Mutational prevalence of chloroquine resistance transporter gene among *Plasmodium falciparum* field isolates in Assam and Arunachal Pradesh, India

J Sharma, M Soni, P Dutta, SA Khan, J Mahanta

**Abstract**

**Objective:** The present study aims to find out the mutational prevalence of *Plasmodium falciparum* chloroquine resistance transporter (*Pfcrt*) gene in Assam and Arunachal Pradesh, India. **Methods:** To fulfill the objective of the study, a total of 54 *P. falciparum* malaria positive samples were attempted for genotyping of *Pfcrt* gene using polymerase chain reaction (PCR) and direct DNA sequencing method. **Results:** The K76T mutation was observed in 77.78% cases followed by M74I (61.11%), N75E (61.11%) and C72S (16.67%). Triple mutant allele M74I+N75E+K76T was found in 61.11% *P. falciparum* field isolates. Double mutant allele C72S+K76T was seen among 16.67% samples. Mutation studies have shown existence of three different haplotypes of which two were associated with chloroquine resistance. The haplotype CVIET was found preponderance and circulated in all four districts. The other haplotype SVMNT was observed only in Karbi Anglong district of Assam. Maximum haplotype diversity was also recorded from Karbi Anglong district of Assam. **Conclusion:** Our study has confirmed high prevalence of mutant *Pfcrt* genotypes associated with chloroquine resistance in Assam and Arunachal Pradesh, India.

**Key words:** Arunachal Pradesh, Assam, chloroquine, haplotype, *Plasmodium falciparum*, polymerase chain reaction

**Introduction**

At present, lots of preventative and treatment strategies are developed for reducing the incidence of malaria. However, malaria is still considered as one of the major health problem in developing countries. The emergence of parasite resistance to newly introduced drugs severely limits the armory of available drugs against protozoal pathogens. Several molecular markers are found to be associated with antimalarial resistance. Analyses of these molecular markers can provide significant information about resistance levels of the different antimalarial drugs. The K76T amino acid substitution in the *Plasmodium falciparum* chloroquine resistance transporter gene (*Pfcrt*) has been shown to be associated with chloroquine (CQ) and amodiaquine resistance.[1,2] Mutations at other amino acid codon such as 72, 74, 75 in *Pfcrt* gene was also detected by many studies in CQ resistance parasite lines.[3-5]

In India, the CQ resistant *P. falciparum* malaria was first reported in 1973 from Diphu area of the Karbi Anglong district in Assam.[6] Gradually it spread to the various parts of the country.[3,7,8] Only limited molecular studies in these aspects are available from North Eastern (NE) region of India, especially from Assam and Arunachal Pradesh. Therefore, the endeavour will certainly bring some outcomes that are very much helpful for understanding the changing pattern of drug resistance, distributions of different haplotypes and their diversity, parasite metabolism and their adaptation in regards to drug pressure. Keeping in mind, the study was conducted in Assam and Arunachal Pradesh, India to find out the mutational prevalence of *Pfcrt* gene among *P. falciparum* isolates.

**Materials and Methods**

**Study sites, blood samples and DNA extraction**

The study was undertaken from December 2011 to December 2013 in some malaria-endemic areas of Assam and Arunachal Pradesh, the NE region of India.
Clinically, suspected malaria cases were eligible for enrolment irrespective of age and sex. Patients who already took antimalarial drugs were not included in our study. Informed consent was obtained from patients or in the case of children from their guardians. Institutional ethical permission was taken from Institutional Ethical Board, Regional Medical Research Centre (Indian Council of Medical Research), Dibrugarh, Assam. Two millilitre of blood samples were collected from suspected malaria patients attended in different primary health centres. Thick and thin blood smears were prepared on a glass slide. Blood films were stained with 10% Giemsa and examined microscopically. Parasite density was assessed by counting asexual parasites in 200 white blood cells on the thick film and then quantified. Parasite genomic DNA was extracted from blood samples using a QIAamp DNA blood kit (Qiagen, Hilden, Germany), according to the manufacturer’s instructions.

A polymerase chain reaction (PCR) method was done to amplify a partial 134 bp portion DNA sequence of PfCRT gene containing the major single nucleotide polymorphisms related to CQ resistance [Figure 1]. The amplification of PfCRT gene fragment was performed with 5 μl of DNA into a 45 μl of master mix containing 2.5 mM of MgCl2, 250 μM of each deoxyribonucleoside triphosphate (dATP, dTTP, dGTP and dCTP), 1.2 unit of taq DNA polymerase and 0.3 μM of each primer CRTD1 (TGT GCT CAT GTG TTT AAA CTT) and CRTD2 (CAA AAC TAT AGT TAC CAA TTT TG).[10] The reaction was settled with an initial hold (95°C/10 min), 40 cycles (94°C/30 s, 48°C/30 s, 72°C/45 s and final extension 65°C/3 min). Products were electrophoresed on 2% agarose gels and visualised under Gel documentation system (Kodak). Amplified products were further purified by using PCR Mini spin columns purification kit (Millipore Corporation). The quantity of purified PCR products was quantified in NanoDrop and depending upon the concentration of PCR products; samples were sent for sequencing in both directions (South Korea through Anshul Biotechnologies, Hyderabad, India). Sequencing products were further analysed by software DnaSP v. 5.10.01 for detection of point mutation, haplotype diversity (Hd), neutrality test and other parameters.

