DEVELOPMENT OF *IN VITRO* MICROTEST FOR THE ASSESSMENT OF *PLASMODIUM VIVAX* SENSITIVITY TO CHLOROQUINE

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INTRODUCTION

Although *Plasmodium vivax* frequently occurs in the same endemic areas as *P. falciparum*, and the two species are often observed in mixed infections (Bruce-Chwatt, 1980), studies on drug sensitivity of the parasites *in vitro* have heretofore focussed only on *P. falciparum*. The major reason for ignoring *P. vivax* in such studies has not been due to the lack of relevance of the species to human health, but to the fact that no rapid method has been available to determine the susceptibility of *P. vivax* to drugs. Studies reported in the past involved human subjects (McChesney *et al.*, 1962) or laboratory animal models (Schmidt, 1978) in which clinical and research facilities as well as experienced clinical investigators were required. Treatment failure in *vivax* malaria infection has often been interpreted as a relapse, which is characteristic of this species. However, careful clinical observations have recently revealed at least 27 human cases in which treatment with chloroquine (total dose 30 mg/kg body weight) could not eliminate all parasites in the peripheral blood within 5 days (Mitra and Kunte, 1977; Gupta, 1978; Talib *et al.*, 1979; Ali and Uppal, 1981). It is thus our interest to develop a model *in vitro*-system to test the susceptibility of *P. vivax* to chloroquine.

MATERIALS AND METHODS

General procedures. From March 1982 to October 1985, 80 samples of human blood naturally infected with *P. vivax* were collected. Thirty-four samples were from individuals who had visited malaria clinics of the Malaria Eradication Center, Unit 2, Pra Phutthabat, Saraburi Province, and from Mae Sod, Tak Province. The other 26 samples were isolated from the out-patients of the Bangkok Hospital for Tropical Diseases, Mahidol University, Bangkok.

All *in vitro* tests were carried out on micro-tissue culture plates which were predosed with chloroquine by technical personnel of the Special Programme for Research and Training in Tropical Diseases, World Health Organization. The chloroquine concentrations of wells B to H were, respectively, 1, 2, 4, 5, 7, 8, 16, 32 p mol. Well A received no drug, thus serving as control. These plates are referred to as microtest plates. The test medium was a mixture of Waymouth and RPMI 1640 (GIBCO) prepared as follows. Each kind of medium was

reconstituted separately to one liter containing 25 mM HEPES (N-2 hydroxyethyl-piperazine-N-2-ethanesulfonic acid), 2.3 mM sodium bicarbonate (Fisher Scientific Co., Pittsburgh, Pa.); one part of Waymouth was added to three parts of RPMI, then supplemented with 15% (v/v) heat inactivated human serum group AB.

P. vivax-infected blood was obtained from patients aged 15 to 30 years who voluntarily donated 2 ml of their blood which was collected by venipuncture. Each sample was taken into 1 ml of Waymouth containing 40 IU of heparin, chilled on ice and sent to the Microbiology Department on the same day. Upon arrival, 1 ml of plasma medium mixture was removed and the settled blood cells agitated. A small volume of 0.2 ml was then mixed with 1.8 ml of the test medium. This infected cell medium mixture was used as 'unwashed' inoculum. The remaining blood in heparin was washed three times in Waymouth medium without serum by repeating centrifugation at 4°C, and finally resuspended with an equal volume of the test medium to make a 50% suspension. A 0.2-ml volume of this suspension was transferred to 1.8 ml of test medium. This was the 'washed' inoculum.

Drug assay. The prepared 'unwashed' and 'washed' inocula were dispensed in 50-µl volumes onto wells of a microtest plate in vertical order from wells A through H, using 4 replicate rows for each inoculum. The plate was shaken gently to redissolve the coated drug and incubated at 39°C in a candle jar atmosphere (Trager and Jensen, 1976). After 24 and 44 hours, thick blood smear was made from the content of each well, stained with Giemsa's stain and examined microscopically. Hence, there were two duplicate smears for each drug concentration at time to 24 or 44 hours.

All parasites appearing in the microscope fields were counted against at least 100 leukocytes. Stage and viability of 200 parasites were recorded on the basis of morphology and intensity of staining. Differential counts were calculated for percentage of mature schizonts (having more than six merozoites).

In another series of experiments, additional 15 isolates of *P. vivax* were collected and incubated in the chloroquine-predosed plates for 30 hours and the counting was done according to the method described by Grab and Wernsdorfer (1983) which is based on the number of plasmodia which have reached the schizont stage. We thus concentrated on three elements, namely, (1) the number of schizonts per 200 asexual parasites, (2) chloroquine concentrations used (converted from p mol values to 0.20, 0.40, 0.80, 1.14, 1.60, 3.20 and 6.40 $\times$ 10^{-6} mole/liter blood), and (3) the number of schizonts determined in each of the seven chloroquine concentrations. The lowest number of schizonts in our controls was 74, whereas the highest was 198 per 200 asexual parasites. These parasites were grouped according to chloroquine concentration and processed using a program for probit analysis of the log dose/response test on a Texas Instrument TI59 calculator and its Applied Statistical Module.

