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 north-eastern 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 artesunate + 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 artemisinin-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-

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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, artesunate + mefloquine for 3 days or artesunate + doxycycline for 7 days were given. Blood samples were centrifuged immediately, the plasma and buffy 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 1640 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...
before storing at −30°C for a maximum of 3 months and then at −80°C until the double-site enzyme-linked pLDH immuno- detection (DELI) assay was performed. **Drug and plate preparation.** Chloroquine diphosphate and quinine citrate (Sigma Chemicals, St. Louis, MO), lumefan- 	rine (Novartis Pharmacia, Basel, Switzerland), sodium arte- sunate 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, artesunate, and dihydroartemisinin were prepared in 70% ethanol (ETOH). Chloroquine stock solution was prepared in deionized water, and lumefantrine 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 ul- trafiltration. Serial dilutions were made in complete RPMI medium. The solvent in the final concentrations had no signif- icant 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, 42.34–2,710.0 
ng/mL (117.33–7,509.0 nmol/L); mefloquine, 1.95–124.7 ng/
/mL (4.70–300.63 nmol/L); lumefantrine, 1.95–124.7 ng/mL 
(3.69–235.73 nmol/L); artesunate, 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, Ox- ford, 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) immunodetec-
tion (DELI) assay was used to assess *P. falciparum* antimal- 
arial drug susceptibility.16–19 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, 
Roskilde, Denmark) pre-coated with a capture monoclonal 
(anti-pLDH, 19G7, which specifically recognizes the pLDH, and 
biotinylated anti-pLDH monoclonal antibody 19E419, which 
specifically recognizes the pLDH, and was added, and the plates 
were incubated for 1 hour at 37°C. After washing three times 
with phosphate-buffed saline (PBS)/0.5% bovine serum album- in (BSA Fraction V; Roche Diagnostics, Mannheim, Germany), 
a second biotinylated anti-pLDH monoclonal antibody 19G719 
was added, and the plates were incubated for 1 hour at 37°C. After removal of unbound antibody by washing three 
times with PBS/0.5% BSA, the plates were incubated at room 
temperature for 30 minutes with a 1:10,000 solution of streptavidin-POD conjugate (Roche Diagnostics). After washing 
the plates three times with PBS/0.5% BSA, the plates were 
incubated for up to 20 minutes at room temperature with a 
peroxidase substrate solution, 3,3′,5,5′-tetramethyl- 
benzidine (KPL, Gaithersburg, MD). The reaction was 
stopped with 1 mol/L phosphoric acid, and color development 
was quantified immediately using a spectrophotometer 
(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 t genotyping.** To study the frequency of the pfcrt mu-
tation, 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 pfcrt gene (pfcr t K76T) using a poly-
merase chain reaction (PCR)-restriction digest assay and 
fluorescent detection of products.20 Briefly, a 132-bp section of 
pfcrt was amplified by fluorescent end-labeled primers using 
semi-nested PCR. The fluorescent end-labeled products from 
the second PCR reaction were digested with Apor l (New 
England Biolabs, Ipswich, MA). Finally the digested products 
were loaded in an ABI 3100 capillary sequencer (Applied 
Biosystems, Foster City, CA). The pfcrt 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 ob-
tain 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.21 The cut-off IC50 values for *in vitro* resistance to 
chloroquine, quinine, and mefloquine were defined as > 100, 
800, and 108 nmol/L, respectively.22–27 IC50 cut-off values for 
artesunate, DHA, and lumefantrine resistance have not yet 
been established. **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, lumefantrine, me- 
floquine, 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) IC50 (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>Lumefantrine</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>37.9–55.7</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>
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</table>
246 (203–298), 1,613 (1,240–2,098), and 146 (105–203) for chloroquine, quinine, and mefloquine, respectively. The geometric mean (95% CI) IC\textsubscript{50} (nmol/L) of isolates defined as sensitive were 63 (54–75), 381 (312–465), and 42 (34.54–50.36) for chloroquine, quinine, and mefloquine, respectively. Because the IC\textsubscript{50} cut-off values for artesunate, DHA, and lumefantrine resistance have not been established, the percentage of isolates resistant to these drugs cannot be calculated. There were no significant correlations between chloroquine \textit{in vitro} IC\textsubscript{50} values and those for artesunate and DHA (r = 0.10; P = 0.41 and r = 0.13; P = 0.46, respectively). There were significant positive correlations between IC\textsubscript{50} values among the other drugs (P ≤ 0.02; Table 2).

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 artemether-lumefantrine had treatment failure (recurrence of parasitemia at day 21) as confirmed by parasite genotyping.\textsuperscript{13} The antimalarial drug IC\textsubscript{508} (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 \textit{pfcr} K76T, and 20 had chloroquine \textit{in vitro} drug susceptibility results. Of all samples genotyped, 48 (91%) were found to be \textit{pfcr} T76 mutant types. One of the 20 isolates was wild-type at \textit{pfcr} and had a chloroquine IC\textsubscript{50} of 167 nmol/L.

