IN VITRO ANTIMALARIAL DRUG SUSCEPTIBILITY AND PFCRT MUTATION AMONG FRESH PLASMODIUM FALCIPARUM ISOLATES FROM THE LAO PDR (LAOS)

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Abstract. Recent drug trials in Laos have shown high levels of Plasmodium falciparum resistance to chloroquine, but there are no published data on in vitro antimalarial drug susceptibility. We used the double-site enzyme-linked pLDH immunodetection (DELI) assay to estimate the in vitro antimalarial drug susceptibility of 108 fresh P. falciparum isolates from southern Laos. The geometric mean (95% confidence interval) 50% inhibitory concentration values (nmol/L) were 152.4 (123.8–187.6) for chloroquine, 679.8 (533.8–863.0) for quinine, 45.9 (37.9–55.7) for mefloquine, 5.0 (4.4–6.4) for artesunate, 6.3 (4.5–8.9) for dihydroartemisinin, and 59.1 (46.4–75.3) for lumefantrine. The proportion of isolates defined as resistant were 65%, 40%, and 8% for chloroquine, quinine, and mefloquine, respectively. Of 53 isolates genotyped for the pfcrt T76K chloroquine-resistance mutation, 48 (91%) were mutants. P. falciparum in Laos is multi-drug resistant; antimalarial immunity resulting from the use of ineffective chloroquine before 2005 probably contributes significantly to the therapeutic responses in clinical trials.

INTRODUCTION

Antimalarial drug resistance in Plasmodium falciparum is a very difficult problem for malaria control in most of the tropical world. This is particularly serious in Southeast Asia where P. falciparum has developed resistance to almost all antimalarial drugs available. In this situation, it is important that monitoring of antimalarial drug efficacies should be carried out regularly so that optimum treatment strategies can be implemented. In the Lao PDR (Laos), P. falciparum malaria remains an important cause of morbidity and mortality, particularly in the southern provinces. Laos and adjacent northeastern Cambodia have been considered to have generally more drug sensitive parasites than elsewhere in the region. Chloroquine (CQ) and sulphadoxine-pyrimethamine (SP) resistance in P. falciparum were first reported in Laos in the late 1960s. Clinical trials of oral CQ and SP for the treatment of uncomplicated falciparum malaria at five different locations in Laos between 2000 and 2003 showed in vivo treatment failures of 35–80% for CQ and 18–35% for SP. In contrast, recent clinical trials have shown that artemisin + mefloquine, artemether-lumefantrine, and dihydroartemisinin-piperaquine are highly efficacious, with treatment failure rates of < 6%. CQ and SP remained the first- and second-line nationally recommended treatments for uncomplicated falciparum malaria until 2005, when the Lao Government changed treatment policy to artemisin-based combination therapy (ACT; artemether-lumefantrine). However, there is no published information on the in vitro antimalarial drug susceptibility of P. falciparum in Laos. We therefore measured the in vitro drug sensitivity of P. falciparum in Savan- nakhet Province and assessed the prevalence of the chloroquine resistance molecular marker pfcrt.

MATERIALS AND METHODS

Study site and sample collection. The study was conducted at Phalanxay District Clinic, Savannakhet Province (~605 km southeast of Vientiane) in parallel with clinical trials of antimalarial drugs between June 2003 and September 2004. Febrile patients presented with P. falciparum parasitemia ≥ 0.5% and without a history of antimalarial drug ingestion in the previous 2 weeks were included in the study provided that they (or their guardians) gave fully informed written consent. The study was approved by the Ethical Committee of the Faculty of Medical Sciences, National University of Laos, and the Oxford Tropical Medicine Research Ethics Committee, University of Oxford, UK.