RESULTS

observations on the growth of *P. vivax* in culture in the presence and absence of chloroquine revealed rapid development of amoeboid and uninucleate stages to young schizonts within 24 hours of incubation. The success of growth into multinucleate schizonts correlated inversely with the level of

Table 1

Growth of *Plasmodium vivax* in the presence of varying concentrations of chloroquine at 24 and 44 hours. Each number represents an average count from four blood films. Numbers in parentheses indicate abnormal looking parasites.

Time (hours)	Well	Drug conc. $x10^{-6}$ M	Number of parasites per 100 leucocytes						
			A	N	2N	>2N	MS	%MS	total
24	A	0	0	71	12	16	0	0	99
	B	0.2	0	70	10	8	0	0	88
	C	0.4	0	66	4	2	1	1.36	73
	D	0.8	0	46	4	0	0	0	50
	E	1.14	0	44	7	2	0	0	53
	F	1.60	0	(56)	0	0	0	0	(56)
44	A	0	0	0	0	21	60	74	81
	B	0.2	0	0	4	7	23	68	44
	C	0.4	0	9	21	16	10	29*	56
	D	0.8	0	25	6	1	1	3.03	33
	E	1.14	0	10	7	8	0	0	25
	F	1.60	0	(8)	0	0	0	0	(8)

A, amoeboid form; N, uninucleate schizonts; 2N, binucleate schizont; >2N, schizonts having 3–6 nuclei; MS, mature schizont harboring merozoites.
*expressed as percent mature schizonts when compared with that of control

chloroquine. The percentage of mature schizonts in the control well was 74% whereas the percentages in wells B through D were 68, 29 and 3.03 respectively (Table 1). The total number of parasites was also lower in comparison with the control well. The difference in the total counts was more pronounced when the incubation period was prolonged to 44 hours. Cultures treated with 0.2×10^{-6} M chloroquine, the lowest concentration used, contained only half as many parasites as control cultures. Effects of chloroquine were more clear cut when calculated for percent relative mature schizonts.

Chloroquine at 0.4 mole/liter blood (mol/1) completely inhibited development of amoeboid stage in 8 out of 23 isolates (Fig. 1). Parasites in the remaining 15 isolates developed to mature schizonts (harboring 8–12 merozoites) at varying rates of success ranging from 5 to 89 percent. At a concentration of 0.4 mol/1, 1.14 mol/1 blood, very few parasites differentiated resulting in an average of 3% mature schizonts.

Only 23 out of 34 isolates used in the experiments could be evaluated for their drug responsiveness because 11 isolates failed to mature in the control wells which

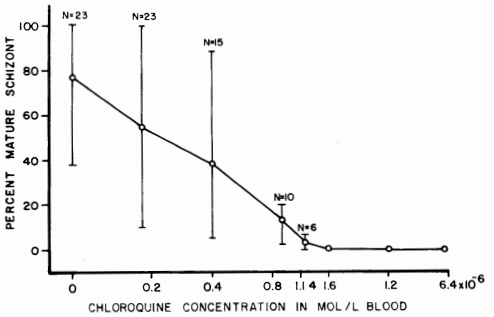

Fig. 1–Chloroquine susceptibility of *Plasmodium vivax in vitro* as evaluated by percentage of schizont maturation.

received no chloroquine. It was thus essential to explore which step(s) of preparing blood samples might be detrimental to the parasites. Table 2 indicates that reproducibility among washed samples was inconsistent. Numbers of developing schizonts tended to be either very high (100%) or extremely low. By contrast, the numbers of developing schizonts among unwashed inocula were generally high although the difference between 'washed' and 'unwashed' group was not statistically significant ($P > 0.05$, pair comparison test).

The results obtained from the additional 15 *P. vivax* isolates tested without washing, and the effective concentration (EC) of the drug was calculated using a program for probit analysis, are summarized in Fig. 2 and Table 3. The effective concentration which gave 50% inhibition of schizont maturation (EC_{50}) was 0.274×10^{-6} mol/1 blood. The EC_{95} value was 1.078 mol/1, and the EC_{99} value was 1.902. The analysis also revealed X^2 value of 0.02 indicating a significant heterogeneity in chloroquine responsiveness among the tested isolates, at the 5% probability level.

