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IN-VITRO SENSITIVITY OF *PLASMODIUM FALCIPARUM* TO CHLOROQUINE, HALOFANTRINE, MEFLOQUINE AND QUININE IN MADAGASCAR

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ABSTRACT

Objective: **To determine how sensitive *Plasmodium falciparum* is to the major antimalarial drugs in Madagascar.**
Design: **Assessment of *Plasmodium falciparum* isolates sensitivity to antimalarials, by use of the *in-vitro* radioisotope method.**
Setting: **Ankazobe and Saharevo in the foothill areas; and Toamasina and Tolagnaro in the coastal areas (between January 1998 and November 1999).**
Subjects: **Primary *Plasmodium falciparum* isolates from patients with uncomplicated malaria attack.**
Results: **Between January 1998 and November 1999, of the 293 *in-vitro* tests done with at least one antimalarial, 70% (205/293) were interpretable. As there was no significant difference between results from the four study sites, the data have been expressed as a whole. All of the successfully tested isolates were sensitive to halofantrine (n = 56) and to quinine (n = 199), 5.8% (12/205) of the isolates were resistant to chloroquine and 2% (4/199) to mefloquine. The geometric mean IC50 was 0.3 µg/L for halofantrine (95% CI = 0.1 - 0.4 µg/L); 9.4 µg/L for chloroquine (95% CI = 7.3 - 10.8 µg/L); 3.8 µg/L for mefloquine (95% CI = 3.3 - 4.3 µg/L); and 26.8 µg/L for quinine (95% CI = 24.3 - 29.4 µg/L). The low positive correlation found between halofantrine and chloroquine IC50s (n=56; r = 0.41, *P* = 0.002) suggests a risk of cross-resistance between these two drugs.**
Conclusion: **The degree and frequency of chloroquine resistance *in-vitro* is stationary in Madagascar compared to previous results during the last decade. The *in-vitro* sensitivity of *P. falciparum* to quinine, mefloquine and halofantrine encourages the use of these drugs as alternative in case of chloroquine treatment failure. Nevertheless, it is important to maintain and to extend malaria and drug sensitivity surveillance in Madagascar.**

INTRODUCTION

Malaria is a major health problem in Madagascar, and chloroquine remains the first line treatment according to the national policy for malaria chemotherapy and chemoprophylaxis. More than 90% of confirmed cases of clinical malaria are due to *Plasmodium falciparum.* Chloroquine resistance R1 type was first discovered in Madagascar in 1980s(1) and R2 resistant type has a very low prevalence(2-3). *In-vitro* studies led to the detection of certain *P. falciparum* isolates with high chloroquine resistance (IC50>38.4µg/L), even though so far no cases of R3 type resistance *in-vivo* have been reported(3). If chloroquine resistance increases, it might become a serious public health problem accompanied with an eventual increase of mortality. Thus, an effective alternative drug is required to cope with (i) the failure of chloroquine treatment and (ii) severe and complicated malaria. As part of an investigation on the effectiveness of the major antimalarial drugs that are commercially available in Madagascar, we assessed *in-vitro* the susceptibility of *P. falciparum* isolates to chloroquine, halofantrine, mefloquine and quinine.

MATERIALS AND METHODS

Plasmodium falciparum isolates were collected (i) from Ankazobe and Saharevo, two rural villages in the foothill areas of the central highlands of Madagascar; (ii) from Esana a rural village in the south east part of the island; (iii) and from the main public urban hospital of Toamasina, on the east coast. This study was performed between January 1998 and November 1999.

For each patient who attended one of the health centres, with suspected malaria, thin and thick blood smears were prepared. The smears were stained with Giemsa's Solution and examined by microscope. *P. falciparum* isolates were included in the study if at least 1,500 trophozoites were detected per microlitre of blood and if patients had not recently (<14 days) taken antimalarial drugs. Blood samples (2 to 5 ml) were drawn by venopuncture from patients with confirmed malaria if they (or their guardians or parents) gave consent and collected in citric acid dextrose-tubes. Patients were then treated for malaria by the local medical team according to the recommendation of the national policies, without additional follow up, the protocols used for the present study were approved by the Madagascar ministry of health.

