Quality development of a reagent kit for enumeration of malarial infected red cells by flow cytometry

Prommalungka K [1)], Saejeng A [2)] and Mundee Y *[1)].

1) Department of Clinical Microscopy, Faculty Associated Medical Sciences, Chiang Mai University 50200.

* Email : myuttana@hotmail.com or Myuttana@netscape.net

2) Vector Born Disease Control Region 10, Chiangmai, Department of Disease Control, Ministry of Public Health.

Abstract

Introduction: Malaria is a transmitted disease that remains one of the public health concerns. The pathogens in human are *Plasmodium falciparum*, *P. vivax*, *P. malariae* and *P. ovale.* The routine diagnostic tests are Giemsa stain on thick and thin blood films together with microscopic examination (ME), which is simple but labor- and time-consuming and depended on the technical skill of the examiners. Flow cytometry (FC) is a new technique, which is labor- and time-saving and subjective. Its sensitivity is higher than ME. In this study, DNA of malaria parasite was stained with fluorescence dyes (thiazole orange, TO; acridine orange, AO and propidium iodide; PI) with and without RNAse. Since these fluorescence dyes can stain both DNA and RNA. Malaria infected erythrocytes then emit green fluorescence of TO, AO and orange fluorescence of PI, while normal erythrocytes do not.

Objective: Study the benefit of RNAse treatment on FC enumeration of malarial infected red cells.

Materials and methods: Twenty two samples from patients with 20 positive and 2 negative on thick blood films were compared with 19 normal samples. All samples were examined on thick and thin blood films and by FC. In FC, samples were fixed with 0.025% (v/v) glutaraldehyde, permeabilized with 0.25 % (v/v) triton X-100, reacted with or without 400 mg/ml RNAse and then stained with either TO, AO or PI.

Result: The percentages of malarial infected red cells (% pos) obtaining from FC using TO with & without, AO with & without and PI with & without RNAse were significantly correlated with the % pos obtaining from ME ($r=0.358$, $p<0.05$; $r=0.497$, $p<0.01$; $r=0.486$, $p<0.01$; $r=0.573$ $p<0.01$; $r=0.317$, $p<0.05$ & $r=0.447$, $p<0.01$, $n=41$). In 20 positive ME patients, the % pos obtaining from FC with and without RNAse were not significantly different. All staining methods gave <0.6 % pos in normal samples. Therefore the % pos of normals were significantly lower than the % pos of the patients ($p<0.01$).

Conclusion: RNAse treatment is not much benefit in FC. However FC is able to replace Giemsa stain because of their good correlation and patient vs normal samples showed significantly different values. Finally, FC is simple, rapid, sensitive and give quantitative results in % malarial infected red cells. Further more the FC method can be improved to be able to count the different stages of malarial parasite.

Key word Malaria, Flow cytometry, RNAse, Thiazole orange, Acridine orange, Propidium iodide.

1. Introduction

Malaria is a Plasmodium protozoan infected disease with Anopheles mosquito as a vector. The primary vectors are *A. minimus*, *A. dirus* and *A. maculatus.* The pathogens in human include *Plasmodium falciparum*, *P. vivax*, *P. malariae* and *P. ovale*, which infect human and the insects alternatively. The conventional method for malarial diagnosis are Giemsa stain on thick and thin blood films accompany with microscopic examination (ME). In this study, we aim to improve the malaria parasite detection. The nucleic acid dyes are used to stain DNA of malaria parasite then detected by flow cytometry (FC). The technique exhibits a higher sensitivity than ME. Flow cytometry can count as high as 50,000 cells within only 2-3 minutes. So it is more rapid than ME. The dyes that are used consisted of thiazole orange (TO), acridine orange (AO) and propidium iodide (PI). Monoclonal anti-glycophorin A (a specific marker of erythroid cell line) is also used to ensure that the analyzed cells be erythrocytes. However the nucleic acid dyes can stain both DNA and RNA. Therefore the RNA within reticulocytes is supposed to interfere the detection system. Using RNAse to eliminate RNA may benefit in reducing the interference. The study also aims to study the benefit of RNAse treatment before flow cytometry.

2. Objective

Study the benefit of RNAse treatment on FC enumeration of malarial infected red cells.

3. Materials and Methods

Blood samples from patients with malarial clinical presentation with positive (n=20) and negative (n=2) thick films were compared with normal blood samples (n=19). The procedure of ME and FC are shown in figure 1 and 2.

