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Original Article

Feasibility and Limitations of Acridine Orange Fluorescence Technique Using a Malaria Diagnosis Microscope in Myanmar

Ye Htut[a], Kyin Hla Aye[a], Kay Thwe Han[a], Myat Phone Kyaw[a], Kunio Shimono[b], and Shigeru Okada[c*]

[a]*Parasitology Research Division, Department of Medical Research (Lower Myanmar), No. 5, Ziwaka Road, Yangon 11191, Myanmar,* [b]*Internal Medicine, Shimono Hospital, Okayama 705-0035, Japan, and* [c]*Department of Pathological Research, Okayama University Graduate School of Medicine and Dentistry, Okayama 700-8558, Japan*

We studied parasite detectability in thick films by an acridine orange fluorescence technique (AO) to test its applicability and the use of a Malaria Diagnosis Microscope (MDM)-ESL in the detection of parasites, compared to the conventional Giemsa staining method. This study was conducted on 1,390 clinically suspected malaria cases of Thaton township, Myanmar. We found sensitivities of 82.8% for *Plasmodium falciparum* (*P. falciparum*) and 100% for *Plasmodium vivax* (*P. vivax*) and specificities of 97.1% for *P. falciparum* and 98.6% for *P. vivax*. AO had a higher sensitivity than Giemsa-stained films at low levels of parasitemia (< 1,000/μl). AO showed lower sensitivity and higher specificity than the Giemsa method at parasite levels of more than 1,000/μl. The results of using the AO method, achieved by both novice and experienced observers, showed no significant difference and required less practice to perform the test as well as to identify the parasite. The acridine orange fluorescence technique using a malaria diagnosis microscope MDM-ESL series is simple, rapid and cost effective. The microscope is conveniently operable using standard AC power or a 12-V DC car battery, and it is easily convertible to a conventional biological microscope. With the exception of species differentiation, which is not possible with this method, this method would be appropriate for both clinical and epidemiological studies.

Key Words: Malaria Diagnosis, acridine orange, fluorescence, *Plasmodium*

A prompt and accurate diagnosis is the key to effective disease management of malaria. In both clinical and epidemiological studies, diagnosis of malaria infection conventionally depends on the microscopical examination of blood films stained with Giemsa or the Field rapid stain method. Diagnosis accuracy depends on the experience of the observer, the quality of the blood smear prepared and the time given for the examination. A simple and rapid method with high sensitivity is needed that could be used in all areas where malaria is a problem. Recent techniques using DNA or RNA probes have high sensitivity and high specificity, and have been tried for the rapid detection of malaria parasites especially of low level, but high cost and difficulty of application in the field are major obstacles to their use. One of the promising methods appears to be fluorochrome staining with acridine orange (AO) dye [1, 2]. With this method, there is a differential staining of nuclear DNA (yellow-green) and cytoplasm (red), which reveals the clear identification of the malaria parasite, but the need for an expensive

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*Corresponding author. Phone: +81-86-235-7141; Fax: +81-86-235-7148
E-mail: okadas@cc.okayama-u.ac.jp (S. Okada)

fluorescence microscope has become its major drawback. With the advancement of appropriate technology, fluorescence microscopy using a standard light microscope with interference and barrier filters was developed to detect malaria parasites in thick and thin blood films using acridine orange staining. This method allows the use of low magnification and permits rapid scanning of large areas. In this study, we used a Malaria Diagnostic Microscope (model MDM-ESL), a product of the Gakken ELIZA Company, Japan. It is equipped with a combination of two barrier filters and a 100-watt halogen lamp, such that used in a projector.

The objective of this study was to determine the parasite detectability in thick films by the acridine orange fluorescence technique (AO), to test its applicability and the use of the MDM-ESL in the detection of parasites, compared to the conventional Giemsa staining method. Tests were evaluated for sensitivity, specificity, accuracy of parasite estimates, time requirement and experience needed.

