THE USE OF FLOW CYTOMETRY AS A DIAGNOSTIC TEST FOR MALARIA PARASITES

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Abstract. A total of 453 clinical blood samples were determined for malaria parasites by flow cytometric assay (FCM) and reagents from Sysmex Corporation, Japan. In this study, the FCM greatly simplified and accelerated parasite detection, with sensitivity of 91.26%, specificity 86.28% and accuracy 87.42%. Overall, the parasite counts by flow cytometric measurement correlated well with the parasitemia measured by microscopic assay (regression coefficient = 0.9409). The detection limit was 0.05-0.1% parasitemia. No evidence of malaria parasites in either blood donor volunteers or other disease patients groups was determined by FCM. However, 48 samples who had been treated with antimalarial drugs and whose parasite microscopic counts were negative, showed false-positive results. When the data of these 48 samples were analyzed, they were found to have high levels of reticulocytes, ranging from 2.0-18.9%. This finding suggested that a high reticulocyte concentration in the blood may interfere with the performance of the FCM. Further improvement, by eliminating this interference, will make the FCM one of the most promising tests for malaria diagnosis.

INTRODUCTION

Microscopic examination of thick or thin blood smears is the most widely used routine method for determining malaria infection in humans, and remains the gold standard test for malaria diagnosis. Although it has good sensitivity and allows species identification and parasite counts, it is time-consuming and the individuals who examine blood films need to be skilled and experienced if they are to identify the parasites accurately.

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Several alternative approaches have been developed, such as microscopy with fluorescent stains (QBC), dipstick antigen detection of HRPs and pLDH (Parasight-F, ICT Malaria Pf, OptiMAL) (Rickman *et al*, 1989; Beadle *et al*, 1994; Kodisinghe *et al*, 1997; Palmer *et al*, 1998; Iqbal *et al*, 1999; 2000). However, there is still no single technique that can replace microscopic examination in the diagnosis and treatment of malaria patients.

Another approach involves the use of flow cytometry, by which it is now possible to count parasites and evaluate malaria-infected red cells (Jackson *et al*, 1977; Brown *et al*, 1980; Hunter *et al*, 1980; Jacobberger *et al*, 1983; Whaun *et al*, 1983; Vianen *et al*, 1993). Recently, Saito-Ito *et al* (2001) described a rapid, simple and sensitive flow cytometric system for detecting *Plasmodium falciparum* by using a newly developed hemolyzing and staining solution. Their system was

proven to be useful and practical for *in vitro* study of *P. falciparum*, but has not yet been used for the laboratory diagnosis of malaria.

Thus, in the present study, flow cytometric assay, and hemolyzing and staining solutions, were further evaluated for the detection of malaria from patients' blood samples. The results were analyzed by comparison with the Giemsa-stained microscopic examination.

MATERIALS AND METHODS

Study group

The study protocols were approved by the Ethics Committee of the Faculty of Tropical Medicine, Mahidol University. A total of 453 blood samples of malaria patients, other disease patients, and blood donor volunteers, were collected from the Hospital for Tropical Diseases, Faculty of Tropical Medicine, Mahidol University. The numbers and criteria for each group are shown in Table 1.

Specimen collection and handling

One milliliter of 5 mM EDTA blood was collected and used within 24 hours for thick and/or thin blood film, Giemsa-stained microscopic examination (MSC), flow cytometric assay (FCM), and complete blood count (CBC). The CBCs were examined by hematology analyzer (SE-9500, Sysmex, Japan). Red blood cell (RBC) count and white blood cell (WBC) count were used for calculating the levels of parasitemia from the MSC. In addition, the reticulocyte count was examined by automated reticulocyte analyzer (RAM-1, Sysmex Corp, Japan).

Microscopic and parasite-density determination

Thick and thin blood films were stained with 5% Giemsa. All forms of presented malaria parasites were counted against 200 WBCs in thick blood films or the percentage of parasitemia was calculated against 1,000 RBCs in thin blood films. Parasite density (parasites per microliter of blood) was calculated by comparison with actual WBC or RBC count per microliter.

