CHLOROQUINE RESISTANT *PLASMODIUM FALCIPARUM* : EFFECT OF RABBIT SERUM AND INCUBATION TIME ON THE *IN VITRO* (MICROTECHNIQUE) PREDICTION OF *IN VIVO* RESISTANCE

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INTRODUCTION

Determination of chloroquine resistance of *Plasmodium falciparum* by *in vitro* methods has been established for over a decade. The microtechnique, as reported by Rieckmann *et al.*, (1978), has greatly simplified this task in that only minute quantities of blood are needed. The development of field incubators such as that described by Eastham and Rieckmann (1981) has permitted determination of drug resistance in the field. These recent advances now allow for rapid *in vitro* screening of *P. falciparum* parasites for resistance or baseline sensitivity to currently available quinoline antimalarials.

The ultimate value of the *in vitro* test for chloroquine resistance is to accurately predict an *in vivo* response. In our studies the results of *in vitro* testing (microtechnique) does not always correlate with the *in vivo* response thus we elected to determine the role of serum and incubation time on the test results. Some investigators (Chen *et al.*, 1980) report the use of human AB serum in the micro-test, whereas others did not use any serum (Rieckmann, 1978; WHO, pers. commun.).

Incubation time is also an important factor for *in vitro* drug testing. A premature harvest of the culture will obviously yield negative results and provides for a possible source of error in correlating *in vitro* and *in vivo* responses. Antunano and Wernsdorfer (1979) suggested an incubation period of 24-30 hours, whereas others (Nguyen-Dinh and Trager, 1980) have used 48 hours.

We report herein the effect of rabbit serum and incubation time on *in vitro* drug testing. These results are correlated with *in vivo* data where possible.

MATERIALS AND METHODS

Patients admitted to the San Lazaro Hospital (Manila, Philippines) with *P. falciparum* infections confirmed by Giemsa-stained thick and thin blood films were entered into the study. Blood was collected by venipuncture (EDTA tubes) from patients prior to treatment (25 mg/kg chloroquine base given over 3 days). Quantiation of malaria parasites was done by counting the number of parasites per 100 leucocytes. Counts were expressed as the number of asexual parasites/µl blood. Patients having a minimum of 1,000 asexual parasites/µl blood were selected for both *in vitro* and *in vivo* evaluation of chloroquine resistance. Those with fewer parasites, mixed

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infections (*P. vivax* and *P. falciparum*), or those with schizonts in the peripheral circulation were excluded from the *in vitro* portion of the study. Parasite counts were followed daily or every other day until negative. Patients whose parasite counts became negative within one week but whose *in vitro* test results predicted chloroquine resistance were encouraged to stay in the hospital for at least two weeks. All discharged patients were requested to return for follow up examinations weekly for one month.

