

## ASSOCIATION OF FAILURES OF SEVEN-DAY COURSES OF ARTESUNATE IN A NON-IMMUNE POPULATION IN BANGUI, CENTRAL AFRICAN REPUBLIC WITH DECREASED SENSITIVITY OF *PLASMODIUM FALCIPARUM*

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**Abstract.** We assessed the efficacy and safety of a seven-day course of artesunate for the treatment of uncomplicated *Plasmodium falciparum* malaria in 55 non-immune patients living in Bangui, Central African Republic. The parasitologic cure rates were 100%, 95%, and 85% on days 14, 28, and 42, respectively. There were no significant differences in parasitemia density, 50% inhibitory concentration of dihydroartemisinin, and frequency of mutant *P. falciparum* multidrug resistance 1 codon 86 between patients who were cured and those who displayed recrudescence. However, the 90% inhibitory concentration for dihydroartemisinin and the number of genotypes isolated were both higher in the recrudescence patients (five- and two-fold, respectively). We found an association between recrudescence and decreased sensitivity. This suggests that the use of artemisinin compounds alone will select resistant strains. We conclude that artesunate should not be used in monotherapy even in seven-day courses, but only in combination with other anti-malarials to prevent the emergence of resistant *P. falciparum*.

### INTRODUCTION

Resistance of *Plasmodium falciparum* to anti-malarial drugs is a major public health problem with therapeutic and prophylactic implications. Decreased sensitivity of the parasite to most of the commonly used anti-malarial drugs has been reported in central Africa.<sup>1–4</sup> In the Central African Republic, resistance of *P. falciparum* to chloroquine (CQ) was first documented in 1983<sup>5</sup> and resistance to sulfadoxine-pyrimethamine (SP) was documented in 1987.<sup>6</sup> The most recent *in vivo* study based on the World Health Organization (WHO) standard protocol (WHO, 2001), conducted in 2002–2004 by the National Malaria Control Program and the Institut Pasteur de Bangui, showed that the overall treatment failure rates with CQ, amodiaquine (AQ), SP, CQ plus SP, and AQ plus SP were 40.9%, 20.0%, 22.8%, 7.2%, and 0%, respectively in Bangui, Central African Republic.<sup>7</sup> Therefore, clinicians have resorted to the use of new drugs for the treatment of *P. falciparum* malaria.

One such drug, introduced for the treatment of multidrug-resistant *P. falciparum* malaria, is artesunate, a derivative of artemisinin. Artesunate is a blood schizontocide that acts by increasing the oxidative stress on intra-erythrocytic parasite stages.<sup>8</sup> The drug is rapidly absorbed and then immediately hydrolyzed into its active metabolite, dihydroartemisinin, which has a short plasma half-life (< 2 hours).

*Plasmodium falciparum* resistance to artemisinin compounds has not been reported.<sup>9</sup> However, several studies have shown high rates of recrudescence when artemisinin derivatives were used in monotherapy for three<sup>10–12</sup> or five days.<sup>13–15</sup> Consequently, since 2000, the WHO and the International Artemisinin Study Group have recommended that uncomplicated *P. falciparum* malaria be treated with artesunate either in combination with another effective blood schizontocide or alone for seven days to prevent recrudescence and to delay the selection of resistant strains.<sup>16,17</sup>

The aims of our study were to 1) determine the efficacy and

the safety of a seven-day course of artesunate (4 mg/kg loading dose on the first day, followed by 2 mg/kg once a day for six days) for the treatment of uncomplicated *P. falciparum* malaria in non-immune patients (European expatriates) living in Bangui, Central African Republic; and 2) to compare host- and parasite-related factors between cured patients and patients in whom treatment failed.

