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Original Research Article

Detection of Pfcrt Point Mutation as a Molecular Marker of Chloroquine Resistance in *Plasmodium Falciparum*, North India

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ABSTRACT

Introduction: The development of chloroquine as an antimalarial drug and the subsequent evolution of drug-resistant Plasmodium strains has major impacts on global public health. In *P. falciparum*, chloroquine resistance is linked to multiple mutations in *pfcrt (Plasmodium falciparum* chloroquine resistance transporter). In particular, specific K76T point mutation is considered to be highly related to chloroquine resistance.
Objective: To detect *pfcrt* gene mutation in *Plasmodium falciparum* by PCR followed by restriction fragment length polymorphism.
Methods: The study was conducted in the Department of Microbiology, J. N. Medical College Hospital, Aligarh over a period of one and half years from February, 2014 to September, 2015. Blood sample was collected from 65 falciparum malaria infected patients. DNA was isolated, and mutation was detected by mutation specific PCR to give a 264-basepair product corresponding to amino acid residues 32 to 119 of the *pfcrt* gene product. Digestion of this PCR product with restriction enzyme Apo I results in two fragments of 128 and 136 basepairs if the CQ-sensitive haplotype (CVMNK) is present.
Results: Out of 65 samples, *pfcrt* K76T mutation was observed in only 23 (35.4%) of clinical isolates investigated. On RFLP analysis, *Apo I* enzyme did not digest any of the 23 PCR products.
Conclusion: PCR and RFLP techniques provide a simple and rapid method of detecting genes that may predict chloroquine resistance. Although the identification of the mutation in the pfcrt provides a significant indicator of CQR, further studies are needed to determine the polymorphisms in the genes.
Key words: Chloroquine resistance, *Plasmodium falciparum*, *pfcrt* mutation

INTRODUCTION

Malaria remains a leading cause of mortality and morbidity worldwide. In India, around 2 million malaria cases and 1000 deaths attributable to malaria are reported annually. [1] Major proportion of malaria cases has been contributed by *Plasmodium falciparum* alone. Chloroquine (CQ) has been the drug of choice in the successful treatment of uncomplicated malaria for a long time. [2] But its heavy and improper use over the years has led to the development of resistance in *P. falciparum.* [3] Emergence of chloroquine resistance is also considered to be responsible for the sudden rise in *P. falciparum* cases which poses a serious challenge to the world's health. [4]

CQ resistance (CQR) was reported for the first time at the Thailand-Cambodia border in 1957 and the Venezuela-Colombia border in 1959 and eventually spread to other countries throughout the world. [5,6] In India, chloroquine resistance in *P. falciparum* was first reported in Assam in 1973 and subsequently reported from several parts of the country. [7-9]

Several studies have shown that chloroquine resistance is related to multiple mutations in *pfcrt gene* that functions as a

transporter in the parasite's digestive in particular K76T point mutation. [10] Such mutations allow the parasite to survive despite the drug pressure. [11] *Pfcrt* K76T mutation has not been observed in CQ responders, and therefore, it has been accepted as a good molecular marker for CQ resistance in *P. falciparum.* [12-15] since only few studies have been conducted in this part of the country, this study was planned to assess the chloroquine resistance distribution in *Plasmodium falciparum* infected patients in an endemic region in Aligarh district of Uttar Pradesh, North India**.** This study could be helpful to revise suitable strategies in the treatment of malaria.

MATERIALS AND METHODS

The present study was conducted in the Department of Microbiology, J. N. Medical College Hospital, Aligarh over a period of one and a half years from February, 2014 to September, 2015. The study groups comprised of 65 patients tested positive for *P. falciparum* malaria either by MP smear, QBC or RDT. All patients were subjected to detailed history regarding fever, associated symptoms, history of anti-malarial drugs, past history of any fever. The study was approved by ethical committee of the Faculty of Medicine, AMU, Aligarh. Informed consent was obtained from the patients or from parents or guardians of patients less than 18 years of age. 5 ml of blood was withdrawn observing all sterile precautions. After 30 minutes, the tubes were centrifuged at 2000 rpm for 5 minutes for separation of plasma. The plasma was aliquoted in labelled vials and stored at -40°C.

