PRACTICAL USES OF ACRIDINE ORANGE FLUORESCENCE MICROSCOPY OF CENTRIFUGED BLOOD (QBC MALARIA TEST™) AND THE QBCII HEMATOLOGY SYSTEM™ IN PATIENTS ATTENDING MALARIA CLINICS IN THAILAND

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Abstract: We evaluated the detection of malaria parasites using acridine orange fluorescence microscopy of centrifuged blood (AOFM/CB or "QBC Malaria Test™") at two government malaria clinics in rural Thailand. In a subgroup of the patients, a QBC Hematology Sytem™ for the determination of complete blood counts was also utilized. A Giemsa-stained thick smear (GTS) reading of 100 (1,000x) microscopic fields was used as standard. The AOFM/CB sensitivities were 97% overall and 95% for *P. falciparum* (Pf). Sensitivity was lower for *P. vivax* (Pv) (76%). Pv sensitivity depended largely on ameboid form density. A threshold for AOFM/CB to consistently detect Pv ameboid forms was estimated to be 10/100 WBC (700/µl blood). AOFM/CB was capable of detecting Pf gametocytes and schizonts more frequently than GTS. The total Pf rings per µl blood estimated from GTS was highly correlated with the number of Pf rings per Paralens™ microscopic field (PMF) suggesting that AOFM/CB could be used quantitatively. From a technical standpoint, the rural tropical settings of Thailand in this study were not an obstacle to the use of QBC Hematology. The system was found to be useful in conjunction with AOFM/CB. However, in patients heavily infected with Pf gametocytes of Pv ameboid forms, their total WBC and lymphocyte counts needed to be appropriately corrected. Overall, AOFM/CB appears to be a promising tool for field diagnosis of malaria if it is affordable to developing countries.

INTRODUCTION

The use of fluorochromes for detection of malaria parasites was first proposed more than 20 years ago (Sodeman, 1970). Only recently acridine orange fluorescent microscopy of centrifuged blood (AOFM/CB) or "QBC Malaria Test™" developed in conjunction with the quantitative buffy coat (QBC) analysis for complete blood count (Wardlaw and Levine, 1983) became available. This malaria diagnostic technique has received considerable attention over the past few years. AOFM/CB is useful in medical clinics where there is no experienced microscopist to read blood films, because identification of *Plasmodium,* especially its ring form, is very easy and requires little training and skill (Spielman *et al,* 1988). The technique may also be useful for large-scale epidemiologic surveys (Wongsrichanalai *et al,* 1991).

Accuracy of AOFM/CB has been reported from populations with various characteristics of malaria infection and different levels of parasite density (Spielman *et al,* 1988, Rickman *et al,* 1989, Wongsrichanalai *et al,* 1991, Pornsilapatip *et al,* 1990). As a practical application, its accuracy in patients attending malaria clinics in Thailand, who might benefit from this technique in the future, has not been reported. In this study, we evaluated the sensitivity and specificity of AOFM/CB in Thai and Myanmar patients at two government malaria clinics in rural areas of Thailand using Giemsa-stained thick smear (GTS) as standard. The use of AOFM/CB and QBCII Hematology System™ (for determination of complete blood count) at that level of rural health setting is discussed in terms of utility and practical application.

MATERIALS AND METHODS

The study was conducted between March and June 1991 at the district malaria clinics in Borai (southeastern Thailand) and Mae Sot (northeastern Thailand). For each patient, 55 µl blood was drawn into a "QBC™ malaria tube" following a single finger prick for the routine preparation of thick smears-required by the malaria clinic. An additional small amount of blood was used to prepare a thick and a thin smear (on the same slide) for reference. About 30-40 patients were recruited into the study per day. Severely ill patients and children under 10 years old were excluded.