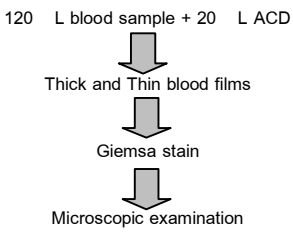

Figure 1 Microscopic examination (ME) procedure

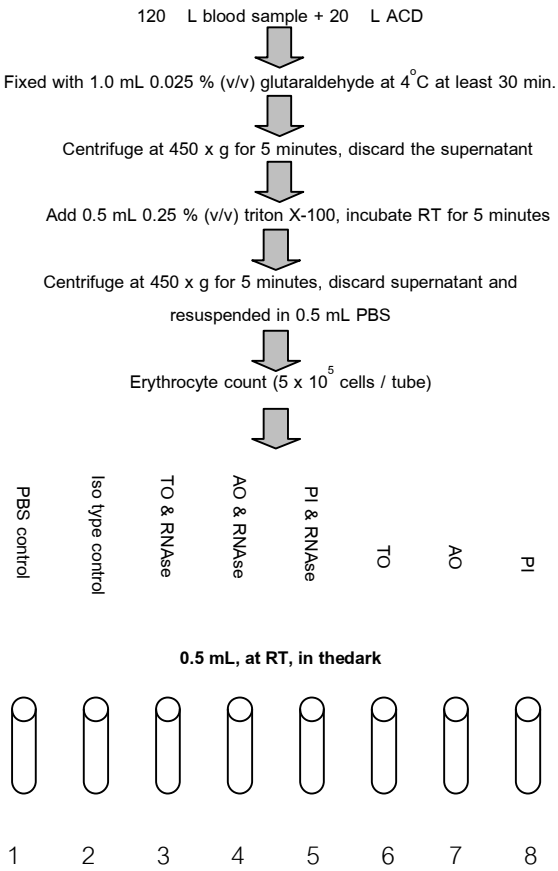

Figure 2 **Flow cytometric (FC) procedure**

Table 1 **Methodology of flow cytometric analysis**

Tube	1	2	3	4	5	6	7	8
RNAse 5 µg/µL (µL)	-	-	1	1	1	-	-	-
RBC (x 10^6 cells)	1	1	1	1	1	1	1	1
Add PBS into every tube upto 25 µL, incubate at RT for 30 minutes, then wash once								
mAb-GPA*RPE (µL)	-	-	1	1	1	1	1	1
Iso RPE(µL)	-	1	-	-	-	-	-	-
Add 0.1 % BSA in PBS into every tube upto 50 µL, incubate at RT for 30 minutes, in the dark box.								
PBS (mL)	0.5	0.5	-	-	-	-	-	-
TO (mL)	-	-	0.5	-	-	0.5	-	-
AO (mL	-	-	-	0.5	-	-	0.5	-
PI (mL)	-	-	-	-	0.5	-	-	0.5