DNA Extraction:

a) Isolation of *P. falciparum* total DNA was done using QIAamp DNA Investigator Kit (QIAGEN) as per manufacturer's instructions. DNA extracts were electrophoresed in 0.8% agarose gel containing EtBr (Ethidium Bromide) and visualised with Bio-rad Gel documentation system. DNA extracts were stored at -20°C in deep refrigerator until further use.

b) Principle:

The QIAamp DNA Investigator procedure consists of 4 steps:

Lyse: sample is lysed under denaturing conditions with proteinase K

Bind: DNA binds to the membrane and contaminants flow through

Wash: residual contaminants are washed away

Elute: pure, concentrated DNA is eluted from the membrane

PCR Amplification:

DNA from 65 whole blood samples from *P. falciparum* malaria was amplified by PCR using the primer pair as described by Vathsala et al in 2004 with some minor modifications [16]

Forward: 5'-GGCTCACGTTTAGGTGGA-3'

Reverse: 5'-TGAATTTCCCTTTTTATTTCCAAA-3'

to give a 264-basepair product (K76T mutation) corresponding to amino acid residues 32 to 119 of the *pfcrt* gene product. PCR was carried out in MJ Mini Biorad Thermal Cycler with cycling conditions consisting of initial denaturation step at 94°C for 5 min, followed by 40 cycles of final denaturation step at 94°C for 30 sec, annealing step at 52°C for 40 sec, initial extension step at 72°C for 45 sec and final extension step at 42°C for 5 min. The PCR products were electrophoresed in 2% agarose gel containing EtBr (Ethidium bromide) and visualised with Bio-rad Gel documentation system.

RFLP for *Pfcrt* Mutation:

Restriction fragment length polymorphisms (RFLP) analysis was done on the PCR products of *pfcrt* gene (that contains K76T polymorphism) with the restriction enzyme *ApoI* (Fermentas) as described by Vathsala et al, 2004. [16] Digestions were conducted at 37ºC for 1 hour in 10µL volume, using 2µL of

restriction endonuclease, 2µL of buffer solution, 4µL of sterile distilled water, and 4µL of the PCR products in each Eppendorf tube. The restriction fragments had the sizes fractionated in agarose gel at 2% at 100 volts for one hour. Later, the agarose gel was stained with ethidium bromide for 30 minutes and the bands were viewed under ultraviolet light and photographed in a photo documentation system.

RESULTS

Study group is divided into 2 groups

Group A (25 patients) included adult patients (age group >15 years)

Group B (40 patients) included paediatric patients (age group 0-14 years).

Out of 25 patients in group A, maximum number of patients 15 (60%) were in age group 15-25 years followed by 3 (12%) in 26-35 years, 36-45 years & 56-65 years each age group and 1 (4%) in age group 46-55 years. Out of 25 patients in study group A, 16 (64%) were males and 9 (36%) were females (Table 1).

Table 1: Age and sex wise distribution of falciparum malaria in adult patients (Study Group A)

Age (years)	Sex		
	Male	Female	Total
15-25	10 (40)	5 (20)	15 (60)
26-35	1 (4)	2 (8)	3 (12)
36-45	3 (12)	0	3 (12)
46-55	1 (4)	0	1 (4)
56-65	1 (4)	2 (8)	3 (12)
>66	0	0	0
Total	**16 (64)**	**9 (36)**	**25 (100)**

In the study group B, majority of the patients 14 (35%) belonged to the age group >10 years. Out of 40 patients in the study group B, 27 (67.5%) were males and 13 (32.5 %) were females (Table 2).

Table 2: Age and sex wise distribution of falciparum malaria in paediatric patients (Study Group B)

Age (years)	Sex		
	Male	Female	Total
0-2	7 (17.5)	1 (2.5)	8 (20)
2-4	5 (12.5)	2 (5)	7 (17.5)
4-6	1 (2.5)	3 (7.5)	4 (10)
6-8	3 (7.5)	1 (2.5)	4 (10)
8-10	2 (5)	1 (2.5)	3 (7.5)
>10	9 (22.5)	5 (12.5)	14 (35)
Total	**27 (67.5)**	**13 (32.5)**	**40 (100)**

Figure 1 shows clinical features in both the study group. In study group A, all the patients 25 (100%) presented with fever and 20 (80%) patients with headache. Vomiting was present in 9 (36%) patients, affected level of consciousness in 7 (28%) patients, and convulsions in 5 (20%) patients. Renal complaints were found in 6 (24%) patients, loose stools in 4 (16%) and jaundice in 3 (12%) patients. In group B also, all the patients 40 (100%) presented with fever and headache in 8 (20%) patients. 21 (52.5%) presented with vomiting, 19 (47.5%) with altered level of consciousness, 18 (45%) with convulsions. Yellow discoloration of eyes and urine & loose stools were found in 11 (27.5%) patients each and only 2 patients presented with renal complaints.