Venous blood (3–5 mL) was collected in sterile heparinized tubes before patients received antimalarial drug treatment according to the protocols of the clinical trials. For patients not included in the trials, artemisin + mefloquine for 3 days or artemisin + doxycycline for 7 days were given. Blood samples were centrifuged immediately, the plasma anduffy coat were removed, and red blood cells were washed three times in RPMI 1640 (ICN; ICN Biomedicals, Costa Mesa, CA). Thin blood smears, stained with Field’s stain, were examined to determine parasite densities. The infected red blood cells were set up in the pre-dosed drug plates in complete RPMI with 10% heat-inactivated AB sera at a parasitemia of 0.5% parasitized erythrocytes and a hematocrit of 1.5%. If parasite densities exceeded 0.5%, samples were diluted with freshly washed uninfected red cells (group O) to obtain an initial parasitemia of 0.5%. Two hundred microliters of the suspension was distributed in each well in 96-well plates prepared with the antimalarial drugs (see below). Plates were incubated in a candle jar at 37°C for 48 hours.
P. falciparum isolates were obtained before storing at –30°C for a maximum of 3 months and then at –80°C until the double-site enzyme-linked pLDH immunodetection (DELI) assay was performed.

**Drug and plate preparation.** Chloroquine diphosphate and quinine citrate (Sigma Chemicals, St. Louis, MO), lumenfantrine (Novartis Pharmacia, Basel, Switzerland), sodium artemunate and dihydroartemisinin (Walter Reed Army Institute of Research [WRAIR], Washington, DC; courtesy of Dr. D. E. Kyle), and mefloquine hydrochloride (Hoffman-LaRoche, Basel, Switzerland) were used. Stock solutions of quinine, mefloquine, artemunate, and dihydroartemisinin were prepared in 70% ethanol (ETOH). Chloroquine stock solution was prepared in deionized water, and lumenfantrine was dissolved in a 1:1 (wt/vol) mixture of ETOH, Triton-X (Sigma), and linoleic acid (Sigma). All drugs were dissolved initially at a concentration of 1 mg/mL and sterilized by ultrafiltration. Serial dilutions were made in complete RPMI medium. The solvent in the final concentrations had no significant effect on parasite growth compared with culture media. The drugs and their respective final concentration ranges in cell-medium mixture were as follows: chloroquine, 10.67–683.0 ng/mL (20.68–1,323.9 nmol/L); quinine, 42.34–7,100 ng/mL (117.33–7,509.0 nmol/L); mefloquine, 1.95–124.7 ng/mL (4.70–300.63 nmol/L); lumenfantrine, 1.95–124.7 ng/mL (3.69–235.73 nmol/L); artemunate, 0.13–8.52 ng/mL (0.35–22.16 nmol/L); dihydroartemisinin (DHA), 0.13–8.25 ng/mL (0.47–29.96 nmol/L). All concentrations, including drug-free controls, were distributed in 25-μL aliquots in duplicate in 96-well tissue culture plates (Falcon; Becton Dickinson, Oxford, UK). The drug plates were made in bulk and stored at –80°C until use (for up to 3 months).

**In vitro drug sensitivity assay.** The double-site enzyme-linked parasite lactate dehydrogenase (pLDH) immunodetection (DELI) assay was used to assess *P. falciparum* antimarial drug susceptibility. In brief, the culture plates were thawed and frozen three times to lyse the cells. One hundred microliters from each well was transferred into 96-well plates (Nunc-Immuno plate; Maxisorb; Nalge Nunc International, Winooski, VT) to determine the optical density at 450 nm (EL800 Universal Microplate Reader; Bio-Tek Instruments, Winooski, VT) to determine the optical density at 450 nm with a reference filter at 690 nm.

**pfcr* genotyping.** To study the frequency of the *pfcr* mutation, parasites from blood spot filter papers from 53 patients from the clinical trial in 2003 were genotyped for the amino acid 76 mutation in the *pfcr* gene (*pfcr* K76T) using a polymerase chain reaction (PCR)-restriction digest assay and fluorescent detection of products. Briefly, a 132-bp section of *pfcr* was amplified by fluorescent end-labeled primers using semi-nested PCR. The fluorescent end-labeled products from the second PCR reaction were digested with *ApoI* (New England Biolabs, Ipswich, MA). Finally the digested products were loaded in an ABI 3100 capillary sequencer (Applied Biosystems, Foster City, CA). The *pfcr* resistant alleles were uncut, giving a peak at 132 bp, whereas wild-type alleles were cut giving a labeled fragment of 101 bp.

