

Evaluation of an in vitro Technique for Detecting
Chloroquine Resistant Falciparum Malaria in Thailand

Principal Investigators : Edward J. Colwell, LTC, MC
Pung Phintuyothin, MG, (Ret)
Narong Sadudee, M.D.*
Wisit Benjapong, M.D.*
Supat Noeypatimanand, M.D.*

Assistant Investigators : Vanchai Numswankijkul, B.Sc.
Amporn Nanakorn
Lek Somchit
Danai Limprayura
Kosol Veivutanapibul
Dumrong Charoendhum

OBJECTIVE: Estimates of the prevalence of resistant strains of Plasmodium falciparum based on clinical responses to an antimalarial agent have several limitations. The supervision of drug intake is often impossible. Prolonged followup examinations may be impractical because of geographic inaccessibility and, perhaps, physical insecurity for the investigators. Moreover, a prolonged followup period can introduce another variable, namely reinfection. Finally, the varying degree of immunity among the population sampled may affect interpretations of the in vivo responses. Conceivably, the interaction of host immunity and an antimalarial agent with relatively resistant parasites may produce a radical cure. It is apparent that a more rapid and reliable technique adaptable to field conditions is desirable for surveys of drug resistant strains of P. falciparum.

Rieckmann and associates have described a relatively simple and rapid technique for the in vitro detection of chloroquine resistant falciparum malaria in human volunteers.¹ More recently, Rieckmann and Lopez-Antuñano examined the potential value of this technique under field conditions and reported that the in vitro technique was successful in detecting resistance of P. falciparum to chloroquine among residents of a Central Brazil community.² The objective of our studies was to evaluate this technique among residents of a malarious area in Central Thailand.

DESCRIPTION: The site selected for study was a district hospital located in the town of Phrabuddhabat, approximately 130 km north of Bangkok. The terrain of this district is mountainous and is located just north of the Chao Phaya River basin and the area is endemic for both vivax and falciparum malaria. Adult and adolescent subjects with falciparum malaria, who were diagnosed at the hospital laboratory or at a nearby Malaria Eradication Center were considered for selection. Those patients who presented with high levels of parasitemia or with renal or cerebral complications were administered immediate quinine therapy and thereby excluded. Subjects who had a history of antimalarial administration within the preceding 5 days were also excluded.

Selected subjects were orally administered 1,500 mg of chloroquine base over a 48 hour interval. Blood smears were obtained daily for the first week and at the end of the second, third and fourth weeks. Asexual parasites were quantified by the method of Earle and Perez and the clinical response to drug administration was classified according to recommendations of the World Health Organization. All subjects were followed on an outpatient basis from the onset of the study or after the first week of hospitalization. Thus, the possibility of reinfection can not be absolutely excluded.

* Phrabuddhabat Hospital

The method described by Rieckmann et al was employed to assess the effect of chloroquine upon the in vitro maturation of asexual erythrocytic forms of P. falciparum.¹ One ml aliquots of a defibrinated, venous blood specimen obtained before treatment were added to 9 ml screw cap vials containing 5 mg of glucose, and either zero (i. e., control) or graded concentrations of chloroquine. The drug treated vials contained 0.4, 0.6, 0.9, 1.35, 2.02 and 3.04 millimicromoles, respectively (1 millimicromole is equivalent to 0.32 μ g base per ml).

With subjects who presented with asexual parasitemias of 20,000 per cmm or greater, a normal saline dilution was employed to adjust the parasite density of the defibrinated specimen to a range of 5,000 to 12,000 per cmm. The vials were mixed and then incubated without agitation for 24 hours at 38–40°C, after which Giemsa stained thick blood films from each vial were prepared and coded. Examination of these smears was accomplished without knowledge of control or drug treated vial concentrations. The degree of maturation for each vial was assessed by counting the number of schizonts per 200 consecutive, asexual parasites and expressed as a percent to the nearest whole integer.

In order to assess the reproducibility of the in vitro technique, examination of 3 to 5 replicate cultures of a single defibrinated specimen was performed on each of 5 subjects. Thick blood smears from each replicate set were randomly allocated on a single slide, coded and examined by different technicians without knowledge of control or drug concentrations of the vials.

PROGRESS: Ninety subjects were studied between July and November, 1970. Of these, both in vitro and in vivo responses were successfully measured in 57 subjects. Because of incomplete followup examinations and/or culture failures, the remaining 33 are not considered in this report. Among the 57 subjects who completed the study, there were 36 males and 21 females, ranging in age from 16 to 67 years.

The clinical responses to conventional chloroquine administration are shown in Table 1. Only 2 of the 57 subjects were radically cured (S). Four subjects were completely resistant (RII). Fifteen subjects demonstrated complete clearing but had recrudescences within the 28 day observation period (RI). Recrudescences in 10 of the latter 15 occurred within 14 days and hence, reinfection is virtually excluded.