Materials and Methods

This study was conducted on 1,390 clinically suspected malaria cases of Thaton township, a malaria endemic area, located 3,600 km from Yangon, Myanmar. Five replicate thick blood films were prepared from each subject. The first slides of every case were stained with Giemsa and checked for malaria parasites by a newly trained grade 3 technician (novice) in the field. Initial Giemsa thick film (GTF) results were confirmed on-site by the experienced technician of the Parasitology Research Division. The other 4 slides were kept dry and transferred to the Department of Medical Research (Lower Myanmar). Examinations of parasites were done within one week by staining the second slide with Giemsa and the third with acridine orange. The remaining 2 slides were examined after one month, using Giemsa or acridine orange solution as before.

The diagnostic test using acridine orange-stained thick film (AOTF) observed under the MDM-ESL was measured against a GTF standard.

Giemsa Staining Method. GTF were stained with 5% Giemsa for 20 min and examined using a standard binocular Olympus BH-2 light microscope under 1000 X magnification.

Acridine Orange Technique. AOTF were stained by placing a small amount of AO solution in a single strip down the center of the cover slip (18 x 18 or 24 x 24 mm) that was laid on a filter paper or a paper towel. Holding the thick blood-smeared slide at both ends with the smear facing downwards, the technician pressed the slide gently against the cover slip with AO stain. Any excess stain squeezed out was adsorbed by the underlying filter paper or paper towel. The blood smear was examined immediately under an MDM-ESL microscope starting from low magnification 200 X, where parasites could be easily spotted. The parasites were best identified under 600 X magnification.

Assessment of Parasitemia. The GTF and AOTF films were scored by counting the number of parasites per 100 white blood cells (WBCs). If no parasite was seen after counting 100 WBCs, examination was continued until we had examined 100 fields. The parasite densities were per microliter.

Any specimens that were expressed as parasite negative by GTF but positive by the AOTF method were re-examined by GTF up to 600 fields. The initial 100-field GTF results examined by the grade 3 technician, the results of the experienced technician and the results of the extended GTF examination were combined and used as the standard. We calculated the sensitivity (true positives/total number of parasite present) and specificity (true negatives/total number of parasite absent).

Results

The sensitivities and specificities of the AOTF method are compared with GTF as the 'gold standard' in Table 1 by species, and discrepancies are shown in Table 2 (A)-(C). We found sensitivities of 82.8% for *Plasmodium falciparum* (*P. falciparum*) and 100% for *Plasmodium vivax* (*P. vivax*) and specificities of 97.1% for *P. falciparum* and 98.6% for *P. vivax*.

AOTF had a higher sensitivity at lower levels of parasitemia than at higher levels of parasitemia (Table 3).

Table 1 Sensitivity and specificity of acridine orange fluorescence-stained thick blood films compared with standard Giemsa-stained thick blood film technique according to species

	P. falciparum	*P. vivax*	Mixed infection
Sensitivity (%)	82.8	100	50.0
Specificity (%)	97.1	98.6	100
Positive predictive value (%)	96.4	75.0	100
Negative predictive value (%)	86.1	100	98.6

Table 2 Discrepancies between acridine orange stain and Giemsa stain methods in the diagnosis of malaria

(A)

P. falciparum			
Giemsa result	Acridine orange result		Total
	Positive	Negative	
Positive	530	20	550
Negative	110	680	790
Total	640	700	1340

(B)

P. vivax			
Giemsa result	Acridine orange result		Total
	Positive	Negative	
Positive	30	10	40
Negative	0	680	680
Total	30	690	720

(C)

Mixed infection			
Giemsa result	Acridine orange result		Total
	Positive	Negative	
Positive	10	0	10
Negative	10	680	690
Total	20	680	700

AOTF showed only 62.5% and 72.7% sensitivity at the parasitemia levels of 1,000-10,000/μl and above 10,000/μl, respectively. The higher the parasitemia level, the greater the specificity observed.

