No Evidence for Spread of *Plasmodium falciparum* Artemisinin Resistance to Savannakhet Province, Southern Laos

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**Abstract.** We conducted an open-label, randomized clinical trial to assess parasite clearance times (PCT) and the efficacy of 4 mg/kg (group 1, n = 22) and 2 mg/kg (group 2, n = 22) of oral artemunate for three days followed by artemether-lumefantrine in patients with uncomplicated *Plasmodium falciparum* malaria at Xepon Interdistrict Hospital, Savannakhet Province in southern Laos. Slides were read in duplicate. The overall mean (95% confidence interval; range) PCT in hours was 23.2 (21.2–25.3; 12–46) and 22.4 (20.3–24.5; 12–46) for the first and second microscopists, respectively (P = 0.57). Ten (23%) patients remained parasitemic on day 1 after treatment (4 [18%] in group 1 and 6 [27%] in group 2; P = 0.47). No patient had patent asexual parasitemia on the second and third days of treatment. The 42-day polymerase chain reaction–corrected cure rates were 100% in both treatment groups. Serious adverse events did not develop during or after treatment in any patients. In conclusion, no evidence of *P. falciparum in vivo* resistance to artemunate was found in southern Laos.

**INTRODUCTION**

Parasitologic responses to artemunate-containing treatment regimens for uncomplicated *Plasmodium falciparum* malaria have recently been shown to be slower in western Cambodia than elsewhere in the world. Both prolonged parasite clearance times (PCTs) and unusually high treatment failure rates after artemesate-mefloquine have been reported despite adequate antimalarial blood concentrations.1–3 Although poor responses to artemesate have been described,4 the recent reports suggest a consistent focal problem. There is also evidence for slightly prolonged PCTs and higher treatment failure rates after artemesate-mefloquine on the Thailand/Myanmar (Burma) border.5,6 In pooled data from 18,699 patients who received artemisinin derivatives, of those who were non-hyperparasitemic, only 1.8–5% had PCTs > 2 days.12–14 These results are reassuring. However, these studies involved daily blood sampling for parasite counts, rather than six hourly counts, which would be required to detect anything other than gross changes in PCT. Furthermore, the last available data were collected in 2008. We therefore conducted a randomized clinical trial comparing oral artemunate (2 mg/kg/day versus 4 mg/kg/day for 3 days) followed by artemether-lumefantrine to detect anything other than gross changes in PCT.
count on day 3 is \( P < 0.05 \) if 20 patients are recruited in each arm.7

Patients with uncomplicated *P. falciparum* malaria were enrolled in the study provided that they gave fully informed written consent, had *P. falciparum* mono-infections with an asexual parasite density of 10,000–175,000 parasites/μL, were \( \geq 10 \) years of age (and for female patients were before menarche or after menopause), had a tympanic temperature \( \geq 37.5^{\circ} \text{C} \) or history of fever during the previous 24 hours, were able to swallow oral medication, and were willing to stay in the hospital until parasite clearance and complete 42-days of follow up. Patients were excluded if they had danger signs or severe *P. falciparum* malaria,15 severe malnutrition, febrile conditions caused by diseases other than malaria or other known underlying chronic or severe diseases, had received antimalarial drugs in the previous 48 hours, had a history of hypersensitivity reactions or contraindications to study medicines, or had had a splenectomy.

On admission, blood samples were taken for parasite count and hemocrit, and three blood spots were collected onto filter paper for polymerase chain reaction (PCR) genotyping in the event of reappearance of *P. falciparum* during follow-up.12–14 The PCR amplification was performed on paired samples for parasite genotyping to distinguish between reinfection and recrudescence by using three parasite loci (merozoite surface protein 1, merozoite surface protein 2, and glutamate-rich protein).16

Patients who met the enrollment criteria were randomized (the treatment allocation was kept in a sealed opaque envelope, which was opened only after the decision to recruit had been made) to oral artesunate (Batch AS091001; Guilin Pharmaceutical Company, Shanghai, China), either 4 mg/kg/day (group 1) or 2 mg/kg/day (group 2) for 3 days, followed by the ACT recommended by the national treatment guidelines; artemether-lumefantrine (20/120 mg) (Coartem®, Novartis, Basel, Switzerland), 1 dose twice a day for three days. Dosing by body weight was 1 tablet if \( < 15 \) kg, 2 tablets if 15–24 kg, 3 tablets if 25–34 kg, and 4 tablets if \( \geq 35 \) kg. The artesunate used was quality controlled by Research Institute for Industrial Pharmacy (Potchefstroom, South Africa). Drug administration was directly observed and if vomiting occurred within one hour of dosing, medication was re-administered. All antimalarial drugs were stored at 6–10°C.