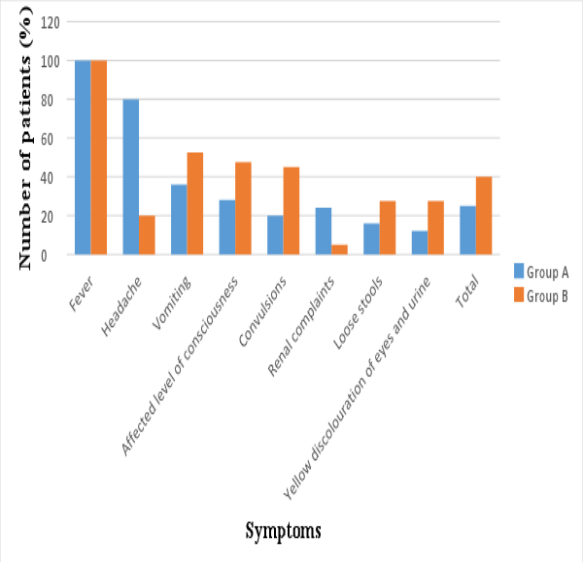

Figure 1: Distribution of clinical symptoms in study group A and group B

Figure 2 shows month wise distribution of cases. Majority of the patients 25 (group A) and 37 (group B) were found in the monsoon season (July–October). In group A, maximum number cases were found in the month of October, 2015 and in group B, in September, 2015.

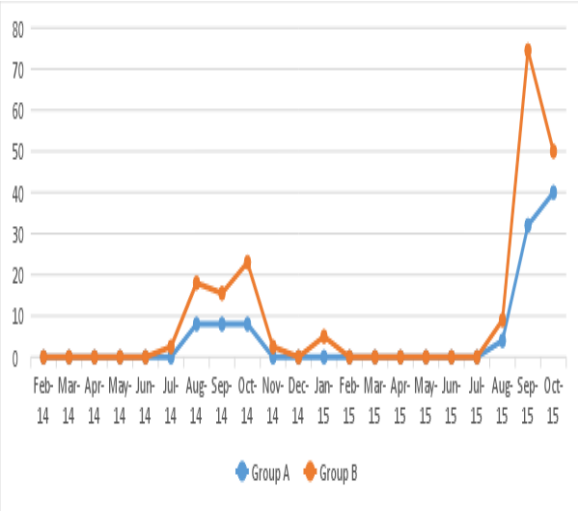

Figure 2: Month wise distribution of cases in study group A and B

DNA isolated from whole blood of a total of 65 falciparum positive patients (using QIAgen DNA Minikit) was amplified by PCR using the primer pair 5'-GGCTCACGTTTAGGTGGA-3' and 5'-TGAATTTCCCTTTTTATTTCCAAA-3' to give a 264-basepair product corresponding to amino acid residues 32 to 119 of the *pfcrt* gene product. Amplicons that resulted from PCR separated by electrophoresis on a 2% agarose gel, run with a 100bp DNA ladder (Fermentas). Figure 3 shows agarose gel electrophoresis image of PCR products. Out of 65 samples, *pfcrt* K76T (lys76Thr) mutation was observed in only 23 (35.4%) of clinical isolates investigated.

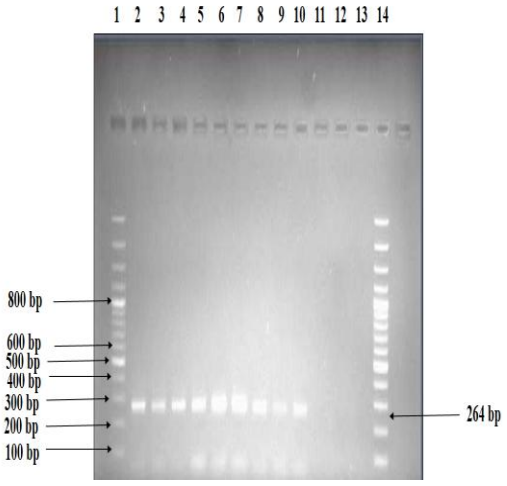

Figure 3: shows agarose gel electrophoresis image of PCR products

PCR products were subjected to digestion with restriction enzyme *Apo I*. Digestion of the PCR product with *Apo I* results in two fragments of 128 and 136 basepairs in the CQ-sensitive haplotype (CVMNK). If K76T mutation is present in the *pfcrt* gene, it renders the fragment resistant to digestion with Apo I. Products of digestion were finally separated by electrophoresis on a 2% agarose gel, run with a 100bp DNA ladder. *Apo I* enzyme did not digest any of the 23 PCR products. Figure 4 shows agarose gel electrophoresis image of RFLP by *Apo I* enzyme . Lane 1 represents 100 bp DNA ladder (Fermentas). Lane 2-8 show *pfcrt* gene (264) PCR product which has not been digested into 128 and 136 bp fragments by RFLP using Apo I restriction enzyme.