Flow cytometric assay

Flow cytometric assay (FCM) was performed according to the manufacturer's instructions (Sysmex Corp, Japan). Briefly, the lysing solution was prepared by mixing 140 µl of 50 mM polyoxyethylene lauryl ether and 500 µl of 120 mM phosphate buffer (pH 9) in a flow cytometry tube. The tube was incubated at 37ºC prior to mixing with 2 µl of blood sample and incubation at 37ºC. Then, 503 µl of the staining solution (1 g/l of dodecyl methyl ammonium chloride and 3 mg/l of acridine orange in 10 mM tricine and 120 mM NaH_2PO_4 at pH 9) were added and incubated at 37ºC for 15 seconds. After incubation, 700 µl of the stained cell suspension was taken up by flow cytometer (SIF II; prototype, Sysmex Corp, Japan). Forward light scatter (FSC) and side scatter (90º) (SSC) were detected simultaneously with green fluorescence (GF) and red fluorescence (RF). At most, 32767 particles were assessed and plotted in two-dimensional scattergrams of two of these four parameters, GF, RF, FSC and SSC. The parasite areas, ring form, trophozoite, and schizont were detected by analyzing of scattergrams from the computer software provided by Sysmex.

Table 1
Number of specimens and criteria of 4 study groups.

Group	No. of specimens	Criteria	Abbreviation
1	103	Malaria patients whose blood was positive by microscopic examination	MP
2	114	Malaria patients who had been treated with antimalarial drugs for not more than 28 days and whose blood was negative by microscopic examination	NP
3	101	Other disease patients proven free of malaria parasites	ODP
4	135	Blood donor volunteers with no history of malaria exposure	BDV

Table 2
Sensitivities and specificities of flow cytometric assays obtained from ROC curve at various cut-off points.

Positive if greater than or equal to	Sensitivity	Specificity
16.0	1.000	0.000
.	.	.
.	.	.
.	.	.
.	.	.
80.5	0.932	0.687
82.5	0.922	0.710
85.5	0.922	0.761
87.5	0.922	0.784
91.0	0.922	0.810
93.5	0.922	0.834
94.5	0.913	0.834
95.5	0.913	0.849
97.0	0.913	0.854
98.5	0.913	0.860
100.0	**0.913**	**0.863**
102.5	0.903	0.863
104.5	0.893	0.866
105.5	0.893	0.869
107.5	0.893	0.871
113.0	0.883	0.875
116.0	0.883	0.878
121.0	0.864	0.886
131.5	0.845	0.895
138.5	0.816	0.898
160.0	0.816	0.932
.	.	.
.	.	.
.	.	.
25,559.0	0.000	1.000

Sensitivity of detection

To determine detection sensitivity, three individual *in vitro* malaria culture samples were two-fold serially diluted in a 1% suspension of uninfected erythrocytes. The parasitemias of cultures 1, 2, and 3 were determined by microscopic examination as 3%, 4%, and 6%, respectively. The infected material was diluted with blood from an uninfected donor to obtain parasitemias ranging from 0.0117 to 6%. Each serially diluted parasite culture sample was then processed in triplicate for flow cytometric analysis, as previously described. The detection limit for each culture was determined by the number of parasites of the corresponding dilution.

Statistical analysis

The data of total parasite counts by FCM were analyzed and plotted in Receiver Operating Characteristic (ROC) curves (Zweig and Campbell, 1993) to find the parasite count cut-off level. Data that were equal to, or greater than, the cut-off level were considered positive by flow cytometric analysis. The sensitivity, specificity and accuracy of the flow cytometry methods were calculated by using microscopic examination as the gold standard test for detecting malaria parasites.

