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

MAYFONG MAYXAY, MARION BARENDS, ALAN BROCKMAN, ANCHALEE JAIDEE, SHALINI NAIR, DAN SUDIMACK, TIENTHONG PONGVONGSA, SAMLANE PHOMPIDA, RATTANAXAY PHETSOUVANH, TIM ANDERSON, NICHOLAS J. WHITE, AND PAUL N. NEWTON*

Wellcome Trust-Mahosot Hospital-Oxford Tropical Medicine Research Collaboration, Mahosot Hospital, Vientiane, Laos; Department of Post Graduate and Research, Faculty of Medical Science, National University of Laos, Laos PDR; Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand; Shoklo Malaria Research Unit, Mae Sot, Tak Province, Thailand; Australian Centre for International Tropical Health and Nutrition, University of Queensland, Brisbane, Australia; Southwest Foundation for Biomedical Research, San Antonio, Texas; Savannakhet Malaria Station, Savannakhet Province, Laos PDR; Centre of Malariology, Parasitology and Entomology, Vientiane, Laos PDR; Centre for Clinical Vaccinology and Tropical Medicine, Churchill Hospital, Oxford, United Kingdom.

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.1 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.2–5 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.6–10 In contrast, recent clinical trials have shown that artesunate + mefloquine, artemether-lumefantrine, and dihydroartemisinin-piperine are highly efficacious, with treatment failure rates of < 6%.11–13 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 artesinin-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-

* Address correspondence to Paul N. Newton, Microbiology Laboratory, Mahosot Hospital, Vientiane, Laos PDR. E-mail: paul@tropmedres.ac

nakhet Province and assessed the prevalence of the chloroquine resistance molecular marker pfcrt.14,15

MATERIALS AND METHODS

Study site and sample collection. The study was conducted at Phalanaxay District Clinic, Savannakhet Province (~605 km southeast of Vientiane) in parallel with clinical trials of antimalarial drugs between June 2003 and September 2004.11,13 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.11,13 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 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 immunodetection (DELI) assay was performed.

**Drug and plate preparation.** Chloroquine diphosphate and quinine citrate (Sigma Chemicals, St. Louis, MO), lumefantrine (Novartis Pharmacia, Basel, Switzerland), sodium artesunate and dihydroartemisinin (Walter Reed Army Institute of Research [WRAIR], Washington, DC; courtesy of Dr. D. E. Kyle), and mefloquine hydrochloride (Hoffmann-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 ultratitration. 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, 42.34–2,710.0 ng/mL (117.33–7,509.0 nmol/L); mefloquine, 1.95–124.7 ng/mL (0.35–22.16 nmol/L); lumefantrine, 1.95–124.7 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* antimarialar 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 antibody 17E419, which specifically recognizes the pLDH, and incubated for 1 hour at 37°C. After washing three times with phosphate-buffered saline (PBS)/0.5% bovine serum albumin (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′-tetramethylbenzidine (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 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.20 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/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.24 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 artemunate, 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 artemunate, chloroquine, DHA, lumefantrine, 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) IC50 (nmol/L) of isolates defined as resistant were

<table>
<thead>
<tr>
<th>Drug</th>
<th>N</th>
<th>Mean (95% CI)</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Artsunate</td>
<td>75</td>
<td>5.02 (4.44–6.43)</td>
<td>0.84–21.9</td>
</tr>
<tr>
<td>Chloroquine</td>
<td>68</td>
<td>152.4 (123.8–187.6)</td>
<td>20.0–1,479</td>
</tr>
<tr>
<td>Dihydroartemisinin</td>
<td>44</td>
<td>6.29 (4.47–8.90)</td>
<td>0.69–23.2</td>
</tr>
<tr>
<td>Lumefantrine</td>
<td>70</td>
<td>59.07 (46.4–75.3)</td>
<td>4.4–251</td>
</tr>
<tr>
<td>Mefloquine</td>
<td>65</td>
<td>45.93 (37.9–55.7)</td>
<td>4.7–223</td>
</tr>
<tr>
<td>Quinine</td>
<td>60</td>
<td>680 (533–883)</td>
<td>100–7,058</td>
</tr>
</tbody>
</table>
Using the same DELI assay technique as used here, the mean IC₅₀ of chloroquine in our study was comparable with that found in Thailand and Senegal, but higher than that reported in Burkina Faso (Table 3). Comparisons between 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 P. falciparum strains in the field. The relatively low recovery of IC₅₀ 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 than those in the adjoining countries. The significantly lower risk of treatment failure among adults compared with children in 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. In higher transmission settings, such a discrepancy between in vitro and 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. 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 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 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 in vivo or 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 IC₅₀ 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.

