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Original Article

Microbiology Section

Simple Molecular Methods for Early Detection of Chloroquine Drug Resistance in *Plasmodium vivax* and *Plasmodium falciparum*

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ABSTRACT

Introduction: Malaria is a human disease of which causes high morbidity and mortality. In *Plasmodium falciparum* malaria, the resistance to antimalarial drugs, especially chloroquine (CQ) is one of the paramount factors contributing to the global increase in morbidity and mortality, due to malaria. Hence, there is a need for detection of chloroquine drug resistance genes i.e., pfcrt-o (*Plasmodium falciparum* chloroquine resistance transporter-o) and *pfmdr-1* (*Plasmodium falciparum* multidrug resistance-1) of *P. falciparum* and *pvcrt*-o (*Plasmodium vivax* chloroquine resistance transporter-o) and *pvmdr-1* (*Plasmodium vivax* multidrug resistance-1) of *P. vivax* by using molecular methods to prevent mortality in malarial cases.

Aim: To standardize chloroquine drug sensitivity testing by molecular method so as to provide reports of chloroquine within 6-8 hours to physicians for better treatment.

Materials and Methods: This study was conducted over a period of one year from January to December 2014. A Total of 300 blood samples were collected from malaria suspected patient attending MGM Hospital, Kamothe, Navi Mumbai, India. Out of 300 blood samples, 44 were malaria positive as assessed by Thick and Thin blood smear stained, by Leishman's method and examination with light microscope. Chloroquine drug sensitivity testing was performed using WHO III plate method (micro test). Nested PCR was done for detection of pfcrt-o and *pfmdr-1* for *P. falciparum* and *pvcrt*-o, *pvmdr-1* genes for *P. vivax*.

Results: Total 44 samples were included in this study, out of which 22 samples confirmed for *Plasmodium falciparum* and 22 samples confirmed for *Plasmodium vivax*. Out of 22 *P. falciparum* 15 (68.18%) samples were chloroquine resistant. *P. vivax* showed chloroquine resistance to 5 samples (22.73%) by method similar to WHO III plate method (micro test) and nested PCR.

Conclusion: Drug resistance testing by molecular methods is useful for early detection of antimalarial drug resistance. *pfmdr-1* along with pfcrt-o can be used as biomarker for chloroquine drug resistance in *P. falciparum* and *pvmdr-1* along with *pvcrt*-o for *P. vivax*.

Keywords: Drug resistant genes, Invitro antimalarial sensitivity, *Plasmodium species*, Nested PCR

INTRODUCTION

The disease Malaria causes high morbidity and mortality in developing countries. According to WHO approximatey 300-500 million malarial cases occur every year, i.e.,90% of the total cases occurring in Africa and Asia. A 700,000 to 2.7 million cases mortality occurr worldwide [1]. According to UNICEF a child dies at every minute from malaria in Africa [2]. A 1.2 billion cases are at risk of malaria, most of whom live in India. However, Southeast Asia contributed 2.5 million cases to the global burden of malaria. Of this, India alone contributed 76% of the total cases [1].

In India around 1.5 million cases of malaria occurr annually. confirmed by clinical examination, radiological examination and laboratory investigations. Chloroquine drug sensitivity can be tested using WHO III plate method [3]. *Plasmodium falciparum* is responsible for 50% of the total incidence occurring in the world. Chloroquine is the choice of drug for prophylaxis of malaria and maximum cases of malaria by *P. falciparum* showed resistance to chloroquine which is used as the first line treatment of malaria [4].

Now-a-days the treatment of malaria with chloroquine in *P. falciparum* cases may cause high morbidity and mortality in patients if treated empirically and without confirmation of the report of antimalarial drug sensitivity testing which is available by both procedures i.e. phenotypic by using WHO III plate (micro test) method and molecular (Polymerase chain reaction) method.

Some studies revealed that chloroquine acts by interfering with heme metabolism in the digestive vacuole of *P. falciparum* and the drug resistance occurred due to decreased concentration of the drug by efflux pump inhibitor of the parasite [5-7].

