Pvdhfrin Plasmodium vivax* are responsible for antimalarial resistance. Initially chloroquine resistant isolates of *Plasmodium vivax* were reported from Papua, Indonesia, and Papua New Guinea [1]. Chloroquine resistance (CQR) is a major determinant of antimalarial resistance worldwide. The major reason for the development of resistance, is due to relapses and recrudescence resulting from incomplete treatment and persistent hypnozoites[2]. The mechanisms involved are mainly by the efflux pump, it pumps out the drug from food vacuole, thus chloroquine does not affect the parasite. *Plasmodium falciparum* being one of the major species causing complications in some endemic countries and its decreased susceptibility to all the currently available anti-malarial drugs (amodiaquine, chloroquine, mefloquine, quinine, sulfadoxine- pyrimethamine) and more recently, resistance to artemisinin derivatives has been a major concern [3]. The present study is aimed in standardizing the molecular methods for

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detecting various resistant genes simultaneous in each species, this can be the basis for further improving of methods, that can detect both species and resistant genes together. These reforms in molecular detection methods can be more sensitive and specific in detecting the prevalence of Plasmodium species and their evolving resistant genes for screening in endemic areas.

MATERIALS AND METHODS:

Study Design:

This study was conducted at the Chettinad Academy of Research Education, Chennai, India from Jan 2019 to Nov 2019. Blood samples were collected from fever and chills categorized patients with symptoms of malaria. Informed written consent will be obtained at the time of sample collection. The Institutional Human Ethical Committee approval was obtained with the no: 361/IHEC/1-19 on Jan. 2019

Sample size and collection:

A total of 64 blood samples were collected from patients with fever and chills, suspected to have malaria symptoms, by following aseptic procedures for blood collection for the diagnosis by molecular technique.

Standardization of multiplex PCR for detection of antimalarial resistance genes:

The resistant genes like Pvcrt-o Pvmdr1 and pfcr-t-o were separately detected by uniplex PCR initially for noting the presence of genes. The primers specific for Plasmodium vivax and Plasmodium falciparum were added to the corresponding positive samples. The PCR cycle was standardized and performed until the resistant genes were detected. A total of 15μl reaction mixture was taken which consists of, 10.5 μl of Emerald master, 1 μl of parasitic DNA, 0.5 μl Nfw, 0.5 μl of pvcr-t-o, pvmdr1, pfcr-t-o forward and reverse primers, respectively. The sequences for Pvcrt-o F-CGCTGTCAAGAGCC and R- AGTTTCCCTCTACACCCG, Pvmdr1 F-GCGAACTCGAATAAGTACTCCCTA and R- R-GGCGTAGCTTCCCGTAAATAA, Pfcr-t-o, F-TGAGAATTAGATAATTTAGTACAAGAAGGAA and R-CGTGAGCCATCTGTAAAGGTC were allowed to be amplified for 35 cycles, at 94°C for 10
min hot start, at 94°C for 50 sec denaturation, at 61.5°C for 1 min annealing and at 72°C for 1 min elongation. The amplified PCR product was loaded on 1% agarose gel for electrophoresis, containing 0.05% Ethidium bromide, in 0.5mM x TAE running buffer with 100V, for 1 hour in gel electrophoresis apparatus. Ladders were procured from Takara bio Home, and DNA bands were visualized by UV transillumination with short length light and documented along with the internal standard controls.

Statistical Analysis
Data was analyzed for calculating the percentage of samples positive by microscopy and molecular methods using SPSS version 2.1.

RESULTS:

Among the 38 Plasmodium species identified, 28 were Plasmodium vivax and 10 samples were Plasmodium falciparum, respectively. In the 28 positive P.vivax strains, 20 were positive for Pvmdr1 gene and 18 were positive for Pvcrt-o gene, whereas from the 10 positive P.falciparum samples, 5 showed the presence of pfcrt-o gene, which were detected by the initial uniplex. After standardizing the multiplex PCR by mixing the primers, out of the 28 Plasmodium vivax positives, 17 showed the presence of both the genes Pvcrt-o and Pvmdr1, 3 showed the presence of Pvmdr1 gene only, and 1 sample showed the presence of Pvcrt-o alone. Among the 10 positives for Plasmodium falciparum 5 showed the presence of Pvmdr1 gene.
Fig/ Table 1: Shows the graphical representation of number of resistance genes in *P. vivax* and *P. falciparum*.
Fig/Table 2: Shows the funnel graph representing the total number of resistance genes in *P. vivax*.