RESULTS

Detection of malaria by flow cytometry

The dots seen in the parasite area in the two-dimensional scattergrams were analyzed by Sysmex computer software. The program was also set up to have parasite areas to differentiate 3 different stages (ring form, trophozoite, and schizont) of *Plasmodium* parasites, according to the sizes and intensities detected by FCM. A representative example of a two-parameter dot-plot for blood samples is shown in Fig 1. *P. falciparum*-positive samples in Fig 1a mostly contained ring stage, while *P. vivax*-positive samples appeared to have a mixture of 3 stages (Fig 1b). Comparing the scattergrams of the parasite-negative samples in Fig 1c to the parasite-positive samples, only a few non-specific dots were found in the parasite areas. Clusters of white blood cells and other non-specific dots were also detected in this scattergram. Correlations between the parasitemia measured by MSC and the number of parasites measured by FCM, from all 453 blood samples, were determined, as shown in Fig 2. Overall, the number of FCM, counts correlated well with the number of parasites detected by MSC with a correlation coefficient (r^2) of 0.9409.

Sensitivity of detection

In order to examine the sensitivity of detection, two-fold dilutions of *P. falciparum in vitro* culture were made and the FCM counts for each dilution were determined. The threshold of para-

site detection in this study was found to be approximately 0.05-0.1% parasitemia, as shown in Fig 3, when the lines of 3 separate dilution experiments leveled off at these points.

Selection of cut-off point for flow cytometric assay

Cut-off point determination was constructed, to distinguish between positive and negative results, by using the Receiver Operating Characteristics (ROC) curve, which was plotted from the results of all 453 samples. A conventional ROC plot is illustrated in Fig 4. The curve displayed FCM sensitivity and specificity at cut-off values ranging from 16-25559. Table 2 shows the test evaluation results with some different cut-off points selected by ROC. When it was considered that the sensitivity and specificity were equally important, a cut-off point at 100 was selected. With this cut-off point, the diagnostic sensitivity, specificity and accuracy of FCM using the MSC as a standard test were 91.26, 86.28, and 87.42%, respectively.

Application of flow cytometry to clinical blood specimens

In order to compare the efficiency of flow cytometry for detecting malaria parasites, the clinical blood samples were classified as MSC-positive and MSC-negative, as shown in Table 3. The MSC-positive samples were divided into 3 levels, according to the degree of parasitemia. In