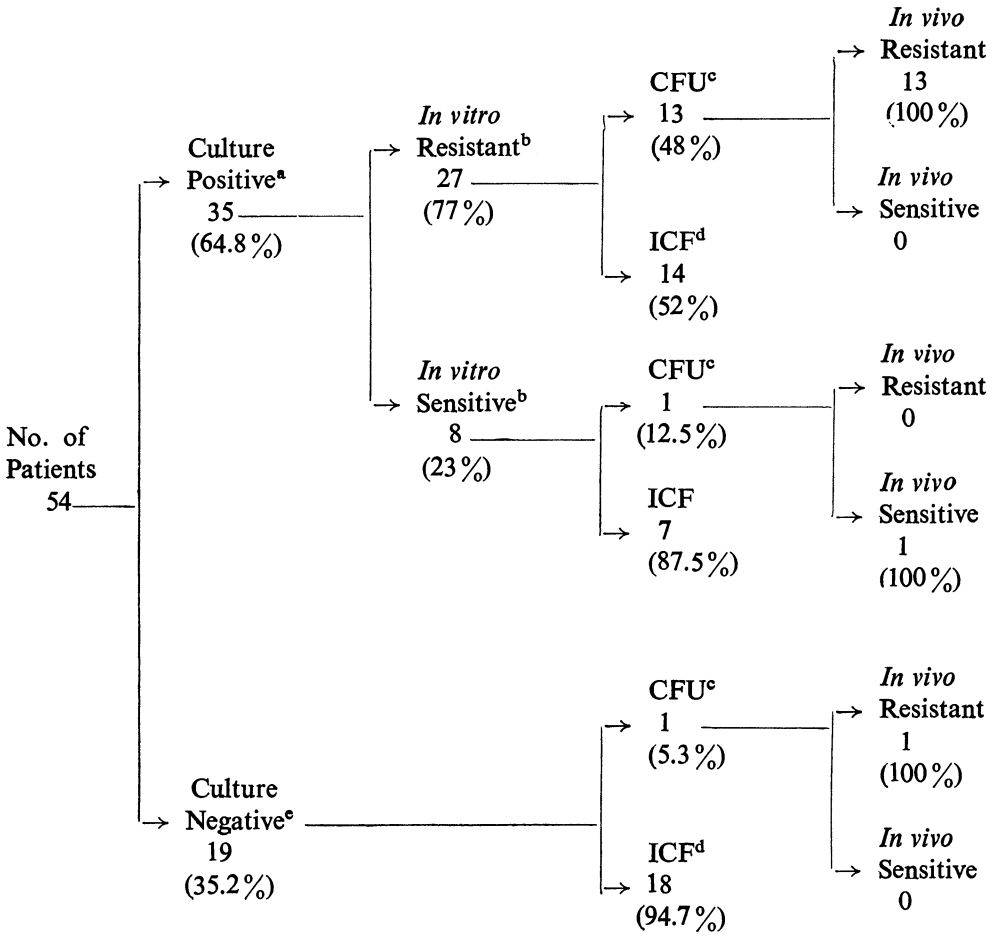
Culture medium used in the *in vitro* test for chloroquine resistance was prepared fresh weekly. RPMI 1640 (260 mg) with L-glutamine was added to 23.0 ml sterile distilled water (USP), plus 1 ml of HEPES (150 mg/ml)/Gentamicin (100 µg/ml) and 1 ml of 7.5% $NaHCO_3$. The medium was sterilized by passage through a .22 µm filter (Millipore Corp.). Serum containing medium was prepared as before except 2.5 ml of sterile, distilled water, was replaced with an equal amount of sterile, normal rabbit serum (M.A. Bioproducts). The serum was added after filtersterilization of the medium and was stored at 4°C.

One hundred µl of patient blood was added to each of 2 vials, one containing 0.9 ml of culture medium without rabbit serum and one containing culture medium plus rabbit serum. The vials were gently swirled to mix the contents. Fifty µl of each suspension was added to wells of flat-bottom tissue culture plates. The plates had been pre-dosed with varying concentrations of chloroquine ranging from 1-32 p-moles by the technical staff of the World Health Organization, Geneva, Switzerland. In addition, similar aliquots of parasitized blood were added to wells of an undosed plate to provide for periodic (approximately every 18-24 hrs) monitoring of parasite growth. The plates were incubated in a candle jar at 37°C. Upon harvest, the supernatant fluid of each well was carefully removed and 5 µl of settled parasitized erythrocytes were used to make thick films. The blood films were airdried for 30 minutes, dipped in reagent-grade acetone to prevent sloughing (Smrkovski and Rodriguez, 1981), stained with 2% Giemsa pH 7.2, and examined for schizonts (3 or more nuclei present). A minimum of 200 oil emersion fields (1,000 ×) were examined before a thick film was scored as negative.

