Quadruple Mutations in Dihydrofolate Reductase of 
*Plasmodium falciparum* Isolates from Car Nicobar Island, India

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Quadruple mutations in the *Plasmodium falciparum* dihydrofolate reductase (PFDHFR) enzyme give rise to the highest level of pyrimethamine resistance leading to treatment failures. We describe here the presence of these quadruple mutations in a majority of *P. falciparum* isolates from Car Nicobar (Andaman and Nicobar) Island, India. Isolates from the mainland, however, continue to show a prevalence of double PFDHFR mutations and some with triple but none with quadruple mutations. In conclusion, the antifolate drug pressure is very high in the island, which should be a cause of concern for the malaria control program in the country.

The antifolate drug pyrimethamine acts on the *Plasmodium falciparum* dihydrofolate reductase (PFDHFR) enzyme and therefore inhibits the folate biosynthesis pathway of the parasite (7, 20). Certain point mutations in PFDHFR reduce its capacity to bind to this drug, resulting in the emergence of resistant parasite strains (4, 7, 11). Pyrimethamine resistance develops in a progressive manner, and treatment failure occurs if there are quadruple mutations (N51I plus C59R plus S108N plus I164L) in this parasite enzyme (11, 17, 19). Monitoring PFDHFR mutations have been proposed for evaluation of the efficacy of this drug (8, 12, 14). Recently, we have described a temporal rise in PFDHFR mutations among Indian *P. falciparum* isolates (1, 16). The mutation rate varied from state to state, and it was lower in Uttar Pradesh (UP) but higher in Assam (1). In the present study, we revisited these high-prevalence and low-prevalence drug resistance areas after a gap of 2 years to monitor the drug pressure. We also included *P. falciparum* isolates from Car Nicobar Island (the Andaman and Nicobar group of Indian islands), where chloroquine resistance was reported a long time ago (5, 6).

Patients with fever attended the malaria clinics in UP (Aligarh), Assam (Kamrup), and Andaman and Nicobar (Car Nicobar). Their blood was screened for the presence of the malarial parasite by light microscopy. Malaria patients were given the prescribed antimalarial treatment according to the national drug policy (18). Briefly, patients from high-risk areas (Car Nicobar) were treated with chloroquine (25 mg/kg of body weight) over a 3-day period, while patients from low-risk areas (Aligarh) received a single dose of chloroquine (10 mg/kg of body weight). Patients from high-risk chloroquine resistance areas (Kamrup) were treated with a single dose of sulfadoxine (25 mg/kg of body weight) and pyrimethamine (1.25 mg/kg of body weight). All patients from high-risk areas also received a single dose of primaquine (0.75 mg/kg of body weight). About 20 to 50 μl of heparinized blood was collected from the *P. falciparum*-positive patients. Informed consent was obtained from the patients prior to the blood collection, following the institutional ethical guidelines. Parasite DNA was extracted and subjected to PCR amplification of a 720-bp fragment of the *pfdhfr* gene as described before (1). An aliquot of the primary amplicon was diluted and subjected to seminested PCR using primers AMP1 (13) and DHFRR2 (5'-ACAGAA ATAATTTGATACTCA-3'). Only 30 cycles were carried out for seminested PCR under the same conditions as for primary PCR. The PCR products were purified and subjected to nucleotide sequencing using an ABI Big Dye Terminator Ready Reaction kit, version 3.1, and the ABI Prism 310 genetic analyzer (PE Applied Biosystems, California) as described before (2, 10).