Many workers reported that the genetic alterations in *P. falciparum* are associated with chloroquine drug resistance i.e. *P. falciparum* multidrug resistance gene (*pfmdr-1*), and the chloroquine resistance transporter gene *pfcrt*. Several point mutations in *pfmdr-1* gene at positions 754, 1049, 3598, 3622 and 4234 result in amino acid changes at codons 86, 184, 1034, 1042 and 1246, respectively. These amino acid changes have been shown to be associated with chloroquine drug resistance [8-15]. A mutation that occurred in codon 86 (from asparagine to tyrosine, N86Y), involved in the substrate specificity of the gene product (P- glycoprotein), appears to be the most important as this may alter the transport activity of the protein [7]. However, some studies have has reported that the *pfmdr-1* gene mutations are also present in chloroquine drug resistance [16].

There are also some variations in point mutations of isolates from different places. The N86Y mutation is present in Southeast Asian chloroquine drug resistant (K1 genotype) isolates whereas it is absent in South American (7G8 genotype) isolates [7].

A study reported that the mutation present in codon 86 has also been evaluated to chloroquine drug resistance in malarial parasites by invitro drug sensitivity testing [16].

The mutations present in the *pfcrt*-o (codon 74, 75, 76, 220, 271, 326, 371) have also been correlated to chloroquine drug resistance by *invitro* drug sensitivity testing of *P. falciparum* in all over the world [17-19]. However, the K76T mutation in *pfcrt*-o gene has not been observed in chloroquine sensitive strains. It can be regarded as a good molecular bio marker for detection of chloroquine drug resistance in *P. falciparum* [13,20–22].

In India, especially in the Northeast, the role of mutations in genes *pfmdr-1* and *pfcrt*-o has not been studied in the emergence of *P. falciparum* chloroquine drug resistance. Studies from other parts of India, reported poor association of chloroquine drug resistance with these gene mutations [23].

In previous days, chloroquine was the recommended first line treatment for uncomplicated malaria in *P. falciparum* endemic areas. However, now a days this has been changed to artemisinin-based combination therapies. Many malaria-affected areas are still using chloroquine drug for treatment of non-complicated malaria [14,24].

There is a need of chloroquine drug sensitivity testing by molecular methods for better treatment of malaria. Malarial parasite population control, genetic studies and determination of the presence of chloroquine drug sensitivity is needed to control the burden of the disease [25].

MATERIALS AND METHODS

This prospective and analytical study was conducted at Department of Microbiology and Central Research Laboratory, MGM Medical College and Hospital over a period of one year from January to December 2014. Total 300 blood samples were collected from malaria suspected patients attending MGM Hospital with symptoms of fever and chills. Patients already on antimalarial treatment were excluded from the study. Out of 300 blood samples 44 were malaria positive. 22 *P. falciparum* and 22 *P. vivax*.

For drug sensitivity and molecular analysis, approximately 5 ml of venous blood was collected from the malaria suspected patients (1 ml for thick and thin smear, 2 ml for invitro antimalarial drug sensitivity testing by WHO III plate method and 2 ml for DNA extraction for detection of drug resistance genes by Nested PCR) who were tested positive for *Plasmodium falciparum* using thick and thin blood smear and stained with Leishman's stain. The blood was stored in cryo vials and stored in at –20°C. The study protocol was reviewed and approved by the Ethical Review Committee of MGM Institute of Health Sciences (Deemed University), Navi Mumbai. Informed written consent was obtained from the patients before start the study.

In vitro drug sensitivity testing: Antimalarial drug sensitivity testing was performed by invitro micro test (Mark III) according to Singh et al., [1] chloroquine drug sensitivity test was performed immediately after the collection of blood. The test was considered valid and interpretable if 10% of the parasites in the control well (drug free well) had developed into the schizonts after 24–36 hours incubation. Isolates were considered resistant if they showed schizont maturation at chloroquine concentrations 8 pmol/well (1.6 mmol/L blood). To evaluate the drug-parasite response, the EC50 value (50% inhibition) was calculated by HN Non Lin (V. 1.01 Beta) Software [26].

DNA extraction: The DNA extraction form above samples was performed by using DNA Mini Kit (Invitrogen) spin column method.

