Preliminary studies on the response of *Plasmodium falciparum* to chloroquine in the Philippines, with the *in vitro* technique

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Previous investigations had shown that resistance to standard regimens of chloroquine occurred in some cases of falciparum infection in the Philippines. More extensive investigations into this phenomenon were planned by the Malaria Eradication Service, by means of the in vitro *technique developed by Rieckmann, in order to determine both the distribution of resistant strains throughout the country and their local incidence. Before these studies were undertaken, a series of observations were made on cases of falciparum malaria encountered in Manila and its environs, to assess the reaction of local strains of the parasite to the* in vitro *test. These cases were also treated with standard doses of chloroquine and some were followed up for 4 weeks to compare the predictions made as a result of the* in vitro *tests with the* in vivo *observations. Of the 34* in vitro *tests carried out, 18 were followed up* in vivo. *In 8 cases, no recrudescence occurred, but in the other 10 recrudescences were detected during the 4-week observation period, thus indicating parasite resistance to the drug. In each of the 18 cases, the* in vivo *response followed the* in vitro *prediction.*

The determination of the degree of susceptibility of *P. falciparum* to chloroquine by the *in vitro* technique is based on the ability of the parasite to develop from the young trophozoite to form schizonts when challenged by increasing concentrations of chloroquine. K. H. Rieckmann and F. J. Lopez Antuñano (unpublished observations, 1970) demonstrated that sensitive strains of *P. falciparum* of African origin failed to form schizonts *in vitro* when the chloroquine concentration was 0.5 nmol or more per ml of blood (1 nmol = 10^{-9} mol). On the other hand, parasites exhibiting chloroquine resistance, such as the Malayan (Camp) strain and the Mato Grosso strains from Brazil, formed schizonts *in vitro* until the chloroquine concentrations were at least 1.5 nmol per ml of blood. These data are invaluable as a guide to the levels of tolerance to the drug that *P. falciparum* may be expected to show. However, it was thought necessary to obtain specific baseline data showing the *in vitro* chloroquine tolerance levels of local Philippine strains before undertaking more extensive field trials. The following two points required particular clarification.

(1) The maximum concentration of chloroquine in which schizogony could occur *in vitro* when *in vivo* treatment and follow-up indicated the strain to be sensitive.

(2) The specific reliability of the individual *in vitro* results. It is important to know if there is an occasional overlap in the chloroquine concentrations in which schizonts of a susceptible strain may develop and in which, therefore, the two types could not always be separated. The possibility of the occurrence of an occasional completely aberrant result was also a matter of concern.

ORGANIZATION OF THE PRELIMINARY STUDIES

The National Malaria Eradication Training Centre and its parasitology laboratory adjoin the San Lazaro Hospital, Manila. For a number of years the laboratory has acted as a malaria diagnostic and reference centre for the hospital. In January 1974

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an agreement was made between the Malaria Eradication Service and the director of the hospital, for patients admitted to the hospital with falciparum infections to be tested in order to determine if the infections were resistant to treatment with 4-aminoquinolines. The *in vitro* technique was to be used. Thus when the *in vitro* test indicated that the parasites had a high degree of tolerance to the drugs, treatment could immediately be changed or supplemented with other drugs if the clinical condition of the patient made this advisable. The Central Verification Laboratory of the Malaria Eradication Service also acts as a passive case-detection post. All falciparum cases detected there are now referred to the parasitology laboratory for *in vitro* study and treatment. In cases where the full course of 4-aminoquinoline treatment was given, follow-up procedures were undertaken in order to confirm the *in vitro* prediction. At the same time this gave the staff experience in handling the technique under favourable conditions.

By the beginning of April 1974 it was considered that the laboratory staff were sufficiently adept in the technique to undertake a limited field trial. The lack of some equipment made it necessary to carry out the trial within easy reach of Manila. The primary objectives were to give the operators experience in carrying out case selection under field conditions and to indicate the personnel and transport requirements for the field team that had yet to be formed. Two locations were selected: (1) the town of Montalban and Wawa Valley, where chloroquine resistance is known to occur, and (2) the area around Antipolo. Both localities are in mountain foothills. They are about 20 km apart and lie some 20 km to the north-east and east of Manila, respectively. Several falciparum infections were detected in each place. The exercise continued for 3 weeks and the results obtained form part of the data given in this report.

