SHORT REPORT: MOLECULAR EVALUATION OF THE EFFICACY OF
CHLOROQUINE TREATMENT OF UNCOMPlicated PLASMODIUM FALCIPARUM
MALARIA IN EAST TIMOR

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Abstract. The efficacy of chloroquine treatment of uncomplicated Plasmodium falciparum malaria in East Timor was investigated via molecular tools. Genotyping of the polymorphic markers msp1 and msp2 was performed to investigate the number and type of parasite alleles in pre- and posttreatment blood samples collected from 48 patients. Patients were infected with a minimum of 8 msp1 and 14 msp2 allelic types of parasite, and 43% of the patients had more than one allelic type before treatment. The genotyping also revealed that 66.7% of the patients were infected with at least one identical allelic type of parasite before and after treatment and therefore were likely to have experienced recrudescence. All parasites in pre- and posttreatment blood samples carried the K76T mutation in pfcrt, regardless of the clinical response to chloroquine. The sequence polymorphism patterns in pfcrt in the majority of parasites examined were identical to those observed in Bougainville, Papua New Guinea.

INTRODUCTION

Chloroquine has been used as first-line therapy for uncomplicated falciparum malaria in East Timor. However, until recently, reliable data on the efficacy of chloroquine in the country were unavailable. In mid-2000, MERLIN (Medical Emergency Relief International) conducted an in vivo chloroquine efficacy study in Lautem district, Los Palos, a hyper endemic area in the eastern sector of East Timor (Ezard N and others, unpublished data). Forty-eight East Timorese patients with uncomplicated Plasmodium falciparum malaria, who were aged between 8 months and 29 years and who lived within a 1-hour car journey, were enrolled. Results of a questionnaire on chloroquine usage before the initiation of the trial revealed that 9 of the 48 patients had taken chloroquine within a 1-month period preceding the trial. Overall, of 48 patients, two-thirds (32 of 48) experienced recurrent parasitemia after treatment with 1,500 mg chloroquine over 3 days, but one patient failed to respond to late treatment (recurrant parasitemia occurred 7–28 days after treatment) (Ezard N and others, unpublished data).

After clinical investigation, molecular analysis was performed on pre- and posttreatment blood samples collected from these patients to investigate whether the recurrent parasitemia resulted from parasites recrudesced under chloroquine treatment or from new infections and whether sequence polymorphism in the pfcrt gene can be used as a molecular marker to predict clinical outcomes after chloroquine treatment in this region.1,2 Blood samples were collected and preserved either on filter paper or in 6 M guanidine hydrochloride on days 0, 14, and 28 after commencing chloroquine treatment, or on the day when symptoms recurred. We extracted DNA via the Wizard Plus Minipreps DNA Purification System (Promega, Madison, WI) according to the manufacturer’s instructions. Msp1 and msp2 were amplified by polymerase chain reaction (PCR) and analyzed by agarose gel electrophoresis.3

Nested PCR was performed to amplify the pfcrt gene. Briefly, the first round of PCR was carried out in a 50-μL reaction mixture containing 1.5 μg/mL of each primer 5’-GTGGAGGTTCTTGTTGGT-3’ and 5’-TCCAGTAGTTCAGGGTTTTCA-3’ and 200 μM of dNTPs, buffer (50 mM KCl, 10 mM Tris-HCl, pH 8.3, 2.5 mM MgCl2), and 1.25 units of AmpliTaq Gold DNA polymerase (Perkin-Elmer, Norwalk, CT). The samples were denatured at 94°C for 10 minutes before 40 cycles of amplification (94°C for 50 seconds, 51°C for 50 seconds, and 70°C for 1 minute). The second round PCR were performed under the same conditions by use of the following primers: 5’-TGTTGCTCATGTGGTTAATACTT-3’ and 5’-AAAGCTCGGTGTTGCCTTC-3’.4

The nested PCR product was purified with a QIAquick PCR Purification Kit (Qiagen, Valencia, CA), then sequenced in an ABI PRISM 370 sequencer (Applied Biosystems, Inc., Foster City, CA).

Genotyping of 2 polymorphic markers, msp1 and msp2, revealed a minimum of 8 (410–580 bp) and 14 (380–800 bp) allelic types, respectively, indicating that many allelic types of P. falciparum were transmitted in the area. Multiple allelic type infections of P. falciparum were detected in 45.8% (22 of 48) of the patients before treatment, indicating a high level of malaria transmission in the area. The genotyping also revealed that 66.7% (32 of 48) of patients had at least one allelic type of parasite in posttreatment blood samples identical to those in pretreatment samples (Table 1, original types). Twenty-eight of these patients came from the in vivo resistant group and 4 from the in vivo susceptible group. Given the number of allelic types in the area, the chance of reinfecting with the same allelic type is low. Therefore, these patients are likely to have experienced recrudescence resulting from parasites that grew in the presence of chloroquine. Approximately 10% (5 of 48) of patients had different allelic types of parasites in their pre- and posttreatment blood samples, indicating new infections (Table 1, new types) during the 28 day follow-up period. Of the 5 patients with new infections, 4 were in the in vivo resistant group and 1 in the susceptible group. In the 16 patients classified as in vivo susceptible to chloroquine, genetic analysis revealed 4 patients to have recrudescent parasites and 1 patient to have a new infection. This finding suggests that these patients were infected with very low number of parasites that were below the limit of microscopic detection.
Allelic types and sequence polymorphisms detected in pre- and posttreatment blood samples and their correlations with in vivo response to chloroquine treatment

<table>
<thead>
<tr>
<th>In vivo chloroquine susceptibility</th>
<th>No. of patients (n = 48)</th>
<th>MSP1/MSP2 in posttreatment samples</th>
<th>Pretreatment Polymorphism in PFCRT protein</th>
<th>Posttreatment Polymorphism in PFCRT protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>MSP/M SP2 types</td>
<td>K76</td>
<td>SVMNT*</td>
</tr>
<tr>
<td>Susceptible</td>
<td>16</td>
<td>Original type</td>
<td>0</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td></td>
<td>New type</td>
<td>0</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Undetected</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>Resistant</td>
<td>32</td>
<td>Original type</td>
<td>0</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td></td>
<td>New type</td>
<td>0</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Undetected</td>
<td>0</td>
<td>40</td>
</tr>
</tbody>
</table>

*SVMNT and CVIET represent amino acids at positions 72 to 76 in PFCRT.

The pre- and posttreatment blood samples were also examined for sequence polymorphisms in the pfcr gene at positions corresponding to amino acids 72, 74, 75, and 76. Mutation K76T in pfcr has been demonstrated to correlate with decreased susceptibility to chloroquine in vitro, and sequence polymorphisms at amino acids 72, 74, and 75 are associated with geographical origins of parasite samples. At amino acid 76, however, a S or C has been observed in the South American/PNG isolates. A synonymous DNA sequence polymorphism of S_AG7 or S_CT7 was also observed in the isolates from mainland PNG. However, in our set of samples from East Timor and Bougainville, PNG, AGT was the only codon type identified in chloroquine-resistant parasites for S72. These findings suggest that majority of the chloroquine-resistant parasites from East Timor and Bougainville, PNG, may be derived from a common origin as a result of the geographical proximity of the 2 islands.

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