High chloroquine treatment failure rates and predominance of mutant genotypes associated with chloroquine and antifolate resistance among falciparum malaria patients from the island of Car Nicobar, India

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Objectives: An in vivo chloroquine efficacy study was undertaken on the island of Car Nicobar because a temporal rise in the Plasmodium falciparum parasite population containing mutations in the chloroquine resistance transporter (PfCRT) protein has been reported there.

Methods: A WHO protocol with a 28 day follow-up schedule was used for chloroquine efficacy studies. Finger-prick blood from P. falciparum malaria patients was used for sequencing the genes encoding PfCRT (exon 2), dihydrofolate reductase (PfDHFR) and dihydropteroate synthetase (PfDHPS).

Results: The majority of patients showed chloroquine treatment failure (60.42%, n = 48). A higher early treatment failure (ETF) rate was recorded among non-responders (23 of 29, 79.31%). Each patient, irrespective of their chloroquine response, was infected with P. falciparum that contained mutated PfCRT (predominantly genotype C72V73I74E75T76) associated with high chloroquine resistance and none with the wild-type pfcr gene. Therefore, mutated PfCRT was also present in the P. falciparum isolates of all the chloroquine responders. The majority of individuals from both groups also contained parasites with a high number of two-locus PDHFR-PDHPS mutations, associated with a high level of antifolate resistance.

Conclusions: There is a predominance of chloroquine- and antifolate-resistant P. falciparum malaria in Car Nicobar, requiring an alternative antimalarial drug treatment policy, such as implementation of artemisunate combination therapy (ACT), for this island.

Keywords: in vivo chloroquine response, sulfadoxine/pyrimethamine resistance, drug resistance markers, point mutations

Introduction

Drug-resistant malaria resulting from continuous drug pressure is a cause of concern for worldwide malaria control programmes and necessitates the discovery of novel antimalarial drugs as well as implementation of combination therapy.1,2 In India, the patterns of antimalarial drug resistance vary from region to region (www.nvbdc.gov.in). The malaria situation in the Andaman and Nicobar Islands is very different from that in mainland India; the disease is transmitted here by a different, but very efficient, vector, Anopheles sundaicus.3–6 We have observed a temporal increase in the Plasmodium falciparum population containing mutant chloroquine resistance transporter (PfCRT) genotypes associated with chloroquine resistance on the island of Car Nicobar of the Andaman and Nicobar Islands, India.5 This has led us to conduct an in vivo chloroquine efficacy study on this island as the last such study was carried out a long time ago.7 We were also interested to know the current status of the mutant PfCRT genotypes among the isolates. Besides PfCRT, we also decided to analyse these isolates for the detection of mutations in dihydrofolate reductase (PfDHFR) and dihydropteroate synthetase (PfDHPS), which are associated with pyrimethamine and sulfadoxine resistance, respectively. This was done to generate the latest baseline data for the implementation of artesunate combination therapy (ACT), since artesunate is combined with sulfadoxine/pyrimethamine in India.
Mutant PFCRT genotypes among chloroquine responders

Materials and methods

Study site and patient recruitment

The study was conducted on the island of Car Nicobar of the Andaman and Nicobar Islands, India, during May to July 2008. The study was approved by the Ethics Committee of the National Institute of Malaria Research, Delhi, and the All India Institute of Medical Sciences, New Delhi. The first contact was with the leaders of the villages (Captains), who then provided access to the patients. Patients and the guardians of the children gave informed consent before inclusion in the study. Patients between 1 and 59 years presenting the signs and symptoms of malaria with fever were screened for malaria parasites. Enrolment of the patients was based purely on the inclusion criteria of the WHO standard protocol. Patients (≥1 year of age) with microscopically confirmed *P. falciparum* infection and a parasitaemia between 1000 and 10000 asexual parasites/μL of blood were selected. Pregnant women and patients with any other concurrent infections or disease were excluded.

Therapeutic treatment

Each patient was orally administered 25 mg of chloroquine/kg of body weight over a period of 3 days, according to the regimen recommended by the National Vector Borne Disease Control Program, Government of India; 10 mg/kg of body weight on day 0 and day 1, and 5 mg/kg of body weight on day 2. The patients were followed-up on days 2, 3, 7, 14, 21 and 28 for clinical and parasitological observations. All patients who failed to respond to chloroquine were treated with quinine or arte-

PCR amplification and nucleotide sequencing

About 200 μL of finger-prick blood from the *P. falciparum*-infected individuals was collected with their full consent according to the Institutional Ethics Committee guidelines. It was used to extract the DNA using an Accuprep genomic DNA extraction kit (Bioneer Corporation) according to the manufacturer’s protocol. This genomic DNA was then used as the template to amplify and sequence the region of *pfdhfr*, *pfhps* and *pfcrt* genes covering codons 16–164, 392–637 and 44–177, respectively. Primer sequences, PCR amplification and sequencing conditions were the same as described previously. The translated amino acid sequences (http://www.expasy.org) were aligned using the online multiple sequence alignment tool Clustalw2 (http://www.ebi.ac.uk/clustalw).