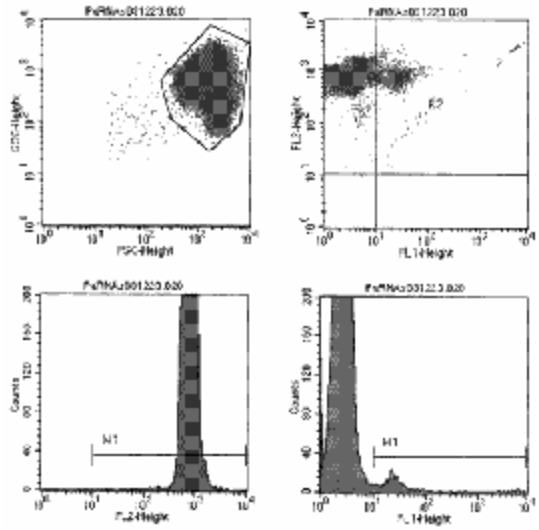

Figure 3 **Flow cytometric histogram of TO without RNAse**

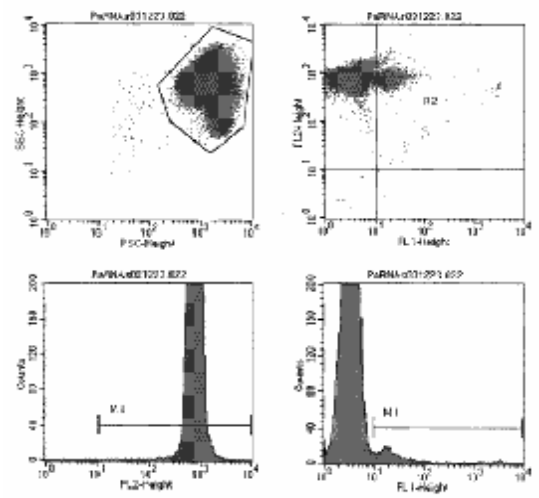

Figure 4 **Flow cytometric histogram of AO without RNAse**

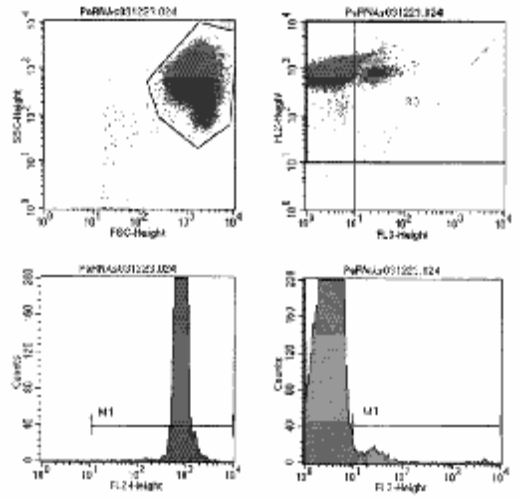

Figure 5 **Flow cytometric histogram of PI without RNAse**

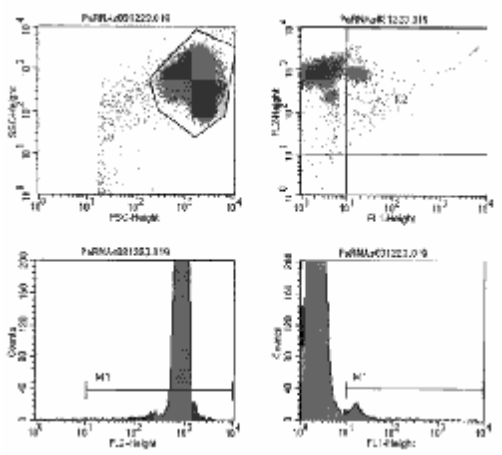

Figure 6 Flow cytometric histogram of TO with RNAse

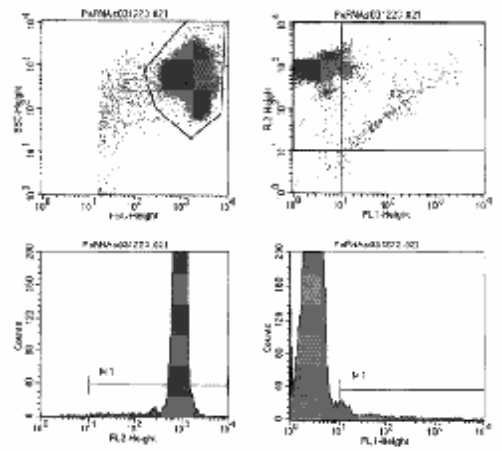

Figure 7 Flow cytometric histogram of AO with RNAse

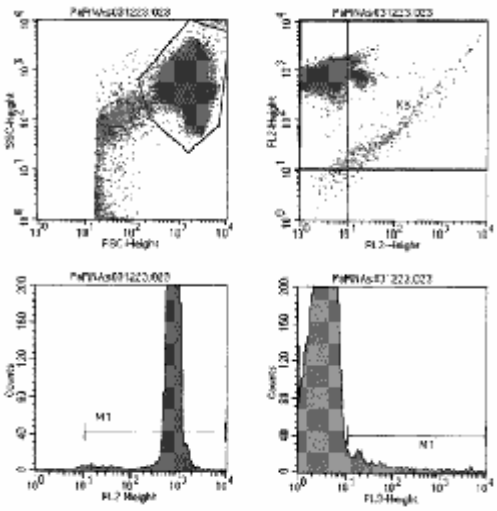

Figure 8 Flow cytometric histogram of PI with RNAse

4. Results

The percentages of malarial infected red cells (% pos) obtaining from FC using TO with & without, AO with & without and PI with & without RNAse were significantly correlated with the % pos obtaining from ME ($r=0.358$, $p<0.05$; $r=0.497$, $p<0.01$; $r=0.486$, $p<0.01$; $r=0.573$ $p<0.01$; $r=0.317$, $p<0.05$ & $r=0.447$, $p<0.01$, $n=41$). In 20 ME positive patients, the % pos obtaining from FC with and without RNAse were not significantly different. All staining methods gave <0.6% pos of normal samples ($n=19$). Therefore the % pos of normal samples were significantly lower than of the % pos of the patients ($p<0.01$).

