In vitro drug sensitivity of *Plasmodium falciparum* in Acre, Brazil

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In Acre, the westernmost state of Brazil in the Amazon region, the sensitivity of Plasmodium falciparum *to chloroquine, amodiaquine, mefloquine, quinine and sulfadoxine/pyrimethamine was determined* in vitro *by the Rieckmann microtechnique. The study was performed between January and June 1987; the* in vitro *parasite responses to all antimalarial drugs were determined according to the recommendations of WHO. Of 83 isolates of* P. falciparum, *all were sensitive to mefloquine and of 87 isolates of* P. falciparum, *84 (97%) were sensitive to quinine. The EC_{50} for mefloquine was 0.27 µmol/l and for quinine 4.60 µmol/l. In contrast, 65 of 89 (73%) and 70 of 83 (84%) isolates were resistant to amodiaquine and chloroquine, respectively; 11 isolates even grew at 6.4 µmol chloroquine/l. The EC_{50} for amodiaquine was 0.34 µmol/l and for chloroquine 0.73 µmol/l. Sulfadoxine/pyrimethamine resistance was seen in 23 of 25 (92%) cases.*

These data clearly indicate that in the western part of the Amazon region the 4-aminoquinolines, as well as sulfadoxine/pyrimethamine, can no longer be recommended for the treatment of P. falciparum *infections.*

Resistance of *Plasmodium falciparum* to antimalarial drugs has been reported in South America since 1961 (*1*, *2*). Resistance to chloroquine was first observed in Colombia and Brazil (*1*, *2*), and is now common in many endemic areas of the Americas (*3–6*). In 1981, resistance to sulfadoxine/pyrimethamine was confirmed in Colombia (*3*). So far, only insufficient information has been available on the resistance of *P. falciparum* to amodiaquine, quinine and mefloquine (*4–6*) on the South American continent. Multidrug resistance is a major public health concern, especially in the Amazon region of Brazil where immigration of non-immunes from the southern part of the country has increased over recent years. It was therefore decided to investigate *in vitro* the response of *P. falciparum* to chloroquine, amodiaquine, quinine, mefloquine and sulfadoxine/pyrimethamine in a "colonization" area in the state of Acre, Brazil.

Patients and methods

The study took place in Rio Branco, Acre, Brazil, which is situated at 68° longitude west and 10° latitude south in the Amazon rain forest. All patients included in the study had a monoinfection with *P. falciparum*, the parasitaemia being >500 and $<90\,000$ asexual parasites per µl of blood. The patients denied having taken any antimalarial medication during the preceding four weeks. Before collection of the blood samples, informed consent was obtained from the patients or, in the case of children, from their parents.

From January to June 1987, 161 patients were admitted to the study. The patients were 2 months to 62 years old and included 37 females and 124 males.

For the *in vitro* tests, the Rieckmann microtechnique (*7*) was applied following the standard procedure[a] and using microtitration plates and culture media provided by WHO. From each patient, 0.2 ml venous blood was collected in heparinized tubes and

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[a] *Instructions for the use of the microtest kit for the assessment of the response of* Plasmodium falciparum *to chloroquine and mefloquine* in vitro. (Unpublished WHO document MAP/82.1, 1982).

Table 1: ***Plasmodium falciparum*** **in Acre, Brazil: sensitivity to chloroquine (83 isolates)**

Drug concentration (μmol/l blood)	No. of isolates showing complete inhibition of schizont maturation	% inhibition of schizont maturation
0.2	0 (0)[a]	8.42
0.4	2 (2.4)	30.02
0.8	9 (10.8)	53.18
1.14	13 (15.7)	64.95
1.6	31 (37.3)	76.27
3.2	54 (65.1)	94.93
6.4	72 (86.7)	99.76

[a] Figures in parentheses are percentages.

mixed with 1.8 ml RPMI 1640 medium previously buffered with 25 mmol/l HEPES and 32 mmol/l $NAHCO_3$. Aliquots (50 μl) of blood-mixture were pipetted into the wells of the microtitration plates, dosed with various drug quantities: namely, 1, 2, 4, 5.7, 8, 16 or 32 pmol chloroquine/well; 0.25, 0.5, 1, 2, 4, 8 or 16 pmol amodiaquine/well; 0.5, 1, 2, 4, 5.7, 8 or 16 pmol mefloquine/well; or 4, 8, 16, 32, 64, 128 and 256 pmol quinine/well.