Mutations in the drug resistance marker genes

Amplification and sequencing of the desired part of the *pfcrt* gene, covering codons 72–76, was achieved for 60 of 65 samples. The majority of isolates (96.67%, *n* = 60) were PCRRT genotype C72V73M74N75T76, while two isolates (3.33%) were PCRRT genotype S72V73M74N75T76 and none was wild-type C72V73M74N75K76. All 16 individuals from the chloroquine responder category (PCR amplification was unsuccessful for 3 individuals) and 26 of 28 (92.86%) from the chloroquine non-responder category (PCR amplification was unsuccessful for 1 individual) were found to contain the mutant genotype C72V73M74N75T76. The remaining two isolates from the chloroquine non-responder group were found to contain the S72V73M74N75K76 genotype. There was no significant difference in the number of patients with the mutant PFCRT genotype C72V73M74N75T76 between the chloroquine responder and non-responder groups.

Sixty of 65 blood samples showed PCR amplification for the *pfhfr* gene. Sequencing revealed the predominance in the parasite population of the quadruple mutation genotype A16N51C59S108L164 (71.67%, *n* = 60). Among 16 cases of chloroquine responders, only 6.25% were wild-type (A16N51S108L164), 81.25% were PFDHFR genotype A16I51R59N108L164 and 12.5% were PFDHFR genotype A16N51R59S108I164. The PFDHFR mutation rate among the chloroquine-resistant patients was also high (96.43%, *n* = 28) with a predominance of genotype A16N51R59S108L164 (75%, *n* = 28).

Only 59 of 65 isolates provided the sequencing data for all the desired codons of the *pfhps* gene. Twenty-three isolates (39.98%) had a wild-type PFDHPS genotype (S436A437K540A581).

Results

In vivo chloroquine response

A total of 1401 individuals with fever were screened for the presence of malarial parasites, of whom 65 were infected with *P. falciparum*, 8 with *Plasmodium vivax* and 3 with *P. vivax* and *P. falciparum*. A total of 51 patients were enrolled in the study, but 3 were lost during follow-up and were excluded. Nineteen of 48 individuals (39.58%) were completely cured by the chloroquine treatment as they remained free of parasites as well as disease symptoms during the 28 day follow-up period. The remaining 29 (60.42%) individuals showed resistance towards the chloroquine treatment; the majority of them (23 of 29, 79.31%) were in the ETF category (Table 1).

Table 1. In vivo chloroquine response among *P. falciparum* malaria patients from the island of Car Nicobar

<table>
<thead>
<tr>
<th>Age (years)</th>
<th>Sex (number of patients)</th>
<th>Haemoglobin concentration on day 0 (g/dL)</th>
<th>Parasitaemia on day 0 (asexual stages/μL)</th>
<th>Clinical outcome (number of patients)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>male</td>
<td>female</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1–4</td>
<td>2</td>
<td>1</td>
<td>9.5–10.0</td>
<td>1912–63529</td>
</tr>
<tr>
<td>5–8</td>
<td>0</td>
<td>0</td>
<td>0–0</td>
<td>0–0</td>
</tr>
<tr>
<td>9–14</td>
<td>3</td>
<td>0</td>
<td>10.0–12.0</td>
<td>1393–11800</td>
</tr>
<tr>
<td>15–59</td>
<td>31</td>
<td>14</td>
<td>9.5–13.0</td>
<td>1520–89756</td>
</tr>
</tbody>
</table>

ETF, early treatment failure; LTF, late treatment failure; ACPR, adequate clinical and parasitological response.
A613), 2 (3.39%) had the PfDHPS genotype S436G437K540A581A613. 4 (6.78%) had the PfDHPS genotype S436G437E540G581A613 and 3 (50.85%) had the PfDHPS genotype A436G437E540A581A613. Among 43 isolates from the in vivo chloroquine efficacy group, with sequence information for all of these codons, the wild-type was present in 41.86% of the isolates (n = 43), while the remaining isolates had either a single mutation [S436G437E540G581A613 (2.33%)] or triple mutations [S436G437E540G581A613 (6.98%) and A16G437E540A581A613 (48.84%)]. Among the chloroquine responder group, of 16 individuals who showed amplification, 43.75% had the wild-type pfdhps gene and 56.25% had genes with triple mutations (50% with A16G437E540A581A613 and 6.25% with S436G437E540G581A613). There was no significant difference in the number of patients with triple PfDHPS mutations between the chloroquine responder and non-responder groups.