RESULTS

Fifty-four patients infected with *P. falciparum* were tested for chloroquine resistance (Fig. 1; Table 1) and only 35 isolates (65%) produced schizonts *in vitro*. Twenty-seven of the 35 culture-positive isolates (77%) showed *in vitro* resistance (schizonts present in wells containing 5.7 p-moles of chloroquine), whereas 8 cultures (23%) were *in vitro* sensitive (schizonts present only in wells containing $\leq$4 p-moles chloroquine). Thirteen of the 27 chloroquine resistant strains (48%) were confirmed to be resistant *in vivo*. The *in vivo* response of the remaining 14 patients was not determined due to incomplete follow up (less than 28 days) or an inability to exclude reinfection. Eleven isolates, of the original 13 (85%) were found to be of the R-1 type, and 2 (15%) were R-II. Three of the 11 (27%) R-1 types showed early recrudescence ($\leq$ 7 days) whereas the remaining 8 (73%) patients did not show recrudescence until 8-20 days. Classification, RI, RII, RIII resistance, is based upon criteria estabished by the World Health Organization (1973).

Among those strains judged *in vitro* sensitive to chloroquine (8 isolates), only 1 complete follow up (28 days) was recorded (No. 206). The *in vivo* response of this patient showed sensitivity to chloroquine while the *in vivo* response of the remaining 7 patients is not known.



[a] In vitro (microtechnique) presence of schizonts in the control wells after examination of 200 oil emersion (1000 ×) fields of view.

[b] Resistance - schizonts present in wells containing $\geq$5.7 p-moles of chloroquine, Sensitive = schizonts in wells with $\leq$ 4.0 p-moles of chloroquine.

[c] CFU = Complete follow up = patient monitored for 28 days for malaria parasite-reinfection excluded as a possibility.

[d] ICF = Incomplete follow up = patient was monitored for less than 28 days.

[e] Absence of schizonts in the control wells.

Fig. 1—*In vitro* and *in vivo* response to chloroquine resistance of *P. falciparum* parasites from 54 patients.

Table 1

In vitro and *in vivo* testing of *Plasmodium falciparum* for chloroquine resistance.

Patient ID No.	Asexual parasites per c. mm. blood	Culture Negative (hrs.)[a]	Cultures Positive (hrs.)[b]	Chloroquine Concentration (p-moles/5µl blood)[c] S	NS	*In Vivo* Prediction	*In Vivo* Response[d] – (#,E,L)
143	69,000	-	42	16	16	R	ICF (5)
145	3,200	-	44	16	8	R	R–II
154	26,000	-	44	NG	8	R	ICF (20)
159	20,000	-	42	2	4	S	ICF (3)
160	16,000	40	63	8	8	R	R–I (E)
161	2,080	40	63	8	16	R	R–I (E)
162	15,000	40	63	8	16	R	ICF (10)
163	1,680	40	63	1	16	R	R–II
166	19,600	-	65	8	NG	R	R–I (L)
185	3,360	42	89	8	5.7	R	R–I (L)
204	8,000	42	69	32	NG	R	ICF (7)
206	21,120	20	42	4	NG	S	S (33)
207	12,000	20	42	8	NG	R	R–I (L)
208	37,500	20	42	8	NG	R	R–I (L)
211	1,350	41	91	4	NG	S	ICF (6)
215	11,200	20	44	4	4	S	ICF (6)
224	60,000	26	70	32	32	R	ICF (8)
225	1,760	-	65	5.7	5.7	R	ICF (4)
233	2,320	44	65	8	8	R	ICF (5)
234	3,840	44	65	5.7	2	R	ICF (0)
238	69,500	-	72	32	16	R	ICF (11)
240	6,720	-	40	32	32	R	R–I (E)
242	2,000	-	40	8	5.7	R	ICF (10)
244	4,720	-	66	8	8	R	R–I (L)
258	8,960	-	64	8	NG	R	R–I (L)
267	24,000	23	46	16	16	R	ICF (9)
268	1,200	23	46	2	NG	S	ICF (7)
278	3,600	-	64	8	NG	R	ICF (5)
280	12,000	-	46	16	16	R	R–I (L)
284	12,080	23	47	4	NG	S	ICF (4)
285	3,440	-	47	32	32	R	R–I (L)
290	20,000	-	48	16	8	R	ICF (9)
291	800	-	66	8	5.7	R	ICF (10)
231	40,000	23	65	1	NG	S	ICF (4)
215	7,200	24,48	64	1	2	S	ICF (16)

[a] Hours of incubation when cultures were checked and no schizonts were found in the duplicate undosed plate.