Test antimalarial drugs: Chloroquine sulphate and quinine base were purchased from sigma chemicals, halofatrine from SmithKline Beecham pharmaceuticals and mefloquine from Roche products. Sterile stock solutions were prepared in methanol/water and serial dilutions were made in distilled water. Test concentrations ranged from 4 to 512 µg/L for chloroquine, from 0.1 to 16 µg/L for halofantrine, 0.9 to 153 µg/L for mefloquine and 8 to 1038 µg/L for quinine.

In-vitro tests: The isolates were transported at +4°C to +8°C to the malaria research unit at the Institut Pasteur de Madagascar in Antananarivo within 48 hours of the blood collection. *In-vitro* chemosensitivity of *P. falciparum* isolates was tested by the determination of the tritium labelled hypoxanthine incorporation(4). The isolates were washed three times with RPMI 1640 (culture medium without serum) buffered with HEPES (25mM) and $NaHCO_3$ (25mM). For the test, the medium was supplemented with 10% non-immune human type AB-positive serum. (3_H)-hypoxanthine was added to a final concentration of 0.5µCi per well. Field isolates with parasitaemia over than 50,000 trophozoites/µl of blood were diluted with human type O-Positive erythrocytes to 25,000 trophozoites/µl of blood before being suspended in culture medium. Erythrocytes were suspended to 2.5% in culture medium and aliquoted into the wells of the appropriate predosed plates (200µl per well ; two or three wells per concentration). The plates were incubated at 37°C for 42 hours in a candle jar atmosphere, with 95% humidity. After incubation, the contents of each well were harvested, by use of an Innotech™ cell harvester, onto the glass-fibre filters which were washed with distilled water and allowed to dry. Each filter was placed in a plastic sample bag (Wallac) containing 4 ml of scintillation fluid (BCS™, Amersham). The radioactivity was counted using a MicoBeta™ Wallac liquid scintillation counter. Tests were considered successful when counts in the control wells (drug free) exceeded 1,000 counts per minute. Quality control for the batches of plates previously treated with test antimalarial drugs was done by testing systematically the chloroquine and pyrimethamine-susceptible *P. falciparum* clone 3D7 and the chloroquine and pyrimethamine-resistant *P. falciparum* clone FCM 29.

Data analysis: The percentage growth relative to the control was calculated. The inhibitory concentrations 50% (IC50), namely the concentration that inhibits the growth of the parasites by 50%, of each test antimalarial were calculated by regression analysis of log concentration/response probit curves. Isolates were considered to be resistant *in-vitro* to chloroquine if the IC50 value was greater than 38.4 µg/L (120 nM). The resistance thresholds for the other antimalarials were as follows: halofantrine IC50>3 µg/L, quinine IC50>194.6 µg/L, and mefloquine IC50>19.1 µg/L(5-6). Results are expressed as the geometric mean of IC50s (GMICS 50s) and the 95% confidence interval (95% CI) are stated. The correlation between *P. falciparum in-vitro* response to two different drugs, based on IC50s, was analysed by Pearson's correlation.

RESULTS

During the study period, 293 isolates were tested *in-vitro* against at least one antimalarial drug. In total, 70% (205/293) of the tests done were interpretable. As there was no significant difference between results from the four study sites, the data have been expressed as a whole (Table 1).

Table 1

In-vitro sensitivity of Plasmodium falciparum to halofantrine, chloroquine and mefloquine, quinine in Madagascar during the malaria transmission 1998/1999

Antimalarial drug	Isolates number	Geometric mean of IC50s in µg/L	95% CI of IC50s Geometric mean*
Halofantrine	56	0.3	0.1-0.4
Chloroquine	205	9.4	7.3-10.8
Mefloquine	199	3.8	3.3-4.3
Quinine	199	26.8	24.3-29.4

* 95% confidence interval

Chloroquine: The GMIC50s was 9.4 ug/L (95% CI = 7.3-10.8 µg/L). Of the 205 *P. falciparum* isolates successfully tested with chloroquine, 12 (5.8%) were resistant i.e. IC50> 38.4µg/L (Figure 1). The IC50 values ranged from 0.1 to 96.3 µg/L, with a median of 9.8 µg/L.