A total of 58 samples yielded sequencing information for both pfdhfr and pfdhps genes. Only one isolate was found to have wild-type genotypes for both of the genes, whereas the rest of the 57 samples contained a mutation in one or both of the genes, resulting in 10 different combined genotypes (Table 2). The majority of the samples (46.55%, n = 58) contained seven PfDHFR–PfDHPS two-locus mutations. Samples containing a total of seven PfDHFR–PfDHPS two-locus mutations were also predominant among chloroquine responders (56.25%, n = 16) and non-responders (44.44%, n = 27).

There was no significant difference in the number of patients between chloroquine responder and non-responder groups containing mutant genotypes associated with either chloroquine or sulfadoxine/pyrimethamine resistance. Genotyping of all the LCF and LF samples collected on day 0 and on the treatment failure day showed the same merozoite surface protein 2, PfCRT, PfDHFR and PfDHPS alleles, suggesting recrudescence of infection and not re-infection among the patients (data not shown).

**Table 2.** Distribution of the two-locus PfDHFR–PfDHPS genotypes among chloroquine responders and non-responders from the island of Car Nicobar

<table>
<thead>
<tr>
<th>Type</th>
<th>PfDHFR–PfDHPS genotype</th>
<th>No. of mutations</th>
<th>Clinical outcome, number of samples/patients (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>total, n=58</td>
</tr>
<tr>
<td>1</td>
<td>A16N51C59S108I164 – S436A437K540A581A613</td>
<td>0</td>
<td>1 (1.72)</td>
</tr>
<tr>
<td>2</td>
<td>A16N51R59I164 – S436A437K540A581A613</td>
<td>2</td>
<td>7 (12.07)</td>
</tr>
<tr>
<td>3</td>
<td>A16N51C59S108I164 – A436G437E540A581A613</td>
<td>3</td>
<td>1 (1.72)</td>
</tr>
<tr>
<td>4</td>
<td>A16N51R59I164 – S436A437K540A581A613</td>
<td>3</td>
<td>1 (1.72)</td>
</tr>
<tr>
<td>6</td>
<td>A16I51R59I164 – S436A437E540A581A613</td>
<td>4</td>
<td>2 (3.45)</td>
</tr>
<tr>
<td>7</td>
<td>A16N51R59I164 – A436G437E540A581A613</td>
<td>5</td>
<td>4 (6.90)</td>
</tr>
<tr>
<td>8</td>
<td>A16N51S108I164 – A436G437E540A581A613</td>
<td>6</td>
<td>1 (1.72)</td>
</tr>
<tr>
<td>10</td>
<td>A16I51R59I164 – A436G437E540A581A613</td>
<td>7</td>
<td>23 (39.66)</td>
</tr>
</tbody>
</table>

ETF, early treatment failure; LTF, late treatment failure; APCR, adequate clinical and parasitological response.

Mutated amino acids are shown in bold.

Discussion

In vivo data from the present study show that a high percentage (60.42%) of individuals living on the island of Car Nicobar of the Andaman and Nicobar Islands were not responding to chloroquine treatment. This treatment failure rate is very high in comparison with the percentage of 10.61% (n = 66) found in our previous study.7 Our recent data from longitudinal studies, using molecular markers, have also predicted a temporal increase in chloroquine resistance in the parasite population of this island,5 which has now been confirmed here by the in vivo chloroquine response data.

All clinical isolates from the chloroquine non-responder group were found to contain the mutant PfCRT genotype. Surprisingly, all isolates from the chloroquine responder group were also found to contain the mutant PfCRT genotype C72V73I74E75T76, which is associated with a higher level of chloroquine resistance.10 Clearance of parasite with chloroquine in this group is intriguing, although other host factors and involvement of other gene products cannot be ruled out. In our previous report, we showed a few isolates from the pre-tsunami group (collected during May–October 2004) with wild-type C72V73I74E75T76 PCRT and none in subsequent years.5 Indeed, the selection of parasites with the PfCRT genotype C72V73I74E75T76 was very prominent among isolates collected during April–May 2008 and afterwards (this study). This decrease in isolates with wild-type PfCRT and the high chloroquine treatment failure rate observed here are a cause of concern and necessitated a change in drug policy for the region.

Based on these results, the Government of India has now changed its drug policy to allow the use of ACT in this region. However, this combination therapy is based on artesunate plus sulfadoxine/pyrimethamine, which we predict may not prove to be very effective as the majority of P. falciparum isolates from this island already contain mutations in the pfdhfr and pfdhps genes associated with a high level of antifolate resistance (Table 2).6,5 We therefore propose the usage of ACT in Car Nicobar, but with a different combination.

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**Transparency declarations**
None to declare.

**References**