For assessment of resistance to sulfadoxine/pyrimethamine, the blood was mixed with RPMI 1640 medium containing lower concentrations of *p*-aminobenzoic acid (pABA) and folic acid.[b] Final concentrations of pyrimethamine were 0.0125, 0.025, 0.05, 0.1, 0.2, 0.4, 0.8, 1.63, 3.13, 6.25 and 12.5 μmol/l blood with a constant ratio of pyrimethamine to sulfadoxine of 1 : 80. Control wells without drug were included in all assays.

The test plates were placed in a candle jar and incubated at 37.5°C for 24 to 32 hours. At the end of incubation, thick blood smears were prepared and stained with Giemsa (5%) for 10 minutes. For each drug concentration tested, the number of schizonts per 200 asexual parasites was determined. Isolates with a schizont maturation of less than 10% in the control wells were not used for evaluation.

In the sulfadoxine/pyrimethamine tests, only schizonts with a normal morphology and at least 8 nuclei were counted.

Drug resistance was assumed if schizont maturation was observed at 1.14 μmol chloroquine/l, 0.4 μmol amodiaquine/l, 3.2 μml mefloquine/l and 51.2 μmol quinine/l, while resistance to sulfadoxine/pyrimethamine was considered if there was less than 90% schizont inhibition at a concentration of 130 μmol sulfadoxine and 1.63 μmol pyrimethamine/l of blood. The results were statistically evaluated using log-dose response probit analysis.[c]

Table 2: ***Plasmodium falciparum*** **in Acre, Brazil: sensitivity to amodiaquine (89 isolates)**

Drug concentration (μmol/l blood)	No. of isolates showing complete inhibition of schizont maturation	% inhibition of schizont maturation
0.05	0 (0)[a]	0
0.1	0 (0)	4.20
0.2	2 (2.2)	23.62
0.4	24 (27.0)	61.56
0.8	71 (79.8)	86.77
1.6	85 (95.5)	98.28
3.2	89 (100.0)	100.0

[a] Figures in parentheses are percentages.

Table 3: ***Plasmodium falciparum*** **in Acre, Brazil: sensitivity to mefloquine (83 isolates)**

Drug concentration (μmol/l blood)	No. of isolates showing complete inhibition of schizont maturation	% inhibition of schizont maturation
0.1	0 (0)[a]	3.59
0.2	2 (2.4)	30.66
0.4	18 (21.7)	74.15
0.8	66 (79.5)	99.21
1.14	80 (96.4)	99.96
1.6	82 (98.8)	99.99
3.2	83 (100.0)	100.0

[a] Figures in parentheses are percentages.

Results

In all, 83 isolates were evaluated for *P. falciparum* sensitivity to chloroquine, 89 for amodiaquine, 83 for mefloquine, 86 for quinine, and 25 for sulfadoxine/pyrimethamine. The response pattern to chloroquine is shown in Table 1. Over 84% of the isolates are chloroquine-resistant (growth at 1.14 μmol/l). The data for various effective concentration (EC) levels are shown in Table 5. They are indicative of high resistance.

A similar frequency of resistance (73%) was observed for amodiaquine (growth at 0.4 μmol/l) (Table 2); the EC data indicate substantial resistance (Table 5). No statistically significant difference could be detected between the response to chloroquine and that to amodiaquine ($P > 0.05$). As for mefloquine, all isolates were fully inhibited at 3.2 μmol/l, indicating high sensitivity to this drug (Table 3). This is also evident from the relatively low EC levels (Table 5).

[b] In vitro *microtest (Mark II) for the assessment of the response of* Plasmodium falciparum *to chloroquine, mefloquine, quinine, sulfadoxine/pyrimethamine and amodiaquine*. (Unpublished WHO document MAP/87.2, 1987).

[c] **Grab, B. & Wernsdorfer, W.H.** *Evaluation of* in vitro *tests for drug sensitivity in* Plasmodium falciparum: *probit analysis of log-dose response test from 3–8 point assay*. (Unpublished WHO document MAL/83.990, 1983).