Primer design: Primers used in this study were designed from published articles and were procured from Eurofins Genomics India [Table/Fig-1].

Optimization of DNA preparation: DNA was extracted from 200 µl of blood in EDTA using the DNA extraction kit (Invitrogen, USA) spin column method and stored at 4°C until PCR could be completed. DNA used for the PCR was standardized through DNA Mini Kit (Invitrogen).

Polymerase Chain Reaction

Nested PCR amplifications were performed in accordance to the procedure as followed by Stephanie P. Johnston et al., within the cycling parameters [Table/Fig-2] by using a PeqSTAR

Sr.	Name of organism	Gene	Sequences 5' – 3'	Gene code	Ref.
1	*P. vivax*	pvmdr1	F-GCGAACTCGAA TAAGTACTCCCTCTA	EU333979.1	[27]
			R-GGCGTAGCTTCC CGTAAATAAA	EU333979.1	
		pvcrt-o	F-CGCTGTCGAA GAGCC	EU333972.1	[28]
			R- AGTTTCCCTCTA CACCCG	EU333972.1	
2	*P. falciparum*	*pfmdr*1	F- TGTATGTGCTGTA TTATCAGGAGGAAC-3'	JN578609.1	[29]
			R-AATTGTACTAAACCTA TAGATACTAATGATAAT ATTATAGG	JN578609.1	
		pfcrt-o	F - TGAGAATTAGATAATTTA GTACAAGAAGGAA	JF520758.1	[17]
			R- CGTGAGCCATCTG TTAAGGTC	AF030694.2	

[Table/Fig-1]: Primers for nested PCR for detection of drug resistant gene in malaria parasites.

Reactiona	Cycling conditions
pvmdr-1	Initial denaturation at 94°C for 10 minutes followed by 35 cycles of denaturation at 94°C for 50 seconds, annealing at 62°C for 1 min, and extension at 72°C for 1 min 30 seconds [30].
pvcrt-o	Initial denaturation at 94°C for 10 minutes followed by 35 cycles of denaturation at 94°C for 50 seconds, annealing at 61°C for 1 min, and extension at 72°C for 1 min 30 seconds [30].
pfmdr-1	Initial denaturation at 50°C for 2 minutes, 95°C for 10 minutes followed by 40 cycles of denaturation at 95°C for 15 seconds, annealing at 60°C for 1 minutes and extension at 72°C for 1 minute [31].
pfcrt-o	Initial denaturation at 94°C for 10 minutes followed by 40 cycles of denaturation at 94°C for 1 minute, annealing at 56°C for 1 min, and extension at 72°C for 1 min 30 seconds [32].

[Table/Fig-2]: Cycling conditions of PCR reactions for detection of drug resistance gene of *P. falciparum* and *P. vivax*.

96Xx Universal Gradient PCR thermal cycler (Peqlab, Germany). According to the procedure master mix "BioMix Red" (Bioline, India), 5µl DNA, 10 pmol of primers were added and mixed to obtain 50µl final volume of the PCR mix. The PCR products along with the appropriate ladder (Bioline, India) and known positive and negative samples from previous malaria diagnosed or uninfected individuals used as controls were subjected to electrophoresis in a 1.5% agarose gel using 1X Tris Acetate EDTA (TAE) buffer. The gel was then placed on the surface of the UV transilluminator (BioEra, India) and visualized in dark. The DNA bands were documented by gel documentation system (BioEra, India).

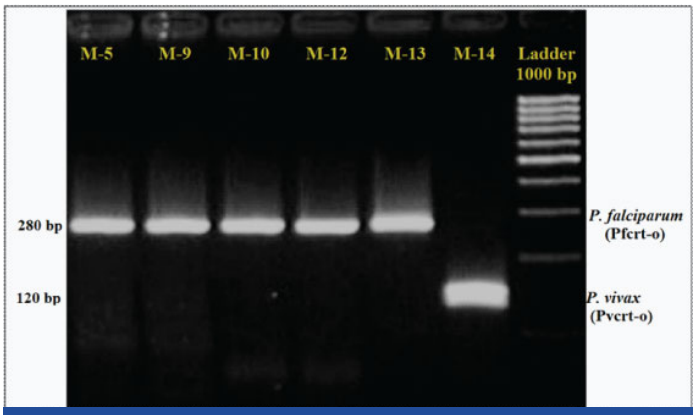
RESULTS

Total 44 malaria positive samples were included in the study, out of which 22 were *P. falciparum* and 22 were *P. vivax*.