TECHNICAL PROCEDURES

The procedures described by Rieckmann (2), Rieckmann et al. (3), Rieckmann (unpublished observations, 1972), and Rieckmann and Lopez Antuñano (unpublished observations, 1970) have been followed fairly exactly, but some observations and minor modifications are discussed under *Technical aspects of interest*, below. As a routine, glassware for sterilization is carefully washed, wrapped in brown paper, and sterilized in an autoclave. Small items, such as culture vials, are not wrapped individually but are packed in groups of 30 in small cardboard containers, which are themselves then wrapped in paper. After sterilization, the articles are placed in an oven to dry, but are not unwrapped until they are required for use.

Culture vials are charged with dextrose solution and chloroquine in lots sufficient to last for 4–6 weeks. The dextrose is added by placing one drop of 50% sterile solution into each vial from a syringe fitted with a 23-gauge Luer-type needle. It has been calculated that this introduces about 6 mg of dextrose into each vial—which has been found to produce excellent growth. The graded solutions of chloroquine are introduced by means of a 20 μl Eppendorf pipette. The range of chloroquine concentrations used, in nmol per ml of blood, is: 0.25; 0.5; 0.75; 1.0; 1.5; 2.0; 2.5; and 3.0.

A full series of tests comprising 8 test vials and a control necessitates the extraction of at least 10 ml of blood. This is frequently not possible, especially in the case of children; consequently, a priority order is followed when placing the blood in the vials. This is: control, 0.5, 1.0, 1.5, 2.0, 0.25, 3.0, 0.75, and 2.5. A 1-ml aliquot of blood is placed in each vial and the vials are incubated at between 38.5°C and 40.0°C for 20–30 h—the exact time depending on the rate of development of the parasites. After 20 h of incubation a sample thin smear is made by carefully withdrawing a small drop of blood from the control vial with a flame-sterilized pipette. The degree of development found in this film acts as a guide in determining the time at which incubation is to be terminated.

For the purpose of determining the most suitable type of smear for reading the results, three separate smears are made from each sample as follows.

(1) Small thick smears are placed serially on a slide so that all samples from a test are located on a single slide. This follows the Rieckmann method.

(2) A thick smear from each sample is placed on a separate slide. This procedure uses more slides than the one given above but was started after parasite transfer was detected on a single occasion in a serial-type smear.

(3) Thin smears made from the interface between blood and serum, i.e., at the buffy coat. This is an attempt to make the smear from blood in which schizonts have become concentrated during infection. The object of investigating the use of the thin smear is to increase the accuracy with which early schizonts can be identified, schizonts can be age-graded, and

degenerate forms can be detected. Thin films also allow accurate readings to be made in the mixed infections that are occasionally encountered.

It was found that thick smears made from defibrinated blood are liable to become fragmented or even to become totally detached from the slide during staining. This occurrence is so significant that we now prefer to keep the films at room temperature for 2–3 days before they are stained.

Standard Giemsa staining was not found to be ideal for detecting schizonts that have been grown *in vitro*—which confirms the view of Rieckmann. We now use the Giemsa saline technique advocated by Shute (5). This produces a very clear background to the film; the cytoplasm stains light blue, and the chromatin is easily detected. We have found, however, that this technique is not satisfactory with several brands of Giemsa stain.