Table 4: ***Plasmodium falciparum* in Acre, Brazil: sensitivity to quinine (86 isolates)**

Drug concentration (μmol/l blood)	No. of isolates showing complete inhibition of schizont maturation	% inhibition of schizont maturation
0.8	0 (0)[a]	0
1.6	0 (0)	3.90
3.2	2 (2.3)	30.50
6.4	8 (11.6)	68.43
12.8	53 (61.6)	94.81
25.6	78 (90.7)	99.88
51.2	83 (96.5)	99.95

[a] Figures in parentheses are percentages.

Resistance to quinine was encountered in three isolates which still showed schizont maturation at 51.2 μmol/l (Table 4). EC data indicate a relatively low sensitivity to quinine (Table 5).

Table 6 presents the results of sulfadoxine/pyrimethamine. Only two out of the 25 isolates showed at least 90% inhibition of schizont maturation at 130 μmol sulfadoxine/l and 1.63 μmol pyrimethamine/l. At the highest concentration of 1000 μmol sulfadoxine/l and 12.5μmol pyrimethamine/l, only 14 out of the 25 isolates showed at least 90% inhibition of schizont maturation.

Table 5: ***In vitro* sensitivity of *Plasmodium falciparum* in Acre, Brazil, 1987: effective concentrations (μmol/l) calculated by probit analysis for 10% (EC_{10}), 50% (EC_{50}), 90% (EC_{90}), 95% (EC_{95}) and 99% (EC_{99}) inhibition**

Drug tested	EC_{10}	EC_{50}	EC_{90}	EC_{95}	EC_{99}
Chloroquine	0.21	0.73	2.50	3.55	6.82
Amodiaquine	0.13	0.34	0.86	1.12	1.83
Mefloquine	0.14	0.27	0.52	0.63	0.91
Quinine	2.06	4.60	10.30	12.94	19.87

Table 6: ***Plasmodium falciparum* in Acre, Brazil: sensitivity to sulfadoxine/pyrimethamine (25 isolates)**

Sulfadoxine/pyrimethamine concentrations (μmol/l blood)	No. of isolates showing 90% inhibition of schizont maturation
1/0.0125	0 (0)[a]
2/0.025	0 (0)
4/0.05	0 (0)
8/0.1	0 (0)
16/0.2	0 (0)
32/0.4	1 (4)
64/0.8	1 (4)
130/1.63	2 (8)
250/3.13	4 (16)
500/6.25	9 (36)
1000/12.5	14 (56)

[a] Figures in parentheses are percentages.

Discussion

The state of Acre in western Brazil is representative of the areas in the South American Amazon jungle which have recently been opened up. The great increase of migration of non-immune people towards the Amazon region and the mobility of populations within the region have led to a considerable rise in malaria transmission.

In 1987, the incidence of malaria in Acre was 3750 cases per 100000 inhabitants (R.M. Rocha, unpublished observation). Appropriate medication is only available in Rio Branco, capital of the state of Acre. In the rural areas, malaria control is limited to visits by Ministry of Health guards, who apply residual insecticides and use amodiaquine for treatment. Self-medication, especially in the rural areas, is therefore frequent: in some instances, chloroquine is even mixed with the food or salt (personal observation). In the present study, a high degree of *P. falciparum* resistance to chloroquine was demonstrated, with EC_{50} and EC_{90} values of 0.73 μmol/l and 6.82 μmol/l, respectively, resulting in a relatively flat regression line. The EC levels observed in this study were much higher compared with the results from Gabon (*8*), but similar to data from Kenya (*9*). The high frequency of chloroquine resistance in the state of Acre is hardly surprising since earlier studies had shown a serious decrease in chloroquine sensitivity in Colombia and eastern Brazil, which was aggravated by uncontrolled population movements (*4, 6, 9, 11*).

In vitro, 73% of the *P. falciparum* isolates were resistant to amodiaquine in this study. *In vivo*, an even higher percentage of amodiaquine resistance was demonstrated in a recent clinical trial in Acre (*5*). This equal inefficiency of both of these 4-aminoquinolines is at variance with other reports from South America, Africa and Asia (*4, 9, 12*), where amodiaquine has been found to be essentially the more effective drug. This difference may be explained by the fact that the earlier observations were made at the time of introducing amodiaquine as an alternative drug, while, in Acre, amodiaquine had already been widely used.

