

Comparative studies of serum-free media and detection techniques for *in vitro* drug sensitivity assessment of *Plasmodium falciparum*

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ABSTRACT

Malaria continues to be a devastating disease. In a previous study, we formulated a chemically defined culture medium that is able to sustain the complete intraerythrocytic growth of *Plasmodium falciparum*. We tested the feasibility of using the medium (CDRPMI) as well as human serum-free media enriched with commercially available human-serum substitutes (GFSRPMI and ALBRPMI) to assess the drug sensitivity of *P. falciparum*, using chloroquine diphosphate (CQ) and dihydroartemisinin (DHART) as conventional antimalarial drugs. Growth inhibition was measured by four different methods: flow cytometry with SYBR Green I (FCM), microscopy (Giemsa method), enzymatic estimation of parasite lactate dehydrogenase (pLDH), and histidine-rich protein 2 (HRPII) determination. In drug sensitivity tests on asynchronous parasites cultured for 96 h in the presence of drugs, the dose-response curves were similar and differences in the 50% growth inhibition concentrations for the drugs, which were estimated by the four methods, were not statistically significant for the three culture media. The effect of the drugs on the growth of synchronous parasites at the ring stage was also assessed in micro-volume tests by three different methods of FCM: tracking fluorescent erythrocytes, schizont test, and merozoite test. Dose-response curves for the drugs were similar, and differences in the 50% growth inhibition concentrations were not statistically significant for CDRPMI and GFSRPMI.

Thus CDRPMI as well as GFSRPMI and ALBRPMI can be similarly useful media for drug sensitivity testing of *P. falciparum*. The FCM, pLDH and HRPII estimations were fast and reliable detection methods, with FCM allowing schizont and merozoite tests to be performed with shorter periods of culture.

Keywords: *Plasmodium falciparum*; Chemically Defined Medium; Drug Sensitivity Test; Flow Cytometry

1. INTRODUCTION

Malaria continues to be a devastating disease, particularly in the tropics, with an estimated annual incidence worldwide of 216 million clinical cases. The annual mortality from malaria, which is caused largely by the protozoan *Plasmodium falciparum*, is estimated to be 0.66 million worldwide [1]. A better understanding of antimalarial treatments is thus needed to allow the development of new medications to combat resistance to conventional antimalarial drugs [2].

The development of *P. falciparum* requires human serum in the original culture medium [3]. To avoid the obstacles associated with the dependence on human serum, including unpredictable risks with infectious agents and difficulties in securing normal human sera, particularly in malaria endemic areas, and in obtaining a particular blood group, human serum substitutes such as a growth-promoting fraction derived from adult bovine plasma (GFS; Wako Pure Chemical Industries Ltd., Japan) and lipid-enriched bovine albumin (AlbuMAX I; Life Technologies, Japan) have been exploited success-

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fully [4,5], and have been employed widely for *in vitro* cultivation of *P. falciparum*. However, sufficient information on the constituents of the serum substitutes is still lacking.

A chemically defined medium is essential for the design of reproducible biochemical, physiological, and genetic studies of microorganisms and various cell types [6,7]. We formulated previously a chemically defined medium that sustained the complete intraerythrocytic growth of *P. falciparum*, based on the results of characterization of the growth-promoting factors in GFS [8]. All stages of the parasite cultured in the chemically defined medium have been comparable to or better than those grown in human serum or GFS-containing medium. This finding implies that the chemically defined medium formulated may be usefully applied in diverse aspects of malaria research, in a similar way to media containing human serum and human serum substitutes.