TECHNICAL ASPECTS OF INTEREST

1. At the beginning of the short field trial, blood was transported from the field to the laboratory on ice. However, it was found that, after the blood had been chilled for 1–2 h, subsequent parasite growth *in vitro* was noticeably less satisfactory than might have been expected, compared with the results achieved with cases selected at the laboratory or in the hospital. Several tests were then carried out in which blood samples taken from the same case in the laboratory were defibrinated, one aliquot of each being subjected to: (*a*) immediate incubation; (*b*) storage at room temperature for 3 h, followed by incubation; and (*c*) storage on ice for 3 h, followed by incubation. The results of these tests showed that virtually the same amount of growth occurred in the samples subjected to (*a*) and (*b*), but that, in the case of (*c*), many of the parasites became compact and failed to develop beyond the late trophozoite stage. It therefore appears that, if it is to be stored for only a few hours, blood is better preserved at room temperature than chilled.

2. The effect of power failures on parasite growth during incubation is a point that was discussed by Colwell (1). It may be especially important in field studies when reliance must be placed on either a generator or a rural power supply. A number of power failures occurred during our observations. It has become fairly clear that a power interruption of 1–2 h is of no great consequence while the parasites are still in the growing trophozoite stage. However, once schizogony proper has started, or possibly earlier than this, even comparatively brief power cuts that cause a fall in the environmental temperature may ruin the culture.

3. It has been observed by Rieckmann that there is a relationship not only between the age of the parasites at the start of incubation and successful culture but also between the latter and the parasite count. We have found that, provided the ring forms are well developed (the " fleshy " parasites of Field showing distinct Maurer's clefts in thin films), a large proportion of the asexual parasites mature even when the parasite counts are of the order of 100 000 per mm^3 of blood. Above this, cultures usually fail and it has been found that the maximum parasite count at which successful culture may be consistently expected is more of the order of 60 000–80 000 per mm^3. When the count is below about 400 per mm^3, the schizonts surviving in the culture vials containing chloroquine may be so few that they may not be detected, thus giving an inaccurate result.

4. An analysis was made of the original chloroquine stock solution obtained from abroad, and of a chloroquine solution prepared locally from technical chloroquine diphosphate powder. Both solutions were reputed to contain 129.6 mg of chloroquine per litre of water. The laboratory of the national Food and Drug Administration performing the analysis quoted the possible error to be within $\pm 5\%$. The analysis showed a content of 152 mg of chloroquine per litre in the original stock solution and of 135.2 mg of chloroquine per litre in the locally prepared solution—i.e., an excess of 17% in the former and of 4% in the latter.

Whatever the reason for the excessive quantity of chloroquine in the original solution—be it due to inaccurate weighing or to subsequent evaporation—there is clearly a need for greater attention to this aspect, which influences all subsequent test results.

5. The possible influence of parasite transfer should not be overlooked, especially when dealing with serial thick smears made from defibrinated blood. As has been mentioned previously, only a single instance of this has been detected. However, parasite transfer is occasionally not detectable, and may then lead to an erroneous reading of drug resistance when in fact it does not exist.

RESULTS

The total number of falciparum cases in which blood samples were assayed by the *in vitro* technique

in the period under review was 51. In 17 of these, schizonts failed to form in the control vials. These samples were therefore discarded. Reasons for the failure were: (*a*) parasitaemia too scanty to enable a subsequent reliable evaluation (2 cases); (*b*) presence of "small" trophozoites only (10 cases); (*c*) power failures occurring at crucial periods during incubation; and (*d*) failure of development of the parasites for unknown reason. In the case of (*a*) and (*b*), the tests were undertaken deliberately, for evaluation purposes.

The result of each test in which there was a successful culture in the control vial is shown in Table 1, giving the number of schizonts detected while counting 300 white cells in the control vials. This figure was taken as the basis for calculating the percentage of schizonts that were detected in each of the vials containing chloroquine. In the first series of tests, the range of chloroquine concentrations was limited to 0.5–2.0 nmol per ml blood, owing to the shortage of equipment. At the end of January 1975, the range was expanded to cover 0.25–3.0 nmol. It is intended to increase the range further to 4.0 and ultimately to 5.0 nmol. However, there have been only 3 cases so far in which concentrations higher than 3.0 nmol would have been of value. This may reflect previous findings in *in vivo* studies which indicated that drug resistance in the Philippines is almost always of the R1 type.[a]

Table 2 shows the comparative *in vitro* and *in vivo* results and the treatment of each case. The lowest concentration of chloroquine at which schizogony was entirely inhibited *in vitro* is indicated, together with our interpretation of the test result and details of the cases followed up. Of the 34 cases in which culture was successful, 18 were followed up for a sufficient time to enable an *in vitro* evaluation to be made. Of these, 8 were susceptible and 10 others produced recrudescences—all of type R1.

