FOLLOW-UP OF THE SUSCEPTIBILITY OF PLASMODIUM FALCIPARUM TO ANTIMALARIALS IN GABON

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Abstract. The sensitivity of Plasmodium falciparum to chloroquine, mefloquine, quinine, quinidine, halofantrine, artemisinin, and sulfadoxine/pyrimethamine was investigated in Lambarene, Gabon in 1994. The development of in vitro susceptibility has been traced from 1983 or 1992 to 1994 for chloroquine, mefloquine, halofantrine, and quinine. Standard in vitro microtests according to World Health Organization methodology were performed. Of 33 isolates tested for susceptibility to chloroquine, 31 were resistant, one was borderline, and one isolate was sensitive (mean 50% effective concentration [EC50] = 1.38 μmol/L of blood). With mefloquine, all isolates were fully inhibited below the threshold of resistance (mean EC50 = 0.51 μmol/L of blood). Of 32 isolates tested with quinine, six had borderline resistance (mean EC50 = 0.54 μmol/L of blood medium mixture). Susceptibility to quinidine was higher with a mean EC50 of 0.15 μmol/L of blood medium mixture. With halofantrine, 26 of 32 isolates matured at 3 nmol/L of blood medium mixture (mean EC50 = 1.64 nmol/L of blood medium mixture), indicating a steep decrease in susceptibility in comparison with 1992. For artemisinin, the mean EC50 was 97.92 nmol/L of blood medium mixture. Sulfadoxine/pyrimethamine showed five of 16 resistant isolates with a mean EC50 of 2.46 nmol/L of blood medium mixture. Whereas chloroquine resistance remained stable with a tendency to decrease, susceptibility to mefloquine and quinine was slightly decreased. A significant increase in the mean EC50 and EC90 in comparison with our previous data from Gabon was found for halofantrine.

Malaria continues to be a major cause of morbidity and mortality in most tropical countries. In Africa alone 140–280 million people suffer from malaria each year and more than one million die. The world malaria situation is aggravated by the fact that an increased prevalence of drug-resistant strains of Plasmodium falciparum continues to reduce the effectiveness of most known antimalarials. Drug-resistant populations of P. falciparum are selected by widespread and improper use of antimalarials; their genetically determined drug resistance is thus promoted and propagated. To plan and improve local malaria control programs, the susceptibility to antimalarial drugs of the local parasite population should be monitored. This can be done by in vivo and in vitro testing; ideally both methods should be combined. In Lambarene, Gabon, initial in vitro data indicating resistance of P. falciparum to chloroquine were reported in the early 1980s. This was subsequently confirmed by in vivo results. A substantial increase in chloroquine resistance in vitro in this area was found in 1992, indicating the highest degree of resistance to that drug reported from central Africa with an 50% effective concentration (EC50) of 1.86 μmol/L of blood. The high in vitro efficacy of mefloquine found by both Burchard and others and Winkler and others was recently confirmed in vivo for the Lambarene area. Halofantrine was found to be highly effective in vitro and in vivo. Positive correlations between the susceptibility to the widely used drug quinine and the less frequently administered mefloquine and halofantrine, together with reports of drug resistance to mefloquine in Cameroon and to halofantrine in the Congo raise the question of emerging resistance to halofantrine in Gabon. Reports of stabilizing chloroquine sensitivity in Cameroon and the Congo necessitate a close follow-up of resistance patterns to this universally used cheap and safe antimalarial. Therefore an in vitro follow-up was carried out in Lambarene, Gabon in 1994. The aim of this study was to monitor the development of the local susceptibility to chloroquine, mefloquine, halofantrine, quinine, quinidine, sulfadoxine/pyrimethamine, and artemisinin and to search for activity correlations between these antimalarials two years after our first study in this area.

PATIENTS AND METHODS

Study area. The study was carried out in the Albert Schweitzer Hospital in Lambarene, Gabon, between April and October 1994. Lambarene is situated in the central African tropical rain forest, a region predominantly hyperendemic for P. falciparum.

Patients. All patients attending the outpatient ward of the hospital were asked to participate in the study if they fulfilled the following criteria: proven monoinfection with P. falciparum, malaria, asexual parasite density between 1,000 and 80,000/μL of blood, no intake of antimalarials in the preceding month, negative urine test results for 4-aminoquinolines and sulfonamides (Wilson-Edeson and Ligninetests), informed consent, and willingness to give a venous blood sample. Ethical clearance for this study was obtained from the Ethics Committee of the International Foundation of the Albert Schweitzer Hospital.