A 15 out of 22 *Plasmodium falciparum* multiplied and grew rapidly in RPMI-1640 medium supplemented with O positive red blood cells under chloroquine pre-coated microtitre plate which were regarded as resistant to chloroquine and 7 out of 22 where sensitive to chloroquine drug. Electrophoresis gel picture shows band of *pfcrt*-o 280 bp and *pvcrt*-o 120bp [Table/Fig-3].

A 5 out of 22 *Plasmodium vivax* multiplied and grew in McCoy 5A medium supplemented with reticulocytes under chloroquine pre-coated microtitre plate which were regarded as resistant to chloroquine and 17 out of 22 where sensitive to chloroquine drug [Table/Fig-4,5].

PCR amplification of extracted DNA of *P. falciparum* and *P. vivax* revealed that chloroquine drug resistant genes (*pfcrt*-o and *pfmdr-1*) were detected in 15 out of 22 P. falciparum. However, 5 out of 22 chloroquine drug resistant genes (*pvcrt*-o and *pvmdr-1*) were detected in *P. vivax*.



[Table/Fig-3]: Gel picture showing band of *pfcrt*-o (*Plasmodium falciparum* chloroquine resistant transporter-o) 280 bp and *pvcrt*-o (*Plasmodium vivax* chloroquine resistant transporter-o) 120 bp.

No. of samples tested	*pfmdr-1*	*pfcrt*-o
Control strain CQS* 3D7 (n=1)	0	0
Patient samples (n=22)	15	15

[Table/Fig-4]: Detection of drug resistance genes of *P. falciparum* by PCR.

No. of samples tested	*pfmdr-1*	*pfcrt*-o
Positive Control strain CQS* (n=1)	0	0
Patient samples (n=22)	05	05

[Table/Fig-5]: Detection of drug resistance genes of *P. vivax* by PCR.
CQS* chloroquine sensitive.

DISCUSSION

Increasing drug-resistance in malarial parasites especially in *Plasmodium falciparum* to chloroquine has created major health problem in the world [18]. Now-a-days in malaria endemic areas chloroquine is taken as prophylactic drug only but still it showed good response in case of vivax malaria. However, the drug sensitivity by moleculars method could help to provide better treatment to patients [1].

The fast rate of emergence of chloroquine drug resistance has become a major burden during malaria control. chloroquine resistance in *P. vivax* was noted for the first time in Papua New Guinea [33] and from there it has spread to other parts of the world. From India also there are now several reports of chloroquine resistance in *P. vivax* [34-36]. Resistance in *P. vivax* is more serious as hypnozoites will cause relapse of resistant parasites and *P. vivax* is a mixture of various strains with respect to incubation period, relapsing pattern and response to primaquine [37] since sulpha drugs are not effective in its treatment.

The development of molecular methods for detection of drug resistant genes in malarial parasites has very important role for screening of the drug resistance, and providing better treatment to patients.

We describe a Nested PCR assay to detect drug resistance genes of *P. falciparum* (*pfcrt*-o and *pfmdr-1*) and *P. vivax* (*pvcrt*-o and *pvmdr-1*). We could successfully find chloroquine drug resistant genes in 15 of the 22 *P. falciparum* and 7 of the 22 *P. vivax* in Navi Mumbai, India. This area has big mountains and becomes malaria endemic during rainy season because it favours mosquito breeding.

