

# Assessment of chloroquine sensitivity of *Plasmodium falciparum* in Choloteca, Honduras

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*During an outbreak of urban malaria in Choloteca, Honduras, the response of local isolates of Plasmodium falciparum to chloroquine was assessed. The 7-day WHO alternative standard field test was used together with three in vitro tests: the Rieckmann macro- and micromethods and a new 48-hour test which underwent its first field trial in this study. No chloroquine resistance was found in in vivo tests in 10 patients or in the in vitro tests on blood samples from 6 patients.*

Although chloroquine-resistant malaria has not so far been found in Central America west of Panama (1), the situation needs to be periodically re-evaluated because of its important implications for control of the disease in the area. Such an assessment was included in the investigations conducted during an outbreak of urban malaria in Choloteca, Honduras. The chloroquine sensitivity of local isolates of *Plasmodium falciparum* was tested by both *in vivo* and *in vitro* methods. One of the *in vitro* methods used was a recently developed 48-hour test (2), which received its first field trial in this study.

## BACKGROUND

Choloteca is a major city of southern Honduras, situated 30 km inland on the Pacific coastal plain. It has a tropical climate characterized by distinct wet and dry seasons. Located on the Panamerican Highway, the city is an important commercial centre around which various agricultural activities have developed. The population of Choloteca has expanded rapidly during the past decade, and was recorded as 34 031 in the 1979 census.

Until 1978, malaria in Choloteca was reported as being at a low to moderate level, and was mainly due to *P. vivax*. Control measures included intra-domiciliary residual insecticide spraying and drug distribution. Since October 1978, there has been a

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dramatic increase in the incidence of malaria as well as in the proportion of cases due to *P. falciparum* (Table 1). This increase was preceded by a heavier than usual rainy season, and the arrival in September 1978 of approximately 1000 Nicaraguan refugees. No house spraying was conducted in the area during the last quarter of 1978 or in 1979. The increased incidence of malaria persisted throughout 1979 in spite of the introduction of mass drug administration, using combination tablets containing 150 mg of amodiaquine and 15 mg of primaquine (3 tablets per adult dose).

In view of the continuing high rate of falciparum malaria despite mass administration of 4-aminoquinolines, and because of unsubstantiated reports of persisting infection in some individuals treated with these drugs, the Ministry of Health of Honduras requested help from the US Centers for Disease Control. Therefore, a project was set up in the area in January 1980 to assess the sensitivity of local strains of *P. falciparum* to chloroquine.

Table 1. Incidence of malaria in Choloteca, Honduras, 1976-79<sup>a</sup>

Year	<i>P. vivax</i>	<i>P. falciparum</i>	Mixed infections
1976	850	41	1
1977	289	9	0
1978 <sup>b</sup>	2995	775	23
1979	2531	913	40

<sup>a</sup> Compiled from the records of the Division of Vector Control, Ministry of Health, Republic of Honduras.

<sup>b</sup> 86% of the *P. vivax*, 97% of the *P. falciparum*, and 100% of the mixed infections recorded in 1978 occurred in October-December.

## MATERIALS AND METHODS

*Selection of patients*

Malaria cases were identified through the existing system of passive case detection, as well as by limited rounds of active case detection. Blood samples were taken from febrile subjects by fingertip puncture; thick and thin smears were made, Giemsa-stained, and examined for the presence of malaria parasites. Patients were selected for study if they were infected with *P. falciparum* only, and had more than 1000 asexual parasites per  $\mu\text{l}$  of blood (or more than 50 asexual parasites per 300 leukocytes, assuming an average leukocyte count of 6000 per  $\mu\text{l}$  of blood). They also had to fulfil the following additional criteria: no critical illness, no history of travel during the past month, no intake of antimalarial drug during the past week, and no 4-aminoquinolines in the urine (tested using the Dill-Glazko method (3)). The study procedures, including the need to take additional blood samples, were explained to the patients (or to the parents of patients less than 15 years old), and consent was obtained before they were included in the study group.