Table 2

Pair comparison test of number of mature schizonts from washed and unwashed *P. vivax* originating from the same isolates. Each number represents number of mature schizonts (segmenters) per 200 asexual parasites after 44 hours of *in vitro* cultivation.

washed	unwashed
24	146
20	200
186	132
200	136
80	104
200	162
130	200
X ±SE 120 ± 30.11	154.29 ± 13.51*

*not significantly higher ($P > 0.05$)
$F_{0.05\ (1,\ 6)} = 4.28$

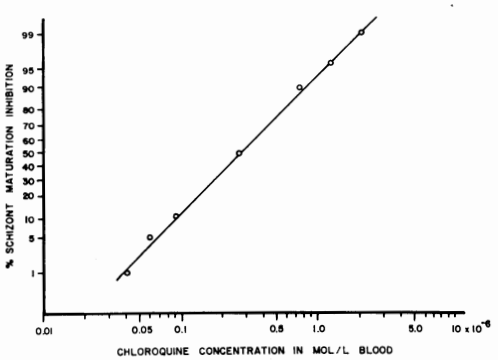

Fig. 2–Regression, confidence limits and observed data points of grouped sensitivity tests of *Plasmodium vivax* to chloroquine.

Table 3

Effective concentrations of chloroquine required to inhibit schizont muturation in *Plasmodium vivax* to varying degrees.

	Effective concentration (x 10^{-6} mol/1)
EC_1	0.0396
EC_{10}	0.0945
EC_{50}	0.274
EC_{90}	0.797
EC_{95}	1.078
EC_{99}	1.902
Inhibition (in probits) at 10^{-6} mol/1	6.55
Slope b	2.77
Variance of slope	0.091

DISCUSSION

This report demonstrates for the first time the application of our short-term culture system for testing the susceptibility of vivax malarial parasites to antimalarials. It is essential to examine the reliability of the system. The technique followed in principle the *in vitro* micro-test described by Rieckmann *et al.*, (1978) to assess *P. falciparum* sensitivity to chloroquine, the major difference being the growth medium used. RPMI 1640 does not support growth of *P. vivax in vitro* (Brockelman *et al.*, 1985). Our newly designed medium mixture (Brockelman *et al.*, 1987) allows the parasite to complete its erythrocytic schizogony to mature schizonts (segmenters). Therefore, evaluation of drug effects can be done simply by microscopic examination of blood films.

There are two major constraints in working with *P. vivax* parasites: the small proportion of infected erythrocytes in patients' blood, and the mixing of stages of development. In this study, we selected only cases that harbored at least 25,000 parasites per mm^3 of blood (0.5% parasitemia) in order to obtain a relatively uniform distribution of infected erythrocytes in the test wells. This permitted reasonably high parasite counts on the blood films. The asynchrony of the inoculum can lead to erroneous results, particularly in cases with high numbers of late schizonts. Thus, only isolates that harbored rings, amoeboid and uninucleate schizonts were included in this report.

Our experiments to evaluate suitability of the test procedure showed that washing of infected blood by centrifugation before culturing did not consistently favor parasite growth. This observation was inconsistent with that reported by Long *et al.*, (1987) on the success of the *in vitro* test to assess *P. falciparum* sensitivity to chloroquine and amodiaquine. One explanation for this difference is that membranes of erythrocytes harboring *P. vivax* tend to be fragile. Washing of *P. vivax* infected erythrocytes by centrifugation in conventional RPMI medium results in a decrease of parasite viability (Brockelman *et al.*, 1984).

Another possible reason is that we used heparin as anticoagulant at a concentration of 20 IU per ml blood. The final concentration of herparin in the test medium was therefore 0.1 IU per 50 µl sample having 2.5% hematocrit. At this concentration, heparin had no unfavorable effects on parasite growth as did EDTA (Brockelman, unpubl.). The latter chemical is known to chelate calcium ions, and addition of calcium blockers was demonstrated to inhibit *P. falciparum* growth *in vitro* (Satayavivad *et al.*, 1987).

In this study the predosed microtest plates were supplied by the Malaria Action Programme, World Health Organization. Hence, technical inconsistency due to quality of drug, type of chloroquine salt, and dilution methods were presumably eliminated. We focussed our efforts on appropriate parameters which would allow effective compilation and processing of drug sensitivity data. In this study, the determination of drug concentration inhibiting schizont maturation at the 50% level was not clear-cut. The drug concentration which led to complete inhibition of schizont maturation appeared higher than reality since there were always one or two parasites that could complete schizogony. To overcome this problem, we have adopted the probit transformation and analysis of log dose/response test developed by Grab and Wernsdorfer (1983). This method has proved to be applicable to our system developed for *P. vivax*. The effective concentration (EC_{50}) obtained represented a true value of all isolates tested, in particular, for a heterogeneous population of parasites such as *P. vivax*.

SUMMARY

An *in vitro* microtest to assess *P. vivax* sensitivity to chloroquine has been developed using a medium mixture which contained RPMI, Waymouth (GIBCO) and 15% (vol/vol) human serum group AB. The rate of success was highest in samples which were not washed by centrifugation before culturing in microtest plates predosed with varying concentrations of chloroquine. Evaluation of the effective concentration of chloroquine using a program for probit analysis of log dose/response test proved superior to simply using the percentage of schizont maturation.

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