COMPARISON OF WASHED AND UNWASHED SPECIMENS IN THE *PLASMODIUM FALCIPARUM IN VITRO* MICROCULTURE DRUG ASSAY

GARY W. LONG, S. MAMA, NUNILON SY, RUPERTO P. SANGALANG, JR.*, and CATHERINE P. RANOA*

U.S. Naval Medical Research Unit No. 2, Manila,
*San Lazaro Hospital, Manila, Philippines.

INTRODUCTION

The *in vitro* assay for drug resistance as described by the World Health Organization (Reickman, 1978; WHO, 1982) is performed adding blood collected from a patient to media and culturing in the presence of various drug concentrations. While this is a proven technique which has given satisfactory results in numerous laboratories, it has been the experience in our laboratory over the past five years, that following this technique results in poor and infrequent growth of isolates. It was found, however, that centrifugation of the sample and washing in RPMI 1640 results in more consistent growth and a markedly better rate of successful growth (Smrkovski, L.L., unpublished data). This study compares the washed specimen technique as practised in our laboratory with the standard World Health Organization method. The methods were compared for rate of successful growth of isolates, end point, and ED_{50} as determined by probit analysis.

This study was supported through funds provided by the Naval Medical Research and Development Command, Navy Department for Work Unit 3M-161102BS10.AF429 and by the Philippine Department of Health.

The opinions and assertions contained herein are those of the authors and are not to be construed as official or reflecting the views of the U.S. Navy Department or the Naval Service at large.

Reprint requests to Publications Office, U.S. Naval Medical Research Unit No. 2, P.O. Box SC#410, Manila, Philippines.

MATERIALS AND METHODS

Specimens were collected by EDTA vacutainer from patients with *Plasmodium falciparum* malaria admitted to San Lazaro Hospital, Manila, Philippines. One aliquot was cultured using the *in vitro* microtechnique of Rieckman (1978). One hundred microliters of blood was added to 0.9 ml of RPMI 1640 with L-glutamine plus $NaHCO_3$, HEPES (150 mg/ml) and Gentamycin (100 μg/ml). Another aliquot was washed twice by centrifugation in RPMI 1640 plus $NaHCO_3$ + HEPES. Packed cells were resuspended 1/10 (v/v) in the complete RPMI 1640 medium plus 10% pooled human AB serum. The cultures were performed in plates dosed with either chloroquine or mefloquine by the technical staff of WHO. Cultures were grown for 24 hours at 37°C in a candle jar. The percentage of schizonts was determined by counting schizonts per 200 asexual parasites.

Probit analysis was performed as described by Grab and Wernsdorfer (1983).

RESULTS

Of 23 cultures performed, 8 (34.8%) grew using the unwashed blood, while 17 (73.9%) grew when the blood was washed before culture (Table 1). Fig 1 compares the end-points (well in which no schizonts were present) of the two culture methods. Using the criteria established by WHO, growth at

1.17 x 10^{-6} m/l blood for chloroquine and 3.2 x 10^{-6} m/l blood for mefloquine, all strains were resistant to chloroquine by both techniques except for one which would have been judged sensitive using the unwashed culture method. All isolates were *in vitro* sensitive to mefloquine by both methods except for one which would be considered resistant using the washed culture technique (Table 2).

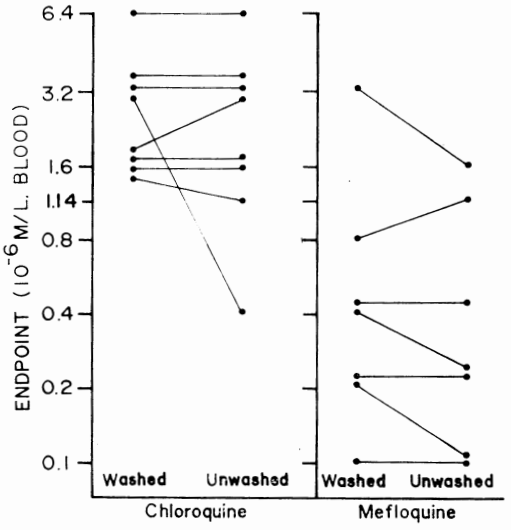


Fig. 1—Comparison of endpoints(drugconcentration at which no schizogony occurs) using washed and unwashed specimens.