The effects of drugs on *P. falciparum* can be assessed both quantitatively and qualitatively by direct examination of erythrocyte (RBC) smears from blood or cultures with a microscope, although this method is tedious and subjective. Numerous assays have been introduced that are more objective, more sensitive, faster and designed to be easier to handle. The most common include isotopic, enzymatic, and enzyme-linked immunosorbent assays, such as the incorporation of ^3H -hypoxanthine into the DNA of the parasite, the estimation of parasite lactate dehydrogenase (pLDH), and quantifying biomolecules (pLDH and histidine-rich protein 2 (HRPII)) by double-site sandwich enzyme-linked immunosorbent assays [9-13]. These methods are relatively reliable and objective, and are well suited for screening large numbers of drugs; however, some of them are associated with unavoidable weaknesses, including insufficient sensitivity and the use of hazardous radioactive material.

Analysis by flow cytometry (FCM) using different fluorescent dyes has also proven useful for analyzing the blood stage of malaria parasites. In particular, FCM using SYBR Green I as a fluorescent dye has been useful in the assessment of the growth of *P. falciparum* and *P. berghei* [14-16]. This system allows visualization of parasitized RBCs (PRBCs) with high accuracy, and can be used to follow the development of *P. falciparum* [14] and merozoite invasion into new RBCs [15; Asahi, unpublished].

In the present study, we tested the feasibility of using a chemically defined medium for *in vitro* drug sensitivity assessment of *P. falciparum*, in comparison with two other commercially available non-human serum media, to obtain a more detailed understanding of this principle of the assessment of antimalarial efficacy. We used chloroquine diphosphate (CQ) and dihydroartemisinin (DHART) as conventional antimalarial drugs, and inhibition of parasite growth was assessed by FCM using

SYBR Green I in comparison with three other assays: the Giemsa method, pLDH estimation, and HRPII quantification.

2. MATERIALS AND METHODS

2.1. Culture Media

The basal medium (CRPMI) consisted of RPMI 1640 containing 2 mM glutamine, 25 mM 4-(2-hydroxyethyl)-piperazine ethansulfonic acid, and 24 mM NaHCO_3 (Invitrogen Ltd., USA), with 25 $\mu\text{g}/\text{ml}$ gentamicin (Sigma-Aldrich Corp., USA), and 150 μM hypoxanthine (Sigma-Aldrich). The complete culture medium termed GFS-RPMI comprised CRPMI with 10% GFS (Daigo's GF21), as reported previously [4,8,12]. The complete medium termed ALBRPMI consisted of CRPMI supplemented with AlbuMAX I at a final concentration of 3 mg/ml. The chemically defined medium termed CDRPMI consisted of CRPMI supplemented with bovine serum albumin free of non-esterified fatty acids at a final concentration of 3 mg/ml, two non-esterified fatty acids (100 μM *cis*-9-octadecenoic acid and 60 μM hexadecanoic acid), and four phospholipids (15 μM 1,2-dioleoyl phosphatidic acid sodium salt, 130 μM 1,2-dioleoyl-sn-glycerol-3-phosphocholine, 25 μM 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine, and 15 μM 1,2-dioleoyl-sn-glycero-3-phosphoserine, sodium salt), as described previously [8]. All the compounds were obtained from Sigma-Aldrich. Dried lipid precipitates were prepared, added to the culture media, and sterilized to reconstitute the lipids, as described previously [8,12].

2.2. Parasite Culture and Synchronization

Cultures of the FCR/FMG (ATCC Catalogue No. 30932, Gambia) strain of *P. falciparum* were used in the experiments. The parasites were maintained routinely by *in vitro* culture techniques using GFSRPMI. The RBCs were preserved in Alsever's solution [4] for 3 - 30 days, washed, and then dispensed in a 24-well culture plate at a hematocrit of 2% (1 ml suspension per well), and cultured under a humidified atmosphere of 5% CO_2 , 5% O_2 , and 90% N_2 at 37°C. Parasitemia was adjusted to 0.1%, except where specified otherwise, by adding uninfected RBCs, and the hematocrit was adjusted to 2% by adding the appropriate volume of culture medium. Cultures were synchronized at the ring stage by three successive exposures to 5% (w/v) D-sorbitol (Sigma-Aldrich) at 41- and 46-h intervals [17]. After the third treatment with sorbitol, residual schizonts and cell debris were removed by isopycnic density centrifugation on 63% Percoll (GE Healthcare Bio-Science Corp., USA).

