Antifolate resistance associated point mutations among asymptomatic malaria cases

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In India, malaria is distributed throughout the country. A large numbers of malaria cases have been reported from Northeastern (NE) states, of which asymptomatic cases shared a major proportion. The presence of asymptomatic malaria cases plays a significant role in malaria transmission. These asymptomatic cases carry malaria gametocytes and act as a long-lasting parasite reservoir that transmits malaria parasites in areas of low or unstable transmission intensity. However, the role of asymptomatic infections in the transmission of malaria parasites with genetic changes conferring drug-resistance is not clear. Many earlier studies have been carried out in India regarding the genetic polymorphisms associated with drug resistance among symptomatic malaria patients. However, no extensive studies were undertaken in India to find out the genetic polymorphisms associated with antimalarial resistance among asymptomatic malaria patients. Keeping this in mind, a study was carried out in few selected malaria endemic areas of Assam and Arunachal Pradesh to observe the point mutations in Plasmodium falciparum dihydrofolate reductase (Pfdhfr) and P. falciparum dihydropteroate synthase (Pfdhps) gene among the asymptomatic P. falciparum malaria cases and drug resistance capability.

Based upon the previous records of malaria, three malaria endemic districts (Karbi-Anglong, North Cachar Hills and Tinsukia) in Assam and two districts (Changlang and Lohit) in Arunachal Pradesh were selected to fulfill the objective of the study. The study was undertaken for a period of three years (from 2012 to 2014). A total of 128 normal healthy (not showing any symptoms of malaria or other disease) participants were recruited for the study irrespective of age and sex. Out of 128, 54 cases had recent malaria infection (before one month), 29 cases had malaria infection before couple of months and remaining 45 cases had malaria infection 1–4 yr ago. The aims and objectives of the study were explained to each participant. Institutional ethical permission was obtained from ethics committee of the Regional Medical Research Centre (ICMR), Dibrugarh, Assam. Written informed consent/assent was taken from all participants prior to recruitment in this study. After obtaining consents/assents 2 ml of blood samples was collected from each participant. Presence of malaria parasite was confirmed by using microscopic examination of the blood smears made from collected samples. Serum was separated from the remaining blood samples and DNA extraction was done using the QIAamp DNA Mini spin columns kit (Millipore Corporation, Qiagen, Hilden, Germany). A 648 bp portion of Pfdhfr gene and 710 bp portion of Pfdhps gene were amplified by polymerase chain reaction (PCR) method in a thermal cycler (Gene Amp®PCR System 9700 and Veriti, Applied Biosystems, Foster City, CA, USA). The amplified products were purified and sequenced commercially in South Korea through Anshul Biotechnologies, Hyderabad, India. The sequencing products were analyzed in BioEdit, DnaSP version v.5.10.01 and Mega 5 software for detection of single nucleotide polymorphisms.

Among the study samples, 45.31% (58/128) cases had P. falciparum monoinfection. Of these P. falciparum positive cases, 22 cases were from Karbi-Anglong, 11 cases from North Cachar Hills and five cases were from Tinsukia district in Assam; whereas 12 cases were detected from the collected field samples in Changlang district and eight from Lohit district of Arunachal Pradesh. The average parasitaemia range varied from 0.1 to 11%, SD ± 2.636 with median value 2 (Table 1). Artemisinin-based combination therapy (artesunate plus sulphadoxine-pyrimethamine) was given as per prescribed protocol to the diagnosed positive patients. Among the asymptomatic

<table>
<thead>
<tr>
<th>Percentage of parasitaemia</th>
<th>Asymptomatic P. falciparum positive cases</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1–1.9</td>
<td>19</td>
</tr>
<tr>
<td>2–6</td>
<td>32</td>
</tr>
<tr>
<td>7–11</td>
<td>7</td>
</tr>
<tr>
<td>Total</td>
<td>58</td>
</tr>
</tbody>
</table>

Table 1. Percentage of parasitaemia among asymptomatic P. falciparum positive patients
atic *P. falciparum* positive cases, 93.10% (54/58) have shown mutations either in *Pfdhfr* or in *Pfdhps* gene. Double mutation C59R+S108N in *Pfdhfr* gene was detected in 77.59% (45/58) samples. Three isolates from Changlang district had shown N51I+C59R+S108N mutations and two isolates had shown C59R+S108N+I164L mutations in *Pfdhfr* gene (Table 2). However, in case of *Pfdhps* gene, 34.48% (20/58) samples had shown S436F mutation. Similarly, 10.34% (6/58) isolates had shown A437G+A581G mutations, 12.07% (7/58) had S436A+A437G mutations and 27.59% (16/58) samples had S436A+A437G+K540E mutations in *Pfdhfr* gene (Table 2). Two *P. falciparum* positive cases in Changlang district of Arunachal Pradesh had N51I+C59R+S108N-S436A+A437G+K540E mutations in *Pfdhfr*-*dhps* two locus genes which may to be associated with treatment failure.

From the above finding it was observed that C59R+S108N mutations in *Pfdhfr* gene and S436F mutation in *Pfdhps* gene are the key point mutations among the asymptomatic *P. falciparum* cases. Apart from this, few cases from Assam and Arunachal Pradesh had shown triple mutations either in *Pfdhfr* or in *Pfdhps* gene. It is an indication that antifolate resistance had reached at an alarming level among asymptomatic carriers. Many earlier studies have reported that these types of mutations are common in symptomatic malaria cases. However, no information is available in case of the asymptomatic malaria cases. These asymptomatic *P. falciparum* cases (having such mutations) serve as a reservoir for malaria parasite having antimalarial resistance capability. Sometimes malaria causing mosquitoes bite such carriers, thereby transmitting the acquired malaria parasitaemia from asymptomatic carriers to a normal healthy person. If malaria parasite enters into a normal population with its already developed antimalarial resistance capability, it could gradually attack the entire population.

So, it is crucial to find out the carriers having asymptomatic malaria infection. For this, the mapping of malaria endemic places in each district is necessary and accordingly mass fever survey is needed in those places at frequent interval. Until and unless we could detect and treat the asymptomatic cases, malaria elimination is not possible. By detecting asymptomatic carriers at an early stage, one can predict reduction in the malaria burden from NE region of India.

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**Conflict of interest**

The authors declare that they have no conflict of interest.

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