NEWER MOLECULAR METHODS FOR THE SURVEILLANCE OF ANTIMALARIAL RESISTANCE BY USING NON-INFRINGEMENT SAMPLES

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ABSTRACT

In Southeast Asian countries, Chloroquine and Primaquine combination therapy is most successful in treating uncomplicated \textit{Plasmodium vivax} infections, for preventing relapses. Later, resistance to Chloroquine has emerged resulting in therapeutic failures, and this encouraged the research to identify causative factors \cite{1}. Several molecular methods have been developed to identify the genetic mechanisms involved in resistance to chloroquine and other
antimalarial drugs. Many attempts were made in the past few years, to overcome the disadvantages of conventional methods in identifying malaria and to prevent treatment failures. Among these methods, multiplex PCR is found to be the most sensitive and specific method providing fast results. Thus, multiplex PCR is trying to replace the conventional methods like microscopy, rapid antigen and QBC, as it can identify the species along with the resistance pattern of antimalarial drugs at the genetic level so that the treatment modalities are improved (2).

**Key words:** Chloroquine, *pfcr*t, *pfmdr*1, *pvcrt-o*, *pvmdr-1*, non-invasive samples, multiplex PCR.


**The burden of antimalarial resistance**

In developing countries, infection with *Plasmodium falciparum* is found to be associated with complications, when compared to the other species. Even though *P. falciparum* infections were treated initially with chloroquine, later resistance was observed and Artemisinin-based combination therapy (ACT) has been recommended as the first-line treatment. In India, malaria is mainly distributed in rural and mountain regions, and the early report of chloroquine resistance was noted in 1973, in the districts of Assam state. Chloroquine accumulates in the digestive vacuoles of the parasite and blocks the heme detoxification pathway. Resistance to Chloroquine is due to mutations of the proteins PfCRT at K76T, which is responsible for primary resistance and N86Y mutation in PfMDR1 protein that enhances the degree of resistance. The two genes *pfcr*t and *pfmdr*1, are used as genetic markers to understand the degree of resistance in various communities like low and high endemic areas, and for mapping the haplotypes. The standardization of advanced and simplified molecular methods are useful for both diagnostic as well as epidemiological purposes. Studies in India were carried out in Odisha and Puducherry, which proved the association of certain genes like *pfcr*t and *pfmdr*1 with Chloroquine resistance in *Plasmodium falciparum*. The mutations at K76T in the *pfcr*t gene showed a strong association with Chloroquine resistance than the *pfmdr* gene mutations. Periodic updating of the genetic markers, understanding their association with resistance pattern, the occurrence of genetic recombination which alters the phenotypic properties is very essential to update the national and international antimalarial treatment...
policies. In *Plasmodium vivax* infections the drug of choice is Chloroquine to kill the blood-stage parasite, and Primaquine given to prevent relapses due to hypnozoites, in some parts of Southeast Asian countries, gradual resistance to Chloroquine has emerged and WHO recommended ACT for treatment of such infections (3). In India, there is a necessity to understand the resistance mechanisms of anti-malarial drugs in endemic regions as a part of research to implement appropriate control measures. The judicious use of Chloroquine therapy is recommended to prevent selective drug pressure by the parasite in endemic countries. Several, approaches were made to understand the rapid gene mutations after chloroquine withdrawal, it was proved by molecular methods that polymorphism is exhibited by the *pfcrt* and *pfmdr1* genes, which are associated with the degree of resistance mechanisms (4).

**Detection of genetic markers and their influence on antimalarial treatment modalities**

Standardization of various molecular methods for identifying the genes like *pfcrt-o* (*Plasmodium falciparum* chloroquine resistance transporter-o), *pfmdr-1* (*Plasmodium falciparum* multidrug resistance-1) of *P. falciparum* and *pvcrt-o* (*Plasmodium vivax* chloroquine resistance transporter-o), *pvmdr-1* (*Plasmodium vivax* multidrug resistance-1) of *P. vivax* have an impact in reducing morbidity and mortality. They are advantageous in identifying the species involved in mixed infections with *Plasmodium vivax* and *Plasmodium falciparum* (5). Apart from the PCR, other molecular methods like RFLP and DNA sequencing clarify the origin, distribution, and circulation of genetic polymorphisms associated with resistant genes (6). In certain regions of the African continent, even though the new treatment policies recommend artemisinin combination therapy (ACT), still Chloroquine is widely used in uncomplicated malaria. In hyperendemic areas of malaria, monotherapies with antimalarial drugs were initially successful and chloroquine was given as a prophylactic drug to prevent *Plasmodium vivax* infections, factors like affordability, ease of availability and poor treatment guidelines followed during therapy, contribute to the emergence of haplotype variations of resistant genes. There is a necessity to strengthen the treatment policies nationally and globally, by improving the research methodologies for the identification of genetic variations in *Plasmodium* species to various antimalarial drugs (7). In a study carried out in Manguluru, one of the endemic west coast regions of India single nucleotide polymorphisms (SNPs) of the associated gene markers was notified, indicating Chloroquine resistance among *Plasmodium vivax* and *Plasmodium falciparum*. In India and neighbouring countries studies revealed the emergence of genotype variations among *Plasmodium vivax* and *Plasmodium falciparum* (8).
Development of innovative detection methods to identify existing and emerging antimalarial genetic markers