### MATERIALS AND METHODS

**Study area.** This study was carried out between March and July 2004 in non-immune patients (European expatriates) who attended the health center of the French Embassy in Bangui (Centre Médico-Social de l'Ambassade de France en République Centrafricaine) with symptoms suggestive of malaria. Bangui is located on the Oubangui River in the Central African Republic, which is north of the Democratic Republic of the Congo (7°N, 21°E). The climate is tropical and the peak period of rainfall is from April to November. The average temperature varies from 19°C to 32°C. The malaria transmission season lasts all year, peaking at the beginning and end of the rainy season. Malaria is hyperendemic and the main parasite responsible is *P. falciparum*.

**In vivo assay.** Patients were included in the study if they met the following criteria: 1) uncomplicated *P. falciparum* infection with an asexual parasitemia between 2,000 and 200,000 parasites/ $\mu$ L, and 2) a temperature > 37.5°C or a history of fever in the previous 24 hours, and 3) provided written informed consent. Exclusion criteria were 1) prior adequate treatment with anti-malarial drugs for the current attack, 2) an inability to drink, sit, or stand, 3) vomiting more than twice within the previous 24 hours, 4) at least one convulsion within the previous 24 hours, 5) a hemoglobin concentration < 8 g/dL, 6) severe underlying disease (cardiac, renal, or hepatic disease, malnutrition, and concomitant disease), and 7) a history of allergy to artemisinins.

Venous blood samples collected on day 0 were used to prepare thick and thin blood films and to determine hematocrit and hemoglobin levels and white blood cells counts using an automated hematology analyzer (T540; Beckman Coulter, Fullerton, CA). The smears were stained with 4%

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Giemsa for 20 minutes. Parasite density was calculated by counting the number of asexual parasites per 200 white blood cells (WBCs) and adjusting for the total WBC count. Thin blood smears were also examined for other *Plasmodium* spp.

Patients received artesunate (50-mg tablets; Sanofi, Gentilly, France) at a dose of 4 mg/kg of body weight on day 0 and 2 mg/kg of body weight once a day for the next six days. If the patient vomited within one hour of the first administration, the full dose was readministered. Patients who vomited more than once were withdrawn from the study and treated appropriately. Follow-up appointments were scheduled for days 1, 2, 3, 7, 14, and 28, and consisted of a physical examination and completion of a standardized form. Patients were encouraged to return to the health center at any time if they felt ill. Blood samples were collected by venous puncture on days 3, 7, 14, and 28 (used to prepare thick and thin blood smears) and whenever necessary based on the results of a clinical examination.

The clinical outcomes were classified into three categories. Early treatment failure (ETF) was defined as the development of danger signs or severe malaria on days 1, 2, or 3 with parasitemia; an axillary temperature  $\geq 37.5^{\circ}\text{C}$  on day 2 with parasitemia higher than on day 0; parasitemia on day 3 with an axillary temperature  $\geq 37.5^{\circ}\text{C}$ ; or parasitemia on day 3  $\geq 25\%$  of count on day 0. Late treatment failure (LTF) was defined as development of danger signs or severe malaria after day 3 with parasitemia without previously meeting any of the criteria for ETF; or parasitemia and an axillary temperature  $\geq 37.5^{\circ}\text{C}$  on any day between days 4 and 14 without previously meeting any of the criteria for ETF. Adequate clinical response (ACR) was defined as absence of parasitemia on day 14, irrespective of axillary temperature, without previously meeting any of the criteria for ETF or LTF; or an axillary temperature  $< 37.5^{\circ}\text{C}$  on day 14, irrespective of parasite count, without previously meeting any of the criteria for ETF or LTF. We also assessed parasitologic failure by dividing the ACR group into two groups: ACR without parasitemia on day 14 (ACR and adequate parasitologic response) and ACR with parasitemia on day 14 but without fever (ACR and parasitologic failure). An adverse event was defined as an unexpected change in the baseline situation, whether or not it was associated with the study drug.