**Data analysis.** Dose–response curves, the concentration of the drugs that resulted in a 50% inhibition of parasite growth (IC50 values), and coefficients of variation were calculated by fitting the data to an inhibitory E-max pharmacokinetic model using WINNONLIN Version 4.1 (Pharsight Corp., Mountain View, CA). To ensure data quality, we rejected all IC50 values with coefficients of variation [(SE × 100)/mean] of estimated IC50 values > 30% and those in which the pLDH production in control wells (parasites, no drug) was less than five times background (red blood cells only). One outlier was removed. For curves from highly resistant or sensitive samples, the range of dilutions was insufficiently high to obtain accurate measures of IC50. In these cases, the curves were “forced” by adding an extra data point (0 indicating no growth or 1 indicating 100% growth) at the next or previous doubling concentration, respectively. This procedure results in conservative IC50 values while allowing us to retain data from interesting parasite isolates with unusually high IC50 values. The cut-off IC50 values for *in vitro* resistance to chloroquine, quinine, and mefloquine were defined as > 100, 800, and 108 nmol/L, respectively.

**RESULTS**

A total of 108 fresh *P. falciparum* isolates were obtained from symptomatic patients with uncomplicated falciparum malaria (48 women and 60 men). The mean (95% confidence interval [CI]) age (years) of the patients was 13.3 (11.4–15.3; range, 2–50 years), and 81% of them were children ≤ 15 years. The geometric mean (95% CI) parasitemia (parasites per microliter) at admission was 55,719 (44,720–69,438).

Of all isolates, 75 (69%), 68 (63%), 44 (41%), 70 (65%), 65 (60%), and 60 (56%) produced interpretable data by DELI assay for artesunate, chloroquine, DHA, lumenfantrine, mefloquine, and quinine, respectively (Table 1). The proportions of isolates resistant to chloroquine, quinine, and mefloquine were 65%, 40%, and 8%, respectively. The geometric mean (95% CI) IC508 (nmol/L) of isolates defined as resistant were

<table>
<thead>
<tr>
<th>Drug</th>
<th>N</th>
<th>Mean</th>
<th>95% CI</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Artesunate</td>
<td>75</td>
<td>5.02</td>
<td>4.44–6.43</td>
<td>0.84–21.9</td>
</tr>
<tr>
<td>Chloroquine</td>
<td>68</td>
<td>152.4</td>
<td>123.8–187.6</td>
<td>20.0–1,479</td>
</tr>
<tr>
<td>Dihydroartemisinin</td>
<td>44</td>
<td>6.29</td>
<td>4.47–8.90</td>
<td>0.69–23.2</td>
</tr>
<tr>
<td>Lumenfantrine</td>
<td>70</td>
<td>59.07</td>
<td>46.4–75.3</td>
<td>4.4–251</td>
</tr>
<tr>
<td>Mefloquine</td>
<td>65</td>
<td>45.93</td>
<td>37.0–53.5</td>
<td>4.7–223</td>
</tr>
<tr>
<td>Quinine</td>
<td>60</td>
<td>680</td>
<td>533–863</td>
<td>100–7,058</td>
</tr>
</tbody>
</table>
Antimalarial drugs were provided to the villages to assess future trends in resistance. Recent clinical trials have shown poor efficacy of chloroquine in different areas of Laos, and in the province where this in vitro study was conducted, the chloroquine treatment failure rate after 42-day follow-up was 36%. This finding is supported by the high level of in vitro chloroquine resistance and that 95% of the isolates were found to carry pfcrtp76t mutant alleles.

