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In vitro response of chloroquine-resistant *Plasmodium falciparum* to mefloquine

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Abstract

The present study was conducted to evaluate the application of the in vitro *microtechnique system in determining the response of chloroquine-resistant* Plasmodium falciparum *to mefloquine.*

Using isolates of P. falciparum *from Boa Vista, Brazil, and Villavicencio, Colombia, mefloquine was more than 7.7, 7.1, 7.1, and 6.4 times more effective than chloroquine* in vitro *at the* ED_{90}, ED_{95}, ED_{99}, *and* $ED_{99.9}$ *levels, respectively.*

Clinical chloroquine resistance of *Plasmodium falciparum* was first observed in Colombia in 1960 (*1*) and subsequently reported from various parts of South America, especially Brazil and Colombia (*2, 3, 4, 5*). The standard *in vitro* test for drug susceptibility described by Rieckmann et al. in 1968 (*6*) has been used to study the geographical distribution of chloroquine-resistant *P. falciparum* (*7, 8*).

Clinical chloroquine resistance is classified by degrees: R-I, recrudescence between days 7 and 28 after complete disappearance of the parasite from the peripheral blood; R-II, failure of the parasite to disappear from the peripheral blood, but reduction below 25% of the pretreatment level of parasitaemia during the first 6 days; and R-III, only slight reduction, maintenance, or increase of parasitaemia during treatment.

In the standard *in vitro* test, complete inhibition of parasite growth at 1.0 nmol of chloroquine per ml of defibrinated blood indicates full sensitivity to the drug (*9*)[a] while growth at 1.25 nmol/ml is a sign of resistance.[b]

Chloroquine resistance of *P. falciparum* is of considerable clinical and epidemiological importance. Patients with resistant strains may continue to exhibit patent parasitaemia and symptoms, or after apparent cure may remain exposed to a potentially fatal infection. In resistant falciparum malaria, the parasite reservoir is maintained thus serving as a source of further transmission and promoting the spread of resistant strains.

The combination of sulfadoxine and pyrimethamine is generally used for the treatment of uncomplicated cloroquine-resistant malaria. This combination is more expensive than chloroquine and fails to cure vivax malaria. The development of operationally useful alternative drugs is essential, since it is likely that resistance to the sulfadoxine/pyrimethamine combination will ultimately occur. One of the most promising drugs developed recently at the Walter Reed Army Institute of Research, Washington, DC, is mefloquine, a well-tolerated compound that is effective against *P. falciparum* both for treatment and for prophylaxis (*10, 11, 12*). Mefloquine is also active against *P. vivax*.

In the Americas, a system of monitoring drug response, using both *in vivo* and *in vitro* test methods, is being developed by the Gorgas Memorial Laboratory in collaboration with the Pan Ameri-

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[a] See also RIECKMANN, K. H. In vitro *assessment of the sensitivity of* Plasmodium falciparum *to chloroquine at Kisumu, Kenya and Lagos, Nigeria* (unpublished document WHO/MAL/72.792).

[b] VALERA, C. V. & SHUTE, G. T. *Preliminary studies on the response of* Plasmodium falciparum *to chloroquine in the Philippines using the* in vitro *technique* (unpublished document WHO/MAL/75.852).

Table 1. Response of chloroquine-resistant *P. falciparum* to mefloquine *in vitro* (micromethod)

Case no.	Number of parasites per µl blood before incubation	Schizonts in control samples (%)	Effective doses (ED) in pmol/5 µl blood							
			Chloroquine				Mefloquine			
			ED_{90}	ED_{95}	ED_{99}	$ED_{99.9}$[a]	ED_{90}	ED_{95}	ED_{99}	$ED_{99.9}$[a]
BVRB 008	28244	88	19.6	21.4	25.8	32	2.3	2.7	3.3	4
BVRB 009	18720	100	17.0	19.0	24.0	32	1.6	1.8	2.0	3
BVRB 010	5280	37	5.0	6.5	9.2	12	1.2	1.3	1.6	2
VMC 009A	3900	70	>32	>32	>32	>32	2.6	2.8	4.4	5.0
VMC 019	4920	90	13.2	13.5	14.9	16	2.5	2.7	3.0	6.0
VMC 023	500	76	15.3	17.0	22.6	32	2.8	3.1	3.5	4.0

[a] The $ED_{99.9}$ is considered to be the first concentration at which complete inhibition is observed.

can Health Organization and the World Health Organization. The present study was conducted to determine the susceptibility of chloroquine-resistant strains of *P. falciparum* to mefloquine in the *in vitro* test system. These trials were carried out in 1978 in Boa Vista, Brazil, and Villavicencio, Colombia.