**In vitro assay.** We used dihydroartemisinin (Sigma, St. Louis, MO) for the *in vitro* assay because this derivative is relatively stable and because all currently used artemisinin derivatives and those that are at advanced phases of development are rapidly transformed into dihydroartemisinin in humans.<sup>18,19</sup> A stock solution of dihydroartemisinin was prepared in methanol. Two-fold serial dilutions of the stock solution were made in distilled water, giving final concentrations between 0.25 and 64 nM. All concentrations, as well as drug-free controls, were distributed in 20- $\mu\text{L}$  aliquots in triplicate in 96-well tissue culture plates.

Venous blood samples were washed three times in RPMI 1640 medium. Each time, the white blood cell interface was removed. The erythrocytes were resuspended in complete RPMI 1640 medium consisting of 10% human serum (batch no. S02909S4190; Serum AbCys, Paris, France), 25 mM HEPES buffer, and 25 mM sodium bicarbonate at a hematocrit of 1.5% and an initial parasitemia of 0.1–0.5%. If the blood sample had a parasitemia  $> 0.5\%$ , fresh uninfected erythrocytes were added to adjust the parasitemia to 0.3%.

The isotopic microtest developed by Desjardins and others<sup>20</sup> was used in this study. A suspension of infected erythrocytes (200  $\mu\text{L}$ ) was added to each well of the 96-well tissue culture plates. To assess parasite growth,  $^3\text{H}$ -hypoxanthine (1  $\mu\text{Ci}$ /well; Amersham Pharmacia Biotech, Orsay, France) was added. The parasites were incubated at  $37^{\circ}\text{C}$  in an atmosphere of 5%  $\text{CO}_2$  for 42 hours. After incubation, the plates were frozen to terminate the *in vitro* assay. The plates were then thawed, and the contents of each well were collected on fiberglass filter papers (Printed Filtermat A<sup>®</sup>; Wallac, Turku, Finland) using a cell harvester (Skatron Instruments AS, Tranby, Norway). The filter disks were transferred into scintillation tubes (4 mL of Pico Pro Vial<sup>®</sup>; Packard Instrument SA) and 2 mL of scintillation cocktail (Ultima Gold F<sup>®</sup>; Packard Instrument SA, Perkin Elmer Life and Analytical Sciences, Inc., Boston, MA) was added. The incorporation of  $^3\text{H}$ -hypoxanthine was quantitated using a liquid scintillation counter (Betamatic<sup>®</sup>; Kontron, Milan, Italy). A CQ-sensitive reference strain (3D7) and a CQ-resistant reference strain (W2) obtained from the Malaria Research Reference Reagent Resource Center/American Type Culture Collection (Manassas, VA) were used as controls in the *in vitro* assay.

The 50% and 90% inhibitory concentrations ( $\text{IC}_{50}$  and  $\text{IC}_{90}$ ) were defined as the drug concentrations corresponding to 50% and 90% of the uptake of  $^3\text{H}$ -hypoxanthine measured in the drug-free control wells. They were determined by non-linear regression analysis of log-transformed concentrations plotted against growth inhibition. For graphic display, the data were adapted to a log-probit model (Excel<sup>®</sup>; Microsoft, Redmond, WA). Data are expressed as geometric mean  $\text{IC}_{50}$  or  $\text{IC}_{90}$  values and 95% confidence intervals (CIs).

**Isolation of DNA.** Parasite DNA was obtained from 100  $\mu\text{L}$  of thawed red blood cell pellets by incubation at  $100^{\circ}\text{C}$  for three minutes with 0.1M NaOH. The supernatant was incubated at  $37^{\circ}\text{C}$  for one hour with 250  $\mu\text{L}$  of lysing solution (15 mM Tris-HCl, pH 7.5, 1% [v/v] Triton 100X, 150 mM NaCl, 1% sodium dodecyl sulfate, 1 mM EDTA) and 20 mg/mL of proteinase K, followed by two extractions with phenol/chloroform (1:1). The DNA was then precipitated with ethanol and resuspended in 100  $\mu\text{L}$  of distilled water.