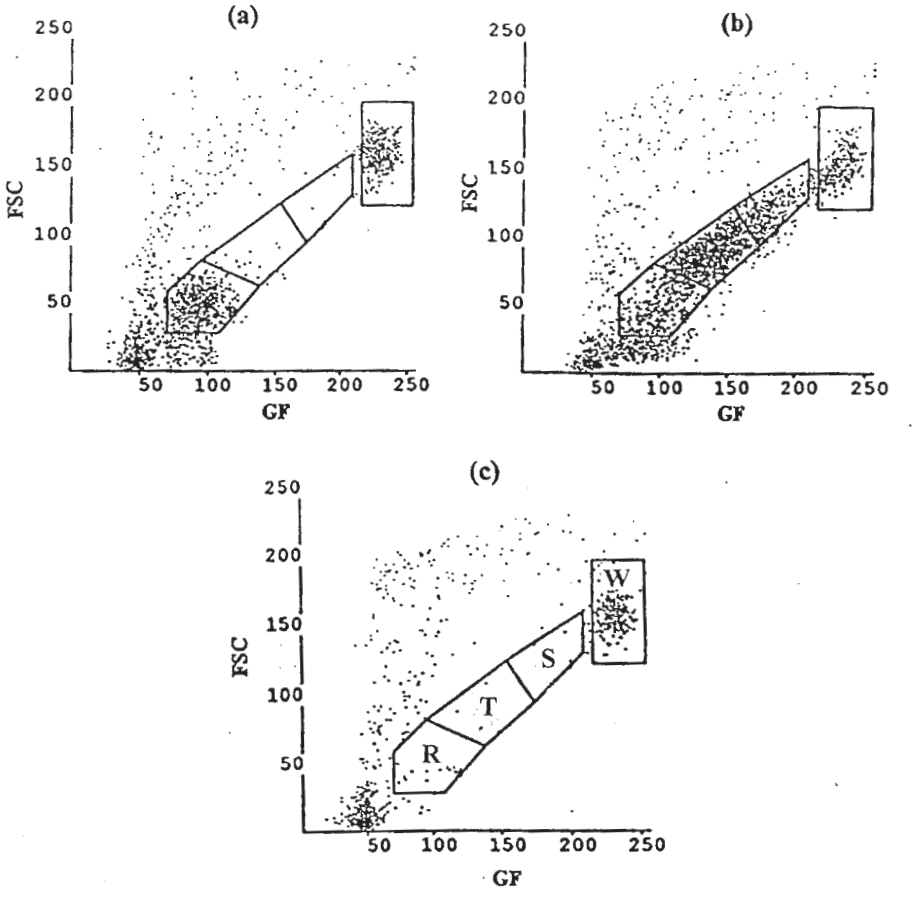

Fig 1–Representation of two-dimensional scattergrams by flow cytometric analysis. (a) Scattergram of a *P. falciparum*-positive sample. (b) Scattergram of a *P. vivax*-positive sample. (c) Scattergram of a malaria-negative sample. The areas in which ring forms (R), late trophozoites (T), schizonts (S) and white blood cells (W) are shown in each scattergram.

FSC = Forward low-angle light scatter; GF = Green fluorescence

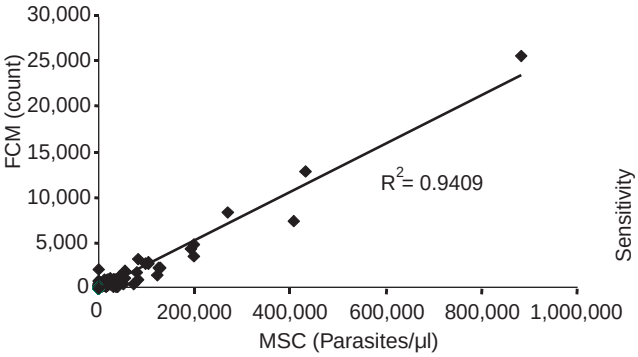

Fig 2–Regression line between the number of parasites counted by microscopic examination and flow cytometric assay from 453 blood samples.

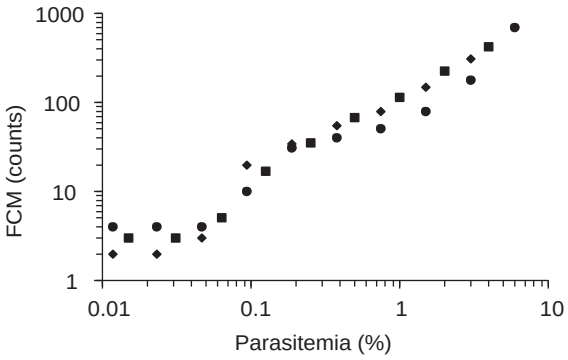

Fig 3–Results from dilution experiments from 3 separated *P. falciparum* cultures (◆ = 3% parasitemia; ■ = 4% parasitemia; ● = 6% parasitemia). Parasites counted by flow cytometry were compared with the parasitemia calculated from the initial parasitemia and the dilution factor.

this study, we found that FCM and MSC corresponded best when the parasitemia exceeded 1,000 parasites/µl; the sensitivities were 100% for both *P. falciparum* (66/66) and *P. vivax* (15/15) infections. When the parasitemia decreased, the sensitivity of FCM decreased as well. At a level of 101-1,000 parasites/µl, the sensitivity for *P. falciparum* detection was 58.33% (7/12), while the two cases of *P. vivax* at this level showed positive FCM results (sensitivity 100%). The FCM results were relatively insensitive, with half of the *P. falciparum* cases (4/8), which had parasitemia of fewer than 101 parasites/µl, not being detected.