All patients with a positive smear were treated with a single oral dose of chloroquine phosphate, 10 mg of base per kg of body weight, whatever the infecting species. This dose of chloroquine was also routinely administered for presumptive treatment of febrile patients at voluntary collaborators' posts in Choluteca. The delay between the initial blood smear and the drug treatment was always less than 10 hours.

*In vivo testing*

The 7-day WHO alternative standard field test was used for preliminary screening, as described elsewhere (4). After the initial blood smear and collection of blood samples for the *in vitro* tests, each patient received a single oral dose of chloroquine phosphate, 10 mg of base per kg of body weight. Parasite counts were made daily for seven days on thick and thin blood smears. On the day following the administration of the drug, a urine sample was collected and tested for the presence of chloroquine (3).

*In vitro testing*

*Rieckmann macromethod.* This method was used as described earlier (5, 6) with minor modifications, using test kits provided by the World Health Organization. Samples of 3–10 ml of intravenous blood were collected and defibrinated, and 1-ml aliquots were distributed into screw-cap vials pre-dosed with 5 mg of glucose and various amounts of chloroquine (to give final concentrations of 0, 0.25, 0.5, 0.75, 1.0, 1.25, 1.5, 2.0 and 3.0 nmol of chloroquine base per ml of

defibrinated blood). After incubation at 37 °C for 24 hours, thick smears were taken from each vial and Giemsa-stained. The maturation of trophozoites into schizonts over the 24-hour period was assessed by counting the number of schizonts per 300 leukocytes. The inhibitory effect of the various concentrations of the drug was measured by comparing the numbers of schizonts in the control vial and in the vials containing chloroquine, and hence the chloroquine sensitivity of the isolate was determined.

*Rieckmann micromethod.* This technique was used as described earlier (7, 8), with some modifications. Where possible, 0.5–0.8-ml aliquots of the defibrinated intravenous blood collected for the macromethod were used for this test. Two patients were not tested by the macromethod, and the necessary samples were obtained from them as 0.5 ml of heparinized intravenous blood or as 0.3 ml of heparinized blood from a fingertip puncture. The blood samples were diluted 10-fold in culture medium consisting of RPMI 1640 medium<sup>a</sup> supplemented with Hepes buffer (30 mmol/litre), resulting in a culture with an erythrocyte suspension of approximately 5% and a serum concentration of about 5% (assuming a haematocrit of 0.5). Aliquots of 50  $\mu\text{l}$  of this preparation were distributed with a micropipette into 6.4-mm flat-bottomed wells, which had been pre-dosed with either 0, 1, 2, 4, 5.7, 8, 16, or 32 pmol of chloroquine base per well. The plates were put in a candle jar and incubated at 37 °C for 24 hours. After incubation, schizont maturation and the inhibitory effect of chloroquine were assessed as for the macromethod.

*48-hour test.* This method, described previously for laboratory-maintained strains of *P. falciparum* (2), was adapted for field conditions as follows. Blood samples were obtained in the same manner as for the micromethod. An initial volume of 0.2 ml of blood was diluted 25-fold by adding 4.8 ml of a complete culture medium consisting of RPMI 1640 medium supplemented with Hepes buffer (30 mmol/l) and 100 ml of AB<sup>+</sup> non-immune human serum per litre. The resulting culture thus contained an erythrocyte suspension of approximately 2% and a 12% serum concentration. Aliquots of 0.5 ml of this preparation were distributed into 16-mm flat-bottomed wells, which had been pre-dosed with various amounts of chloroquine, to give final concentrations of 0, 0.01, 0.03, 0.1, 0.3, and 1.0 nmol of chloroquine base per ml of culture medium. The plates were agitated to ensure resuspension and uniform settling of the erythrocytes, placed in a candle jar, and incubated at 37 °C for a total of 48 hours. After 24 hours' incubation, the jars were opened, the plates again agitated, and the atmosphere regenerated to ensure optimal

<sup>a</sup> From GIBCO Laboratories, Grand Island, New York, USA.