Mefloquine: Of the 293 *P. falciparum* isolates, 287 were tested against mefloquine. Four (2%) of the 199 assessable tests were resistant. The IC50s ranged from 0.5 to 23.9 µg/L. (95%CI = 3.3-4.3 µg/L). The median IC50 was 3.8 µg/L.

Quinine: Of the 287 tests done for quinine, 199 were assessable. All of the successfully tested isolates

were susceptible to quinine. The highest quinine 1C50 value recorded was 106.5 µg/L. The GMIC50s was 26.8 µg/L (95% CI = 24.3-29.4 µg/L), and the median IC50 was 27 µg/L. Quinine was effective against the 12 chloroquine and the four mefloquine-resistant isolates.

Halofantrine: Of the 293 *P. falciparum* isolates, only 74 were tested against halofantrine. Tests were assessable for 56 (76%) isolates. All of the successfully tested isolates were sensitive to halofantrine, and the IC50s ranged from 0.005 to 1.9 µg/L (Figure 2). The median IC50 was 0.45 µg/L. The GMIC50s was 0.3 µg/L (95% CI: 0.1- 0.4µg/L).

Figure 1

In-vitro response of Plasmodium falciparum isolates to chloroquine (n=205)

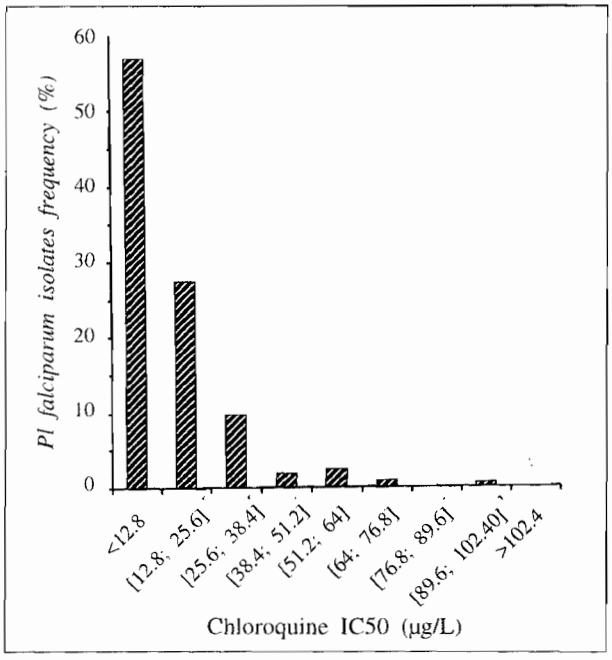
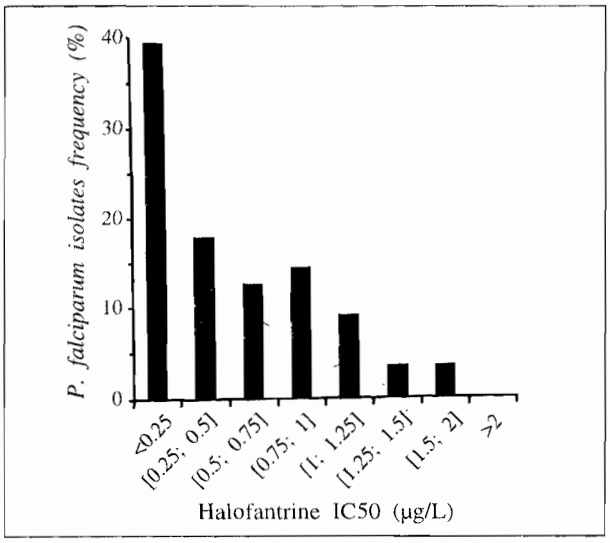


Figure 2

In-vitro sensitivity of Plasmodium falciparum isolates to halofantrine (n=56)



Correlation between drug activities: A low but significant positive correlation was observed between the responses of *P. falciparum* to halofantrine and mefloquine (n = 55; r = 0.22 *p* = 0.03); halofantrine and chloroquine (n = 56; r = 0.41; p = 0. 002); chloroquine and quinine (n = 199; r = 0.18; p = 0.01); chloroquine and mefloquine (n = 199; r = 0.17; *p* = 0.02) and mefloquine and quinine (n = 199; r = 0.43; *p* = 0.0001). There was no significant correlation between the responses to halofantrine and quinine. Halofantrine was almost 13 times more potent than mefloquine *in-vitro*. No cases of cross resistance were observed between chloroquine and mefloquine.