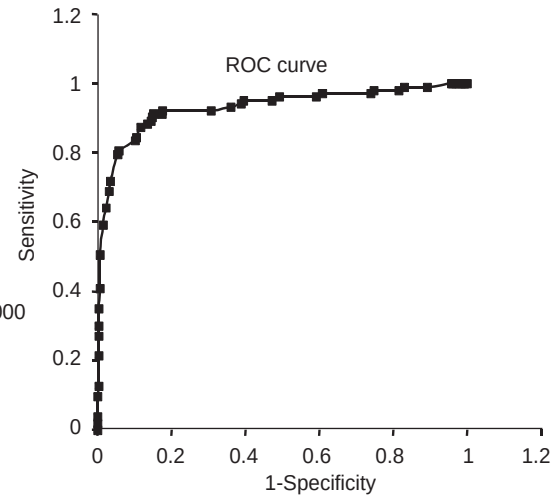

Fig 4–Receiver Operating Characteristic (ROC) curve for flow cytometric assay based upon 453 blood samples. A blood sample was defined as malaria-positive if the flow cytometric count was 100 or greater. The curve displays the sensitivity and specificity of flow cytometric assays at cut-off values ranging from 16-25559.

The MSC-negative samples were comprised of 3 groups. The blood donor volunteers and other disease patient groups gave negative results by FCM, with a specificity of 100%. The third group was malaria-negative patients, which gave an unexpected 48 false-positive results when measured by FCM (specificity 57.89%). Upon hematological data analysis, it was found that all of these 48 samples had high levels of reticulocytes, ranging from 2.0-18.9%, while the normal range was only 0.5-1.5%.

DISCUSSION

The need for improved malaria diagnostics has long been recognized. Local diagnostic needs may vary markedly, and it is unlikely that a single diagnostic test would be ideal for all situations. Recognizing these needs, many flow cytometric assays using different fluorochromes have been tried. Several reports show the potential of this technique in determining *Plasmodium* spp in large numbers of samples. Bianco *et al* (1986) used Hoechst 33258, a DNA-specific fluorescent dye,

Table 3
Sensitivities and specificities of flow cytometric assays between the MSC-positive and MSC-negative groups.

Group		Species	No. of samples	FCM		Sensitivity	Specificity
MSC positive (parasites/μl)	MSC negative			Positive	Negative		
1-100		*P. falciparum*	8	4	4	50%	NA
		P. vivax	0	0	0	NA	NA
101-1,000		*P. falciparum*	12	7	5	58.33%	NA
		P. vivax	2	2	0	100%	NA
>1,000		*P. falciparum*	66	66	0	100%	NA
		P. vivax	15	15	0	100%	NA
	MN	-	114	48	66	NA	57.89%
	BDV	-	135	0	135	NA	100%
	ODP	-	101	0	101	NA	100%

MSC= Microscopic examination; MN= Malaria-negative patients; BDV = Blood donor volunteers; ODP = Other disease patients; NA= Not applicable

to detect the parasites in malaria culture. It appears to have high sensitivity, but it can not differentiate between uninfected RBCs and unstained parasites in low parasite conditions. Other investigators have used acridine orange (Jackson *et al*, 1977; Whaun *et al*, 1983), cyanine dye DiOC1 (Bauer and Dethlefson, 1980), YOYO-1 (Barkan *et al*, 2000), and fluorescein-labeled immunoglobulins (Hunter *et al*, 1980) for parasite detection. However, few studies have reported examples for laboratory diagnosis. Recently, Saito-Ito *et al* (2001) and Sysmex Corporation developed a simple procedure that is quick and suitable for clinical testing, a flow cytometric method to measure the number of parasites using acridine orange with hemolysis, but without centrifugation. Reported sensitivity was sufficiently high, especially for *in vitro* cultured samples. Therefore, we conducted a preliminary study to evaluate their system for detecting malaria in clinical blood specimens.