DISCUSSION

The majority of the cases that were tested were inpatients of San Lazaro Hospital. The routine treatment for malaria in the hospital was with amodiaquine and cases Nos. 2/1–9/1 were thus treated. In order to facilitate the *in vitro* studies the medical authorities at the hospital agreed to treat all falciparum cases with chloroquine as from the end of January 1974. Although all patients are weighed for our records, the drug dosage given to adults in the hospital is standardized at 1 500 mg per treatment (spread over 3 days). Patients that were encountered elsewhere and treated by the authors either at the laboratory or at their homes were given 25 mg of chloroquine per kg of body weight. For this reason, a number of the cases shown in Tables 2 and 3 were given more than 25 mg/kg. However, this does not appear to have caused any anomalies in the studies. Previous observations (4) also indicated that an increased dosage of 4-aminoquinolines fails to inhibit recrudescences in the resistant strains so far encountered in the Philippines.

Malaria transmission does not occur in the city of Manila and most of its suburbs. The patients recorded here had acquired their infections in the provinces and, following treatment, the majority wished to return there. This is the primary reason why the follow-up results are rather scanty in relation to the number of cases actually tested. However, sufficient results are available for a tentative premise to be made that parasites that are totally inhibited from forming schizonts in concentrations of 0.75 nmol or less per ml of blood are susceptible to standard chloroquine treatment. Those in which total inhibition occurs only at concentrations of 1.5 nmol or more per ml are resistant. So far 3 cases have been encountered in which total inhibition of schizogony occurred at the concentration of 1.0 nmol per ml. The significance of these results cannot be assessed at present as only one of these cases (No. 1/5) was followed up effectively. This case proved to be susceptible.

Table 3 divides the cases that were followed up into resistant and susceptible groups as determined by *in vivo* study. These are shown against the range of chloroquine concentrations *in vitro* at which total inhibition to schizogony occurred. It may be seen that, in the 18 cases reviewed, the predictions of drug sensitivity by *in vitro* testing were confirmed *in vivo*. So far, no anomalies or completely aberrant results have been observed. It is intended to continue these observations to accumulate more detailed baseline data.

Tables 1 and 3 show that there is a wide range of minimum concentrations of chloroquine that are required to cause total inhibition of schizogony in individual cases even in this small series of observations. On the other hand, the response of the parasites to challenge by increasing concentrations of chloroquine in each test series is fairly consistent, as demonstrated by the reasonably straight curves

[a] Clearance of asexual parasitaemia as in sensitivity, followed by recrudescence.

Table 1. Survival to schizogony of culture samples, shown as a percentage of survival in the control counts against 300 white blood cells