In this study, all 44 samples were subjected to PCR for amplification of *pfmdr-1* and pfcrt-o for *P. falciparum* and *pvmdr-1* and *pvcrt*-o for *P. vivax*. *pfmdr-1* and *pvmdr-1* are multidrug resistance genes for *P. falciparum* and *P. vivax*. *pfcrt*-o and *pvcrt*-o are chloroquine drug resistance genes for *P. falciparum* and *P. vivax*.

pfmdr-1 and pfcrt-o genes were not detected in 3D7 control strain which is *P. falciparum* chloroquine sensitive strain. However, 15/22 (68.18%) patient samples showed presence of both *pfcrt*-o and *pfmdr-1* genes confirming chloroquine drug resistance by molecular methods. In vito analysis of PCR and chloroquine susceptibility of *pfmdr-1* and pfcrt-o polymorphisms in *P.falciparum* have revealed that chloroquine resistance has been linked to the mutations in pfcrt and *pfmdr*1 genes [38].

pvmdr-1 and *pvcrt*-o mutant genes were not detected in chloroquine sensitive strain of *P. vivax* by *in vitro* drug sensitivity testing. However, 5/22 (22.73%) patient samples showed presence of both *pvcrt*-o and *pvmdr-1* genes confirming chloroquine drug resistance. This validates the findings of invitro drug sensitivity testing. Findings from a previous study suggests that increased expression levels of the *pvcrt* o and *pvmdr-1* genes are strongly associated with clinical severity and chloroquinone resistance in *P.vivax* infections [39]. *pvmdr*1 Y976F mutation has been identified as a possible genetic marker for chloroquine resistance in *P.vivax*. in a study that has investigated the association between the polymorphisms of pvmdr1 and *pvcrt* o as markers of chloroquine resistance. pvmdr 1 Y976F mutation was detected only in 7/30(23.3%) *P.vivax* isolates. Chloroquine resistance phenotype in *P.falciparum* are strongly associated with the point mutations in pfcrt genes especially K76T [40].

In our study, 68.18% samples showed presence of pfcrt-o drug resistance genes. This finding is closer to Shrivastava SK et al., from India (86.95%), Sutar SKD et al., from Odisha, India (80%) and, Anvikar AR et al., from India (90.47%) [32,41,42]. Lim P et al., from Cambodia reported in all isolates of chloroquine resistance [43]. Babiker HA et al., from Sudan reported association of chloroquine resistance transporter gene (pfcrt-o) with in vivo and in vitro resistance [44]. Jalousian F et al., from Iran however reported less value of 23.1% [45]. Chloroquine in combination with primaquine or alone is still effective against *P.vivax* malaria according to a study from Kolkata in which the in vivo efficacy of chloroquine (CQ) and chloroquine plus Primaquine was determined [46]. For comparison of genetic determinants of chloroquine resistance in both *P.falciparum* and *P.vivax*, identification of difference in the orthologous genes is important. The development of resistance may be different in both *P.falciparum* and *P.vivax* however the mechanism of chloroquine resistance is probably similar in both of these two species [40].

In our study, we found 22.73% resistant genes of *pvcrt*-o and *pvmdr-1*. However, Lu F et al., from Central China, they did not find *pvcrt*-o and *pvmdr-1* resistant genes [30]. A direct relationship between unusual mutation in *pfmdr-1* and pfcrt genes and prevalence of chloroquine resistance has been determined with early treatment failure cases [47] chloroquine resistance monitoring through molecular markers is thus a useful tool that can be used for future control [40]. Such molecular markers that are associated with the increase in chloroquine resistance and disease severity in *P.vivax* are needed [39].

LIMITATION

In this study we could not study resistance in other drugs like Artemisinin, Mefloquine, Quinine, Sulphadoxine/Pyrimethamine and Primaquine.

Thus, further studies are required to enable the detection of resistance against these drugs, which can help in the treatment of multi drug resistance cases of malaria. Nevertheless, given the advantages of this method, nested PCR could serve as a useful tool for detection chloroquine drug resistant in malaria in endemic area.

CONCLUSION

Drug sensitivity testing by molecular methods is useful for early detection of drug resistance of chloroquine and will help physician to provide better treatment which decreases morbidity and mortality of patients. *pfmdr-1* along with pfcrt-o can be used as

biomarker for chloroquine drug resistance in *P. falciparum* and *pvmdr-1* along with *pvcrt*-o for *P. vivax*.