The sensitivity of detection in this study was determined and it was found that *P. falciparum* parasites were reproducibly detected at a percentage of 0.05-0.1% parasitemia (Fig 3). This sensitivity, however, was not as high as that reported by Saito-Ito *et al* (2001), who found that the sensitivity was very high, at 0.002-0.003% parasitemia. The reason for this is unclear. It may be due to a new set-up of Sysmex's analysis program, in which they tried to reduce the non-specific background caused by platelet interference. This may reduce detection sensitivity, as well. Nevertheless, the result presented here was in agreement with Bianco *et al* (1986) when using Hoechst 33258 dye for the rapid quantification of parasitemia in fixed malaria culture by FCM. They demonstrated that the parasites could be detected with parasitemias of 0.06-0.15%.

In this study, FCM and MSC showed good correlation in measuring the number of parasites, with a regression coefficient of 0.9409. This is one advantage of FCM. It allows for semiquantitative assessment of parasite levels comparable with MSC, which is not easily done with other simple methods. In addition, parasites at different erythrocytic stages appeared as isolated clusters on a scattergram, and thus *P. falciparum* and other malaria species can be distinguished by ring, trophozoite, or schizont stages.

Receiver operating characteristic (ROC) analysis is the standard method to demonstrate the co-variation of sensitivity and specificity (conventionally expressed as 'false-positive fraction' 1-Sp) for systematically changed cut-off values (Sondik, 1982). The suitable point, with balanced sensitivity and specificity based on the data in

Table 2, was determined as 100. This was the best cut-off point for the target population, according to the data from this study. By using the above cut-off point, the FCM assay gave a high sensitivity (91.26%) with 86.28% specificity and 87.42% accuracy.

Our results indicate that the FCM would be useful in clinical diagnosis. The study shows that when parasitemia is greater than 1,000 parasites/μl, the FCM is 100% sensitive. At lower levels of parasitemia, sensitivity decreases, as shown in Table 3. As demonstrated by Vianen *et al* (1993) in their field studies, flow cytometry allows for the reproducible detection of 50 parasites/μl of human blood, which is ~0.001% parasitemia (assuming $5x10^6$ RBC/μl). In comparison with Vianen *et al* (1993), detection of malaria parasites in this study was somewhat less sensitive. The explaination for this may be due to the set-up of the flow cytometric analysis program by Sysmex, as mentioned previously, to different studied populations, or to the small number of samples tested in some levels. Larger trials with more representative samples in each level are needed to establish more reliable sensitivity and specificity.

Studies of blood donor volunteers and other disease patient groups indicate that false-positivity is not a common finding. No false-positive results were found in these two groups. Interestingly, when FCM was used to determine samples from 114 malaria patients who had been treated with various antimalarial drugs for not more than 28 days, and for whom no malaria parasites appeared in thick and/or thin blood films by MSC, an unexpected 48 false-positive samples was observed. An important question is what caused these false-positive results? Platelets or reticulocytes were the prime suspects. Following investigation of hematologic data, we found it unlikely to be platelets but rather caused by nucleic acids from the reticulocytes, which were falsely considered to be nucleic acids from the parasites contaminated in the ring form area. The evidence for this was that all 48 samples had high reticulocyte percentages, ranging from 2.0-18.9%, while normal reticulocyte counts were only 0.5-1.5%. Reticulocyte interference was observed by Laurencet *et al* (1997) and Barkan *et al* (2000). The reason for the high level of reticulocytes released into the peripheral blood may be a compensatory mechanism of the host response to malaria infection.

In conclusion, the FCM described in this study could detect the presence of, and differentiate, plasmodial parasites in whole blood specimens. It could be used as a screening assay, even though the sensitivity of detection was not as high as the thick blood smear technique. The advantage of FCM is that less than 4 minutes per sample are needed to analyze a specimen, including sample preparation time. Thus, it is time-saving when a large number of specimens needs to be analyzed at the same time. Moreover, data are processed in a standardized way, and the data remain available for re-examination. In the near future, when measurement conditions are optimized, together with the application of automated blood cell analysers, this FCM should be very helpful in diagnosing and treating patients with uncomplicated and complicated malaria, in epidemiologic studies, and in field trials of vaccines and new chemotherapeutic agents.

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