Each QBC™ malaria tube was immediately tilted and rolled between fingers to mix the contained blood with the precoated anticoagulants and acridine orange. The tube was then placed upright of a rack at room temperature, which varied from 40°C around noon time in March to 26°C on a rainy day in June. About 1-2 hour after collection, a batch of 20 tubes was centrifuged at 12,000 rpm (with a centrifugal force of 14,387 *g*) for 5 minutes with a battery-operated portable QBC centrifuge ("Parafuge™"). Centrifugation results in a blood specimen being separated into red cell column, buffy coat and plasma. In the buffy coat, a granulocyte layer is located just above the red cell column, a lymphocyte layer in the middle and a platelet layer just under the plasma. The tubes were returned to the rack, which was then placed in a cool thermos until examined. Examination was made 3-6 hours after collection by one AOFM/CB reader (CW). We used Paralens™, which is a 60x fluorescent-adaptor objective attachable to a regular light microscope. Each tube was examined for 15 Paralens microscopic fields (PMF), 10 of which were in the red blood cell column not to exceed the width of five fields below the buffy coat (to search for ring-form parasites) and five of which were in the lymphocyte layer (to search for trophozoites, schizonts and gametocytes). The average number of ring-form parasites per PMF was recorded. If there were 150 or more parasites per PMF, this was recorded as 150/PMF. Other forms of parasites were graded as "few," "many," or "abundant." The "lymphocyte layer" (as recognized by QBC Hematology) of specimens heavily infected with Pf gametocytes or Pv contains, in fact, lymphocytes plus the infected red blood cells. We also estimated the percentage of the area of this layer that was taken up by the parasite-infected cells during AOFM/CB examination.

A subgroup of 79 Myanmar patients attending Mae Sot Malaria Clinic were asked to donate an additional 55 µl blood for complete blood count by quantitative buffy coat analysis (Wardlaw and Levine, 1983). Blood was drawn into a QBC™ hematology tube. Unlike the QBC™ malaria tube, the hematology tube is precoated with a monoclonal antibody specific for a ubiquitous antigen on the red blood cell membrane in addition to acridine orange and anticoagulants. This makes the red cells sediment according to their mean density. Specimens were processed in the same way as those for AOFM/CB. They were read with QBCII Hematology System™ about 3-4 hours later. The system reports eight hematology values: hematocrit, hemoglobin, total white blood cell (WBC) per µl blood, percent and total granulocytes, percent and total lymphocytes + monocytes (combined), and total platelets.

Blood films were stained and transported to AFRIMS laboratory in Bangkok where they were read by an experienced microscopist blinded to the AOFM/CB results. Each thick smear was read for 100 microscopic fields (1,000x magnifying power) before declaring it as negative. When species differentiation was questionable, the matched thin smear was carefully examined. These results will be referred to as AFRIMS GTS standard. In a portion of the specimens, malaria clinic thick smear results were copied and saved in our database for a comparative analysis. Generally, the maximum number of microscopic fields read by malaria clinic microscopists before declaring a thick smear as negative was also 100.

RESULTS

AOFM/CB performance compared to microscopy standards

There were a total of 317 specimens. Parallel malaria clinic diagnoses were available in 199 of them (Table 1). Using malaria clinic results as standard, the overall AOFM/CB sensitivity was 96.3% (68 + 2 + 3 + 31/73 + 35 or 104/108). As expected, the sensitivity was higher for Pf (93.2%, 68/73) than for Pv (88.6%, 31/35). Speci-

ficity was 80.2% (73/91). Except where indicated, all the other analyses were done in reference to AFRIMS GTS standard.

Using AFRIMS GTS standard (Table 2), the overall sensitivity was 96.8% (117 + 1 + 12 + 47 + 1 + 3 + 1/123 + 61 + 4 or 182/188). Sensitivity was 95.1% (117/123) for Pf but only 75.8% (47/61) for Pv. Twelve of the 61 Pv specimens were misdiagnosed. Specificity was 79.8% (103/129). There were 26 specimens positive by AOFM/CB but negative by thick smear ("false positive") (Table 3), 23 were AOFM/CB positive for Pf and 3 for Pv.