**DISCUSSION**

We studied the \textit{in vitro} susceptibility of \textit{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 \textit{in vitro} study was conducted, the chloroquine treatment failure rate after 42-day follow-up was 36%.\textsuperscript{8} This clinical finding is supported by the high level of \textit{in vitro} chloroquine resistance and that 95% of the isolates were found to carry \textit{pfcr} mutant types. Studies in the north and the far south of Laos have also shown a high prevalence (64–100%) of \textit{pfcr} K76T mutants.\textsuperscript{8,14,15,28} Using the same DELI assay technique as used here, the mean IC\textsubscript{50} of chloroquine in our study was comparable with that found in Thailand and Senegal\textsuperscript{18,29} but higher than that reported in Burkina Faso (Table 3).\textsuperscript{17} Comparisons between \textit{in vitro} susceptibility studies, especially those using different methods, are difficult, and there are no other published data from Southeast Asia using the DELI technique. Additional limitations of the study include the absence of quality control data to allow more accurate comparisons between studies, because we could not maintain cultures of control \textit{P. falciparum} strains in the field. The relatively low recovery of IC\textsubscript{50} information, particularly for DHA, probably result from the stringent criteria we used for acceptance of data (coefficient of variation < 30%).

Therapeutic responses to chloroquine are generally better in Laos than in adjacent Thailand, Cambodia, and Viet Nam, but the parasites causing these infections seem to be as resistant or more resistant as those in the adjoining countries.\textsuperscript{30–32} The significantly lower risk of treatment failure among adults compared with children in \textit{P. falciparum} clinical trials in Laos strongly suggests that patient immunity plays a considerable role in the response to chloroquine and other antimalarial drugs there.\textsuperscript{33} In higher transmission settings, such a discrepancy between \textit{in vitro} and \textit{in vivo} susceptibility is a usual observation, but transmission intensities in southern Laos are low and not significantly higher than other parts of the region.\textsuperscript{34} In adjacent Thailand, chloroquine was abandoned nearly 30 years ago because of very high treatment failure rates. The acquisition of significant immunity in Laos has resulted presumably from the widespread use of chloroquine and SP as antimalarials until last year and the consequently greater individual exposure to \textit{P. falciparum} parasites. The mapping of the geographical distribution of molecular markers of resistance suggests that the falciparum parasites in Savannakhet Province are more sensitive to chloroquine and folate drugs than elsewhere in the country (Mayxay and others, unpublished data). Falciparum parasites in northern and southern Laos are therefore likely to be less sensitive \textit{in vitro} than those described here.

In Laos, quinine is an alternative for the treatment of both severe and uncomplicated malaria. It has been used alone extensively in villages by village health volunteers and in hospitals. However, no studies have been carried out to assess the \textit{in vivo} or \textit{in vitro} efficacy of this drug. In this study, 40% of the parasite isolates showed resistance to quinine based on a cut-off of 800 nmol/L. This supports the recommendation that quinine should not be used alone in Laos, but combined with doxycycline. The mean IC\textsubscript{50} of quinine in this study was similar to that given in a recent report from the western border of Thailand using the same DELI technique.\textsuperscript{18}

Mefloquine is very rarely used in Laos, except by tourists as prophylaxis, because it is expensive and rarely available. Consistent with this observation, the proportion of mefloquine resistant \textit{P. falciparum} found in this study was low. The few mefloquine-resistant parasites found in southern Laos may reflect intrinsically resistant parasites in this area or flow of parasites from southern Vietnam or Cambodia/Thailand. The \textit{P. falciparum} IC\textsubscript{508} for artesunate and DHA were higher in Laos than in Thailand (Table 3), although these differences are not clinically significant. The geometric (SD) \textit{in vitro} arte-

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**Table 2**

<table>
<thead>
<tr>
<th>Drug 1</th>
<th>Drug 2</th>
<th>N\textsuperscript{*}</th>
<th>r†</th>
<th>P\textsuperscript‡</th>
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</thead>
<tbody>
<tr>
<td>Artesunate</td>
<td>Chloroquine</td>
<td>62</td>
<td>0.41</td>
<td>0.10</td>
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<tr>
<td>DHA</td>
<td>Artesunate</td>
<td>42</td>
<td>0.84</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Lume...</td>
<td>DHA</td>
<td>61</td>
<td>0.72</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Mefloquine</td>
<td>Chloroquine</td>
<td>63</td>
<td>0.69</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Quinine</td>
<td>Mefloquine</td>
<td>50</td>
<td>0.53</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>DHA</td>
<td>Lume...</td>
<td>32</td>
<td>0.13</td>
<td>0.46</td>
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<td>Mefloquine</td>
<td>Lume...</td>
<td>55</td>
<td>0.34</td>
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<tr>
<td>Quinine</td>
<td>Mefloquine</td>
<td>56</td>
<td>0.32</td>
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<tr>
<td>DHA</td>
<td>Quinine</td>
<td>49</td>
<td>0.45</td>
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<tr>
<td>Lume...</td>
<td>Mefloquine</td>
<td>35</td>
<td>0.56</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Mefloquine</td>
<td>Lume...</td>
<td>34</td>
<td>0.50</td>
<td>0.003</td>
</tr>
<tr>
<td>Quinine</td>
<td>Lume...</td>
<td>28</td>
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<tr>
<td>DHA</td>
<td>Quinine</td>
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<tr>
<td>Mefloquine</td>
<td>Quinine</td>
<td>50</td>
<td>0.55</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

\textsuperscript{*} N, number of paired results.
\textsuperscript{†} Correlation coefficient (R) was calculated from linear regression analysis of logarithmic IC\textsubscript{50}.
\textsuperscript{‡} Probability (P) refers to the significance level of the test.
sunate IC$_{50}$ of P. falciparum in Cambodia was, at 1.25 (2.8) nmol/L, slightly lower than those collected in Laos. However, because the $^3$H-hypoxanthine technique was used, these data cannot be reliably compared with those from Laos. The IC$_{50}$ of lumefantrine in this study was similar to that from northwestern Thailand using the same DELI technique.\textsuperscript{18}

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 markers 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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