In vitro test procedure. To assess the susceptibility of different strains of P. falciparum to chloroquine, mefloquine, halofantrine, quinine, quinidine, sulfadoxine/pyrimethamine, and artemisinin the World Health Organization (WHO) microtest technique was used and the inhibition of schizont maturation was measured microscopically (WHO, 1990, unpublished data). Venous blood (0.5 ml) from each patient was drawn into a sterile, heparinized tube (Vacutainer; Becton Dickinson, Rutherford, NJ). This was immediately added to 4.5 ml of RPMI 1640 low p-aminobenzoic acid and low folic acid content culture medium (Gibco, Paisley, United Kingdom). Fifty microliters of the blood-medium mixture...
(BMM) were pipetted into each well of the predosed tissue culture plates (Mark II; WHO, Manila, The Philippines) and incubated at 37.5°C in candle jars for 28 hr according to standard methodology. The chloroquine plates were dosed with 1, 2, 4, 8, 16, 32, and 64 μmol/well. The mefloquine plates were dosed with 2, 4, 8, 16, 32, 64, and 128 μmol/well. The quinidine plates were dosed with 4, 8, 16, 32, 64, and 128 μmol/well. The quinine plates were dosed with 2, 4, 8, 16, 32, 64, and 128 μmol/well. The mefloquine plates were dosed at 0.08, 0.16, 0.32, 0.64, 1.28, 2.56, and 5.12 μmol/L of BMM. The halofantrine plates were dosed with 0.1, 0.3, 1, 3, 10, 30, and 100 nmol/L of BMM. The artemisinin plates were dosed with 0.003, 0.01, 0.03, 0.1, 0.3, 1, and 3 μmol/L of BMM. The sulfadoxine/pyrimethamine plates were dosed with an 80:1 mixture of sulfadoxine and pyrimethamine, yielding pyrimethamine concentrations of 2.5, 7.5, 25, 75, 250, 750, and 2,500 nmol/L of BMM.

After incubation parasites were harvested and Giemsa-stained thick blood films were prepared. The number of mature schizonts was counted per 200 asexual parasites. For the chloroquine, mefloquine, halofantrine, quinidine, and quinidine tests schizonts with more than two nuclei were defined as mature, for the sulfadoxine/pyrimethamine test maturation was defined as having more than seven nuclei. Isolates with less than 10% of mature schizonts in the control well were excluded. Drug concentrations of 1.6 μmol/L of blood for chloroquine, 6.4 μmol/L of blood for mefloquine, and 5.12 μmol/L of BMM for quinine were considered as a threshold for in vitro resistance to these drugs. For halofantrine and artemisinin the threshold of resistance remains to be determined. For sulfadoxine/pyrimethamine the threshold was set at < 90% of schizont maturation inhibition at a pyrimethamine concentration of 75 nmol/L of BMM.

**Statistical analysis.** Effective concentrations (EC) and regression parameters were calculated using a computer adapted probit analysis of log-dose responses based on the methods of Litchfield and Wilcoxon. Discrete data were compared using the chi-square test. For all tests, P < 0.05 was considered to be significant.

### RESULTS

Sixty isolates of *P. falciparum* were tested in *vitro* yielding 33 valid tests for chloroquine, artemisinin, mefloquine and quinidine (55%), 32 for halofantrine and quinine (53%), and 16 for sulfadoxine/pyrimethamine (27%). The results are summarized in Tables 1 and 2.

**Chloroquine.** Thirty-one isolates (94%) showed schizont maturation at 1.6 μmol/L of blood, thus indicating resistance to chloroquine. One isolate was in the borderline area between reduced sensitivity and resistance. Only one isolate was completely inhibited at 0.8 μmol/L of blood, indicating chloroquine-sensitivity. If this finding is not due to an antimalarial not detectable by the Wilson-Edeson and Lignin tests, there may be a slightly lower level of resistance to chloroquine in comparison with 1992, when all isolates were fully resistant to the drug. This would be consistent with slightly lower mean EC50 and EC90 values in 1994 of 1.38 and 3.90 μmol/L of blood, respectively, compared with 1.86 and 4.18 μmol/L of blood, respectively, in 1992 (Table 1).

**Mefloquine.** Thirty-three isolates were fully inhibited at 6.4 μmol/L of blood. This is consistent with the findings in 1992 when all isolates were inhibited well below the threshold of resistance. The mean EC50 and EC90 in 1994 were 0.51 and 1.03 μmol/L of blood, respectively, slightly higher than in 1992 (Table 1).