Table 1

AOFM/CB sensitivity and specificity for *P. falciparum* (Pf) and *P. vivax* (Pv) based on malaria clinic Giemsa-stained thick smear (GTS) standard*.

		Malaria Clinic GTS			
		Neg	Pf	Pv	
AOFM/CB	Neg	73	3	1	
	Pf	13	68 (93.2%)	3	
	Pv	5	2	31 (88.6%)	
		91	73	35	199

* Examination of 100 microscopic fields (1,000x) of thick smears by malaria clinic microscopists.

Sensitivity of AOFM/CB for Pv

Results were generally similar whether AFRIMS GTS or malaria clinic standard was used. However, AOFM/CB sensitivity for Pv was higher when using the malaria clinic as opposed to AFRIMS blood film results as standard (88.6% vs 75.8%). This was due to misclassification of Pv as Pf by malaria clinic microscopists. Since AOFM/CB also tended to misclassify Pv cases in the same direction, an erroneously high sensitivity was obtained. As a routine procedure for malaria clinics, no thin smear is prepared and diagnosis is based solely on a thick smear. When only ring-form parasites were detected, there was a tendency to report the case as Pf.

Table 2

AOFM/CB sensitivity and specificity for *P. falciparum* (Pf) and *P. vivax* (Pv) based on AFRIMS Giemsa-stained thick smear (GTS) standard*.

		AFRIMS GTS				
		Neg	Pf	Pv	Mixed	
AOFM/CB	Neg	103 (79.8%)	5	1	-	
	Pf	23 (17.8%)	117 (95.1%)	12 (19.7%)	3	
	Pv	3 (2.3%)	1	47 (75.8%)	1	
	Mixed	-	-	1	-	
		129	123	61	4	317

* Examination of 100 microscopic fields (1,000x) of thick smears by experienced AFRIMS microscopists. If species differentiation is questionable the corresponding thin smear is examined.

In the 12 Pv cases diagnosed as Pf by AOFM/CB, none showed an ameboid form in the tube. In all cases, ameboid forms were found in the corresponding thick smears with densities of $<10/100$ WBC ($<700/\mu l$ blood).

AOFM/CB detection of Pf gametocytes

A systematic search of five PMF in the lymphocyte layer on each QBC tube in this study revealed that AOFM/CB was capable of detecting the sexual stage of Pf malaria more frequently than the conventional thick smear. There were 52 specimens positive for Pf gametocytes by either or both of the two techniques, half (26) of which were detected by AOFM/CB alone (Table 4).

AOFM/CB detection of Pf schizonts

Schizonts of Pf are rarely observed on blood films of outpatient malaria cases in Thailand. In this study, AOFM/CB detected 8 cases with Pf

Table 3

Results of Giemsa-stained thick smear (GTS) review of "false positive" specimens by AOFM/CB.

"False positivity"	N	GTS review		
		Confirmed Pf	Confirmed Pv	False positive?
GTA -ve, AOFM/CB + ve for Pf	23	3	2	18
GTS -ve, AOFM/CB + ve for Pv	3	2	1	-

Table 4

Comparative diagnosis of *P. falciparum* (Pf) gametocytes by AOFM/CB and Giemsa-stained thick smear (GTS).

Total	Negative for Pf gametocytes	Positive for Pf gametocytes by			
		Total	AOFM/CB only	GTS only	Both
317	265	52	26 (50%)	7 (13%)	19 (37%

schizonts while thick smear detected only one of them. Similar to Pf gametocytes, false positivity by AOFM/CB was unlikely for schizonts. However, it was not always possible to distinguish a schizont of Pf from that of Pv. As a general rule, nuclei of Pf schizonts appeared more tightly packed than those of Pv. In a Pv infection, schizonts were often numerous and usually accompanied by trophozoites. Pf schizonts were almost always scarce in number and Pf trophozoites were extremely rare.