Tympanic temperature, parasite counts, and hemocrit were measured every 6 hours until parasite clearance and then daily until discharge and on days 7, 14, 21, 28, 35, and 42. A standard physical examination was performed on day 0 (before drug administration), and the patients were reviewed daily until parasites cleared, then weekly for 42 days from the start of treatment, or at other times if he or she felt unwell. At each visit, finger prick blood was taken for a malaria smear and hemocrit. Three blood spots were collected onto filter paper from those with reappearance of asexual parasitemia. Patients with *P. falciparum* recurrence were re-treated with artesunate, 4 mg/kg/day for 7 days plus mefloquine, 15 mg base/kg on day 1 and 10 mg base/kg on day 2 and followed up for an additional 42 days. Those who had *P. vivax* appearance during follow-up were treated with chloroquine (25 mg base/kg) for 3 days and further followed-up. The incidence of any adverse event was recorded.

Written informed consent was obtained from all participants. Ethical clearance for the study was granted by the Lao National Ethics Committee for Health Research, the Oxford University Tropical Medicine Research Ethics Committee, United Kingdom and the Research Ethics Review Committee of the World Health Organization. The trial registration number is ISRCTN12741594. The trial was monitored by the Clinical Trials Support Group of Mahidol-Oxford Tropical Medicine Research Unit, Bangkok, Thailand.

**Laboratory investigations and outcome measures.** Parasite counts for thick and thin blood films, stained with Field’s stain, and hemoglobin were determined every six hours until parasite clearance for two consecutive slides and then daily until patients’ hospital discharge and weekly after day 7.12–14 Two qualified microscopists (Level 1/expert malaria microscopists certified by the World Health Organization, Geneva, Switzerland, and the Asian Collaborative Training Network for Malaria, Manila, the Philippines) read all the slides independently. The PCR amplification was performed on paired samples for parasite genotyping to distinguish between reinfection and recrudescence.17

The primary study outcome was the number of patients with PCT > 72 hours. The PCT was defined as the time in hours from the first treatment dose to the first of two consecutive thick blood films that were negative for asexual *P. falciparum* parasites after checking ≥ 200 oil-immersion fields. The secondary outcomes were the PCR-corrected adequate clinical and parasitologic response at day 42,18 fever clearance times, time in hours from the start of treatment at which the tympanic temperature first decreased to < 37.5°C and remained below 37.5°C for 48 hours), the frequency of adverse events and fractional changes in hemocrit after antimalarial treatment.

**Statistical analysis.** Data were entered into a database by double independent data entry and cross-validated. Data were analyzed by using SPSS version 11.0 (SPSS Inc., Chicago, IL). Comparisons between the two treatment groups were made by using the Mann–Whitney U test, Student’s *t*-test, chi-square test, and Fisher’s exact test as appropriate. Parasite clearance rates were calculated as the slope of the linear portion of the log-normalized parasite clearance curve, and this was used to estimate the times to clearance of 50% and 90% of the parasite load for each patient. The parasite reduction ratio was defined as the proportion of admission parasites that remained after 24 hours.

**RESULTS**

Patient screening and enrollment are shown in Figure 1. Baseline characteristics of the two study groups were similar (Table 1). The male preponderance reflects the decision of the Institutional Review Board of the World Health Organization that women of childbearing age could not be recruited. Because three patients were found after randomization to have taken antimalarial drugs before recruitment, four additional patients were randomized so that 20 patients without prior antimalarial drug use were recruited into each arm. All patients (100%) completed 42 days of follow-up (Figure 1).