Case No.	Asexual parasite count per m^3 of blood	Control (schizonts/ 300 white blood cells)	Percentage of schizonts in test vials against controls (chloroquine concentration in nmol/ml blood)							
			0.25	0.5	0.75	1.0	1.5	2.0	2.5	3.0
2/1/74	86 320	574	—	0	0	0	0	0	—	—
3/1/74	12 560	532	—	0	0	0	0	0	—	—
4/1/74	55 200	118	—	86	53	52	5.9	1.6	—	—
5/1/74	8 320	35	—	17	11	2.8	0	0	—	—
6/1/74	5 920	186	—	80	45	31	13	5.3	—	—
8/1/74	31 520	106	61	82	49	63	47	35	3.7	2.8
9/1/74	6 160	212	59	7.5	1.8	0.4	0	0	—	—
1/2/74	1 480	6	16	0	0	0	0	0	0	—
2/3/74	204 000	4	100	75	25	0	0	0	0	—
3/2/74	25 000	13	77	69	60	61	30	30	15	0
4/3/74	109 000	147	97	89	4	0.66	0.6	0.6	0	0
5/3/74	3 440	131	27	3	1.5	0.8	0.4	0	0	0
1/4/74	32 000	7	57	14	0	0	0	0	0	0
2/4/74	13 200	557	41	0.9	0	0	0	0	0	0
3/4/74	22 000	160	82	77	71	39	34	21	17	—
4/4/74	14 160	37	0	0	0	0	0	0	—	0
6/4/74	12 400	25	100	64	60	32	24	4	—	0
7/4/74	600	16	100	31	31	0	(0.6)[a]	0	0	0
8/4/74	62 000	107	81	67	21	24	3.7	2.8	—	0
13/4/74	28 140	78	93	4	0	0	0	0	—	0
14/4/74	3 040	112	0	0	0	0	0	0	—	0
15/4/74	1 160	26	0	0	0	0	0	0	0	0
16/4/74	5 720	92	14	0	0	0	0	0	0	0
1/5/74	28 960	827	—	12	—	0	0	0	—	—
2/5/74	9 320	172	82	78	41	36	0	0	0	0
3/5/74	7 640	312	78	15	0	0	0	0	0	0
6/5/74	31 200	139	84	50	15	2.8	0	0	0	0
7/5/74	31 040	14	100	29	0	0	0	0	0	0
9/5/74	1 400	36	2.8	0	0	0	0	0	0	0
10/5/74	3 200	334	—	71	—	10	—	2	—	0
2/6/74	8 480	284	—	89	80	43	4.2	0.4	—	0
4/6/74	8 160	122	—	94	—	39	1.6	0	—	0
5/6/74	5 760	45	2	2	0	0	0	0	0	0
6/6/74	2 700	185	0	0	0	0	0	0	0	0

[a] Not valid: parasite transfer detected.

Table 2. *In vitro* indication of parasite susceptibility to amodiaquine (A.) and chloroquine (C.), and *in vivo* treatment and follow-up results

Case No.	Weight (kg)	Drug and dosage (mg)	*In vitro*		*In vivo* observations		
			lowest C. concentration in which schizonts not detected	*in vitro* prediction	period of follow-up (days)	follow-up result	day of recrudescence
2/1	54.5	A. 1 500	0.5	S	4	—	—
3/1	20.9	A. 900	0.5	S	4	—	—
4/1	44.5	A. 1 500	2.0	R	17	R	17
5/1	54.5	A. 1 500	1.5	R	—	—	—
6/1	42.7	A. 1 500	2.0	R	17	R	17
8/1	56.0	A. 1 500	3.0	R	13	R	13
9/1	55.0	A. 1 500	1.5	R	15	R	15
1/2	—	other [a]	0.5	S	—	—	—
2/3	63.0	C. 1 500	1.0	?	36	S	nil
3/3	51	C. 1 500	3.0	R	—	—	—
4/3	58	C. 1 500	2.5	R	14	—	—
5/3	58	C. 1 500	2.0	R	15	—	—
1/4	54	C. 1 500	0.75	S	45	S	nil
2/4	48	C. 1 500	0.75	S	12	—	—
3/4	49	other [a]	2.5	R	—	—	—
4/4	15	C. 450	0.25	S	30	S	nil
6/4	48	C. 1 088	3.0	R	33	R	20–33
7/4	48.6	C. 1 238	1.0 [b]	?	6	—	—
8/4	20	C. 525	3.0	R	14	R	14
13/4	—	C. 1 500	0.75	S	28	S	nil
14/4	50.3	C. 1 275	0.25	S	28	S	nil
15/4	46.3	C. 1 200	0.25	S	28	S	nil
16/4	51.0	C. 1 275	0.5	S	15	—	—
1/5	50.0	C. 1 500	1.0 [c]	?	26	S	nil
2/5	36.0	C. 1 500	1.5	R	24	R	18–24
3/5	50.0	C. 1 500	0.75	S	8	—	—
6/5	44.0	other [a]	1.5	R	—	—	—
7/5	39	other [a]	0.75	S	—	—	—
9/5	40	C. 1 500	0.5	S	28	S	nil
10/5	41	C. 1 950	3.0	R	20	R	20
2/6	51.8	C. 1 275	3.0	R	28	R	28
4/6	41.2	C. 1 050	2.0	R	21	R	19–21
5/6	39.5	C. 1 500	0.75	S	cases under current observation		
6/6	41.0	C. 1 500	0.25	S			