Density of Pf ring-forms determined by AOFM/CB

The total ring-form parasites per µl blood was calculated from the following formula:

Total Pf rings per µl blood
= (no. of Pf rings per 100 WBC on thick film/100) x total WBC in µl blood.

There were 36 Pf cases for which total WBC (per µl blood) estimated by QBC Hematology was available. A plot of the calculated Pf rings per µl blood against the average number of Pf rings per PMF in the area just below the buffy coat (Fig 4) showed the relationship to be linear with a correlation coefficient (r) of 0.89. Pf ring-form density on blood smears of $\geqslant$ 101,000/100 WBC was set to 101,000/100 WBC and average Pf rings/PMF of $\geqslant$ 150 was set to 150. A regression analysis showed the following relationship:

$$\text{Density} = 550.5 * \text{QPFR} - 3733.4,$$

Where "density" = Pf rings/µl blood and "QPFR" = average number of Pf rings/PMF.

Performance of QBC Hematology under malaria clinic conditions

Repeated readings of QBC Hematology specimens showed that hematology values remained consistent over 12 hours after collection. Among the 79 specimens for which QBC Hematology was performed, acceptable results were obtained in 75 of them. We were unable to read the other 4 speci-

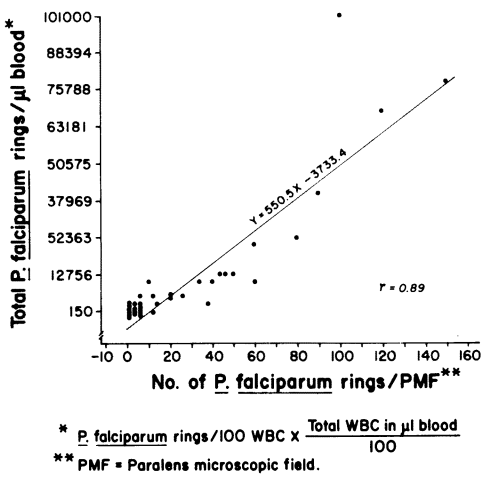

Fig 1—Plot of the total Pf rings per µl blood (calculated as the number of Pf rings/100 WBC on a thin smear and the total WBC/µl blood obtained from QBCII Hematology System™) against the number of Pf rings per Paralens microscopic field (PMF). Pf rings/µl blood of ⩾ 101,000 was set to 101,000 and Pf rings/PMF of ⩾ 150 was set to 150.

mens because of poor band formation or absence of a granulocyte band. All of them had very low hematocrits. Data for the 75 subjects revealed a mean hematocrit of 36.4 (± 7.0 SD) and a mean hemoglobin of 11.7 gm% (± 2.5 SD). Heavy infection with Pf gametocytes or Pv was a problem in QBC Hematology reading. Eighteen of the 75 specimens fell into this category. Among the remaining 57 specimens, the mean total WBC was 8,169/µl blood and the mean total platelets was 186,410/µl blood. The mean proportions of granulocytes and lymphocytes were 60% and 40%, respectively.

In the 18 individuals heavily infected with Pf gametocytes or Pv, microscopic examination of the lymphocyte layer with the Paralens™ showed abundant Pf gametocytes, Pv trophozoites or Pv schizonts. The infected cells either formed an isolated band between granulocytes and lymphocytes or were interspersed amongst the lymphocytes. From our experience, when these cells accounted for 20% or more (heavily infected) of what was recognized as the "lymphocyte" layer by QBC Hematology, the total WBC and total (and %)

Box 1

Example of how to correct lymphocyte and total WBC values obtained from QBCII Hematology System™ in specimens heavily infected with Pf gametocytes or Pv ameboid forms.

Specimen No. 249 (Mae Sot, 26 June 1991)

AOFM/CB result:

PF with abundant gametocytes, taking up about 70% of what was recognized as the lymphocyte layer by QBC Hematology.