Table 1

Comparison of successfully grown isolates of *P. falciparum* from washed and unwashed specimens.

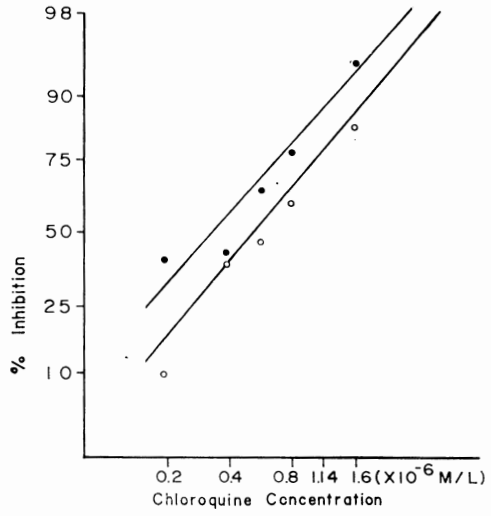
		Number cultures (%) Unwashed	Washed
Insufficient growth	≤10%*	15 (65.2%)	6 (26.1%)
Growth	>10%	8 (34.8%)	17 (73.9%)

* Percent schizonts per 200 asexual parasites.

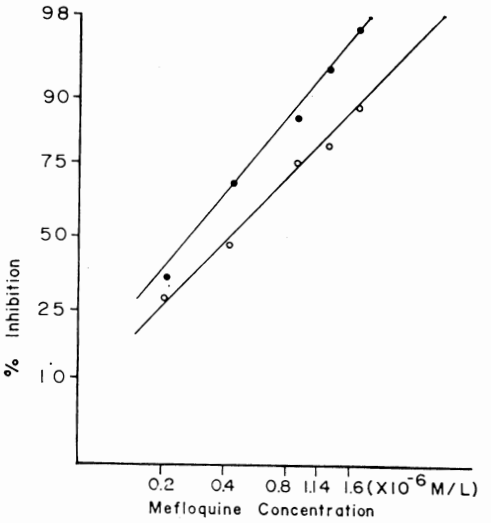
Table 2

Schizont production at 1.14 × 10^{-6} m/l blood for chloroquine and 3.2 x 10^{-6} m/l blood for mefloquine.

	Prediction of drug response			
	Unwashed		Washed	
	Sensitive	Resistant	Sensitive	Resistant
Chloroquine	1	7	0	8
Mefloquine	7	0	6	1



(a)



(b)

Fig. 2a Probit analysis of chloroquine dose response of washed (o) and unwashed (•) specimens. 2b. Mefloquine response.

Probit analysis (Fig. 2a, b) revealed similar slopes for both methods in both drugs. However, the line for washed cultures shifted to the right. The ED_{50} for the cultures are shown in Table 3.

Table 3

Comparison of ED_{50} and ED_{90} of washed and unwashed isolates.

	Chloroquine		Mefloquine	
	ED_{50}	ED_{90}	ED_{50}	ED_{90}
Unwashed	0.26*	0.80	0.72	2.6
Washed	0.40	1.30	1.10	3.8

*10^{-6} m/l blood

DISCUSSION

The culture method described here, centrifugation of the specimen, washing in RPMI 1640, and cultivation in RPMI 1640 plus 10% human AB serum was devised as a result of the low rate of successful growth in this laboratory of isolates using the standard WHO assay. The reason for the low rate of success is unknown although, in many cases, serum drug levels may be responsible. Self treatment is very common in the Philippines. Antimalarials may be bought over the counter at drug stores and neighborhood markets. Forty-eight percent of patients admitted to San Lazaro Hospital for malaria are positive by the Dill-Glazko test (Long *et al.*, unpublished paper). The immune and nutritional status of the host may also be involved.