[b] Total hours incubation (harvest time) till schizonts detected.

[c] Highest concentration of chloroquine (p-moles) in which schizonts were detected (NG = no growth detected, S = 10% rabbit serum, NS = no serum)

[d] Observed patient response after chloroquine therapy (25 mg/kg chloroquine base) (# = days of follow up, E = Early recrudescence = 0-7 days, L = late recrudescence, 8-28 days, ICF - incomplete follow up = $\leq$ 28 days), S = sensitive, R-I and R-II = type of clinical response of resistance (WHO, 1973).

Table 2

Statistical analysis of the effect of serum on *in vitro* culture of *Plasmodium falciparum*.

Chloroquine p-moles	# Positive cultures (10% serum)	Cumulative total (%)	# Positive cultures (no serum)	Cumulative total (%)	p-value*
1	3	3 (6)	0	0 (–)	NS
2	2	5 (9)	2	2 (4)	NS
4	4	9 (17)	2	4 (7)	NS
5.7	2	11 (20)	4	8 (15)	NS
8	13	24 (44)	6	14 (26)	0.02
16	5	29 (54)	7	21 (39)	0.04
32	5	33 (63)	3	24 (44)	0.02
Negative	20	54 (100)	30	54(100)	-
Total	54				

* McNemar's Test
NS = not significant at 5% level

Nineteen isolates of *P. falciparum* (35%) failed to produce schizonts *in vitro*. Eighteen of the patients, from whom these isolates were obtained were not available for the complete 28 day follow up. One patient (of the 19 studied) showed R-II resistance.

Table 1 summarizes the *in vitro* data of the 35 isolates that were culture-positive for schizonts. The parasitemia levels of the study group ranged from 800 to 69,500/c.mm blood (mean - 15,749). Nineteen cultures, when examined for schizogony at incubation periods ranging from 20-48 hrs (mean = 32), were negative. In contrast, all 35 cultures were positive for schizonts when examined at times ranging from 42-91 hrs (mean - 57).

The *in vitro* response of the 35 isolates to varying concentrations of chloroquine with or without serum is shown in Table 2. The presence or absence of rabbit serum in the culture medium showed no significant difference ($p > 0.05$, McNemar's tests) with regard to positivity among the wells containing ≤ 5.7 p-moles of chloroquine. In contrast, at higher levels of chloroquine (≥ 8 p-moles), the presence of serum resulted in a statistically significant (p-value 0.02 = 0.04) increase in culture positivity. In addition, the response of the individual isolates to varying p-moles of chloroquine appeared to be different. For example, in isolate No. 234 schizonts were detected at 5.7 p-moles of chloroquine when cultured in medium containing rabbit serum. The same isolate was *in vitro* chloroquine sensitive (growth at 2 p-moles) when cultured in the absence of rabbit serum. The *in vivo* response of the parasites from this patient was unknown due to the inability to obtain follow-up specimens. Isolates No. 154 and No. 163 are of interest since growth was apparently inhibited by the presence of rabbit serum. Some isolates that demonstrated schizogony, whether the culture medium contained serum or not, showed comparable results. For example; 11 isolates (Nos. 143, 160, 215, 224, 225, 233, 240, 244, 267, 280, 285) manifested identical endpoint growth (schizonts detected at the same chloroquine concentrations for serum and no serum) and 10 isolates (No. 145, 159, 161, 162, 185, 238, 242, 290, 291, 215) were similar (+ or – at a

single chloroquine dilution). On the other hand, 11 isolates were culture-positive only when grown in the presence of serum; in the absence of serum no schizogony was observed. Six of these strains demonstrated *in vitro* resistance of which 4 were *in vivo* - confirmed R-1 cases.

DISCUSSION

In vitro testing of isolates of *P. falciparum* for drug resistance has been an important part of the World Health Organization's global malaria programme for many years. Data acquired from such studies is important in detecting the emergence of drug resistance in specific regions. In addition, such information is useful for monitoring the shifting pattern of sensitivity or resistance of geographical strains already identified.