[a] Alternative or combined treatment schedules given.
[b] See details of this parasite transfer case in Table 1.
[c] Limited observation *in vitro* owing to shortage of blood for testing. Total inhibition to schizogony may have occurred at the 0.75 nmol concentration.

Table 3. Cases that have been confirmed as susceptible or resistant by *in vivo* follow-up, classified according to the concentration of chloroquine that completely inhibited schizogony *in vitro*

In vivo results	Minimum chloroquine concentration (nmol/ml) to prevent schizogony *in vitro*								
	0.25	0.5	0.75	1.0	1.5	2.0	2.5	3.0	3+
susceptible	3	1	2	2	0	0	0	0	0
resistant (R1)	0	0	0	0	2	1	2	4	1

obtained when plotting the responses on log-scale graphs. It would appear, therefore, that several different strains of parasite may have been encountered. There are already indications that certain patterns of response may be more common in different localities. As an example the patterns found in cases from Montalban and Antipolo are compared in Fig. 1 and 2, respectively. It is a little difficult to understand how localized strains could occur so close together (these localities being only 20–30 km apart), although it would not be so surprising to find this in the islands. It may be significant, in the example given, that during the past 20 years many immigrants from Panay Island (which itself is influenced by immigration from the highly malarious island of Palawan) have settled in the Montalban

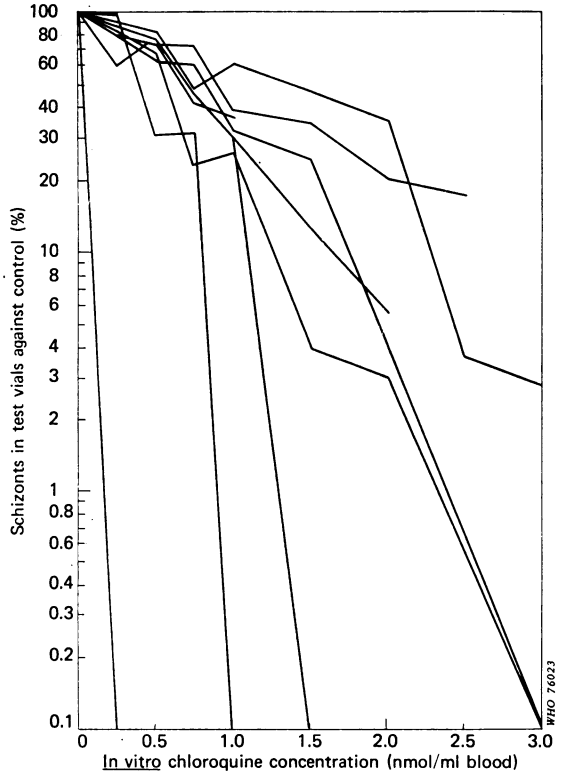

Fig. 1. Patterns of susceptibility of *P. falciparum* to chloroquine *in vitro* (Montalban).