**Admission parasitemia, parasite reading, and clearance.** The overall admission geometric mean (95% CI) parasitemia/μL was 45,113 (35,473–57,372) (Table 1). The percentage (95% CI) agreement in reading slides (as positive or negative) between the first and second microscopists was 96.4% (94.4–97.6%) (κ = 0.92), which is classified as very good agreement.19
The slide-reading results from the first microscopist were used to calculate the slopes and parasite reduction ratios (Figure 2). The overall mean (95% CI; range) PCTs (hours) were 23.2 (21.2–25.3; 12–46) and 22.4 (20.3–24.5; 12–46) for the first and second microscopists, respectively (P = 0.57). Ten (23%) patients remained parasitemic on day 1 after treatment (4 [18%] and 6 [27%] in group 1 and group 2, respectively; P = 0.47). No patient had patent asexual parasitemia on day 30.

Figure 1. Participants in a study comparing 4 mg/kg/day versus 2 mg/kg/day of oral artesunate for treatment of *Plasmodium falciparum* Malaria in Laos. *Pf* = *P. falciparum*; *Pv* = *P. vivax*; PCR = polymerase chain reaction.

The slide-reading results from the first microscopist were used to calculate the slopes and parasite reduction ratios (Figure 2). The overall mean (95% CI; range) PCTs (hours) were 23.2 (21.2–25.3; 12–46) and 22.4 (20.3–24.5; 12–46) for the first and second microscopists, respectively (P = 0.57). Ten (23%) patients remained parasitemic on day 1 after treatment (4 [18%] and 6 [27%] in group 1 and group 2, respectively; P = 0.47). No patient had patent asexual parasitemia on day 30.

Table 1
Admission demographics and clinical and laboratory details for patients enrolled in a study comparing 4 mg/kg/day versus 2 mg/kg/day of oral artesunate for three days followed by three days of artemether-lumefantrine for treatment of *Plasmodium falciparum* malaria in Laos*

| Variable                              | Treatment groups |
|---------------------------------------|------------------|
|                                       | All (n = 44)     | 4 mg/kg/day (n = 22) | 2 mg/kg/day (n = 22) |
| Male sex, no (%)                      | 38 (86)          | 20 (91)              | 18 (82)              |
| Age, years, median (range)            | 28 (11–66)       | 28 (11–66)           | 25 (12–65)           |
| Height, cm                            | 155.4 (151.6–159.2) | 158.9 (155.6–162.2) | 151.9 (145.1–158.7) |
| Body weight, kg                       | 49.8 (45.8–53.8) | 51.7 (46.7–56.7)     | 47.9 (41.5–54.4)     |
| Tympanic temperature, °C              | 38.5 (38.1–38.9) | 38.3 (37.8–38.8)     | 38.7 (38.1–39.2)     |
| Patients without fever on admission, no. (%) | 9 (20)           | 6 (27)               | 3 (14)               |
| Systolic blood pressure, mm Hg        | 109.1 (106.2–112.1) | 108.6 (104.7–112.6) | 109.5 (104.9–114.2) |
| Diastolic blood pressure, mm Hg       | 77.0 (74.5–79.6) | 76.8 (73.1–80.9)     | 77.3 (73.4–81.2)     |
| Pulse, beats/min                      | 86.8 (84.6–88.9) | 86.2 (82.4–89.9)     | 87.4 (84.8–89.9)     |
| Respiratory rate/min                  | 24.3 (23.3–25.4) | 24.3 (22.7–25.8)     | 24.4 (22.8–25.9)     |
| Splenomegaly, no. (%) patients        | 2 (4.5)          | 1 (4.5)              | 1 (4.5)              |
| Hepatomegaly, no. (%) patients        | 1 (2.3)          | 0                    | 1 (4.5)              |
| Parasitemia, geometric mean (95% CI) parasites/µL | 45,113 (35,473–57,372) | 45,112 (30,853–65,948) | 45,123 (32,509–62,632) |
| Hematocrit, %                         | 42.1 (40.4–43.8) | 42.9 (40.1–45.7)     | 42.1 (40.4–43.8)     |

*Values are mean (95% confidence interval) unless otherwise indicated.
the second and third days of treatment. The mean [95% CI] PCT (hours) was significantly shorter in group 1 than in group 2 (21.0 [18.7–23.3] versus 25.4 [22.1–28.8]; \( P = 0.029 \)) for slides read by the first microscopist but not for the second microscopist (\( P = 0.10 \)). For the 41 patients with no evidence of prior antimalarial drug use, the mean (95% CI; range) PCT (hours) was 23.6 (21.5–25.7; 14–46) and 22.5 (20.3–24.6; 12–46) for the first and second microscopists, respectively.