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 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 P. falciparum IC₅₀₈ₐ for artemunate and DHA were higher in Laos than in Thailand (Table 3), although these differences are not clinically significant. The geometric (SD) in vitro arte-

### Table 2

<table>
<thead>
<tr>
<th>Drug 1</th>
<th>Drug 2</th>
<th>N*</th>
<th>r†</th>
<th>P†</th>
</tr>
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<tbody>
<tr>
<td>Artesunate</td>
<td>Chloroquine</td>
<td>62</td>
<td>0.41</td>
<td>0.10</td>
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<td></td>
<td>DHA</td>
<td>42</td>
<td>0.84</td>
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<tr>
<td></td>
<td>Lumefantrine</td>
<td>61</td>
<td>0.72</td>
<td>&lt; 0.001</td>
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<tr>
<td></td>
<td>Mefloquine</td>
<td>63</td>
<td>0.69</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td></td>
<td>Quinine</td>
<td>50</td>
<td>0.53</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Chloroquine</td>
<td>DHA</td>
<td>32</td>
<td>0.13</td>
<td>0.46</td>
</tr>
<tr>
<td></td>
<td>Lumefantrine</td>
<td>55</td>
<td>0.34</td>
<td>0.01</td>
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<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>Lumefantrine</td>
<td>35</td>
<td>0.56</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td></td>
<td>Mefloquine</td>
<td>34</td>
<td>0.50</td>
<td>0.003</td>
</tr>
<tr>
<td></td>
<td>Quinine</td>
<td>28</td>
<td>0.60</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Lumefantrine</td>
<td>Mefloquine</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>
<tr>
<td>Mefloquine</td>
<td>Quinine</td>
<td>46</td>
<td>0.59</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 IC₅₀.‡ Probability (P) refers to the significance level of the test.
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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Authors’ addresses: Mayfong Mayxay, Nicholas J. White, and Paul N. Newton, Wellcome Trust–Mahosot Hospital–Oxford Tropical Medicine Research Collaboration, Mahosot Hospital, Mahosot Road, Vientiane, Lao PDR, Telephone: 85621-250752, Fax: 85621-242168, E-mail: paul@tropmedres.ac. Mayfong Mayxay, Department of Medicine, Faculty of Medical Science, National University of Laos, Lao PDR, Telephone: 85621-250752, Fax: 85621-242168, E-mail: mmayxay@yahoo.com. Marion Barends, Alan Brockman, and Anchalee Jaidee, Shoklo Malaria Research Unit, Mae Sot, Tak Province, Thailand. Marion Barends, Nicholas J. White, and Paul N. Newton, Centre for Clinical Vaccinology and Tropical Medicine, Churchill Hospital, Oxford, UK. Marion Barends, Alan Brockman, and Nicholas J. White, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand. Marion Barends, Alan Brockman, and Nicholas J. White, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand, 420/6 Rajvithi Road, Bangkok, 10400, Thailand, Telephone: +66 23549172; Fax: +66 23549169; E-mail: nickw@tropmedres.ac. Alan Brockman, Australian Centre for International Tropical Health and Nutrition, University of Queensland, Brisbane, Australia. Shalini Nair, Dan Sudimack, and Tim Anderson, Southwest Foundation for Biomedical Research, San Antonio, Texas, Telephone: +210-2389596, Tiengkham Pongvongsa, Savannakhet Provincial Malaria Station, Savannakhet Province, Lao PDR, Phone: 85621-214040, Fax: 85621-218131.

**REFERENCES**


2. Ebisawa I, Muto T, Kameko S, Mitsui G, 1970. Response of...