DISCUSSION

This study demonstrated that 5.8% (12/205) of the successfully tested isolates of *P. falciparum* were resistant to chloroquine. The median chloroquine IC50s was 9.8µg/L, suggesting that *P. falciparum* is generally susceptible to chloroquine overall. The rate of *in-vitro* chloroquine resistance has not changed much for the last decade, ranging from 0 to 15% regardless of the regions in Madagascar(2,3,6). The *in-vivo* efficacy of this drug is satisfactory. No cases of R3 have been observed despite years of surveillance and early treatment failure with chloroquine is still rare(3,7). The fact that Madagascar is an island seemed to mean that it was protected from the rapid dissemination of resistant malaria for years compared to what happens in Africa for example(8-10). However, recent improvements in commercial exchanges between Madagascar and its neighbouring countries in the Indian ocean region that have prevalent chloroquine-resistant isolates, and between Madagascar and Asia are likely to increase the probability of importing drug resistant malaria. Up to today, the surveillance of the chloroquine resistance in Madagascar has been based mainly on the *in-vivo* and the *in-vitro* test. Djimdé *et al* have shown the role that some mutations may play in *Pfmdr* and *Pfcrt* genes to predict the risk of chloroquine treatment failure(11). The lack of *Pfcrt* mutation lysine—> threonine-76 even in *in-vitro* chloroquine resistant isolates of Madagascar *P. falciparum* encourages the use of the genotyping-based surveillance of drug resistance for the future(12).

All of the *P. falciparum* isolates tested in this study were sensitive to quinine. Quinine is usually used to treat severe malaria in Madagascar. There has never been a documented case of *in-vitro* or *in-vivo* quinine resistance(6,13-15). This drug is then a valuable alternative to chloroquine for the treatment of falciparum malaria in cases of treatment failure with chloroquine.

The *in-vitro* mefloquine test revealed that 2% of *P. falciparum* isolates were resistant, which confirms that the prevalence of mefloquine resistant isolates are still very low as we already reported(16). However, there is a need of monitoring mefloquine effectiveness against *P. falciparum* because mefloquine-prophylaxis

failure have been reported in Madagascar(17-18).

Our results have shown as well that halofantrine is effective against Madagascan *P. falciparum* . The only documented case of halofantrine treatment failure against Madagascan *P. falciparum* occurred in France. It was a late recrudescence of the parasites in a French patient who contracted malaria during his journey in Madagascar(19). As halofantrine post-treatment dosage was not done, we cannot easily draw conclusion about the resistance of this isolate. But halofantrine does not have a well defined role in antimalarial chemotherapy policy in Madagascar, except perhaps as a stand-by treatment for foreign travellers. This drug is available in commercial pharmacies in Madagascar. Different authors reported that this drug remains an effective means of treating falciparum malaria regardless of the prevalence of chloroquine-resistance(8,20-21). However, this drug is costly for an average patient, and is not used in the primary and secondary health care centres in Madagascar. The correlation between halofantrine and chloroquine *in-vitro* activities suggests anyway a potential risk of cross-resistance. This positive correlation noticed in Madagascar contrasts with the situation described by Ouédraego *et al*(5) in Burkina Faso.

In conclusion, the degree and frequency of chloroquine resistance *in-vitro* is stationary compared to our previous results during the last decade. The sensitivity of Madagascan *P. falciparum* to quinine and halofantrine encourages mainly the use of these drugs as alternative in case of chloroquine treatment failure. These informations on the current status of drug sensitivity are vital to general practitioners, and therefore needs to be continually updated. Further *in-vivo* and *in-vitro* studies are required to monitor in real time the sensitivity of Madagascan *P. falciparum* to major antimalarial drugs.

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