**Quinine and quinidine.** With quinine, no isolate exhibited maturation at 5.12 μmol/L of BMM, i.e., at the threshold of resistance, but six (19%) of 32 were borderline with maturation at 2.56 μmol/L of BMM. This indicates a slight decrease in quinine sensitivity in comparison with 1992, when all isolates were sensitive at lower EC50 and EC90 levels (Table 1). The mean EC50 and EC90 were 0.54 and 1.30 μmol/L of BMM in 1994 and 0.51 and 1.10 μmol/L of BMM in 1992 based on the meth...
Figure 1. *In vitro* response of *Plasmodium falciparum* to quinine and quinidine in Lambarene, Gabon in 1994. Values are in μmol/L of blood medium mixture. SMI = inhibition of schizont maturation.

1992, respectively. All but four of the isolates tested for quinidine were fully inhibited at 0.64 μmol/L of BMM, with the remaining isolates being inhibited at 1.28 or 2.56 μmol/L of BMM. The mean EC$_{50}$ and EC$_{90}$ were 0.15 and 0.30 μmol/L of BMM (Table 1). When quinine was compared with quinidine, the higher sensitivity to the latter was evident (Figure 1). The two regression lines were parallel within experimental error since the factor for the slope ratio was greater than the slope ratio (1.23 and 1.14). Isolates were significantly more sensitive to quinidine than to quinine since the factor for the potency ratio was greater than the potency ratio (3.69 and 1.43).

Halofantrine. With halofantrine, only six isolates (19%) were inhibited at 3 nmol/L of BMM, and 26 isolates (81%) matured at concentrations ≥ 3 nmol/L of BMM, indicating a steep decrease in susceptibility in comparison with 1992, when all isolates were fully inhibited at 3 nmol/L of BMM. The mean EC$_{50}$ and EC$_{90}$ were 1.64 and 7.62 nmol/L of BMM in 1994 and 0.41 and 0.94 nmol/L of BMM in 1992, respectively. (Tables 1 and 3) The four-fold increase of the mean EC$_{50}$ from 1992 to 1994 was significant ($P < 0.05$) (Figure 2).

Artemisinin. Twenty-three isolates (70%) tested for artemisinin were fully inhibited at 300 nmol/L of BMM, and 10 isolates (30%) were inhibited at 1,000 nmol/L of BMM. The mean EC$_{50}$ and EC$_{90}$ were 97.92 and 363.58 nmol/L of BMM (Table 1).

Sulfadoxine/pyrimethamine. With sulfadoxine/pyrimethamine, five (31%) of 16 isolates showed less than 90% inhibition at a pyrimethamine concentration of 75 nmol/L of BMM. This is not different from 1992, when 30% were found to be resistant. The mean EC$_{50}$ and EC$_{90}$ were 2.46 and 142.07 nmol/L of BMM (Table 1). Table 4 shows the *in vitro* response to sulfadoxine/pyrimethamine in 1994 compared with 1992.

**DISCUSSION**

Chloroquine is a cheap, safe, and universally used antimalarial. Initial observations of chloroquine resistance were reported in the early 1960s from South America and Southeast Asia. In Africa, chloroquine sensitivity remained stable until 1977 when the first case of chloroquine resistance occurred in Kenya. In the following decade it has spread over the whole tropical zone of Africa. In the countries neighboring Gabon, chloroquine resistance has been observed *in vitro* and *in vivo*. In 1985, initial indigenous cases were reported from Cameroon. Several *in vivo* studies yielded prevalences from 30% to 60% of intermediate (RII) and high-grade (RIII) resistance to chloroquine in Cameroon. Brasseur and others found a remarkably higher prevalence of chloroquine resistance in southern Cameroon compared with the northern part of the country. In the Congo, the prevalence of resistance to chloroquine ranges from 29% in the plains to 62% in the capital. From both countries, chloroquine resistance is reported to be stabilizing or even decreasing. In neighboring Equatorial Guinea, the prevalence of *in vitro* resistance to chloroquine is reported to be only 16%. In Gabon, our first *in vitro* study revealed a dramatic increase in the prevalence of chloroquine resistance and in the mean EC$_{50}$ from 1983 to 1992.