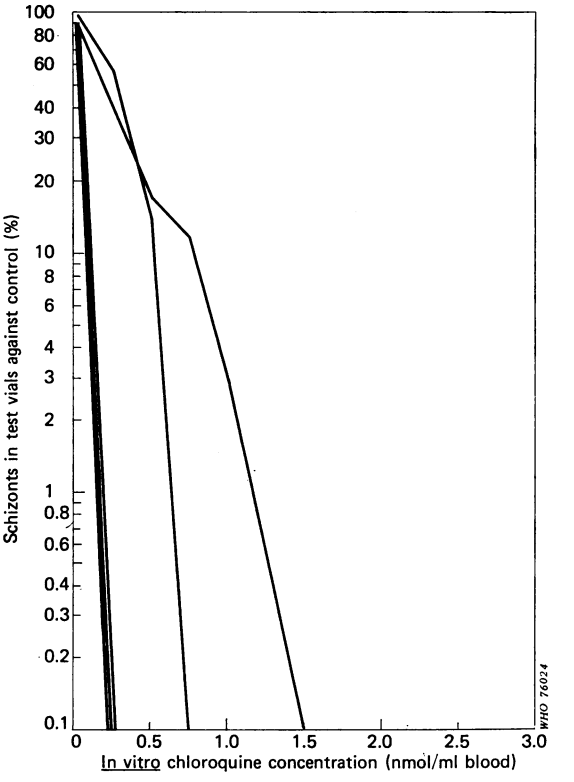

Fig. 2. Patterns of susceptibility of *P. falciparum* to chloroquine *in vitro* (Antipolo).

area and that it was in this immigrant community that falciparum resistance to chloroquine was found to be common (4). It may be, therefore, that Montalban itself now contains more than one strain of *P. falciparum*, including one or more strains of fairly recent importation that have not yet been disseminated or overwhelmed by the indigenous strains. Grouping of results by locality will be continued.

In all but two cases, in which the patients were unable to urinate, the Dill–Glazko test was applied to determine if there was excretion of 4-aminoquinoline derivatives at the time that the *in vitro* test was to be started. This has proved to be of considerable value in excluding patients who have previously been given 4-aminoquinolines but who either may not be aware of or may not admit to it. As a trial, the *in vitro* test was performed on a case in which the urine showed the strong red colouration indicative of the presence of the drug. The *in vitro* result was quite erroneous. The infection appeared to be susceptible to chloroquine, schizonts being found in decreasing numbers in the three lowest concentrations (0.25, 0.5, and 0.75 nmol per ml of blood), as well as in the control. Nevertheless, a recrudescence occurred on the 12th day of follow-up.

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RÉSUMÉ

ÉTUDES PRÉLIMINAIRES SUR LA SENSIBILITÉ DE *PLASMODIUM FALCIPARUM* À LA CHLOROQUINE AUX PHILIPPINES, ÉVALUÉE PAR LA TECHNIQUE *IN VITRO*

Le laboratoire central du service d'éradication du paludisme des Philippines procède régulièrement à des essais *in vitro* de sensibilité de *Plasmodium falciparum* à la chloroquine afin de comparer les résultats *in vitro* avec ceux du traitement à la chloroquine et de pouvoir faire des prédictions valables à partir des résultats *in vitro*. Des échantillons de sang provenant de 51 infections à falciparum, toutes contractées en dehors de Manille, ont été testés. Dans 17 cas, il n'y a pas eu formation de schizontes dans les flacons de contrôle; les échantillons ont donc été rejetés. Le phénomène s'explique par trois raisons: dans la plupart des cas, il n'y avait au départ que de très jeunes trophozoïtes; dans certains, la parasitémie était trop faible; dans d'autres enfin des pannes de courant se sont produites à des périodes critiques de l'incubation.

Au total, 18 cas ont été suivis pendant 4 semaines. On a constaté que des parasites dont la transformation en schizontes est totalement inhibée sous des concentrations de chloroquine égales ou inférieures à 0,75 nmol par ml de sang sont sensibles au traitement standard par la chloroquine. Les parasites dont l'inhibition totale exige des concentrations de chloroquine égales ou supérieures à 1,5 nmol par ml sont tous résistants au degré R1. On n'a pas encore relevé de résultats complètement discordants.

La comparaison des profils de sensibilité dans deux localités de l'île de Luzon, situées à 30 km l'une de l'autre, a révélé des différences importantes.

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