Malaria is considered a neglected tropical disease, owing to the hurdles like antimalarial resistance, and failure to develop an effective vaccine due to antigenic variations of the parasite. Comparative studies were carried out in Thailand from the DNA obtained from samples like saliva and urine apart from the blood, by using microscopy and molecular methods. These, non-invasive samples are handy for screening Plasmodium species and genetic markers resistant to antimalarial agents, along with species identity in mixed infections by the PCR method (9). The DNA yield may be less in saliva when compared with the blood samples, so methods with more sensitivity, targeting specific genes are to be developed. In a study done in Cameroon, a comparison was done by detecting the 18S rRNA gene by conventional PCR in peripheral blood and mitochondrial cox3 gene and varATS genes by primer-based PCR it was helpful in malaria surveillance. In a collective study done in India, to understand the prevalence of CQ resistance, a mutational analysis of K76T and N86Y in pfcrtn and pfmdr1 genes inferred the presence of 11 haplotypes among 30 Southeast Indian strains of Plasmodium falciparum. Sequence analysis of DNA in two different populations showed genetic diversity among the haplotypes, a strong correlation was found with CQ resistance and various parameters like alteration of molecular genotypes, in vitro CQ resistance, and treatment efficacy of CQ. Pfmdr1 polymorphisms were noted in the western part of the Indian subcontinent among Plasmodium falciparum strains when compared to those prevalent in Southeast Asian and South American countries. Similarly, a comparison was made with the non-invasive samples preserved in ice and ethanol, which outperformed with blood samples by nested PCR (1,10). The non-invasive samples for the detection of malaria are more advantageous as there is no pain of drawing blood and less danger of contaminated sharps disposal. When mass screening is required for epidemiological purpose non-invasive samples are more ideal, as the chance of needlestick injuries can be avoided, this might help in bringing down the prevalence of healthcare-associated blood-borne pathogenic infections like HIV, HBV, and HCV. Research studies using highly specific primers like cytochrome b gene (cytb), cox3, and varATS are more promising in identifying species as well as antimalarial resistance by PCR. These three primers eliminate the risk of nonspecific human DNA amplification which is the major disadvantage with traditional 18S rRNA gene-based PCR methods (10).

With the introduction of Artemisinin-based combination therapies (ACTs), the prevalence of resistance is associated with treatment failures in Plasmodium falciparum infections and there is a
need to evaluate the occurrence of haplotype variations in endemic regions (11). The consistent development of resistance to widely used antimalarial drugs has made various National Malaria control programs to make reforms in antimalarial treatment regimens. In the past few years resistance to Artemisinin compounds is gaining significance and there is a need to regularly survey the antimalarial drug resistance pattern. Many studies described innovative methods for sample collection, sample type, nucleic acid extraction, and amplification, for detecting the emergence of novel genetic markers. Filling the gap in analysis, the standardization of innovative malaria detection methods may help in comparing global scenarios of antimalarial resistance. In collaboration with Public Health sectors and academic expertise, FIND (Foundation for Innovative New Diagnostics) was developed internationally, which involved the participation of various Nations across the world. A program called Target product profile (TPP) was set up which decided the basic qualities of advanced methods to identify the genetic markers associated with antimalarial resistance. A detailed description was given by TPP on various innovative methods, by including parameters like personnel, reagents, and equipment required, duration of assays from extraction to result in analysis, cost per test, the reliability of positive and negative controls, and limitations in detection of copy number variations. Among the assays described by TPP, PCR and Micro assays are found to be rapid and economical, these testing facilities can be accommodated in Research Laboratories and National Reference Laboratories. The intended use of these assays is targeted to detect mainly the artemisinin resistance in *Plasmodium falciparum* infections. The target users can be a Research Laboratory, National Reference Laboratory, Academic Institution with Research Scholars, and Technical staff. The performance of these assays should meet the criteria like analytical sensitivity and specificity. The minimal parasitic detection limit must be less than 200 parasites per microliter of the blood sample, the sensitivities of which can be compared with Next-generation sequence (NGS) and RT-PCR. The test specificity should be targeted to a species of the parasite with 90-95% comparing with gold-standard Sanger Sequencing. The quality indicators like repeatability and reproducibility should be standardized to meet the challenges (12-18).

CONCLUSION

Epidemiological studies on a large scale will help in identifying the diversity of novel mutations occurring in specific genes, responsible for antimalarial resistance in Plasmodium species. Surveillance methods like comparing the microscopy, molecular methods, and
sequencing using both invasive and non-invasive samples in endemic regions may throw some light, to prevent indiscriminate use of antimalarial agents and in reforming treatment regimens in complicated and uncomplicated cases. A synchronized effort in research by comparing various geographical regions can help in designing specific resistance markers, for developing rapid molecular methods, and preferring simple non-invasive sample collection techniques with maximum yield avoiding biohazardous manoeuvres.

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