

## In vitro susceptibility of *Plasmodium falciparum* to four antimalarial drugs in the Central Province of Papua New Guinea

FRANCIS W. HOMBHANJE<sup>1</sup>

Department of Basic Medical Sciences, University of Papua New Guinea, Port Moresby

### SUMMARY

The susceptibility of *Plasmodium falciparum* to chloroquine, quinine, mefloquine and halofantrine was investigated in the Central Province of Papua New Guinea between March 1995 and September 1996, when chloroquine resistance was widely present in the country. The standard World Health Organization in vitro microtest methodology was used in the study. Of the 30 isolates tested for chloroquine susceptibility all were resistant to chloroquine with median IC<sub>50</sub> of 1.15 µmol/l (range 0.54 to 4.24), indicating a high prevalence and degree of resistance. Three isolates each for quinine (3/31) and halofantrine (3/28) showed resistance at concentrations of 51.2 µmol/l and 10 nM respectively, while all 31 isolates tested for mefloquine were fully susceptible. The comparative analysis of median IC<sub>50</sub> values between isolates resistant and susceptible to chloroquine showed chloroquine-resistant isolates to be less susceptible to quinine and halofantrine while fully susceptible to mefloquine. It seems that the evolution of chloroquine resistance together with increased use of quinine treatment of *P. falciparum* malaria may increase the risk of emergence of quinine resistance and possibly of halofantrine resistance as well. The development of mefloquine resistance, however, is independent of chloroquine resistance.

### Introduction

Chloroquine-resistant strains of *Plasmodium falciparum* are now widespread in Papua New Guinea (PNG) (1,2) since such a strain was first reported in 1976 (3,4). These resistant strains have now been reported from almost every part of the country with increasing frequency (5-8). As a result, quinine alone or in combination with sulfadoxine-pyrimethamine (Fansidar) has been used more frequently. Unfortunately, resistance to these and other alternatives has been reported (9-11), posing a serious challenge to any efforts to treat malaria effectively in the region. To meet the challenge, alternative antimalarials need to be sought and evaluated as to their suitability against local malarial parasite strains.

Mefloquine and halofantrine are the next generation of alternative antimalarial drugs awaiting approval from the Health Department

for clinical use in PNG. To date, very little information on their antimalarial activity against local strains or isolates of *P. falciparum* is available to form the basis for important therapeutic decisions. The present study was conducted to assess the susceptibility pattern of local *P. falciparum* isolates to all four drugs, chloroquine, quinine, mefloquine and halofantrine. These data set the baseline for subsequent studies in monitoring the evolving pattern of drug resistance in the Central Province.

### Patients and Methods

The study was carried out in the Central Province of PNG from March 1995 to September 1996. Patients presenting themselves at the Adult Outpatients Department of Port Moresby General Hospital were selected if they had asexual forms of *P. falciparum* with a density between 1000 and

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<sup>1</sup> Department of Basic Medical Sciences, Faculty of Medicine, University of Papua New Guinea, PO Box 5623, Boroko, NCD 111, Papua New Guinea

90,000 per  $\mu\text{l}$  of blood with no history of antimalarial drug ingestion within three weeks of presentation. The recently developed ParaSight-F test (12) was applied for confirmation of *P. falciparum* species in questionable cases.

Pretreatment blood samples (5 ml) were collected into a heparinized tube by venipuncture and transported immediately to the laboratory. Following centrifugation at low speed (150-200 rpm) the plasma was discarded and infected erythrocytes were washed three times in complete culture medium (RPMI 1640 with 25 mM HEPES buffer and 25 mM  $\text{NaHCO}_3$ ) before assaying. Samples with high parasitaemia were diluted with fresh washed human type O-positive red blood cells to reach a parasitaemia of 1%. All chemicals for parasite culture, including nonimmune human sera, were obtained from Sigma (USA) except for RPMI 1640 (Gibco).

### **In vitro microtest technique**

The drug susceptibility of *P. falciparum* was determined using the World Health Organization (WHO) standardized in vitro microtest system (13) developed by Rieckmann et al. (14), adapting the methodology used for the cultivation of *P. falciparum* (15). The technique involves quantitating schizont maturation following cultivation of infected erythrocytes in plates charged with defined quantities of drug. This technique has been widely applied in many countries of the world to assess the in vitro susceptibility of *P. falciparum* to chloroquine, amodiaquine, quinine, mefloquine, pyrimethamine, halofantrine and other newer agents.