Table 2. Chloroquine sensitivity of *P. falciparum* isolates from Choluteca, Honduras, by the macromethod

Patient no.	Age (years)	Sex	Pre-incubation parasitaemia (asexual parasites per $\mu$ l of blood) <sup>a</sup>	No. of schizonts per 300 leukocytes (control sample)	Schizont maturation in test vials <sup>b</sup>	
					Chloroquine concentration (nmol/ml of blood)	
					0.25	0.5
1	23	Female	5 700	190	38	0
4	8	Male	1 700	94	32	
7	12	Female	13 760	54	0	0
10	47	Female	13 900	60	12	0

<sup>a</sup> Obtained as 20 x number of asexual parasites per 300 leukocytes.

<sup>b</sup> Expressed as 100 x no. of schizonts in test vial/no. of schizonts in control sample.

growth. Parasite counts were made on Giemsa-stained thin smears taken at the beginning and end of the incubation period. By comparing the increase in parasitaemia in the control well and in the wells containing chloroquine, the inhibition of parasite growth by various concentrations of the drug was measured and used to determine the chloroquine sensitivity of the isolate tested.

## RESULTS

The study group comprised seven female and three male patients, with an age range of 6–47 years (mean age: 18.3 years). Their parasite counts ranged from 1220 to 63 200 asexual parasites per  $\mu$ l of blood (geometric mean count, 7382).

### In vivo testing

All ten patients completed the 7-day observation period required for the *in vivo* test. Uniformly rapid clinical improvement was reported, and all blood smears were negative for asexual parasites 2–4 days after administration of the single dose of chloroquine (mean parasite clearance rate, 2.5 days). No recrudescence was noted during the 7-day observation period.

### In vitro testing

**Macromethod.** Blood samples were collected from eight patients for testing by this method (two 6-year-old children were excluded from this part of the study). Satisfactory schizont formation was obtained in the control vials of four samples, with more than 5% of the parasites maturing into schizonts. These

Table 3. Chloroquine sensitivity of *P. falciparum* isolates from Choluteca, Honduras, by the micromethod

Patient no.	Age (years)	Sex	Pre-incubation parasitaemia (asexual parasites per $\mu$ l of blood) <sup>a</sup>	No. of schizonts per 300 leukocytes (control sample)	Schizont maturation in test wells <sup>b</sup>			
					Chloroquine concentration (pmol/well)			
					1.0	2.0	4.0	5.7
1	23	Female	5 700	189	88	102	17	0
4	8	Male	1 700	24	50	46	25	0
5	6	Female	1 280	15	53	73	40	0
7	12	Female	13 760	143	48	52	49	0
10	47	Female	13 900	47	112	108	66	0

<sup>a</sup> Obtained as 20 x no. of asexual parasites per 300 leukocytes.

<sup>b</sup> Expressed as 100 x no. of schizonts in test well/no. of schizonts in control sample.



obtained for five samples, since the parasite count in the control wells increased during the 48-hour incubation period. In these samples, there was no increase in parasitaemia in wells containing 0.03 nmol of chloroquine base per ml of medium. This endpoint was similar to that previously determined for laboratory-maintained, chloroquine-sensitive strains (2) (Table 4).

#### DISCUSSION

No chloroquine resistance was detected in the isolates of *P. falciparum* tested in Choluteca. In all ten patients participating in the *in vivo* studies, the rapid clearance of the parasites following treatment with a single dose of chloroquine suggested that the parasites were sensitive to the drug; the limited duration of the follow-up, however, did not exclude the possibility of an RI type of resistance with delayed recrudescence.

The combined results of the *in vitro* studies established chloroquine sensitivity in isolates from six of the ten patients. These isolates showed a pattern of inhibition by the drug similar to that previously established for known chloroquine-sensitive strains of *P. falciparum*. The results of tests on the four remaining isolates were not interpretable since parasites in the control samples failed to develop.