**Genotyping of merozoite surface protein 1 (*msp-1*) and merozoite surface protein 2 (*msp-2*).** The *msp-1* and *msp-2* genes were genotyped to distinguish cases of recrudescence from new infections by comparing matched pairs of parasite isolates obtained at time of inclusion and on the day of reappearing parasitemia. The polymorphic regions of *msp-1* (block 2) and *msp-2* (block 3) were amplified by a nested polymerase chain reaction (PCR). First-round PCR primers corresponded to conserved sequences flanking these regions.<sup>21</sup> Second-round PCR primers were used to amplify the K1, MAD20 and RO33 allelic families of *msp-1*,<sup>22</sup> and the 3D7 and FC27 allelic families of *msp-2*.<sup>23</sup> Nested PCR products were analyzed for size polymorphism by electrophoresis using 3% agarose gels. The DNA was stained with ethidium bromide, visualized under ultraviolet light, and photographed. Samples from an individual patient were run in adjacent lanes.

Genotyping results were used to calculate PCR-corrected cure rates. We classified parasitemia as re-infection if all bands seen on gels on the day of reappearing parasitemia were distinct from those seen on the day of inclusion (the samples were tested in parallel). We also calculated multiplic-

ity of infection (MOI) or the number of genotypes per isolate, i.e., the highest number of genotypes at either of the two *msp* loci.

**Plasmodium falciparum multidrug resistance 1 (*pfmdr-1*) PCR-restriction fragment length polymorphism.** The *pfmdr-1* gene was analyzed as described by Duraisingh and others.<sup>24</sup> Parasite DNA was amplified with specific primers flanking codon 86 in two rounds of outer and nested PCRs. The *pfmdr-1* alleles were identified for codon 86 by using *Afl* III, which cuts the coding sequence at allele Tyr86 (mutant) but not at Asn86 (wild type).

**Statistical analysis.** We calculated that 50 patients were required to obtain an estimate of the cure rate (estimated to be 90%) within a 95% binomial CI. Qualitative variables were compared by using either the chi-square test or Fisher's exact test. Quantitative variables were compared by analysis of variance (normal data with Bartlett's test) or the Kruskal-Wallis test (non-normal data with Bartlett's test). The 95% CIs of percentages were calculated using the exact binomial test. The level of significance *P* was fixed at 0.05 for all statistical tests. The data were analyzed using the Epi-Info 2000 program (Centers for Disease Control and Prevention, Atlanta, GA) and MedCalc® version 7.4.3.0 software (Franck Schoojans, Department of Endocrinology, University Hospital of Ghent, Ghent, Belgium, available from <http://www.medcale.be>).

**Ethical approval.** Since there is no national ethical committee in the Central African Republic, this study was reviewed and approved by the expert committee for the anti-malarial drug policy and the Ministry of Health in this country. Written informed consent was obtained from all patients.

## RESULTS

Fifty-five non-immune patients were treated with artesunate. The outcomes for all 55 patients could be evaluated and

no protocol violations, exclusions, or losses to follow-up occurred. All patients were living in Bangui at least one year. Three patients were receiving CQ-proguanil prophylaxis (one in the recrudescing group and two in the non-recrudescing group).

Parasites were eliminated within 72 hours in all cases. The geometric mean time to parasite clearance was 32 hours (95% CI = 28–36 hours). Fever was also rapidly cleared (temperature < 37.5°C), with a geometric mean clearance time of 24 hours (95% CI = 22–26 hours). Artesunate was well tolerated and no serious adverse events were recorded.

The treatment and parasitologic cure rate was 100% on day 14, 93% on day 28 (51 of 55 patients, 95% CI = 83–98%), and 80% on day 42 (44 of 55 patients, 95% CI = 68–89%). The PCR showed that in 3 of the 11 cases of apparent recrudescence, the parasites recovered at baseline subsequently had different *msp-1* and *msp-2* genotypes. These patients were therefore considered re-infected and were classified as cured. These re-infections occurred from days 21 to 28 in one case and from days 28 to 42 in two cases. The PCR-corrected parasitologic cure rate was therefore 95% on day 28 (52 of 55 patients, 95% CI = 86–98%) and 85% on day 42 (47 of 55 patients, 95% CI = 74–93%). All recrudescing patients were symptomatic.