Forty (37%) patients were eligible and consented to the clinical trials of antimalarial drugs with 42-day follow-up. Of these, one patient who was treated with artesunate-lumefantrine had treatment failure (recurrence of parasitemia at day 21) as confirmed by parasite genotyping. The antimalarial drug IC_{50} (nmol/L) of the parasite isolate from this patient was 1.45, 639, 1.65, 23.1, 20.3, and 1,340 for artesunate, chloroquine, DHA, lumefantrine, mefloquine, and quinine, respectively.

Fifty-three isolates were available for genotyping of pfcrtp76t and 20 had chloroquine in vitro drug susceptibility results. Of all samples genotyped, 48 (91%) were found to be pfcrtp76t mutant types. One of the 20 isolates was wild-type at pfcrtp76t and had a chloroquine IC_{50} of 167 nmol/L.

**DISCUSSION**

We studied the in vitro susceptibility of *P. falciparum* to antimalarial drugs to provide information on antimalarial drug resistance patterns at a clinical trial site in Laos and as a necessary baseline to assess future trends in resistance. Recent clinical trials have shown poor efficacy of chloroquine in different areas of Laos, and in the province where this in vitro study was conducted, the chloroquine treatment failure rate after 42-day follow-up was 36%.

This finding is supported by the high level of in vitro chloroquine resistance and that 95% of the isolates were found to carry pfcrtp76t mutant alleles.

**Correlation of the in vitro responses of Lao *P. falciparum* isolates among antimalarial drugs**

<table>
<thead>
<tr>
<th>Drug 1</th>
<th>Drug 2</th>
<th>N*</th>
<th>r†</th>
<th>P‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Artesunate</td>
<td>Chloroquine</td>
<td>62</td>
<td>0.41</td>
<td>0.10</td>
</tr>
<tr>
<td>DHA</td>
<td>DHA</td>
<td>42</td>
<td>0.84</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Lumefantrine</td>
<td>Lumefantrine</td>
<td>61</td>
<td>0.72</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Mefloquine</td>
<td>Mefloquine</td>
<td>63</td>
<td>0.69</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Quinine</td>
<td>DHA</td>
<td>50</td>
<td>0.53</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>Lumefantrine</td>
<td>55</td>
<td>0.34</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td>Mefloquine</td>
<td>56</td>
<td>0.32</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td>Quinine</td>
<td>49</td>
<td>0.45</td>
<td>0.001</td>
</tr>
<tr>
<td>Dihydroartemisinin</td>
<td>Lumenfantrine</td>
<td>35</td>
<td>0.56</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Mefloquine</td>
<td>Mefloquine</td>
<td>34</td>
<td>0.50</td>
<td>0.003</td>
</tr>
<tr>
<td>Quinine</td>
<td>Mefloquine</td>
<td>28</td>
<td>0.60</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>Quinine</td>
<td>57</td>
<td>0.74</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>Quinine</td>
<td>50</td>
<td>0.55</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

* N, number of paired results.
† Correlation coefficient (r) was calculated from linear regression analysis of logarithmic IC_{50}.
‡ Probability (P) refers to the significance level of the test.

Types of antimalarial drugs in Laos are not clinically significant. The geometric (SD) IC_{50}s for artesunate and DHA were higher in Laos than in Thailand (Table 3), although these differences are not clinically significant. The geometric (SD) in vitro arte-

in vitro
sunate IC₅₀ of *P. falciparum* in Cambodia was, at 1.25 (2.8) nmol/L, slightly lower than those collected in Laos. However, because the ³H-hypoxanthine technique was used, these data cannot be reliably compared with those from Laos. The IC₅₀ of lumefantrine in this study was similar to that from northwestern Thailand using the same DELI technique.

In conclusion, *in vitro* data suggest that high levels of resistance to chloroquine and quinine but not to mefloquine, lumefantrine, and the artemisinin derivatives have developed in this area of Laos. More information on the *in vivo* response to quinine therapy in Laos is needed. Regular monitoring of antimalarial drug efficacy, with mapping of the distribution of molecular makers of drug resistance, needs to be carried out in Laos to monitor the pattern of antimalarial drug resistance and assist in determining the rational antimalarial policy.

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