2.3. Assessment of Parasite Growth Inhibition

The GFSRPMI was replaced by other culture media

containing graded concentrations of CQ (Sigma) or DHART (Fluka-Sigma-Aldrich, Japan) to test for growth inhibition. Stock solutions (10 mM) were dissolved initially in either 50% ethanol (for DHART) or distilled water (for CQ), diluted, and dispensed at optimal volumes. Asynchronous PRBCs were dispensed in a 48-well culture plate at a hematocrit of 2% (500 μ l suspension per well), and cultured for 96 h (2 cycles).

For micro-volume tests, synchronized PRBCs were dispensed in a 96-well culture plate containing graded amounts of CQ or DHART, at a hematocrit of 2% (100 μ l suspension per well), and cultured for 25 or 45 h.

The drug concentrations required to inhibit growth of the parasite and schizont/merozoite formation by 50% in comparison with drug-free controls (GIC_{50} and EC_{50} , respectively) were extrapolated from the concentration-response curves.

The culture wells were run in triplicate or quadruplicate in all experiments, and each experiment was repeated two to four times.

2.4. Techniques for Measuring Antimalarial Activity

2.4.1. Growth Rate (Giemsa Method)

Thin smears were prepared on microscope glass slides, which were stained with Giemsa. Parasitemia was determined microscopically based on the examination of more than 10,000 PRBC/RBCs. The growth rate was estimated by dividing the parasitemia of the test sample, after incubation for the indicated times, by the initial parasitemia.

2.4.2. Tracking Fluorescent PRBCs (FPRBCs) and Released Merozoites

After fixation by the addition of 1% paraformaldehyde combined with Alsever's solution, PRBCs (8×10^5 cells in a 16 μ l aliquot of 0.5% PRBC/RBC suspension) were stained by mixing with 0.5 - 1 ml SYBR Green I ($\times 1$ dilution, Invitrogen) prepared in Tris-saline solution consisting of 20 mM Tris (hydroxymethyl) aminomethane hydrochloride at pH 8.8 and 138 mM NaCl. Parasitemia and each developmental stage were determined by examining more than 10,000 PRBC/RBCs. The numbers of PRBCs and each developmental stage of the parasite were measured by FCM using either a PAS flow cytometer (PAS, Partec Co. Ltd., Germany) or a FACSCalibur (Becton Dickinson Immunocytometry Systems, USA), as described previously [14]. Analysis was performed using FCS express software (De Novo Software Inc., Canada).

In the schizont test, the number of high-fluorescent FPRBCs (schizonts) was measured by FCM after rings of the parasite had been cultured for 25 h [14].

In the merozoite test, merozoites released from mature

schizonts into the surrounding medium were counted by FCM after rings of the parasite had been cultured for 45 h [14]. The number of merozoites was counted in more than 5000 PRBCs.

2.4.3. pLDH Enzymatic Assay

The pLDH enzymatic assay was performed according to the methods described by Makler and Hinrichs [9] and Asahi *et al.* [12]. Briefly, at the indicated incubation times, PRBC/RBCs in culture were hemolyzed by three freeze-thaw cycles, and 15 μ l aliquots were transferred to each well of a 96-well microtiter plate. Subsequently, 100 μ l of Malstat reagent (Flow Inc., USA), 10 μ l of 1 mg/ml nitroblue tetrazolium (Wako), and 10 μ l of 1 mg/ml diaphorase (Wako) were added to each well. The plated contents were allowed to stand for 40 min at 37°C, and the reaction was stopped by adding 50 μ l of 5% (v/v) acetic acid. The absorbance at 650 nm (OD_{650}) was read on a plate reader. The initial OD_{650} value, measured when the assay reagents were first added, was subtracted from the endpoint reading.