The demographic, clinical, and parasitologic characteristics of baseline isolates from recrudescing and non-recrudescing patients are summarized in Table 1. There were no significant differences in parasitemia, IC<sub>50</sub> for dihydroartemisinin (on available blood samples with parasitemias ≥ 0.1%), and frequency of mutant *pfmdr-1* codon 86 between the two groups of patients. However, the IC<sub>90</sub> for dihydroartemisinin was five-fold higher and the IC<sub>50</sub>:IC<sub>90</sub> ratio for dihydroartemisinin was eight-fold lower, in isolates from recrudescing patients. The IC<sub>50</sub> and IC<sub>90</sub> for dihydroartemisinin of recrudescing and non-recrudescing patient isolates are shown in Figure 1. We

TABLE 1  
Demographic, clinical, and parasitologic characteristics of baseline isolates from recrudescing and nonrecrudescing patients\*

	Patients							<i>P</i>
	Recrudescing			Non-recrudescing				
	No.	Value	95% CI	No.	Value	95% CI		
Patient characteristics								
No. of males/no. of females	8†	7/1		47‡	25/22		NS§	
Age (years), mean	8	34.6	25.4–43.7	47	36.7	31.5–41.9	NS¶	
Weight (kg), mean	8	69.0	59.0–79.0	47	66.9	63.6–70.1	NS¶	
Temperature (°C), mean	8	38.7	38.4–39.0	47	38.5	38.3–38.7	NS¶	
Hematology								
Hemoglobin concentration (g/dL), mean	8	14.4	12.2–16.7	47	13.7	13.1–14.4	NS¶	
Hematocrit (%), mean	8	42	36–48	47	40	38–42	NS¶	
Leukocyte count (10 <sup>3</sup> /μL), mean	8	6,560	5,605–7,514	47	5,443	4,853–6,033	NS¶	
Isolates at the baseline								
Parasitemia (no. of parasites/μL), mean	8	5,430	1,516–9,343	47	7,902	3,581–12,222	NS#	
MOI, mean	8	3.25	1.78–4.72	47	1.52	1.15–1.89	0.003¶	
IC <sub>50</sub> % DHA (nM), mean	5	0.97	0.15–1.79	17	0.95	0.42–1.48	NS#	
IC <sub>90</sub> % DHA (nM), mean	5	46.2	13.1–79.3	17	8.9	2.6–15.2	0.02#	
IC <sub>50</sub> %/IC <sub>90</sub> % DHA (nM), mean	5	0.017	0–0.038	17	0.134	0.068–0.193	0.007#	
<i>pfmdr-1</i> 86, Tyr %	8	0	0–39.3	47	17.8	6.8–35.2	NS¶	

\* CI = confidence interval; NS = not significant; MOI = multiplicity of infection; IC<sub>50</sub>% (nM) = geometric mean 50% inhibitory concentrations in nanomoles/liter; DHA = dihydroartemisinin; IC<sub>90</sub>% (nM) = geometric mean 90% inhibitory concentrations in nanomoles/liter; *pfmdr-1* = *Plasmodium falciparum* multidrug resistance-1.

† Polymerase chain reaction (PCR)-corrected. Recrudescences occurred in two cases between day 14 and day 21, in three cases between day 21 and day 28, in three cases between day 28 and day 42.

‡ PCR-corrected (three patients were reinfected and classified as cured). Reinflections occurred in one case between day 21 and day 28 and in two cases between day 28 and day 42.

§ By Fisher's exact test.

¶ By analysis of variance.