Using the results of slide reading from the first microscopist, we determined that the estimated overall median (range) time (hours) to 50% parasitemia and the clearance rate of parasites was 5.1 (0.6–17.8) and 0.16 (0.09–0.26), respectively (Table 2). The mean [95% CI] PCT (hours) was longer in those \( \leq 15 \) years of age (\( n = 7 \)) than in those > 15 years of age (\( n = 37 \)), but this was not statistically significant (26.1 [17.5–34.6] versus 22.7 [20.6–24.8]; \( P = 0.23 \)). None of the patients had patent gametocytemia on admission, and gametocytemia did not develop after treatment.

**Cure rates, fever clearance, and change in hematocrit.** The mean (95% CI) multiplicity of infection was 1.33 (0.92–1.75) and this was found in 30% of the total samples for this study. One patient in group 2 had *P. falciparum* reappearance at day 30 and the PCR analysis indicated that this was a reinfection. Therefore, the 42-days PCR-corrected cure rates were 100% in both treatment groups. Two patients (one in each group) had *P. vivax* appearance at day 35 and day 27 and were successfully treated with oral chloroquine.

All patients had a history of fever during the 24 hours before enrollment and 35 (79.5%) of them were febrile at presentation, as defined by a tympanic temperature \( \geq 37.5 \)°C. After treatment, all patients were afebrile by day 2. The overall mean (95% CI) fever clearance time was 20.0 (16.2–23.8) hours, and this finding was not significantly different between the two groups. The admission mean (95% CI) hematocrit was significantly higher than those on other days (\( P < 0.001 \)), but the fractional changes in hematocrit from admission over time did not differ significantly between the groups (Table 2).

**Adverse events.** Severe malaria or serious adverse events did not develop in any patients during or after treatment. The frequency of patients with symptoms and signs, before treatment, that may subsequently be confused with drug adverse events were similar between the two groups (Table 3).

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**Figure 2.** Parasite clearance curves for Lao patients compared with those from previous studies conducted in Pailin, Cambodia and Wang Pha, Thailand.3 Lao data are log_{10}-normalized median parasite densities over time among the 22 patients in each treatment group. Error bars indicate 50% of interquartile ranges (IQR).

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**Table 2**

Outcome measures for the treatment of patients enrolled in a study comparing 4 mg/kg/day versus 2 mg/kg/day of oral artesunate for treatment of *Plasmodium falciparum* malaria in Laos*

| Variable | Treatment groups | All (n = 44) | 4 mg/kg/day (n = 22) | 2 mg/kg/day (n = 22) | \( P \) |
|----------|-----------------|-------------|---------------------|---------------------|-------|
| 42-day cure rate, no. (%) patients | 44 (100) | 22 (100) | 22 (100) | – |
| Fever clearance time, mean hours | 20.0 (16.2–23.8) | 20.6 (15.7–25.5) | 19.5 (13.3–25.7) | 0.77 |
| Patients remained febrile at day 1, no. (%) | 7 (16%) | 2 (9%) | 5 (23%) | 0.41 |
| Patients remained febrile at day 2, no. (%) | 0 | 0 | 0 | – |
| Parasite clearance time by first microscopist | 23.2 (21.2–25.3) | 21.0 (18.7–23.3) | 25.4 (22.1–28.8) | 0.029 |
| Parasite clearance time by second microscopist | 22.4 (20.3–24.5) | 20.8 (18.4–23.1) | 24.1 (20.6–27.6) | 0.10 |
| Positive parasitemia at day 1, no. (%) patients | 10 (23) | 4 (18) | 6 (27) | 0.47 |
| Positive parasitemia at day 2, no. (%) patients | 0 | 0 | 0 | – |
| Time to 90% clearance of parasite density, hours | 5.1 (0.6–17.8) | 4.4 (0.6–15.8) | 6.4 (1.2–17.8) | 0.01 |
| Time to 50% clearance of parasite density, hours | 9.7 (4.5–28.1) | 9.0 (4.5–18.1) | 11.6 (5.2–28.1) | 0.02 |
| Parasite reduction ratio at 24 hours | 0 (0–10) | 0 (0–0.6) | 0 (0–10) | 0.38 |
| Parasite clearance rate (slope of curve for log_{10}-normalized parasite clearance)† | 0.16 (0.09–0.26) | 0.17 (0.13–0.26) | 0.16 (0.09–0.22) | 0.02 |
| *P. vivax* appearance after treatment of *P. falciparum*, no. (%) patients | 2 (4.5) | 1 (4.5) | 1 (4.5) | – |