2.4.4. HRPII Assay

The HRPII assay was performed according to the manufacturer's instructions for the Malaria Ag CELISA (Cellabs Pty Ltd., Australia), with some modifications. Briefly, 5 μ l aliquots of thawed samples from a 2% hematocrit were transferred to each well of the CELISA plate. These were topped up with 95 μ l of phosphate-buffered saline, and then allowed to stand for 1 h at room temperature. The plate was washed five times, 100 μ l of the diluted conjugate was added, and the plate was incubated again for 1 h at room temperature. After five washes, 100 μ l of the substrate was added and incubated in the dark at room temperature for 15 min. The reaction was stopped by adding 50 μ l of stopping solution. The absorbance at 450 nm (OD_{450}) was read on a plate reader.

2.5. Statistical Analysis

The significance of the differences between means was evaluated using multifactorial analysis of variance. All calculations were performed using GraphPad PRISM 5 (GraphPad Software, USA). The *P* value for significance was 0.05, and all pairwise comparisons were made post hoc with Bonferroni's test. Error bars were added to the y-axes on the graphs to indicate the standard deviation for each point.

3. RESULTS

3.1. Drug Sensitivity of Asynchronous Parasite Culture

The effects of CQ and DHART on the growth of parasites cultured asynchronously in CDRPMI, GFSRPMI

and ALBRPMI for 96 h were assessed by four methods: FCM, the Giemsa method, and estimations of pLDH and HRPII. The dose-response curves obtained for all culture medium-estimation method combinations were similar at all concentrations of CQ and DHART (**Figure 1**). Also, differences in GIC_{50} values that were estimated by the four methods were not statistically significant among the three culture media (**Table 1**). However, slightly higher values of near total parasite killing concentrations of CQ and DHART were detected by the HRPII estimation method (**Figure 1**).