# By Kruskal-Wallis test.

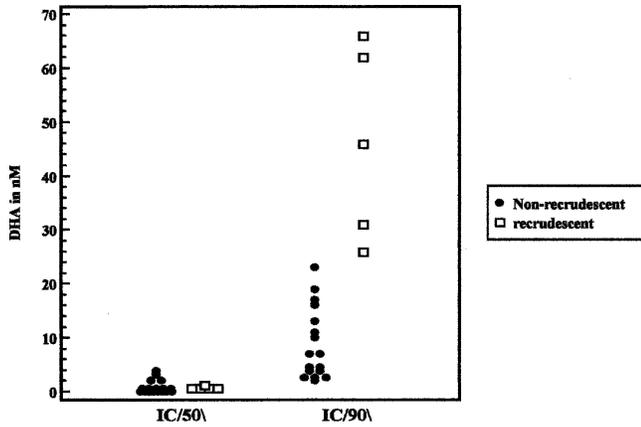


FIGURE 1. 50% and 90% inhibitory concentrations ( $IC_{50}$  and  $IC_{90}$ ) for dihydroartemisinin (DHA) of recrudescent and non-recrudescent patient isolates.

also found that the multiplicity of infection was significantly 2-fold higher in isolates from recrudescent patients. The parasitologic characteristics of isolates at the baseline and at the failure treatment from recrudescent patients are shown in Table 2.

## DISCUSSION

The first objective of this study was to determine the efficacy and safety of seven-day courses of artesunate (4 mg/kg loading dose on the first day, followed by 2 mg/kg once a day for six days) for the treatment of uncomplicated *P. falciparum* malaria in Africa. We studied non-immune European expatriates for two main reasons: 1) to see if the cure rates were due to the effect of artesunate alone because humoral immunity can control low-level parasitemia and therefore potentially prevent treatment failure,<sup>25</sup> and 2) to facilitate long-term follow-up.

We confirmed that artesunate was well tolerated and rapidly cleared parasites and fever. We also demonstrated that a seven-day course of artesunate showed a high cure rate by day 14 (100%). However, the PCR-adjusted cure rate was 92% on day 28 and 85% on day 42.

Previous studies have shown high rates of recrudescence when artemisinin derivatives were used as monotherapy for three days (46–80% in non-immune patients on day 28 in Thailand and China<sup>11,12</sup> and 72% in children on day 28 in Gabon<sup>10</sup>) and five days (80% in patients in Tanzania and Nigeria<sup>13–15</sup>). Few studies have investigated the effects of extending the duration of artemisinin monotherapy. In a Viet-

namese study that compiled results of different non-comparative studies, the recrudescence rate was highest in patients treated for *P. falciparum* malaria with artemisinin for  $\geq 5$  days (50%), but ranged from 10% to 23% for patients receiving the drug for 5–10 days.<sup>26</sup> Our study confirmed the results of another Vietnamese study,<sup>27</sup> which showed that extending the duration of artesunate treatment from five to seven days is not useful, and that a seven-day course is no better than a five-day course. We conclude that a seven-day course of artesunate is not sufficiently effective for the treatment of uncomplicated *P. falciparum* malaria in non-immune patients in Bangui (European expatriates and central African citizens less than five years of age).

Our study indicates that the parasitologic cure rates on day 42 are potentially a more sensitive marker of the *in vivo* efficacy of an anti-malarial drug than the cure rates on days 14 or 28. This is particularly true for drugs with long plasma half-lives, but is also applicable, although to a lesser extent, to drugs with very short plasma half-lives such as artemisinin compounds. Use of validated PCR protocols<sup>28</sup> to distinguish recrudescent infections from new infections increases the specificity of the *in vivo* test, as is recommended in the latest WHO *in vivo* protocol.<sup>29</sup> The fact that the PCR-corrected cure rates decreased over time (from 100% to 85%) in our study clearly shows that the *in vivo* test was most sensitive on day 42. This confirms that 28 days should be the minimum follow-up period when assessing the efficacy of drugs against *P. falciparum* malaria.<sup>30</sup>

The second objective was to identify host- and parasite-related factors that differed between cured patients and patients in whom treatment failed. We observed no significant differences in host-related factors (age, sex, weight, temperature, hemoglobin concentration, hematocrit, or leukocyte count) between these two groups (Table 1).