### **Drug preparation**

Pre-dosed plates of chloroquine, quinine and mefloquine were obtained from WHO in Manila. Halofantrine (racemate) was obtained from Smith Kline & Beecham (Welwyn, UK) and the drug plates for halofantrine were prepared as follows: a stock solution ( $10^{-2}$  M) was prepared by dissolving 53.7 mg in 10 ml of 70% alcohol solution. From the stock solution serial dilutions were prepared to give the final test concentrations of 0.1, 1.0, 2.5, 5, 10, 50 and 100 nmol per well. Aliquots of 100

$\mu\text{l}$  of each drug concentration were dispensed into the wells of a 96-well sterile microtitre plate, one concentration per row of 12 wells. The first row was the control, containing drug-free culture medium, 100  $\mu\text{l}$  per well.

### **In vitro microtest**

100  $\mu\text{l}$  of washed infected erythrocytes was mixed aseptically with 900  $\mu\text{l}$  of complete culture medium (1:10) and 50  $\mu\text{l}$  of the blood-medium mixture was pipetted into each well of the pre-dosed plates. For halofantrine, an aliquot (10  $\mu\text{l}$ ) of washed parasitized erythrocytes was added to the drug solutions (100  $\mu\text{l}$ ) in each well. The plates were incubated in low oxygen conditions in a candle jar for 24-36 hours. All the susceptibility assays for each drug were done in duplicate. Depending on the schizont maturation stages following incubation of the test plates, the supernatant in each well was removed and thin blood films were prepared. These blood smears were left to dry, fixed in methanol and stained with buffered (pH 7.2) 20% Giemsa for 15-20 minutes. The schizonts with two or more nuclei were counted against 100 asexual parasites. The percentage for each drug concentration was calculated by dividing the schizont count per 100 parasites by the schizont count per 100 parasites of the control and multiplying by 100. A test was considered assessable if 20% or more schizonts were in the control well. Resistance was indicated if schizonts appeared in the presence of 1.14  $\mu\text{mol/l}$  (5.7 pmol/well) of chloroquine, 51.2  $\mu\text{mol/l}$  (256 pmol/well) of quinine, 3.2  $\mu\text{mol/l}$  (16 pmol/well) of mefloquine and 10 nmol/well of halofantrine.

### **IC<sub>50</sub> (50% inhibitory concentration)**

The IC<sub>50</sub> represents the concentration at which 50% of the isolates were inhibited from maturing to schizonts. The IC<sub>50</sub>s for the individual isolates were determined by the linear extrapolation method previously described (16). The threshold value for chloroquine resistance (1.14  $\mu\text{mol/l}$ ) was used as a break-point to subdivide the isolates into two groups, chloroquine-susceptible (IC<sub>50</sub> <1.14  $\mu\text{mol/l}$ ) and chloroquine-resistant (IC<sub>50</sub>  $\geq$ 1.14  $\mu\text{mol/l}$ ), in order to evaluate the drug susceptibility pattern for quinine, mefloquine

**TABLE 1**  
 RESULTS OF IN VITRO MICROTTESTS WITH CHLOROQUINE (CQ), QUININE (Q), MEFLOROQUINE (MEF) AND HALOFANTRINE (HF) OF CENTRAL PROVINCE ISOLATES OF *P. FALCIPARUM*

Drug dose pmol/well	Drug concentration µmol/l	No (%) of isolates totally inhibited				% inhibition of schizont maturation			
		CQ	Q	Mef	Hf	CQ	Q	Mef	Hf
1.0	0.20	0 (0)				11.7			
2.0	0.40	0 (0)				21.5			
4.0	0.80	0 (0)				34.8			
5.7	1.14	0 (0)				48.4			
8.0	1.60	1 (3)				67.3			
16.0	3.20	7 (23)				79.5			
32.0	6.40	15 (50)				95.6			
4.0	0.80		0 (0)				15.2		
8.0	1.60		0 (0)				35.3		
16.0	3.20		0 (0)				57.1		
32.0	6.40		1 (3)				78.7		
64.0	12.80		12 (39)				93.2		
128.0	25.60		23 (74)				97.9		
256.0	51.20		28 (90)				99.7		
0.5	0.10			0 (0)				15.1	
1.0	0.20			0 (0)				33.4	
2.0	0.40			0 (0)				56.8	
4.0	0.80			6 (19)				84.0	
5.7	1.14			19 (61)				96.2	
8.0	1.60			24 (77)				98.5	
16.0	3.20			31 (100)				100.0	
<b>nanomol/well</b>									
0.1					0 (0)				17.1
1.0					0 (0)				47.0
2.5					3 (11)				63.3
5.0					15 (54)				92.6
10.0					25 (89)				98.9
50.0					25 (89)				99.7
100.0					28 (100)				100.0
<b>No of isolates tested</b>		30	31	31	28	30	31	31	28

TABLE 2

DRUG SUSCEPTIBILITY PROFILE OF ISOLATES OF *PLASMODIUM FALCIPARUM* WITH RESPECT TO CHLOROQUINE RESISTANCE

Drug	Chloroquine-susceptible isolates			Chloroquine-resistant isolates		
	No	Median IC <sub>50</sub>	Range (µmol/l)	No	Median IC <sub>50</sub>	Range (µmol/l)
Chloroquine	13	0.80	0.54 - 1.12	14	1.46	1.17 - 2.24
Quinine	13	1.98	1.16 - 9.77	14	2.80	1.03 - 5.43
Mefloquine	13	0.37	0.15 - 0.92	14	0.35	0.23 - 0.83
Halofantrine*	13	0.83	0.07 - 3.34	14	1.65	0.58 - 3.75

IC<sub>50</sub> = 50% inhibitory concentration (µmol/l)

\* nanomolar (nM) concentration

and halofantrine against these two parasite subpopulations.