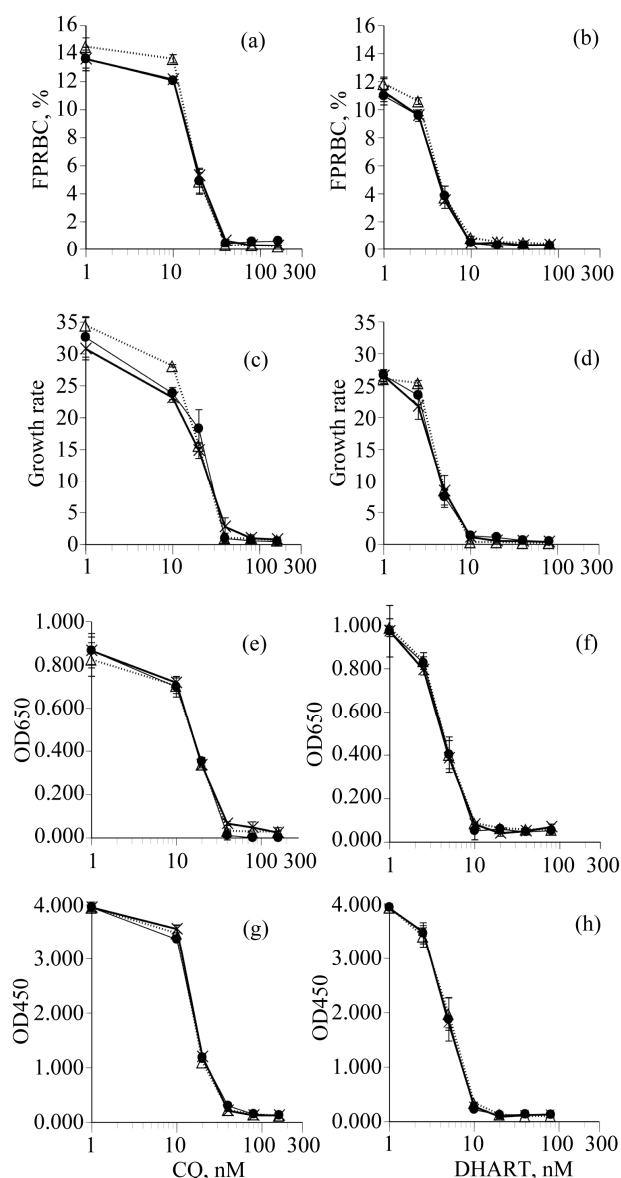


Figure 1. Growth inhibition curves of *P. falciparum* parasites cultured asynchronously in CDRPMI (—●—), GFSRPMI (—X—) and ALBRPMI (...△...) in the presence of CQ and DHART. Parasite growth was assessed by FCM (a, b), Giemsa method (c, d), pLDH levels (e, f), and HRPII assay (g, h). The initial parasitemia was adjusted to 0.3%.

3.2. Microtitration Analyses on Synchronized Parasite Cultures

The effects of CQ and DHART on the growth of ring forms of the parasite cultured in CDRPMI and GFSRPMI for 25 or 45 h were assessed by three different types of FCM: (1) tracking FPRBCs for parasitemia; (2) tracking high-fluorescent FPRBCs (schizont test); and (3) counting released merozoites (merozoite test) in a 96-well microplate containing graded concentrations of the antimalarial drugs, because clinical blood samples from individuals infected with *P. falciparum* contain predominantly ring forms. The dose-response curves obtained for all culture medium-estimation method combinations were similar at all concentrations of CQ and DHART (**Figure 2**). In addition, the GIC_{50} (parasitemia) and EC_{50} (schizont test and merozoite test) values for the two drugs in the two culture media were not statistically different (**Table 2**). Further, only the schizont and merozoite tests showed near total parasite killing: at 40 nM for CQ and 10 nM for DHART (**Figure 2**).

4. DISCUSSION

The advent of *in vitro* culture with the use of human serum has led to unprecedented advances in diverse aspects of malaria research, including studies on drug resistance, vaccine development, genetics and parasite biochemistry [3]. Although the potential complexity of human serum supplementation has posed an enormous challenge, the successful exploitation of human serum-free culture media using alternatives to human serum, such as GFS and AlbuMAX I, has avoided the disadvantages of human serum. Sufficient information on the constituents of the serum alternatives is, however, still lacking. Culture of malarial parasites in chemically defined media may not only avoid any unpredictable effects that can arise when human serum and substitutes are used, but also secure reproducible study designs and thus increase the opportunities for interdisciplinary research on malaria.

We formulated previously a chemically defined medium (CDRPMI) that sustained complete intraerythrocytic growth of *P. falciparum* [8]. The CDRPMI consisted of paired non-esterified fatty acids, phospholipids with specific fatty acid moieties, and specific proteins dissolved in the basal medium RPMI-1640, supplemented with hypoxanthine [8,18]. All stages of the parasite cultured in the CDRPMI have been comparable to those grown in GFSRPMI. Also, on genome-wide transcriptome profiling, only slight differences in transcript levels have been detected between the parasites cultured in CDRPMI and GFSRPMI [19]. In the current study, the feasibility of using CDRPMI, as well as GFSRPMI and ALBRPMI, for drug sensitivity tests on *P. falciparum* was investigated. The growth curves were similar for all three media after

Table 1. Comparison of GIC₅₀ determined by FCM, Giemsa method, pLDH levels, and HRPII levels. *P. falciparum* was cultured in CDRPMI, GFSRPMI, or ALBRPMI in the presence of graded concentrations of CQ or DHART.