We also determined the  $IC_{50}$  and  $IC_{90}$  of dihydroartemisinin for baseline isolates from the two patient groups (Figure 1). The range of *in vitro*  $IC_{50}$  values observed (0.15–1.79 nM) was similar to those previously reported for Africans isolates,<sup>31</sup> but lower than those reported for Asian isolates.<sup>32,33</sup> We observed no significant differences in  $IC_{50}$  for dihydroartemisinin between the two groups. However, our study provides the first evidence that recrudescence is linked to diminished sensitivity of isolates to dihydroartemisinin. Indeed, the  $IC_{90}$  and the  $IC_{50}:IC_{90}$  ratio for dihydroartemisinin of isolates from recrudescent patients were five-fold higher and eight-fold lower, respectively, than that of isolates from non-recrudescent patients. Unlike previous studies,<sup>34</sup> we did not find that the increase in the  $IC_{90}$  and the decrease in the  $IC_{50}:IC_{90}$  ratio of dihydroartemisinin were associated with a mutation at codon 86 of the *pfmdr-1* gene (N86Y).

TABLE 2  
Parasitologic characteristics of isolates at the baseline and at treatment failure from recrudescent patients\*

	Isolates at baseline			Isolates at treatment failure			P
	No.	Value	95% CI	No.	Value	95% CI	
Parasitemia (no. of parasites/ $\mu$ L), mean	8	5,429	910–9,947	8	3,355	2,762–3,947	NS†
MOI, mean	8	3.25	1.78–4.72	8	1.14	0.88–1.40	0.01‡
<i>pfmdr-1</i> 86, Tyr %	6	0		6	0		NS‡

\* CI = confidence interval; NS = not significant; MOI = multiplicity of infection; *pfmdr-1* = *Plasmodium falciparum* multidrug resistance-1.

† By Kruskal-Wallis test.

‡ By analysis of variance.

No significant differences in parasite density were found between the two groups of patients, which is consistent with the findings of Ittarat and others<sup>35</sup> or previous studies on quinine<sup>36</sup> and mefloquine,<sup>37</sup> which showed that recrudescence patients tended to have higher parasitemias before treatment than non-recrudescence patients. This difference could be explained by the limited size of our population and a resulting missed power in the statistical analysis.

Conversely, we found that the number of genotypes (MOI) was two-fold higher in the baseline isolates from recrudescence patients than in those from cured patients, and that only one survival genotype was found in the treatment failure isolates in all cases of recrudescence. This observation, combined with the higher IC<sub>90</sub>% observed in recrudescence patients, suggests that isolates from recrudescence patients contain three or four clones of which one is slightly diminished sensitivity to dihydroartemisinin. The presence of this slightly diminished sensitivity to dihydroartemisinin clone in recrudescence isolates did not modify IC<sub>50</sub>% values for dihydroartemisinin but increase CI<sub>90</sub>% values. We suspect that this clone was not completely eliminated with the seven-day courses of artesunate treatment and was responsible for the recrudescence cases. Unfortunately, the parasite density was too low in the treatment failure sample to measure the sensitivity of the isolate, so we cannot confirm this hypothesis.

In conclusion, our study demonstrates that a seven-day course of artesunate does not prevent recrudescence. Furthermore, the association between recrudescence and decreased sensitivity suggests that the use of artemisinin compounds alone will select resistant strains. Therefore, artesunate should not be used alone, even in seven-day courses as is recommended by the WHO, but only in combination with other anti-malarials to prevent the emergence of resistant *P. falciparum*.

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