Fig/Table 3: Shows gel documentation of the standardized multiplex PCR method with, 20 bands at 762 bp region for *Pvmdr 1* gene, 18 bands at 1186 bp region for *Pvcrt o* gene, and 5 bands at 280 bp region for *Pfcrto* gene.
DISCUSSION:

PCR is one of the molecular techniques gaining significance in understanding the epidemiological aspects of Plasmodium species and antimalarial resistance pattern. Prophylactic chloroquine therapy and a combination with primaquine as a followup therapy is recommended in endemic areas where *Plasmodium vivax* is known to cause relapses, this was the main reason for the development of chloroquine-resistance among *P. vivax* (4,5). Initially, resistance by *P. falciparum* to chloroquine was seen in neighboring countries like Pakistan which slowly started spreading to India (6). Sulfadoxine and pyrimethamine are alternative drugs to chloroquine used for treatment of malaria, but resistance to these drugs developed due to mutations. In some countries like Africa, *Plasmodium falciparum* is quite common, and treatment polices recommend Artemisinin Combination Therapy as first line, later molecular marker Kelch 13 protien (*K13* propeller gene) has been identified which caused resistance (7,8). According to Hiasindh Ashmi Antony in 2016, the prevalence of *pfcrt* K76T mutation was 40% and 73.33% in Puducherry and Odisha population, respectively. Similarly, the N86Y mutation in the *pfmdr1* gene was found to be 13.33% and 6.67% by PCR-RFLP in Puducherry and Odisha population, respectively (9,10). In the present study, attempt was made to develop multiplex PCR where the primers to the corresponding resistant genes were mixed with the sample DNA, so that all the genes belonging to the same species can be identified simultaneously. In our study from a total of 64 samples collected 38 were positive by microscopy and conventional PCR which were considered for the detection of resistant genes initially by uniplex PCR and later by standardized multiplex PCR. Among the 38 samples positive, 28 were *Plasmodium vivax* and 10 belong to *Plasmodium falciparum*. About 17 samples of *Plasmodium vivax* showed the presence of both *Pvmdr 1* and *Pvcrt o* genes, while three showed the presence of *Pvmdr 1* genes only and one sample *Pvcrt o* gene alone. Among the 10 samples positive for *Plasmodium falciparum*, 5 showed the presence of *Pfcrt o* gene. This study can be further widened by standardizing with...
emerging genes to various antimalarial agents, to understand the prevalence of resistance so that treatment modalities can be reformed.

Prevalence studies conducted in Guyana on antimalarial resistance among *Plasmodium falciparum* showed a constant presence of both Chloroquine sensitive and resistance in endemic populations(11). As per the molecular studies it is found that there is a significant association between *Pfdmr1* and *Pfcrt-o* in causing various levels of resistance among *Plasmodium falcoiparum* species (12). There are two haplotypes, SVMNT and CVIET which are known to undergo increased mutational variations which is responsible for high-level CQ resistance among Plasmodium species (13). The whole genomic sequencing of Plasmodium species in identifying the diversity of resistant gene proteins like *dhfr*, *dhps*, *mdr1* and *crt-o* play an important role in understanding the different haplotypes and point mutations. These observations help in understanding the disease endemicity and the diversity in various geographical areas (14).

Alternatively, the LAMP assay was developed which proved to have similar sensitivity to that of microscopy and a specificity better than that of microscopy, and it yielded results similar to those of nested PCR for the detection of four species of human malaria parasites (15). With the microscopy as a gold standard, the gene expression studies of *Pvcrt-o* showed a threefold increase in the isolates with the ring stage of parasites when compared to the trophozoite stage (16). The emergence of CQ resistance in *P.vivax* and ACT in *P.falciparum* is one of the annoying incidences in antimalarial therapy in endemic areas like India. To understand these factors, it is necessary to study mutational variations of different proteins responsible for antimalarial resistance and approaches for regular whole genomic studies and gene expression studies.

CQ is the routinely recommended antimalarial agent that is safe for administration in the pediatric age group, CQ resistance in endemic areas like Africa, showed an increased rate of treatment failures (17,18). CQ is the drug of choice in developing countries as it is more economical, but the emerging CQ resistance is an annoying factor, in African countries like Uganda, extensive molecular genotypic studies were done and it was found that *K76T* protein is responsible for the development of chloroquine resistance (19,20). The wild type *Pfdmr1* is associated with resistance to Mefloquine, Hydroxychloroquine and Artemisinin drugs, there must be different haplotypes where further studies are needed (21). The *P.vivax* strains, in Thailand are found to be effective to chloroquine therapy, when compared to the prevalence in surrounding CQ resistance countries like Indonesia and Papua. Modified schizont maturation assay studies

inferred the presence of \textit{Pvmdr1} polymorphism among \textit{P.vivax} which showed variation between CQ resistance and CQ sensitive samples at Y976F region of the gene (22). In India, \textit{P.vivax} isolates from Mangaluru are susceptible to CQ, but the occurrence of both Y976F mutants and K10 insertions, even though at a low rate may be an indication that chloroquine resistance in this geographical area, will surface in near future (23).

\textbf{CONCLUSION:}

In India, antimalarial treatment failures are varying in different regions, the distribution of antimalarial resistance is reported in some areas, studying the resistant pattern of various species by molecular methods and standardization of such methods for implementing in routine diagnosis is very important to decrease the mortality rate.

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\textbf{Conflict of Interest} - Nil.

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