Culture Media	GIC ₅₀			
	CQ, nM			
	FCM	Giemsa	pLDH	HRPII
CDRPMI	20.77 ± 0.34	20.99 ± 0.25	19.96 ± 1.12	20.20 ± 0.16
GFSRPMI	21.34 ± 0.40	20.56 ± 0.29	20.68 ± 0.23	20.77 ± 0.25
ALBRPMI	21.33 ± 0.83	20.85 ± 0.27	20.98 ± 0.73	20.24 ± 0.78
DHART, nM				
CDRPMI	5.15 ± 0.41	5.05 ± 0.17	5.26 ± 0.14	5.53 ± 0.16
GFSRPMI	4.97 ± 0.94	5.01 ± 0.10	5.19 ± 0.11	5.52 ± 0.13
ALBRPMI	5.24 ± 0.47	5.23 ± 0.12	5.38 ± 0.10	5.64 ± 0.16

GIC₅₀: The drug concentrations required for 50% inhibition of parasite growth.

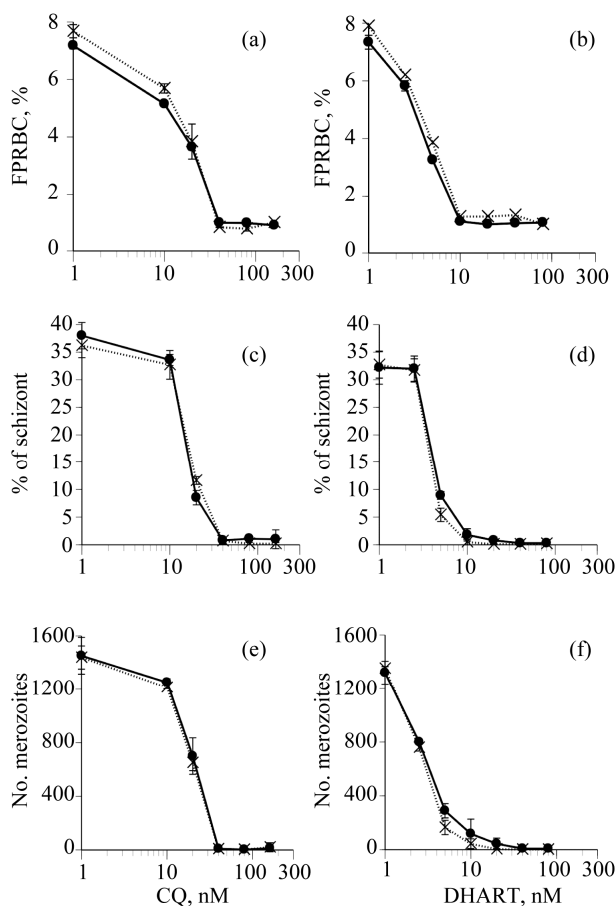


Figure 2. Growth inhibition curves of synchronized *P. falciparum* cultured in CDRPMI (—●—) or GFSRPMI (...X...) in the presence of CQ and DHART. Parasite growth was assessed by micro-volume tests using three types of FCM: tracking FPRBC after 45 h culture (a, b), tracking schizonts after 25 h culture (c, d), and counting the number of merozoites released after 45 h culture (e, f). The initial parasitemia (rings) was adjusted to 1%.

exposing asynchronous parasite culture to CQ and DHART for 96 h. Likewise, microtitration analyses using the schizont test and merozoite test on synchronized parasite culture showed a similar trend in parasite growth inhibition in the presence of graded concentrations of the drugs. These findings support the feasibility of using CDRPMI for drug sensitivity tests, in a similar way to GFSRPMI and ALBRPMI.

Affordable and reliable analytical techniques to test growth-promoting and antimalarial effects on plasmodia are needed to improve tracking of malaria parasites. FCM has a particular advantage in determining populations of dividing stages of the parasite. FCM that uses different intercalating dyes has already been used successfully to test human and murine samples [14,20,21]. However, some of the dyes lack sufficient sensitivity and/or require complicated preparation procedures that make their use problematic. To overcome those limitations we selected SYBR Green I as a DNA intercalating dye to take advantage of the excellent bright signal of SYBR Green I and the simple preparation procedure for FCM [14].