Whatever the case, the washing of the parasites before culture results in an increased success rate for cultures in our laboratory when compared to the method described by W H O. Seventy-four percent of isolates grew (greater than 10% schizonts) using the former method while only 34.8% grew when the isolates were not centrifuged before culture.

In this study, eight isolates grew using both techniques. The results for mefloquine was lost for one of these. The drug resistance assay is interpreted by evaluating growth of the isolate at a predetermined drug level. Growth (one schizont) at 1.14×10^{-6} m/l blood level for chloroquine is considered resistance *in vitro*. Comparison of the endpoints for chloroquine and mefloquine show that in only one case did the endpoint differ by more than one well. In that case, the difference was four wells. The endpoint of the washed cultures was lower by one well in five assays and greater by one in two. For the two drugs, one of the eight chloroquine and one of the seven mefloquine tests would have been interpreted differently, a resistant isolate to chloroquine in the uncentrifuged method and a resistant to mefloquine using the washed isolates.

Probit analysis of the growth curves revealed a shift to the right for the growth of the washed cultures. The ED_{50} for the washed cultures was about 50% higher than that observed with the unwashed specimens (53.8% in chloroquine and 52.8% in mefloquine). This is less than a one well difference in the assay system.

One of the primary purposes of the *in vitro* assay is to study trends of changing response in a population. The washing of specimens increases successful cultivation of isolates by 100% in this laboratory. An additional advantage is that the growth conditions are consistent among isolates giving a better indication of the properties of the parasite, separated from the complicating factors of serum drug levels, immune factors and host nutritional status.

The *in vitro* drug assay can provide valuable information when used as a monitoring tool. A good example of this is our experience with the assay as performed in this laboratory in

the monitoring of the response to amodiaquine (Long *et al.*, unpublished data). Over the period from 1982 to 1984 we observed an increase in *in vitro* resistance. This was the first indication of increased resistance to this drug since 1968 (Shute *et al.*, 1970) and was soon verified *in vitro* (Watt *et al.*, 1987).

One obvious disadvantage of the technique is that centrifugation is not possible in many field situations. In spite of this, where centrifugation is possible, we feel it is a reasonable technique to improve the performance of the *in vitro* assay in areas where poor growth is a problem.

SUMMARY

The dose response of *Plasmodium falciparum* isolates in the standard *in vitro* assay for drug resistance was compared using blood specimens which were centrifuged and washed before cultivation. Washing of the cultures increased the success of cultivation by greater than 100%. Eight cultures which grew using both methods gave similar results in the determination of resistance or sensitivity. The ED_{50} as determined by probit analysis, was approximately 50% higher in parasites which had been washed before cultivation.

REFERENCES

GRAB, B. and WERNSDORFER, W.H., (1983). Evaluation of *in vitro* tests for drug sensitivity in *Plasmodium falciparum:* probit analysis of logdose/response test from 3-8 points assay. *W.H.O./MAL/ 83.990*: 1-18.

LONG, G.W., WATT, G., SY, N., BUCK, R.L., SANGALANG, R.P., JR. and RANOA, C.P., (1987). *In vitro* drug response of *Plasmodium falciparum* in the Philippines: Increased resistance to amodiaquine. (NAMRU-2 Report).

REICKMAN, K.H., (1978). Drug sensitivity of *Plasmodium falciparum* and *in vitro* microtechnique. *Lancet, i* : 22.

SHUTE, G.T., RAY, A.P., and SANGALANG, R., (1970). Preliminary studies of a Philippine strain of *Plasmodium falciparum* resistant to amodiaquine. *J. Trop. Med. Hyg., 75* : 125.

WATT, G.H., (1987). Amodiaquine less effective than chloroquine in the treatment of falciparum malaria in the Philippines. *Amer. J. Trop. Med. Hyg., 36* : 3.

WHO, (1982). Instructions for use of the micro test kit for the assessment of the response of *Plasmodium falciparum* to chloroquine and mefloquine *in vitro*. *MAP/82.1,*