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REFERENCES

[1] Kumar A, Valecha N, Jain T, Dash AP. Burden of Malaria in India: Retrospective and Prospective View. *Am J Trop Med Hyg*. 2007;77(6):69–78.
[2] http://www.unicef.org/prescriber/eng_p18.pdf
[3] Singh G, Urhekar AD, Raksha. Invitro Antimalarial Drug Sensitivity Testing For *Plasmodium falciparum* and *Plasmodium vivax*. *IOSR Journal of Dental and Medical Sciences*. 2015;14(4):49-55.
[4] National Drug Policy on Malaria (2013) Directorate General of National Vector Borne Disease Control Programme, Ministry of Health & Family Welfare, Government of India. New Delhi: 1-15.
[5] Fitch CD. *Plasmodium falciparum* in owl monkeys: drug resistance and chloroquine binding capacity. *Science*. 1970; 169(942):289-90.
[6] Douki JBL, Boutamba SDD, Zatra R, Edou SEZ, Ekomy H, et al. Increased prevalence of the *Plasmodium falciparum pfmdr*1 86N genotype among field isolates from France ville, Gabon after replacement of chloroquine by artemether–lumefantrine and artesunate–mefloquine. *Infect Gen Evo*. 2011;11:512-17.
[7] Sanchez CP, Dave A, Stein WD, Lanzer M. Transporters as mediators of drug resistance in *Plasmodium falciparum*. *Int J Parasitol*. 2010;40:1109-18.
[8] Foote SJ, Kyle DE, Martin RK, Oduola AM, Forsyth K, et al. Several alleles of the multidrug-resistance gene are closely linked to chloroquine resistance in *Plasmodium falciparum*. *Nature*. 1990;345(6272):255–58.
[9] Basco LK, Le Bras J, Rhoades Z, Wilson CM. Analysis of *pfmdr*1 and drug susceptibility in fresh isolates of *Plasmodium falciparum* from sub-Saharan Africa. *Mol Biochem Parasitol*. 1995;74(2):157–66.
[10] Cox-Singh J, Singh B, Alias A, Abdullah MS. Assessment of the association between three *pfmdr*1 point mutations and chloroquine resistance invitro of Malaysian *Plasmodium falciparum* isolates. *Trans R Soc Trop Med Hyg*. 1995;89(4):436–37.
[11] Adagu IS, Dias F, Pinheiro L, Rombo L, do Rosario V, Warhurst DC. Guinea Bissau: association of chloroquine resistance of *Plasmodium falciparum* with the *Tyr*86 allele of the multiple drug-resistance gene *pfmdr*1. *Trans R Soc Trop Med Hyg*. 1996;90(1):90–91.
[12] Duraisingh MT, Drakeley CJ, Muller O, Bailey R, Snounou G, et al. Evidence for selection for the tyrosine-86 allele of the *pfmdr* 1 gene of *Plasmodium falciparum* by chloroquine and amodiaquine. *Parasitol*. 1997;114:205-11.
[13] Chaijaroenkul W, Ward SA, Mungthin M. Sequence and gene expression of chloroquine resistance transporter (pfcrt) in the association of invitro drugs resistance of *Plasmodium falciparum*. *Malaria J.* 2011;10:42.
[14] Murambiwa P, Masola B, Govender T. Anti-malarial drug formulations and novel delivery systems: A review. *Acta Tropica1*. 2011;18:71–79.
[15] Atroosh WM, Mekhlafi HM, Mahdy MAK, Surin J. The detection of pfcrt and *pfmdr*1point mutations as molecular markers of chloroquine drug resistance, Pahang, Malaysia. *Malaria J*. 2012;11:251.
[16] Pickard AL, Wongsrichanalai C, Purfield A, Kamwendo D, Emery K, et al. Resistance to antimalarials in Southeast Asia and genetic polymorphisms in *pfmdr*1. *Antimicrob Agents Chemother.* 2003;47:2418–23.
[17] Fidock DA, Nomura T, Talley AK. Mutations in the *P. falciparum* digestive vacuole transmembrane protein *pfcrt* and evidence for their role in chloroquine resistance. *Mol Cell*. 2000;6(4):861–71.