### Results

A total of 39 falciparum isolates were collected but only 30 isolates were evaluated for chloroquine, 31 for quinine and mefloquine, and 28 for halofantrine. The reasons for discarding the other tests were bacterial contamination and failure of schizonts to mature satisfactorily. The drug susceptibility data are presented in Tables 1 and 2.

#### Chloroquine

All the isolates tested showed schizont maturation at a concentration of 1.14 µmol/l of chloroquine, indicating some degree of resistance to the effect of the drug in all the parasite cultures at this concentration (Table 1). Above this concentration some isolates were totally inhibited and at a concentration of 6.4 µmol/l 50% of the isolates were inhibited. The remaining 50% showed some schizont maturation at 6.4 µmol/l, indicating in these isolates a high degree of in vitro chloroquine resistance. The median IC<sub>50</sub> of chloroquine was 1.15 µmol/l (range 0.54-4.24), a value close to the threshold value which has been used to define in vitro resistance. 50% of the parasite population was classified as chloroquine susceptible (<1.14 µmol/l) and the other 50% as chloroquine resistant (≥1.14 µmol/l).

#### Quinine

Schizont maturation was inhibited in 90% (28/31) of the isolates at 51.2 µmol/l of quinine concentration (Table 1). 3 isolates showed minor maturation at that concentration with the isolates overall demonstrating more than 99% inhibition of trophozoite maturation to schizonts. The median IC<sub>50</sub> was 2.76 µmol/l (range 1.03-9.77), well below the threshold value for quinine resistance. The chloroquine-resistant isolates were less susceptible to quinine than the chloroquine-susceptible isolates (Table 2).

#### Mefloquine

All the isolates were inhibited at 3.2 µmol/l of mefloquine, indicating a high susceptibility to the drug (Table 1). At this concentration maturation of trophozoites to schizonts was totally inhibited in 100% of the isolates. The low median IC<sub>50</sub> values (0.35; range 0.15-0.77 µmol/l) indicate high efficacy of mefloquine in vitro. Chloroquine-resistant and susceptible isolates were equally susceptible to mefloquine in vitro (Table 2).

#### Halofantrine

Not all the isolates were susceptible to halofantrine. 89% (25/28) of the isolates demonstrated inhibition of schizont maturation at a concentration of 10 nM (Table 1). The remaining 3 isolates (11%) showed schizont

maturation at and above that concentration but with eventual inhibition of schizont maturation for all the isolates at 100 nM concentration of halofantrine, the highest drug concentration tested in this system. The  $IC_{50}$ s (0.07 to 3.7 nM; median = 1.02) for the individual isolates were lower than the defined threshold. The chloroquine-resistant isolates were less susceptible to the drug than the chloroquine-susceptible isolates (Table 2).

### Correlation of responses

The individual responses to chloroquine and halofantrine, and to chloroquine and quinine, showed no correlation. However, a positive correlation was found between the individual responses to quinine and mefloquine, and between the responses to mefloquine and halofantrine. An inverse correlation was found between chloroquine and mefloquine.

### Discussion

This is the first report of an in vitro susceptibility assessment of *P. falciparum* isolates to chloroquine, quinine, mefloquine and halofantrine in the Central Province of PNG. Among the isolates collected and assessed from the study area, a high frequency and degree of chloroquine resistance in vitro was observed. In all the isolates tested at 1.14  $\mu\text{mol/l}$  concentration, the accepted threshold for chloroquine resistance in vitro, chloroquine was unable to inhibit schizont maturation. Even at the maximal drug concentration of 6.4  $\mu\text{mol/l}$ , only 50% of the isolates showed complete inhibition of schizont maturation, suggesting that the level of chloroquine resistance is relatively high by in vitro criteria. The findings are consistent with recently available data from some areas of the country (8,17). The level of in vitro chloroquine resistance observed here would be expected to exceed R1/R2 levels of resistance by in vivo methods. Although in vitro susceptibility of the isolates may not directly predict the in vivo outcome, such a high level of chloroquine resistance in the area will put increasing demand on quinine use as an alternative.