QBC Hematology reading:

Total WBC	6,100/µl blood
Granulocytes	2,400/µl blood (39%)
Lymphocytes and monocytes	3,700/µl blood (61%)

Thin smear result:

Granulocyte: Lymphocyte ratio	125 : 100

By interpolation, the number of lymphocytes should be corrected to: (2,400 × 4)/5 = 1,900/µl blood,
and the total WBC to: (2,400 + 1,900) = 4,300/µl blood.

lymphocyte counts reported by QBC Hematology needed to be corrected. Box 1 demonstrates how to correct these values.

DISCUSSION

In previous studies, sensitivity of AOFM/CB was found to increase with levels of parasite density, being highest in hospital patients suspected of having malaria (Rickman *et al,* 1989, Pornsilapatip *et al,* 1990) and lowest in asymptomatic individuals from an endemic population (Wongsrichana lai *et al,* 1991). For species-specific diagnosis, sensitivity was higher for Pf than for Pv (Spielman *et al,* 1988; Rickman *et al,* 1989; Pornsilapatip *et al,* 1990; Wongsrichanalai *et al,* 1991).

In this study, we evaluated the practical use of AOFM/CB at two malaria clinics in rural Thailand. We determined the test accuracy in patients attending the malaria clinics and further explored other aspects of the technique such as the threshold parasite density for AOFM/CB at which Pv could be consistently detected and the relationship between Pf ring-form densities as determined by AOFM/CB and blood smear.

It was evident in a tropical setting that when specimens were not read right away and an air-conditioned environment was not available, optimal results for AOFM/C B and QBC Hematology could still be achieved by storing the specimens in a cool container. This was important especially for specimens collected for QBC Hematology because, in a tropical area like rural Thailand, blurred boundaries of cell layers and thus inaccurate test values, could develop within a few hours of collection if specimens were left at room temperature.

The overall sensitivity of AOFM/CB observed in this study was high. Specificity was more difficult to interpret. specificity of 80% was satisfactory and the inability to attain a higher level could be partly explained by the fact that a thick smear reading of 100 microscopic fields was not an ideal standard. It was evident that AOFM/CB was capable of detecting more malaria cases than thick smear in this study. Several cases with low-density parasitemia that were missed by thick smear were apparently picked up by AOFM/CB. These cases were nonetheless considered "false positive" by AOFM/CB according to our analysis. We did not read more than 100 fields or try to make a more rigorous search for parasites in the AFRIMS GTS standard. Rather, our intent was to mimic as much as possible the actual blood film reading criteria practised at the malaria clinics while obtaining results that were reliable and not affected by work overload and time constraints encountered at malaria clinics.

After reviewing the blood films, diagnosis of malaria was confirmed in 8 of the 26 originally thick smear-negative and AOFM/CB positive specimens ("false positives"). Based on our three-year experience with AOFM/CB, we think that about half of these 26 specimens were probably true cases of malaria. It would have been useful to perform polymerase chain reaction (PCR) or malaria culture to help confirming the AOFM/CB findings in this study.

Our results were less sensitive for Pv than for Pf and implied that Pv sensitivity depended largely on its ameboid-form density. The data suggested that a threshold for AOFM/CB to consistently detect ameboid forms of Pv was about 10/100 WBC (700/μl blood). We suspected that a lot of the ameboid forms were located in the granulocyte layer and were difficult to detect because of the glaring background. Those that floated into the lymphocyte layer were easier to detect because of the homogeneous light-green background. When no ameboid form was seen, detection of Pv became difficult. Although, ring forms of Pv are generally larger than those of Pf, it was not always possible to distinguish Pv from Pf based on ring forms alone. Since red blood cells are not visible in AOFM/CB, we cannot use morphology of the infected cells to support species differentiation such as is possible with the thin blood smear.