[18] Sidhu AB, Verdier-Pinard D, Fidock DA. chloroquine resistance in *Plasmodium falciparum* malaria parasites conferred by pfcrt mutations. *Science*. 2002;298(5591):210-13.
[19] Vathsala PG, Pramanik A, Dhanasekaran S. Widespread occurrence of the *Plasmodium falciparum* chloroquine resistance transporter (*pfcrt*) gene haplotype SVMNT in *P. falciparum* malaria in India. *Am J Trop Med Hyg*. 2004;70(3):256-59.
[20] Ojurongbe O, Ogungbamigbe TO, Beyioku AFF. Rapid detection of *pfcrt* and *pfmdr1* mutations in *Plasmodium falciparum* isolates by FRET and in vivo response to chloroquine among children from Osogbo, Nigeria. *Malaria J*. 2007;6:41.
[21] Mekhlafi AMA, Mahdy MAK, Mekhlafi HM. High frequency of *Plasmodium falciparum* chloroquine resistance marker (pfcrt T76 mutation) in Yemen: An urgent need to re-examine malaria drug policy. *Parasit Vect*. 2011;4:94.
[22] Veiga MI, Ferreira PE, Jornhagen L. Novel polymorphisms in *Plasmodium falciparum* ABC transporter genes are associated with major ACT antimalarial drug resistance. *PLoS ONE*. 2011;6(5):e20212.
[23] Bhattacharya PR, Pillai CR. Strong association, but incomplete correlation, between chloroquine resistance and allelic variation in the *pfmdr-1* gene of *Plasmodium falciparum* isolates from India. *Ann Trop Med Parasitol. 1999;93(7):679–84.*
[24] *Ranjitkar S, Schousboe ML, Thomsen TT. Prevalence of molecular markers of* anti-malarial drug resistance in *Plasmodium vivax* and *Plasmodium falciparum* in two districts of Nepal. *Malaria J*. 2011;10:75.
[25] Alam MT, Souza DK, Vinayak S. Selective sweeps and genetic lineages of *Plasmodium falciparum* drug -resistant alleles in Ghana. *J Infect Dis*. 2011;203:220-27.
[26] http://www.meduniwien.ac.at/user/harald.noedl/malaria/download.html.
[27] Lekweiry LM, Boukhary AOMS, Gaillard T, Wurtz N, Bogreau H, Hafid JE, et al. Molecular surveillance of drug-resistant *Plasmodium vivax*using *pvdhfr, pvdhps* and *pvmdr1* markers in Nouakchott, Mauritania. *J Antimicrob Chemother*. 2011;1-8. doi:10.1093/jac/dkr464.
[28] Suwanarusk R, Russell B, Chavchich M, Chalfein F, Kenangalem E, et al. chloroquine Resistant *Plasmodium vivax*: Invitro Characterisation and Association with Molecular Polymorphisms. *PLoS ONE*. 2007;2(10):e1089. doi:10.1371/journal.pone.0001089.
[29] Purfield A, Nelson A, Laoboonchai A, Congpuong K, McDaniel P, Miller RS, et al. New method for detection of *pfmdr*1 mutations in *Plasmodium falciparum* DNA using real-time PCR. *Malaria J*. 2004;3(9).
[30] Lu F, Wang B, Cao J, Sattabongkot J, Zhou H, Zhu G, et al. Prevalence of drug resistance-associated gene mutations in *Plasmodium vivax* in Central China. *Korean J Parasitol*. 2012;50(4):379-84.
[31] Pickard AL, Wongsrichanalai C, Purfield A, Kamwendo D, Emery K, Zalewski C, et al. Resistance to antimalarials in Southeast Asia and Genetic polymorphisms in *pfmdr* 1. *Antimicrob agents Chemother*. 2003;47(8):2418-23.
[32] Shrivastava SK, Gupta RK, Mahanta J, Dubey ML. Correlation of Molecular Markers, *pfmdr*1-N86Y and *pfcrt*-K76T, with Invitro chloroquine Resistant *Plasmodium falciparum*, Isolated in the Malaria Endemic States of Assam and Arunachal Pradesh, *Northeast India*. PLoS ONE. 2014;9(8):e103848. doi:10.1371/journal.pone.0103848.