There was no evidence of *in vitro* resistance to mefloquine. All parasites were completely inhibited at 3.2 μmol/l; 80% of the isolates were already fully inhibited at 0.8 μmol/l and the EC_{50} and EC_{90} were 0.27 μmol/l and 0.91 μmol/l, respectively, indicative of high sensitivity. Furthermore, the steep regression is a sign of relatively high sensitivity to mefloquine. These data are similar to those recently obtained in Thailand (*13*).

The EC_{50} and EC_{99} levels for quinine were 4.60 and 19.87 μmol/l of blood, respectively. These values

are also similar to data from Thailand where a relatively high incidence of quinine resistance has been reported (*11*). In Acre, however, 97% of the isolates were inhibited by quinine concentrations below the generally accepted threshold of 51.2 μmol/l for *in vitro* resistance. This agrees with the results of quinine treatment in the study area. Virtually all cases of falciparum malaria correctly treated with a 10-day quinine regimen were cured.

In the test system for measuring sensitivity to sulfadoxine/pyrimethamine, schizonts were considered to be mature only if they had at least eight nuclei of normal appearance. Moreover, optimized conditions were used for sulfadoxine/pyrimethamine testing as described by Sabchareon et al. (*14*). In a different test system, there was evidence that *in vitro* growth of *P. falciparum* at the concentration of 3.22 μmol sulfadoxine/l and 0.1 μmol pyrimethamine/l correlated *in vivo* with recurrent parasitaemia (*15*). In the present study, data obtained with the WHO standard test system permitted a prediction of resistance to sulfadoxine/pyrimethamine in all *P. falciparum* infections tested. After a single-dose treatment with sulfadoxine/pyrimethamine, maximum plasma concentrations between 0.8 and 1.63 μmol pyrimethamine per litre of blood are reached (*16*). If the latter concentration is taken as the threshold level for *in vitro* resistance, then 92% of the cases investigated were resistant. These findings correspond closely with clinical observations from neighbouring areas in Colombia (*4*).

Conclusions

The observations made in the present study indicate that in the western part of the Amazon area of Brazil the 4-aminoquinolines, as well as the combination of sulfadoxine/pyrimethamine have practically lost their efficacy in the treatment of *P. falciparum* infections. In this area, quinine remains an effective drug when used correctly. However, compliance problems often arise due to the side-effects of quinine. Prospects of overcoming these constraints by combining a short course of quinine with other drugs are limited because of the lack of suitable partner compounds. Single-dose treatment with mefloquine is a feasible alternative, but this should be strictly based on an unequivocal microscopic diagnosis of falciparum malaria followed by the administration of an adequate mefloquine dose so as to prevent undue drug pressure and thus delay the occurrence of resistance.

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Résumé

Pharmacosensibilité *in vitro* de *Plasmodium falciparum* dans l'état d'Acre (Brésil)

Dans l'Etat d'Acre, l'Etat le plus occidental du Brésil, situé dans la région de l'Amazonie, la sensibilité de *Plasmodium falciparum* à la chloroquine, l'amodiaquine, la méfloquine, la quinine et l'association sulfadoxine/pyriméthamine a été mesurée *in vitro* à l'aide de la microtechnique développée par Rieckmann. Dans le cadre de cette enquête, les épreuves ont été faites entre les mois de janvier et juin 1987. Pour tous les antipaludiques soumis à l'épreuve *in vitro*, la réponse du parasite a été mesurée suivant les recommandations de l'Organisation mondiale de la Santé. Sur 83 isolements de *P. falciparum*, tous étaient sensibles à la méfloquine et sur 87 isolements, 84 (97%) l'étaient à la quinine. La concentration efficace à 50% (CE50) était de 0,27 μmol/l de sang pour la méfloquine et de 4,60 μmol/l pour la quinine. En revanche, 65 isolements sur 89 (73%) étaient résistants à l'amodiaquine et 70 sur 83 (84%) à la chloroquine. Il a été observé dans 11 isolements une maturation du parasite à une concentration de 6,4 μmol de chloroquine par litre de sang. Une résistance à la sulfadoxine/pyriméthamine a été démontrée dans 23 cas sur 25 (92%).

Les résultats de cette enquête indiquent que, dans la partie occidentale de l'Amazonie, l'utilisation des amino-4 quinoléines ou de l'association sulfadoxine/pyriméthamine ne peut plus être recommandée pour le traitement des infections à *P. falciparum*.

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