**Hematocrit**

| Day | All (n = 44) | 4 mg/kg/day (n = 22) | 2 mg/kg/day (n = 22) |
|-----|-------------|---------------------|---------------------|
| Day 0 | 42.1 (40.4–43.8) | 42.9 (40.1–45.7)† | 42.1 (40.4–43.8)† | 0.36 |
| Day 7 | 37.1 (35.6–38.5) | 37.4 (35.3–39.5) | 37.1 (35.6–38.5) | 0.63 |
| Day 14 | 37.4 (35.9–38.8) | 37.7 (35.4–39.9) | 37.4 (35.9–38.8) | 0.67 |
| Day 21 | 38.3 (36.9–39.7) | 38.5 (36.6–40.4) | 38.3 (36.9–39.7) | 0.75 |
| Day 28 | 38.8 (37.6–39.9) | 38.7 (37.0–40.4) | 38.8 (37.6–39.9) | 0.90 |
| Day 35 | 39.2 (37.9–40.4) | 39.7 (37.9–41.4) | 39.2 (37.9–40.4) | 0.39 |
| Day 42 | 39.5 (38.3–40.7) | 39.7 (38.0–41.4) | 39.5 (38.3–40.7) | 0.76 |

*Values are mean (95% confidence interval) unless otherwise indicated.
†Defined as the slope of linear portion of the log_{10}-normalized parasite clearance curve.
‡Significant difference from other days \( P < 0.001 \).
Possible adverse events found in patients enrolled in a study comparing 4 mg/kg/day versus 2 mg/kg/day of oral artesunate for the treatment of Plasmodium falciparum malaria in Laos

| Symptoms and signs | Before treatment (n = 44), no. (%) | 4 mg/kg/day (n = 22), no. (%) | 2 mg/kg/day (n = 22), no. (%) |
|--------------------|-----------------------------------|-----------------------------|-----------------------------|
| Weakness           | 43 (98)                           | 0                           | 1 (4.5)                     |
| Anorexia           | 38 (86)                           | 1 (4.5)                     | 3 (14)                      |
| Nausea             | 27 (61)                           | 0                           | 1 (4.5)                     |
| Vomiting           | 18 (41)                           | 0                           | 1 (4.5)                     |
| Abdominal pain     | 11 (25)                           | 0                           | 1 (4.5)                     |
| Diarrhea           | 7 (16)                            | 0                           | 1 (4.5)                     |
| Headache           | 44 (100)                          | 1 (4.5)                     | 3 (14)                      |
| Insomnia           | 27 (61)                           | 1 (4.5)                     | 1 (4.5)                     |
| Nightmares         | 6 (14)                            | 0                           | 0                           |
| Palpitations       | 25 (57)                           | 0                           | 1 (4.5)                     |
| Dyspnea            | 12 (27)                           | 0                           | 1 (4.5)                     |
| Dizziness          | 36 (82)                           | 0                           | 1 (4.5)                     |
| Vertigo            | 14 (32)                           | 0                           | 0                           |
| Tinnitus           | 13 (29.5)                         | 0                           | 0                           |

**DISCUSSION**

The study showed no evidence of *P. falciparum* resistance to artesunate, as defined by prolonged PCT, approximately 400 km, as the crow-flies, from the focus of *P. falciparum* resistance to artemisinin derivatives in western Cambodia. None of the study patients were parasitemic on days 2 or 3 after treatment with either 4 mg/kg/day or 2 mg/kg/day of oral artesunate for 3 days, which is reassuring for the continued use of the currently recommended ACT regimen in Laos. The mean PCT for 3 days, which is reassuring for the continued use of the currently recommended ACT regimen in Laos.

The absence of artemisinin resistant *P. falciparum* parasites resistant to artemisinin in the southern Laos, especially closer to the Cambodian border, is required.

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