The reliability of *in vitro* testing to predict correctly the *in vivo* response is of obvious importance and is dependent upon the adaptability of the parasite population to an *in vitro* environment. Our data suggest that for the geographical isolates of *P. falciparum* tested, serum is an important ingredient for *in vitro* drug testing. This is supported by our observations that 11 isolates tested failed to undergo schizogony in the absence of serum and others (10 isolates) showed different chloroquine end-point growth patterns when cultured in the presence or absence of serum. Only 11 isolates of *P. falciparum* showed no differences in their *in vitro* response to chloroquine when cultured in the presence of or absence of serum. Of interest are the two strains that were actually inhibited by the presence of the rabbit serum. This observation suggests that the use of human AB serum may be preferred over that of rabbit serum.

The growth pattern of the majority of the isolates cultured in the presence of serum resulted in a correct prediction of the *in vivo* response, whereas the growth pattern of some isolates that were cultured in the absence of serum did not. For example, 13 of the 27 *in vitro* resistant cases confirmed by *in vivo* follow up, four cases would not have been predicted *in vitro* resistant had they not been cultured in the presence of serum. The remaining isolates tested (8=62%), which were also found to be *in vitro* and *in vivo* resistant, showed no detectable differences in growth whether cultured in the presence or absence of serum. Other isolates of *P. falciparum* had a marked *in vitro* preference for serum but since their *in vivo* response was not accurately assessed, due to an incomplete follow up, one can only speculate as to the significance. The reason(s) why serum is necessary for *in vitro* schizogony of some primary isolates of *P. falciparum* is unknown, but may be a reflection of strain variation of some malaria parasite populations.

The data further suggest that various primary isolates of *P. falciparum* have different *in vitro* growth cycles. For example, some strains failed to produce detectable schizonts when examined from 20-48 hours but were positive at a later time. Harvesting of these isolates prematurely would have yielded false negative results. These data suggests that once a culture is positive for schizonts it will probably remain so in the ensuing hours and even days since mature schizonts will rupture leading to a new cycle of reinvasion and growth (WHO, 1979). The practical advantage of this is that cultures can be harvested anytime after 48 hours; an obvious advantage for laboratories where weekend work is not practical or possible. Thus, cultures can be established on a Friday and harvested the following Monday.

Data on the testing of urine for the presence of 4-aminoquinolines (Dill Glasko test) or other antimalarials may or may not be important in the interpretation and subsequent correlation of *in vitro* test results. It has been

our experience that in most instances, strains of *P. falciparum* that have been confirmed chloroquine resistant by *in vivo* testing, also show *in vitro* resistance regardless of a positive urine test. In this study, 3 (No. 185, 207, 208) of the 13 patients confirmed *in vivo* and *in vitro* chloroquine resistant, had a positive urine test for 4-aminoquinolines. On the other hand, we have also noted that those isolates found *in vivo* chloroquine sensitive normally do not produce schizonts *in vitro* (even in the control wells) when the urine is test-positive. This may explain in part why some primary isolates of *P. falciparum* fail to undergo schizogony when placed in an *in vitro* environment.

It is concluded that the accuracy of *in vitro* drug resistant data to predict an *in vivo* response is increased if (a) the primary parasite isolates are cultured in the presence of serum (rabbit or human AB) and (b) the cultures are incubated for at least 42 - 48 hours.

SUMMARY

A study of chloroquine resistance of 54 isolates of *Plasmodium falciparum* is reported. Sixty-four percent of the isolates tested produced schizonts *in vitro* (micro-technique), whereas the remaining 36 percent did not. The accuracy of the *in vitro* test to predict *in vivo* resistance was increased when the primary parasite isolates were cultured in the presence of rabbit serum and when the cultures were allowed to incubate for more than 48 hours. Thirteen isolates of *P. falciparum* that showed *in vitro* resistance were confirmed *in vivo* resistant. Eleven of these cases were identified as R-I and two as R-II. Only one case of *in vivo* resistance (R-II) was observed among the 19 isolates that failed to produce schizonts *in vitro*.

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