Table 2 Results of 19 patients with positive ME

Patient	Percentage of malarial infected RBC by FC						Thin film	
	TO & RNAse	TO	AO & RNAse	AO	PI & RNAse	PI	%	Species
29	1.1	1.2	2.5	1.2	1.9	1.3	0.5	P.f.
30	0.4	0.3	1.1	1.3	1.4	0.5	0.5	P.f.
MC23	3.3	1.6	3.2	3.5	1.1	0.5	1.0	P.v.
MC24	1.3	0.9	0.8	0.7	1.0	0.3	0.9	P.v.
MH4	1.4	2.2	3.6	1.9	0.5	1.7	1.0	P.f.
MH5	0.8	0.7	1.3	1.2	1.3	1.3	0.7	P.f.
Pm	1.8	2.1	1.9	2.1	2.7	2.1	2.0	P.m.
151	0.8	1.0	1.7	2.0	0.5	0.9	5.0	P.f.
MH7	0.7	0.3	1.5	1.6	1.0	0.4	0.5	P.f.
MH8	0.7	1.1	1.4	1.7	0.8	1.4	0.8	P.f.
MC2	0.9	0.9	1.2	1.1	0.6	0.5	0.8	P.f.
MC8	0.6	0.5	0.7	0.2	0.5	0.1	0.7	P.v.
MC17	0.7	0.1	0.6	0.8	0.3	0.7	0.1	P.f.
MC19	0.6	1.6	0.1	2.9	0.7	3.0	0.1	P.f.
V1	1.0	1.5	1.1	1.0	0.9	0.7	0.3	P.v.
F1	1.4	1.0	0.6	1.4	0.8	0.1	0.3	P.f.
MC25	0.6	0.7	0.5	0.7	0.3	0.1	0.2	P.f.
MC26	0.4	0.8	0.5	1.1	0.1	0.5	0.2	P.f.
F3	0.4	5.0	0.6	4.6	0.5	3.4	2.0	P.f.
F5	1.7	2.5	1.9	4.4	0.6	1.2	2.0	P.f.

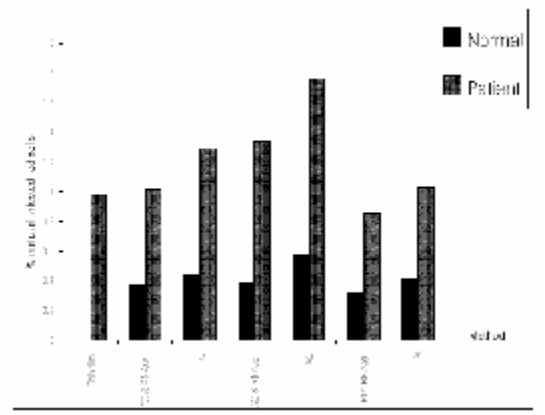

Figure 9 Comparison of all results (mean +/- S.D.)

5. Discussion

Flow cytometric method using only DNA dye (TO, AO, PI) can not give an accurate percentage of malarial infected red cells (% pos). Since DNA dyes can stain both DNA of malarial parasite and RNA in the reticulocytes. This problem can be solved by RNAse treatment before the staining. This is supposed to give more accuracy, because RNA in the reticulocyte was degraded. In 20 patients with positive microscopic examination, the % pos obtaining from flow cytometry with and without RNAse were not significantly different. Since the reticulocyte levels in these patients were quite low. Thus, using RNAse treatment in flow cytometric method does not show any change of % pos. However using DNA dyes to stain the parasite DNA does not restricted to only malaria DNA, but DNA in another stages of erythroid cells e.g. orthrochromatic normoblasts can also be stained. Therefore RNAse treatment can not solve this problem. This may give false positive of % malarial infected red cells. In the next development, monoclonal anti-malarial antibodies that can react to malarial antigens expressed on red cell membrane should be used to improve specificity. The percentage of malarial infected red cells should be more accurate than the one obtained in this study. The DNA staining accompany with hemolysing step followed by flow cytometry shows a promising results of staging differentiation on malarial diagnosis. The future trend of flow cytometric malarial detection is pointed toward the typing and staging of the infected parasite.

6. Conclusion

RNAse treatment is not much benefit in FC. However FC is able to replace Giemsa stain because of their good correlation and patient vs normal samples showed different values. Finally, FC is simple, rapid, sensitive and give quantitative results in % malarial infected red cells. The FC method can be developed further to count the different stages of malarial parasite.

7. Acknowledgement

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