The effects of chloroquine upon the in vitro maturation responses of P. falciparum in 4 subjects who were completely resistant to chloroquine therapy are shown in Table 2. All exhibited in vitro schizogony at a dose level of 2.02 millimicromoles of chloroquine. A dose—response relationship is suggested wherein the degree of in vitro schizogony generally decreases as a function of increasing drug concentration.

Because of the large number of subjects in the RII and RI response groups, the in vitro findings at a given drug level for specimens from each clinical response group were summarized and are shown in Table 3. At the 0.9 millimicromole dose level, all specimens in the RI group and 35 of 36 in the RII group exhibited in vitro schizogony. Moreover, schizogony was frequently observed at the higher dose levels of 2.02 and 3.04 millimicromoles. As shown in Table 3, there was a considerable range of the percent of schizonts at a given drug level. Fourteen specimens in the RII group and 9 specimens in the RI group exhibited less than 20% of schizont maturation in the control vials. It was considered inappropriate to normalize quantities less than 20% by a percent reduction transformation (ratio of drug—treated vial response to corresponding control vial response).

The effects of chloroquine upon the in vitro maturation responses of P. falciparum in two subjects who were clinically sensitive to chloroquine are shown in Table 4. Complete arrest of schizont formation occurred at dose levels of 2.02 and 1.35, respectively. Similar to the RIII group shown in Table 2, a dose—response relationship is suggestive in case No. 56.

Replicate examinations were accomplished on defibrinated blood specimens obtained from 5 subjects. The results from one are shown in Table 5. There was little variation among the replicates at a given drug level. The experimental error was considered well within acceptable limits.

SUMMARY: A simplified in vitro technique was evaluated for reliability and reproducibility in detecting chloroquine resistant strains of P. falciparum in Central Thailand. Fifty-five of 57 subjects had parasites which were resistant both in vitro and in vivo to chloroquine. Two subjects who were cured with conventional chloroquine administration had parasites which were resistant in vitro. The major technical limitations of the in vitro test were growth failures with high parasite densities and with immature trophozoites. With these limitations, the technique gives a reliable and reproducible qualitative index of chloroquine resistance in falciparum malaria.

Table 1.
Classification of Clinical Responses to Conventional Chloroquine Administration in 57 Thai Residents with Falciparum Malaria

Response	No.	%
R II	36	63.1
R I	15	26.3
R III	4	7.0
S	2	3.5

Table 2.
Effect of Chloroquine Upon in vitro Maturation of P. falciparum in Subjects with an RIII Clinical Response to Chloroquine

Case No.	Parasite count (per cmm)	Chloroquine concentration*						
		0	0.4	0.6	0.9	1.35	2.02	3.04
52	1,210	58†	62	55	37	38	29	0
53	790	55	57	52	42	46	35	28
54	3,280	52	33	37	31	15	3	0
55	206,950 [‡]	5	3	2	2	1	1	0

* Milimicroles per ml of added inoculum.

† % schizonts

‡ 1/15 saline dilution

Table 3.
Frequency and Range of in vitro Schizogony at a Given Drug
Level Among the RII and RI Response Groups

Clinical response	No. of subjects	Chloroquine concentration						
		Control	0.4	0.6	0.9	1.35	2.02	3.04
RII	36	36*	36	36	35	33	27	19
		3-80†	6-66	3-60	0-55	0-40	0-35	0-35
		23.5 ⁺ †	20	13	12	5.5	2.5	1
RI	15	15	15	15	15	12	9	6
		3-80	1-79	2-75	1-59	0-42	0-10	0-23
		12	10	9	9	3	2	0

* No. of vials at the given drug level exhibiting in vitro schizogony.

† † Range and median values, respectively of % schizont maturation at the given drug level.

Table 4.
Effect of Chloroquin Upon in vitro Maturation of P. falciparum
in Subjects Who Were Clinically Sensitive to Chloroquine

Case No.	Parasite count (per cmm)	Chloroquine concentration*						
		0	0.4	0.6	0.9	1.35	2.02	3.04
56	900	32†	26	24	17	12	2	0
57	1,440	3	3	2	2	2	0	0

* Millimicromoles per ml of added inoculum.

† % schizonts

Table 5.
Results of Replicate Examinations of a Defibrinated Blood
Specimen from a Single Subject

Chloroquine concentration*	Replicate No.			
	I	II	III	IV
Control	78†	78	77	80
0.4	56	56	32	60
0.6	45	43	20	49
0.9	26	8	19	9
1.35	3	3	3	3
2.02	0	0	0	0
3.04	0	0	0	0

* Millimicromoles per ml of added inoculum.

† % Schizonts.

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