![Figure 1](https://example.com/figure1.png)

**Table 3**

<table>
<thead>
<tr>
<th>EC in nmol/liter of BMM</th>
<th>Mean</th>
<th>95% confidence limit</th>
</tr>
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<tbody>
<tr>
<td>EC$_{50}$ (1992)</td>
<td>0.41</td>
<td>0.29–0.51</td>
</tr>
<tr>
<td>EC$_{50}$ (1994)</td>
<td>1.64</td>
<td>1.08–2.47</td>
</tr>
<tr>
<td>EC$_{90}$ (1992)</td>
<td>0.94</td>
<td>0.60–1.34</td>
</tr>
<tr>
<td>EC$_{90}$ (1994)</td>
<td>7.62</td>
<td>4.53–12.81</td>
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*EC = effective concentration; BMM = blood medium mixture.*
present follow-up study, the EC₅₀ was similar to the level observed in 1992. The local drug pressure has not changed, with the overall amount of chloroquine prescribed at the Albert Schweitzer Hospital approximating 10 kg/year from 1992 to 1994 (Pharmacy of the Albert Schweitzer Hospital, Lambarene, Gabon, 1994, unpublished data).

Figure 3 shows the overall development of the EC₅₀ of chloroquine, mefloquine, halofantrine, and quinine in Lambarene, Gabon from 1983 to 1994 or from 1992 to 1994. Chloroquine is the only drug showing a slight tendency towards a lower resistance between 1992 and 1994, while the EC₅₀ of halofantrine, mefloquine, and quinine are increasing. This is consistent with reports of a genetically determined cross-resistance between mefloquine, halofantrine, and quinine and an inverse correlation between chloroquine and mefloquine. Resistance to mefloquine and susceptibility to chloroquine was reported to be linked to the \textit{P. falciparum} multiple drug resistance (\textit{mdr}) gene. A positive correlation between the \textit{in vitro} responses to mefloquine, halofantrine, and quinine was observed in the Lambarene area in 1992.

\textit{In vitro} and \textit{in vivo} resistance to the antimalarial mefloquine has been reported from Cameroon and found to be correlated with resistance to quinine. Even before the introduction of the drug to west Africa, resistant strains were found there. In \textit{in vitro} resistance to this drug in one of 34 isolates has been found in Brazzaville in the Congo. In the present study, the high susceptibility to mefloquine could be confirmed in the study area since the mean EC₅₀ was only slightly higher than in 1992 and none of the isolates exhibited signs of resistance. This is consistent with our \textit{in vivo} findings in 1994, in which none of 21 children showed recrudescence after a single dose of 15 mg/kg mefloquine. The present \textit{in vitro} and \textit{in vivo} findings indicate that mefloquine is an effective antimalarial in mild \textit{P. falciparum} malaria in Gabon. Nevertheless, in the light of the above mentioned cross-resistance between mefloquine, quinine, and halofantrine the slight increase in the EC₅₀ for mefloquine deserves further investigation and close follow-up.

The same is true for quinine, which is still the most important antimalarial for the treatment of severe and complicated malaria on the African continent. In Cameroon, \textit{in vitro} resistance to quinine in local populations of \textit{P. falciparum} was first observed in 1985 and found to have remained relatively stable in 1988. Quinine resistance has sporadically occurred in travelers returning from francophone west Africa and east Africa. In Gabon, Winkler and others reported full sensitivity \textit{in vitro} to quinine. In a recent \textit{in vivo} study these findings were confirmed since no RII and RIII responses were observed with the standard seven-day quinine regimen for severe malaria in children. In the present study, no isolate exhibited maturation at concentrations above the threshold of resistance, but six (19%) of 32 isolates had borderline to resistance with maturation at 2.56 \(\mu\)mol/L of BMM. Quinine sensitivity has thus decreased in comparison to 1992, indicating emerging resistance to quinine. This would be consistent with findings from Nigeria, where emerging resistance to quinine was reported. If a
substantial level of resistance to quinine occurred in Africa, the treatment of severe and complicated malaria would certainly have to be reflected since the emergence of clinically important resistance to quinine in Thailand has been demonstrated. When quinine is compared with its diastereoisomer quinidine, the latter is clearly more effective, as shown in Figure 1. However, the clinical implications of this finding are unclear since quinidine has also more adverse effects on the cardiovascular system and therefore necessitates electrocardiographic monitoring during treatment.

Artemisinin has been found to be an effective antimalarial in Africa both in vivo and in vitro. However, the number of in vitro studies is limited. Mean EC₅₀ values range from 16 to 21 nmol/L. In our study, 23 isolates (70%) tested for artemisinin were fully inhibited at 300 nmol/L of BMM and 10 isolates (30%) were inhibited at 1,000 nmol/L of BMM. The mean EC₅₀ and EC₉₀ were 98 and 364 nmol/L of BMM (Table 1).