This preliminary trial indicated that the 48-hour test, which had been used previously to determine drug sensitivity in laboratory-maintained, asyn-

chronous strains of *P. falciparum*, was also applicable to the synchronous parasitaemias characteristically found in the field. It thus offers a useful complement to the Rieckmann macro- and micromethods for the field assessment of drug resistance. Its main technical disadvantage is the need to supplement the medium with non-immune serum, but this is balanced by its simplicity and the relatively small amount of blood required for each test.

The 48-hour test can be used to compare the drug sensitivity of malaria parasites before and after cultivation in the laboratory, which would be of interest for *in vitro* chemotherapy studies. This has been illustrated in additional work with the isolate collected from patient 10, which was adapted to culture as strain Honduras 1/CDC (9). After six weeks' cultivation *in vitro*, the strain was tested by the 48-hour test and showed a chloroquine sensitivity identical to that found in the original isolate.

The 48-hour test covers the entire asexual cycle of *P. falciparum*, and thus can be adapted to various drugs acting on different stages of the parasite cycle. The response to pyrimethamine of both laboratory-maintained strains (9) and freshly collected parasites has been determined by this test (Nguyen-Dinh & Campbell, unpublished data). In view of the continuing spread of resistance to an increasing number of antimalarials, the development of a test which can be adapted to a variety of drugs and which is equally applicable in the field and the laboratory, is an important achievement.

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#### RÉSUMÉ

##### ÉVALUATION DE LA SENSIBILITÉ À LA CHLOROQUINE CHEZ *PLASMODIUM FALCIPARUM* À CHOLUTECA, HONDURAS

En janvier 1980, au cours d'une épidémie de paludisme urbain à Choluteca (Honduras), la sensibilité à la chloroquine des isolements locaux de *Plasmodium falciparum* a été évaluée par des méthodes tant *in vivo* que *in vitro*. Dix malades ont fait partie des études *in vivo*, pour lesquelles a été utilisée la variante en 7 jours de l'épreuve pratique type de l'OMS. Après administration de 10 mg/kg de chloro-

quine base, on a noté une élimination rapide des parasites (moyenne: 2,5 jours) et aucune recrudescence n'a été décelée pendant la période d'observation de 7 jours. A partir des mêmes malades, on a recueilli des échantillons de sang pour déterminer la sensibilité des parasites à la chloroquine par trois méthodes *in vitro* différentes. Le matériel provenant de 8 échantillons a été utilisé pour des déterminations par la

macrométhode de Rieckmann; des résultats interprétables ont été obtenus dans 4 épreuves: inhibition complète de la formation de schizontes dans les flacons contenant 0,25 nanomole (1 échantillon) ou 0,5 nanomole (3 échantillons) de chloroquine base par ml de sang défibriné. Le matériel provenant des 10 échantillons a été utilisé pour des déterminations par la microméthode de Rieckmann; des résultats interprétables ont été obtenus dans 5 épreuves: inhibition complète de la formation de schizontes dans les cupules contenant 5,7 picomoles de chloroquine base par cupule. Le matériel provenant de 9 échantillons a été utilisé pour des déterminations par une épreuve en 48 heures récemment mise au point; des résultats interprétables ont été obtenus dans 5 épreuves: inhibition complète de la multiplication des

parasites dans les cupules contenant 0,03 nanomole de chloroquine base par ml de milieu. Les points finals observés dans les épreuves *in vitro* étaient semblables à ceux qui avaient été antérieurement établis pour des isolements sensibles à chloroquine.

Ainsi, aucune indication d'une résistance à la chloroquine n'a été décelée à Choluteca dans cette enquête. Le succès de l'essai pratique de l'épreuve en 48 heures accroît la valeur de cette dernière pour les études de chimiothérapie, car cette épreuve couvre la totalité du cycle asexué de *P. falciparum* et peut être utilisée pour les parasites synchrones trouvés dans le sang des malades comme pour les parasites asynchrones qui existent après adaptation à la culture *in vitro*.

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