We have analyzed the nucleotide sequence of the *pfdhfr* gene from 117 Indian *P. falciparum* isolates. There was no isolate with the A16V and S108T mutations in PFDHFR; this is the target for cycloguanil, which is not being used in India. Further, there was no isolate with Bolivia repeats and the C50R mutation, as has been found elsewhere (12). The maximum number of isolates (96.58%) showed a mutation at codon 108 (S108N) followed by the C59R mutation (Fig. 1A). The majority of the isolates were found to contain double PFDHFR mutations (Fig. 1B). The double mutation C59R plus S108N was much more common (43.58%) than the N51I plus S108N mutation (0.85%). Triple mutations C59R plus S108N plus I164L or N51I plus C59R plus S108N were not very common, as they were found only in 5.12% and 7.69% of isolates, respectively. Surprisingly, quadruple mutations N51I plus C59R plus S108N plus I164L were found in more isolates (28.20%) than the triple mutations (12.82%) (Fig. 1B). The regional distribution of the total number of PFDHFR mutations showed a wide variation (Fig. 2A). Quadruple mutations were predominantly present only in Andaman and Nicobar isolates, while double mutations were common in UP and Assam. Triple mutations were present in Assam and in Andaman and Nicobar but not in UP. Conversely, single mutation was specific to UP. There were a total of six different combinations of *pfdhfr* mutations (genotypes) among these isolates. Some of these genotypes showed a regional bias (Fig. 2B).
We describe here pfdhfr mutations among P. falciparum isolates from three different geographical regions of India. These regions are far apart from each other and show a different level of drug resistance and malaria transmission patterns viz. a lower level of drug resistance and malaria transmission in UP, where Plasmodium vivax is prevalent, and a higher level of drug resistance and intense perennial malaria transmission in Assam and in Andaman and Nicobar, where P. falciparum is predominant (5, 6, 9, 15). The pfdhfr mutation pattern observed here was also different for these regions, as it was lower in UP but higher in the other two areas (Fig. 2). Therefore, it seems that there is an association between the degree of malaria transmission and the number of pfdhfr mutations. This is similar to the previous observations in which greater numbers of Pfcrt mutations, associated with a higher level of chloroquine resistance, were found among isolates from these high malaria transmission areas (10). The PFDHFR mutations in UP and Assam isolates were similar to those reported earlier, except that at present there were more isolates with triple C59R plus S108N plus I164L mutations in the latter state (1). Still, there was no isolate with quadruple mutations in Assam, although sulfadoxine-pyrimethamine has been used there for more than two decades (9, 15). Surprisingly, quadruple mutations were found only in Car Nicobar (Andaman and Nicobar) Island, and they were present in a majority of the isolates from this region (Fig. 2). In fact this is the first time that we are reporting the quadruple PFDHFR mutations from India (Table 1). Such quadruple mutations have been reported from the high drug resistance areas of Southeast Asia (3, 7). It is possible that the P. falciparum parasite from Andaman and Nicobar with these quadruple mutations has evolved independently here, as these islands are physically separated from the main-
Parasites with these quadruple PFDHFR mutations show the highest level of antifolate resistance (11, 17, 19). This indicates that the drug pressure in this Indian island is much higher than on the mainland. For some reason, this has gone unnoticed by the national malaria control program. Unfortunately, the national drug policy for this island has remained the same despite chloroquine resistance having been reported since 1981 (5, 6). This warrants a systematic study on in vivo drug resistance followed by a change in the drug policy for this island.

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### TABLE 1. DHFR mutations in Indian isolates of *P. falciparum*

<table>
<thead>
<tr>
<th>Type</th>
<th>Genotype</th>
<th>No. of mutations</th>
<th>Geographical location of isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>A16N, C51S, G108S, L164</td>
<td>0</td>
<td>Delhi, Uttar Pradesh, Assam, Orissa</td>
</tr>
<tr>
<td>II</td>
<td>A16N, C51S, G108D, I164</td>
<td>1</td>
<td>Delhi, Uttar Pradesh, Orissa, Assam, Goa</td>
</tr>
<tr>
<td>III</td>
<td>A16N, R51N, G108D, I164</td>
<td>2</td>
<td>Delhi, Uttar Pradesh, Orissa, Assam, Goa, Andaman and Nicobar</td>
</tr>
<tr>
<td>IV</td>
<td>A16I, C51S, G108D, L164</td>
<td>2</td>
<td>Uttar Pradesh, Delhi, Assam, Goa</td>
</tr>
<tr>
<td>V</td>
<td>A16N, R51N, G108D, L164</td>
<td>2</td>
<td>Uttar Pradesh, Assam</td>
</tr>
<tr>
<td>VI</td>
<td>A16N, R51N, G108L, I164</td>
<td>3</td>
<td>Assam, Andaman and Nicobar</td>
</tr>
<tr>
<td>VII</td>
<td>A16N, R51N, G108L, L164</td>
<td>3</td>
<td>Assam, Andaman and Nicobar</td>
</tr>
<tr>
<td>VIII</td>
<td>A16I, R51N, G108L, L164</td>
<td>4</td>
<td>Andaman and Nicobar</td>
</tr>
</tbody>
</table>

*Genotype A16I, R51N, G108L, L164 is from the present study, while other genotypes are from a previous report (1). Mutated amino acids are underlined.
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REFERENCES