Thus far, treatment failure with quinine has not been reported in the Central Province. Nevertheless, the results of the present study indicate the presence of a few isolates of

*P. falciparum* with diminished susceptibility to quinine. Approximately 10% (3/31) of the isolates tested showed minor schizont maturation at the threshold quinine concentration of 51.2  $\mu\text{mol/l}$ . It is yet to be determined what it means in vivo for these isolates since Central Province strains of *P. falciparum* are, in general, highly susceptible to quinine by in vivo observations. In other countries, in parallel with the emergence of chloroquine resistance, some authors found diminution of quinine susceptibility (18-21) while others (20,22,23) have reported full susceptibility of *P. falciparum* to quinine in vitro. Such discrepancies call for further studies to define the geographical variation of response in vitro to these drugs. Although the picture of chloroquine resistance appears gloomy in PNG, including the Central Province, it is important to note that quinine resistance is present in some foci at generally a low level and that quinine still largely retains its therapeutic efficacy in semi-immune populations.

The Central Province isolates of *P. falciparum* are highly susceptible to mefloquine. This finding is expected as mefloquine has never been openly prescribed for malaria treatment in the country. The results of the present study are consistent with the high in vitro susceptibility to the drug reported elsewhere (21,23-25), although some investigations (18,20,22,26-29) have demonstrated the occurrence of mefloquine resistance in vitro, even in areas where the drug has never been introduced (28,30-33). Chloroquine resistance has not influenced mefloquine's antimalarial activity or susceptibility profile in any way (see Table 2).

Halofantrine, a close relative of mefloquine, failed to inhibit all the isolates tested in vitro: 3 of 28 isolates (11%) grew in a halofantrine concentration of 10 nM, indicating resistance according to the definition of in vitro resistance in this study. The findings are unexpected because halofantrine has not been introduced into the country except for research purposes (34). It is likely that a spontaneously resistant gene pool exists in the local parasite population. Reduced in vitro susceptibility of *Plasmodium falciparum* to halofantrine has

been reported in some parts of the world (22,35,36) while others have reported high susceptibility to the drug (30,37). This wide variability in drug response across different geographical areas may reflect the genetic diversity of malarial parasites. However, the findings in this study of in vitro halofantrine resistance should not be overstated, since there have not been many studies which have examined the correlation between in vitro and in vivo results.

With respect to the correlation of responses, the individual isolate responses to chloroquine and halofantrine, and to chloroquine and quinine, showed no correlation. Apparent lack of correlation between chloroquine and quinine is not consistent with the findings of a previous study (33) where strong positive correlation existed between the drugs; the discrepancies may be due to differences in methodology or the characteristics of the isolates tested. Lack of correlation between chloroquine and halofantrine could imply that their antimalarial activities are independent of each other, at least against these isolates. However, a positive correlation was found between quinine and mefloquine, and between mefloquine and halofantrine. Since all the three drugs (aminoalcohols) are structurally related, cross-resistance between them is likely and has been found elsewhere (26,38). When the isolates were grouped into chloroquine-susceptible and chloroquine-resistant (Table 2), different implications emerge, with quinine and halofantrine both showing higher  $IC_{50}$  levels in chloroquine-resistant isolates, whereas mefloquine showed no difference. On individual isolate responses, in fact an inverse correlation was found between chloroquine and mefloquine, which may imply that mefloquine would be generally more active against chloroquine-resistant than chloroquine-susceptible isolates of *P. falciparum* in the Central Province. This observation confirms the findings of some studies (38,39) but does not support conclusions reached by others for chloroquine and mefloquine susceptibility (29). Such discrepancies may indicate geographic variations in parasite susceptibility patterns across different malaria-endemic areas, both within and between different countries.

It must be emphasized that the in vivo implications of these in vitro findings are not

immediately known. It is a common experience that antimalarial susceptibility tests of malaria parasites may not reflect the expected response of patients to therapy. For example, the parasites may be reported as resistant in vitro to a particular drug but the infection is cleared upon treatment. On the other hand, treatment may be ineffective despite high susceptibility of the infecting strains in laboratory tests. There are many possible reasons for such discrepancies. The methods used for determining drug susceptibility may be one of those. Others may include host immunity and the diversity in parasite biology (40,41). So the in vitro drug susceptibility data do not necessarily predict clinical outcome though they may strongly suggest the presence of a certain degree of resistance in vivo and be useful for monitoring changes in drug susceptibility patterns.

In conclusion, the results of this study revealed a high level and degree of chloroquine resistance in vitro in this study area. Chloroquine resistance may have compromised quinine's antimalarial activity in vitro but it is not clear to what extent and degree this observation might apply in vivo. As for the third-line antimalarials, mefloquine may be of greater value than halofantrine against *P. falciparum*. The parasites' more general genetic and biological constitution and behaviour at the time of the study cannot be ignored and may have partly influenced the drug susceptibility patterns demonstrated here.

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