Materials and methods

In these investigations, the *in vitro* microtechnique of Rieckmann et al. (*13*) was used with some modifications.

Flat-bottomed 8 cm × 12 cm tissue culture plates[c] were dosed with 0.1 mg of the disodium salt of ethylenediamine tetraacetic acid (EDTA) in well 1; wells 2 and 3 were left untreated for the controls; wells 4–12 were dosed with either 1–32 pmol of chloroquine or 1–16 pmol of mefloquine. The plates were dried at 37 °C. Before use, wells 2–12 were charged with 50 µl/well of freshly prepared, sterile growth medium containing 10.4 g of RPMI 1640,[d] 2 g of sodium bicarbonate, 6 g of HEPES buffer powder, and 4 mg of gentamicin in 1 litre of double-distilled water. The plates were gently agitated in order to dissolve the drugs. Parasitized blood was taken from a finger-prick by means of a 100-µl sterile, calibrated capillary tube and ejected into well 1. After brief stirring, the blood was transferred aseptically in 5-µl aliquots to wells 2–12 by means of an Eppendorf pipette. The plate was covered with a sterile lid, again gently agitated, and placed on a rack in a static water bath, and a lighted candle made of pure paraffin was used to produce the correct CO_2–air mixture. Finally, the slanting lid was put firmly in place on the water bath and sealed with plastic and silicone grease. The unit remained sealed at 38 °C for the incubation period of 24–30 hours.

After incubation the plates were taken from the water bath and the supernatant culture medium/plasma mixture removed from the individual wells by means of capillary tubes. Thick blood films were prepared from the sediment, dried for 2–24 hours, and stained for 10 minutes using a modified Romanowsky stain (*14*). The number of schizonts per 200 asexual parasites was determined in the samples from control and drug wells. The average of the two control readings was used as a basis for the calculation of proportional growth in the drug wells according to the standard procedure.[e]

Results

Comparative tests were carried out with mefloquine in the blood samples of 6 patients with chloroquine-resistant *P. falciparum*. Relatively high schizont counts in the controls were obtained in all cases. The values of ED_{90}, ED_{95}, ED_{99}, and $ED_{99.9}$, i.e., the doses effecting a 90%, 95%, 99%, and 99.9% inhibition of schizont formation, are given in Table 1.

On the basis of the geometric mean values (pmol doses), mefloquine was more than 7.7, 7.1, 7,1, and 6.4 times as effective as chloroquine at the ED_{90}, ED_{95}, ED_{99}, and $ED_{99,9}$ levels, respectively. In case BVRB 009, with a highly chloroquine-resistant parasite population, the $ED_{90\text{-}99,9}$ values of chloroquine were, consistently, more than 10 times as high as those of mefloquine. In cases BVRB 010 and VMC 019, which were less resistant to chloroquine, the difference was less marked, as was to be expected.

[c] Microtest II, Falcon Plastics, Division BioQuest, 5500 West 83rd Street, Los Angeles, CA 90045, USA.

[d] Gibco, Grand Island, NY, USA.

[e] World Health Organization, *Instructions for use of the WHO test kit for the assessment of the response of* Plasmodium falciparum *to chloroquine* (unpublished document MPD/77.4).

Discussion

The *in vitro* microtechnique, modified as described, can easily be performed by well trained technicians. It requires only a small quantity of blood, which can be drawn from a finger-prick. In contrast to the standard macromethod it can be performed with blood containing more than 100 000 parasites per microlitre and its results are less dependent on the availability of large rings.

Difficulties with blood inoculation into the wells are overcome by the use of EDTA as an anticoagulant. Earlier observations showed that it does not significantly interfere with parasite growth, adherence of blood to the microscope slides, or the staining properties.

The use of a sealed water bath for incubation proved to be highly effective and convenient, since the correct level of the medium/plasma mixture was maintained throughout incubation. Previous work with the conventional candle jar had resulted in drying of the wells, especially when operating in areas with low relative humidity.

The results obtained *in vitro* indicate that mefloquine is fully effective against chloroquine-resistant *P. falciparum* from Boa Vista, Brazil, and Villavicencio, Colombia.

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