So far there have been limited data on AOFM/CB sensitivity for Pf gametocytes. Low sensitivities were reported by earlier studies including ours (Wongsrichanalai *et al,* 1991), but all were based on small sample size. In this study AOFM/CB detected Pf gametocytes more frequently than thick smear. False positivity by AOFM/CB is unlikely because acridine orange-stained gametocytes appear very unique in their shape, size and characteristics. For an experienced AOFM/CB microscopist, these characteristics are evident even when the parasitized cells are located

in the fluorescent lymphocyte layer. The number of specimens positive for Pf gametocyte by AOFM/CB was higher than by thick smear.Compared to malaria clinic results, AOFM/CB picked 60% more cases with Pf gametocytes (16/17 vs 10/17). The higher capability of AOFM/CB to detect Pf gametocytes in this study suggested that this technique may be a useful malariometric tool for malaria control.

The high correlation between the number of Pf rings per PMF and the total ring forms per µl blood suggests that AOFM/CB may be used quantitatively. Based on our data, successful quantitation was confined to the ring stage of Pf and to parasite densities under 101,000 per µl blood. In spite of these limitations, AOFM/CB may be quite useful in severity grading of falciparum malaria, the most serious form of human malarias, and may serve as a prognostic indicator in the treatment of falciparum malaria patients.

The ability to detect Pf schizonts more frequently than thick films appears to be an advantage of AOFM/CB, in which the infected cells are concentrated. It is possible that some schizonts are lost during the Giemsa staining process (Dowling and Shute, 1966; Trape, 1985). Detection of schizonts in severe malaria may prove useful in monitoring antimalarial drug effects and in the assessment of severity of infection. This requires further evaluation.

This study showed that the QBCII Hematology System^{TM} worked well in a tropical setting provided that optimal environmental conditions were created and maintained. There were, however, limitations when applied to malaria patients. Prior observations indicated the percentages of unreadable QBC tubes for clinical outpatients and hospital patients varied from 1.5 to 10 depending on the pathology of the patient populations (Operator's Manual 1987, Becton Dickinson Co). In this study with an anemic malaria endemic population, 5.1% (4/79) of the QBC tubes were unreadable. Moreover, for the readable tubes of malaria patients, there was a need to correct for total WBC and lymphocyte counts if Pf gametocytes or Pv involoved 20% or more of the "lymphocyte" layer. The effect of floating parasitized erythrocytes in the buffy coat on hematocrit values should be minimal or negligible because the portion of red blood cells around the float is only about 0.1% (Becton Dickinson Co, unpublished data) of the total red cell volume.

In spite of the problems and limitations, AOFM/CB appears to be a promising tool for field diagnosis of malaria provided it is affordable to developing countries. The market price of the QBC Malaria System^{TM} in Thailand is about four times that of a regular light microscope. The QBC^{TM} capillary tubes have been estimated to cost more than five times the price of stained blood slides (Anonymous, 1992). AOFM/CB is the only fluorescence microscopic technique based on the principle of sedimentation gradient of cells. It has the advantages of quick and easy preparation, concentration of the parasitized cells, prominent staining of the parasites and easy detection at a magnifying power much lower than that used for Giemsa-stained blood films. In this study, we observed AOFM/CB in a practical field application to have advantages that include high overall sensitivity, ability to detect Pf gametocytes and schizonts more frequently than the routine thick smear and, when used together with a QBC Hematology System^{TM}, ability to estimate Pf ring-form density. Although, the technique detected several cases of malaria which were negative by thick smear (100 microscopic fields), it is still not known why some cases were missed. We suspect that a large enough number of parasitized cells have to be present in the tube for AOFM/CB to be able to consistently detect them because a certain portion of the infected cells must have floated into buffy coat and therefore escaped detection. Improvement of the technique either by creating a gradient between the lighter infected cells and WBC or by differential staining of these cells would increase its accuracy.

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