For sulfadoxine/pyrimethamine, our findings indicate a stabilizing level of resistance of approximately 30% of all isolates. This is consistent with levels of 29% in Nigeria and 23% in Ghana. In Equatorial Guinea, only 3% of all tested isolates were reported to be resistant. At the Albert Schweitzer Hospital, sulfadoxine/pyrimethamine is the treatment of choice and the most frequently administered antimalarial despite 30% in vitro resistance and the possibility of severe side effects because it is a cheap drug and easy to administer. However, in a recent in vivo study in Lambarene, only one of 40 school children exhibited low-grade resistance to sulfadoxine/pyrimethamine. Reports of in vivo resistance to sulfadoxine/pyrimethamine in 28 of 38 children in Tanzania and 37% of RII/RIII resistance in Ghana indicate an emerging resistance to sulfadoxine/pyrimethamine in Africa in areas of high and sustained drug pressure.

The most striking result of the present study is the steep decrease in the susceptibility to halofantrine, with a four-fold increase in the mean EC₅₀ and an almost eight-fold increase in the EC₉₀ (Figure 2). Previously, halofantrine was found to be highly effective in vivo in its micronized formulation. Halofantrine is a frequently used, relatively safe antimalarial in Gabon, and there are no reports of clinical treatment failures so far in this country. Our own experience in Lambarene indicated a marked sensitivity of the parasite to halofantrine in Gabon in 1992. Nevertheless, the question of emerging resistance to halofantrine in Gabon was raised in 1992 because of positive correlations between the susceptibility to the widely used drug quinine and mefloquine and halofantrine in Lambarene. Several investigators have found a positive correlation among the responses to these drugs.

Halofantrine is a frequently used, relatively safe antimalarial in Gabon, and there are no reports of clinical treatment failures so far in this country. Our own experience in Lambarene indicated a marked sensitivity of the parasite to halofantrine in Gabon in 1992. Nevertheless, the question of emerging resistance to halofantrine in Gabon was raised in 1992 because of positive correlations between the susceptibility to the widely used drug quinine and mefloquine and halofantrine in Lambarene. Several investigators have found a positive correlation among the responses to these drugs. Drug resistance to mefloquine in Cameroon and to halofantrine in the Congo, as well as a decreasing efficacy of quinine, made the emergence of a reduced sensitivity to halofantrine in Gabon a matter of time. A reduced sensitivity to halofantrine was observed in west Africa in 1990. In Lambarene, all isolates were fully inhibited at 3 nmol/L of BMM in 1992. In 1994, 81% of the isolates matured at that concentration. In Thailand, the susceptibility to halofantrine had already decreased before it was commercially available there, probably as a result of increased mefloquine pressure and resistance to this latter drug. In Gabon, the emergence of reduced sensitivity to halofantrine is unlikely to have been triggered by widespread use of mefloquine because this drug is too expensive and available only as mefloquine/sulfadoxine/pyrimethamine (two tablets of Fansimed® [E Hoffmann-La Roche, Basel, Switzerland] = 4,700 Franc de la Communaute Financiere Africaine [FCFA] in 1994). Halofantrine itself is a frequently sold and self-administered antimalarial in Gabon, despite its high cost (six tablets of Halfan® [SmithKline Beecham, Brentford, United Kingdom] cost 6,800 FCFA in 1994). Because of the high cost, self-treatment with halofantrine is frequently underdosed. This, in combination with intense transmission and selection due to the presence of residual drug, is likely to result in a reduced sensitivity to halofantrine. Nevertheless, a reduced sensitivity to halofantrine in vitro will not express itself as treatment failures as long as...
an appropriate threshold has not been reached. Since there are no data concerning the in vivo response to halofantrine from studies in Gabon in 1994 and no clinical treatment failures were recorded at the Albert Schweitzer Hospital, clinical studies to monitor a possible change in the in vivo response of *P. falciparum* to halofantrine seem to be necessary. Quinine is still widely used and is the second most frequently prescribed antimalarial drug in the Albert Schweitzer Hospital. Halofantrine and quinidine drug pressure may select and sustain an emerging resistance to halofantrine and eventually mefloquine. The sensitivity to quinine and mefloquine has also decreased in the study area, but to a much lesser degree as compared with halofantrine. The fast increase in the EC₅₀ of halofantrine confirms the observed positive correlations between quinine, mefloquine, and halofantrine and necessitates a close follow-up of the susceptibility to those drugs in Lambaréne, Gabon.

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