[33] Rieckman KH, Davis DR, Hutton DC. *Plasmodium vivax* resistance chloroquine? *Lancet* 1989;2:1183–84.
[34] Potkar CN, Kashersagar NA, Kathwria R. Resurgence of malaria and drug resistance in *P. falciparum* and *P. vivax* species in Bombay. *J Assoc Phys India*. 1994;43:336–38.
[35] Garg N, Gopinath P, Bodhe P, Kshersagar A. Vivax malaria resistant to chloroquine: case reports from Bombay. *Trans R Soc Trop Med Hyg.* 1995;89:656–57.
[36] Dua VK, Kar PK, Sharma VP. Chloroquine resistant *P. vivax* in India. *Trop Med Intl Hlth*. 1996;1:816–19.
[37] Adak T, Sharma VP, Orlov VS. Studies on *P. vivax* relapse pattern in Delhi. *Am J Trop Med Hyg*. 1978;59:175–79.
[38] Thomas SM, Ndir O, et al. Invitro chloroquine susceptibility and PCR analysis of pfcrt and *pfmdr*1 polymorphisms in *Plasmodium falciparum* isolates from Senegal. *Am J Trop Med Hyq*. 2002;66(5):474-80. (http://www.ncbi.nlm.nih.gov/pubmed/12201579)
[39] Melo GC, Monteiro WM, et al. Expression levels of *pvcrt*-o and *pvmdr-1* are associated with chloroquinone resistance and severe *P.vivax* malaria in patients of the Brazilian Amazon – (http://journals.plos.org/plosone/article?id=10.1371/journal.pone.0105922)
[40] Rungsihirunrat K, Muhamad P, et al. *Plasmodium vivax* drug resistance ; pvmdr1 and *pvcrt*-o polymorphisms in relation to chloroquine sensitivity from a malaria endemic area of Thailand. *Korean J Parasitol*. 2015;53(1):43-9. (http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4384798/)
[41] Sutar SKD, Gupta B, Ranjit M, Kar SK, Das A. Sequence analysis of coding DNA fragments of pfcrt and *pfmdr-1* genes in *Plasmodium falciparum* isolates from Odisha, India. *Mem Inst Oswaldo Cruz, Rio de Janeiro*. 2011;106(1):78-84.
[42] Anvikar AR, Sharma B, Sharma SK, Ghosh SK, Bhatt RM, et al. Invitro assessment of drug resistance in *Plasmodium falciparum* in five States of India. *Indian J Med Res*. 2012;135:494-99.
[43] Lim P, Chy S, Ariey F, Incardona S, Chim P, Sem R, et al. pfcrt Polymorphism and chloroquine Resistance in *Plasmodium falciparum* Strains Isolated in Cambodia. Antimicrob. *Agents Chemother*. 2003;47(1):87-94.
[44] Babiker HA, Pringle SJ, Abdel-Muhsin Mackinnon AM, Hunt P, Walliker D. High-Level chloroquine Resistance in Sudanese Isolates of *Plasmodium falciparum* Is Associated with Mutations in the chloroquine Resistance Transporter Gene pfcrt and the Multidrug Resistance Gene *pfmdr*1. *The Journal of Infectious Diseases*. 2001;183:1535–38.
[45] Jalousian F, Dalimi A, Samiee SM, Ghaffarifar F, Soleymanloo F, Naghizadeh R. Mutation in *pfmdr*1 gene in chloroquine-resistant *Plasmodium falciparum* isolates, Southeast Iran. *International Journal of Infectious Diseases*. 2008;12:630-34.

[46] Guanguly S, Saha P, et al. In vivo therapeutic efficacy of chloroquine alone or in combination with primaquine against viavx malaria in *pvmdr1* and *pvcrt* o genes.(http://www.ncbi.nlm.nih.gov/pubmed/23262997).

[47] Das S, et al. Association between prevalence of chloroquine resistance and unusual mutations in *pfmdr-1* and pfcrt genes in India. Am J Trop Med Hyg. 2013;88(5):828-34. (https://www.researchgate.net/publication/236061526_Association_between_Prevalence_of_chloroquine_Resistance_and_Unusual_Mutation_in_*pfmdr*-I_and_pfcrt_Genes_in_India).

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