Results

During the study period, a total 164 clinically suspected malaria patients were enroled from malaria-prone areas of Assam and Arunachal Pradesh. *P. falciparum* malaria parasite was detected among 35.37% (58/164) cases. The mean age groups among the malaria *P. falciparum* positive cases was found 25.21 (ranging from 1.6 year to 56 years). Mean parasite count level was found as 4.38% (standard deviation [SD] ±3.60 with a median value of 1.00). The two-tailed *P* < 0.0001 considered extremely significant having *t* = 6.209 with 25% of freedom. Low haemoglobin concentration was recorded in 86.21% (50/58) *P. falciparum* positive cases.

Mutations at the amino acid position from 72 to 76 of PfCRT gene encoding protein were analysed in 54 samples to find out the drug resistance competency among the *P. falciparum* isolates in Assam and Arunachal Pradesh. PfCRT double mutant allele C72S+K76T was found in 16.67% (9/54) samples and triple mutant allele M74I+N75E+K76T was noticed in 61.11% (33/54) *P. falciparum* field isolates from Assam and Arunachal Pradesh. The prevalence of PfCRT triple mutant allele M74I+N75E+K76T revealed a high level of CQ resistance in NE region. Depending upon the mutation at amino acid position, a total of three different PfCRT haplotypes (CVMNK, SVMNT and CVIET) were identified, of which SVMNT and CVIET were found to be associated with CQ resistance. The haplotype (gene) diversity was (Hd: 0.560), the variance of Hd (0.00314) and SD of Hd (0.056). Maximum Hd was found in the *P. falciparum* field isolates of Karbi Anglong district in Assam (Hd: 0.692) with a value of SD of Hd 0.027. A total of 5 numbers of polymorphic (segregating) sites, (S: 5) and total number of mutations, (Eta: 5) were observed among the analysed samples of PfCRT gene [Table 1].

Mutation analysis at nucleotide level showed that at nucleotide position 214–216, TGT-AGT (C72S), at location 220–222, ATG-ATT (M74I), at 223–225 nucleotide positions, AAT-GAA (N75E) and at nucleotide position from 226 to 228, AAA-ACA (K76T) mutation leads to the occurrence of different haplotypes associated with CQ resistance. Nucleotide diversity and average number of nucleotide differences among the PfCRT genome was found little beat high in the field isolates of Karbi Anglong district of Assam as compared to other study areas. The *P. falciparum* field isolated in Changlang district had shown no genetic differentiation [Table 2]. This is because all the analysed samples in Changlang district of Arunachal Pradesh had shown M74I + N75E + K76T mutation. A

![Figure 1: Amplification of 134 bp portion of PfCRT gene (Lane M represents 50 bp ladder, S1, S2, S3… represents sample nos.)](image)
positive significant value of Tajima’s D and Fu and Li’s F (FLF) was observed among the field isolates of Assam whereas the analysed samples from Arunachal Pradesh had shown the negative significant value of Tajima’s D, Fu and Li’s D and FLF [Table 2].

Discussion

In the past, CQ had been considered as an effective antimalarial drug in India against malaria.[7,11,12] However, after the occurrence of CQ resistance *P. falciparum* malaria parasite in Assam, the drug policy was changed and Sulphadoxine–Pyrimethamine (SP) combination was introduced by Government of India in 1982.[15,7,8] Again, SP resistance *P. falciparum* malaria cases were detected in Delhi and Assam (Karbi Anglong district) in 1987.[11,13-16] Accordingly in 2010, Government of India had introduced artemisinin-based combination therapy (ACT) for the treatment of all uncomplicated *P. falciparum* malaria cases.[13,14] However, nowadays, the NE region is facing the problem of reduced susceptibility to the ACT regime also. Keeping this in mind, the National Vector Borne Disease Control Programme (NVBDCP), Government of India has recommended artemether-lumefantrine combination for treatment of *P. falciparum* malaria cases in NE region in 2013.[13] This combination therapy is believed to be beneficial for the people of NE states.

Our study has provided significant information on mutational prevalence of *Pfcr* gene among *P. falciparum* isolates in Assam and Arunachal Pradesh, India. In our result, *Pfcr* 76T mutation was found in 77.78% cases. This data indicated high prevalence of CQ resistance *P. falciparum* malaria parasites in NE region of India. It has also shown resemblance with other studies conducted in different part of India as well as in neighboring countries.[16-24]

Table 1: Haplotype analysis for *Plasmodium falciparum* chloroquine resistance transporter gene among the isolates of Assam and Arunachal Pradesh at district level

<table>
<thead>
<tr>
<th>CRT gene Assam-Arunachal</th>
<th>Karbi Anglong</th>
<th>Tinsukia</th>
<th>Overall Assam</th>
<th>Changlang</th>
<th>Lohit</th>
<th>Overall Arunachal Pradesh</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample size</td>
<td>26</td>
<td>9</td>
<td>35</td>
<td>10</td>
<td>9</td>
<td>19</td>
</tr>
<tr>
<td>Number of haplotypes</td>
<td>3</td>
<td>2</td>
<td>3</td>
<td>1</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>H≥</td>
<td>0.692</td>
<td>0.389</td>
<td>0.671</td>
<td>0.222</td>
<td>0.105</td>
<td></td>
</tr>
<tr>
<td>Variance of H≥</td>
<td>0.00073</td>
<td>0.02703</td>
<td>0.00097</td>
<td>0.02764</td>
<td>0.00846</td>
<td></td>
</tr>
<tr>
<td>Standard deviation of H≥</td>
<td>0.027</td>
<td>0.164</td>
<td>0.031</td>
<td>0.166</td>
<td>0.092</td>
<td></td>
</tr>
</tbody>
</table>

CRT: Chloroquine resistance transporter, H≥: Haplotype diversity

our study has shown high prevalence of CQ resistance *P. falciparum* malaria cases in NE states of India. Continuous molecular surveillance and regular monitoring.
will help the policy makers for better management of malaria in near future.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

References