FCM using SYBR Green I has been shown to enable not only visualization of PRBCs with high accuracy, but also to allow development of the parasite to be followed during its life cycle. In the current study, when investigating the effect of antimalarial drugs on synchronized cultures using FCM, the readings of the number of high-fluorescent FPRBCs (schizonts) in 25-h cultures gave comparable results to microscopic examination of slides stained with Giemsa. This suggests that readings from a number of high-fluorescent FPRBCs could be used to assess the drug-sensitivity of the parasite, in a similar way to the conventional microscopic method that uses changes in the percentages of schizonts after 25 - 28 h of culture.

Table 2. Comparison of GIC₅₀ and EC₅₀ determined by microtitration using tracking FPRBCs (parasitemia, 45 h culture), schizont test (25 h culture), and merozoite test (45 h culture). *P. falciparum* was cultured in CDRPMI or GFSRPMI in the presence of graded concentrations of CQ or DHART.

Method (FCM)	GIC ₅₀ or EC ₅₀			
	CQ, nM		DHART, nM	
	CDRPMI	GFSRPMI	CDRPMI	GFSRPMI
Parasitemia	20.99 ± 0.27	20.96 ± 0.26	5.43 ± 0.20	5.00 ± 0.10
Schizont test	19.65 ± 0.63	20.66 ± 0.58	5.45 ± 0.96	5.19 ± 0.94
Merozoite test	21.53 ± 1.02	20.90 ± 1.08	4.84 ± 0.63	4.11 ± 0.64

The drug concentrations required for 50% inhibition of parasite growth (GIC₅₀) and of schizont/merozoite formation (EC₅₀).

The merozoite test (counting the number of released merozoites) using FCM has also been described to be a valuable tool for assessment of the effects of antimalarial drugs on synchronous growth of the parasite after 45 h of culture [14]. The schizont and merozoite tests involving FCM described here consistently detected the sensitivity of the parasite to CQ and DHART in shorter culture periods, even in micro-volume tests. However, the merozoite test may have limitations in the assessment of parasite growth in the case of some antimalarial agents, such as antifolates, that begin acting at a later stage in parasite development. Parasites under the influence of these drugs tend to produce relatively high numbers of merozoites, which then stop growing before reaching the next stage of the cycle, including invasion into new RBCs.

The correlations among FCM, the Giemsa method, pLDH estimation, and HRPII assay were excellent. Although the HRPII estimation method is highly sensitive and reliably detects growth inhibition in drug-sensitivity tests, the amounts of HRPII produced have been known to reflect not parasite viability but the presence of the bi-molecule. This implies that the HRPII of killed parasites may be measured, and may mean that the near total parasite killing concentrations required in the HRPII assay were somewhat higher than those for other methods. On the other hand, levels of pLDH have been known to correlate well with viable development of *P. falciparum* [9,11]. Regardless of the estimation of parasite viability, the differences in GIC₅₀ values for the long culture method were not statistically significant among the assays employed here.

Concerns about artemisinin resistance have been reported recently [22], and all malaria-endemic countries have been warned to be more vigilant in monitoring antimalarial drug efficacy, to allow the early detection of artemisinin resistance and aid global malaria control [23-25]. Furthermore, most rapid diagnostic test kits currently available on the market are based on HRPII and pLDH (as a biomolecule). The finding that the concentrations that show near total killing of the parasite in the HRPII assay are variable should be taken into account in monitoring antimalarial drug efficacy.

5. CONCLUSION

This study confirmed that CDRPMI, as well as the human serum-free media GFSRPMI and ALBRPMI, was useful for assessment of the drug-sensitivity of *P. falciparum*. FCM, using SYBR Green I as a fluorescent dye, and pLDH and HRPII estimations were fast and reliable detection methods. Furthermore, FCM allowed schizont and merozoite tests to be performed with shorter periods of culture.

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