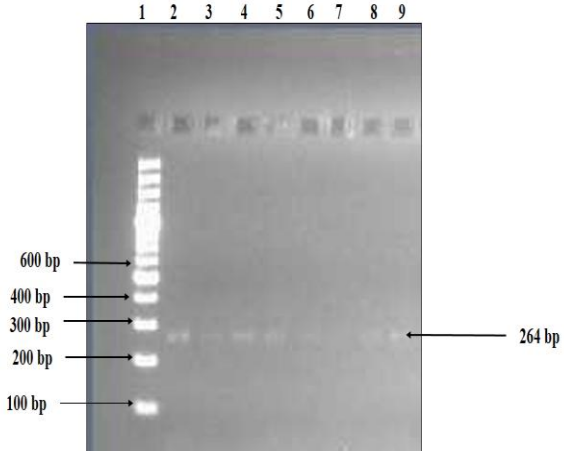

Figure4: shows agarose gel electrophoresis image of RFLP by restriction enzyme Apo I.

DISCUSSION

Malaria continues to be a major public health problem world-wide. The most vulnerable groups with high chances of morbidity are children under five years of age, pregnant women and non-immunes. India has the largest population under the risk of malaria in the world, with 85% living in malarious region. [17] The sex distribution in our study groups were in favour of males. In study group A, 64% patients were males and in study group B, 67.5% patients were males. All the study participants belong to male category in a study by Sharma et al, 2017. [18] Kumar et al (2007) also reported

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higher burden in men than in women in all age groups. [19]

In our study, maximum number of cases in both the study groups (25 patients in group A and 37 patients in group B) were found in monsoon season [temperature (18-39°C), humidity (34-83%)]. A study by Alemu et al (2011) also reported the importance of ambient temperature in life cycle of malaria vector. [20] Dhiman et al (2008) reported that the minimum temperature required for development of *Plasmodium falciparum* parasite ranges from 16.5-19°C. [21] However, the best conditions for development of the malaria parasite are 20-30°C temperature and 60% relative humidity (RH). Alemu et al (2011) reported that the monthly total rainfall was the most significant factor that affects the transmission cycle of malaria. [20] In a study done by Alemu et al (2011), the season with the highest number of cases of malaria was spring (September, October, and November) and the minimum number of cases were found during winter (December, January and February). [20]

The presenting symptoms of patients were similar as reported by other workers. [22] All the patients in both the study groups presented with fever. Convulsions were found in 20% patients in group A and 45% patients in group B as reported by White et al, 1987. [23] Vomiting was found in 36% patients in group A and 52% patients in group B. Genton et al, 1994 reported a high incidence of vomiting in malaria. [24]

CQ-resistance has been linked to both the *pfcrt* and *pfmdr-1* genes but the *pfcrt* (*P. falciparum* chloroquine transporter) gene is primarily important for CQ-resistance in *P. falciparum.* In our study, a total of 65 *P. falciparum* isolates of unknown sensitivity were analysed for the presence of K76T mutation in the *pfcrt* gene (264 bp) by PCR.

Out of 65 *P. falciparum* isolates, only 23 (35.4%) gave positive results for *pfcrt* gene and 42 (64.6%) gave negative results. To establish the presence of the K76T mutation, these polymerase chain reaction (PCR) products from 23 *P. falciparum* isolates were enzymatically digested with ApoI restriction enzyme. If restriction digestion took place and divided the PCR product into two fragments (128 and 136 bp), the isolate was considered CQ-sensitive. [16] In our study, none of the 23 PCR samples produced the expected 128 and 136 bp amplicons and resisted the enzymatic digestion with ApoI, indicating the presence of the K76T mutation in these samples and absence of wild-type chloroquine-sensitive haplotype (CVMNK), because the presence of K76T mutation renders the fragment resistant to digestion with Apo I enzyme. In a study conducted by Vathsala et al (2004) on 73 *P. falciparum* DNA isolates collected from different parts of India, 70 (95.9%) had the *pfcrt* K76T mutation. [16] In another study conducted by Sutar et al (2011), in Odisha, 32 (80%) of the 40 *P. falciparum* isolates possessed the K76T mutation on *pfcrt* gene. [25] Interestingly, the K76T mutation in *Pfcrt* gene was not observed from any of the multiple mutant *P. falciparum* cases. [18] Thus, the high prevalence of mutation at codon 76 of the *pfcrt* gene (*pfcrt* K76T) is a key indicator of *P. falciparum* CQ-resistance.

CONCLUSION

From the study, it is confirmed that chloroquine-resistant *P. falciparum* malaria parasites are prevalent in and around Aligarh region. Our results confirm that PCR-RFLP technique provides a simple and rapid method of detecting polymorphisms in genes that may predict chloroquine-resistance. Although the identification of the polymorphism in the pfcrt gene provides a significant indicator of chloroquine resistance, further studies are needed to determine the role of these polymorphisms in the in vivo and in vitro responses to drug treatment.

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