Time factors. The total time taken by the AO method was 7 min, which included 2 min of staining time and 5 min for slide examination. The Giemsa staining method took 15 min to prepare, when samples were processed in batches. Regardless of whether the result was negative or positive, it took about 10 min to declare a specimen negative by microscopy. Total handling time, calculated as the preparation time per specimen plus the time it took to declare a specimen negative, decreased for all techniques as specimens were processed in larger batches. The total handling time for the AOTF technique was therefore lower, whether specimens were processed singly or in batches.

Table 3 Sensitivity and specificity of acridine orange fluorescence staining according to malaria parasitemia, compared with standard Giemsa-stained thick blood film technique

Parasite/μl	No. of samples	Sensitivity (%)	Specificity (%)
$<$ 100	600	89.3	96.9
100–500	270	83.3	93.3
500–1000	110	100	100
1000–10000	160	62.5	100
$>$ 10000	200	72.7	100

Table 4 Comparison of two diagnostic techniques for *P. falciparum* infection; proportion of replicates recorded as positive at decreasing parasite densities

Parasite/μl	No. replicates	Observer			
		Experienced		Novice	
		GTF	AOTF	GTF	AOTF
$>$ 10000	4	1	1	1	1
1000–10000	4	1	1	1	1
500–1000	4	1	1	1	1
100–500	4	1	1	0.75	1
$<$ 100	4	1	1	0.25	0.5

Practice requirement. The 100% sensitivity cut-off points for the inexperienced technician (novice) were higher than those for the experienced technician (Table 4), and the novice recorded 80 false positive results among the 680 uninfected controls. The microscopic examination times were also affected by the experience of the microscopist. The novice took 15 min to declare a specimen negative with GTF and 10 min to do so with AOTF, whereas the experienced microscopist took 10 min with GTF and 3 min with AOTF.

Discussion

The AO staining method was found to be more sensitive under lower magnification (600X) than the Giemsa staining method (1000X), when slides were observed by the novice (Table 4).

There were cases of mixed infection (*P. falciparum* and *P. vivax*) in GTF as read by the experienced technician that were difficult to identify in the AO slide, which accounted for the sensitivity levels of 62.5% and 72.7%

at the parasitemia levels of 1,000-10,000 and above 10,000, respectively (Table 3). The results demonstrated the higher specificity and greater rapidity of AO when compared to Giemsa-stain films, confirming the results of other studies [3, 4]. However, careful preparation of the acridine orange solution is essential, because either under-stained or over-stained specimens will produce poor results. Once AO stain is prepared, the smear should be freshly examined or the slide must be kept in a moist chamber or box to prevent it from drying up. Apart from this, one advantage observed from the study was that the parasites were still detectable even if the glass slides were kept unstained up to one month and then examined using the AO technique, and the results were not significantly different from those of slides stained and examined within one week.

The technique necessitates a fluorescence system, which can pose logistic problems for field investigations. That was why Kawamoto [1] proposed the use of daylight-illuminated microscopes fitted with interference filters, which could have significant advantages for field use, except that they do not allow for species differentiation. [5]. The AO technique is not, however, satisfactory for specific diagnosis, as the precise morphology of the red cell is not clearly visible. Although erythrocytes were visible with the AO method, stippling (Schuffner's or James's dots) was not stained clearly and differentiation was therefore difficult. As the morphology of the parasites is not apparent with the AO technique, it was difficult to differentiate between *Plasmodium malariae* (*P. malariae*) and *Plasmodium ovale* (*P. ovale*). Gay *et al*., [6] (1996) noted that the AO method allowed the diagnosis of species difference of *Plsamodium* in 85% of samples. Wongsrichanalai *et al*. [7] and Agabani *et al*. [8] suggested that the AO technique of Kawamoto [1] was as good as, or better than, the use of Giemsa stain. If species differentiation is required, in areas with a higher proportion of species other than *P. falciparum*, misclassification might lead to suboptimal therapy. The infection with *P. falciparum* occupies about 95%, and *P. vivax*, about 5%, and the infection with *P. malariae* and/or *P. ovale* is very rare in Myanmar. Therefore, differentiation from those infections is not a practical problem. The results of using the AO method achieved by the novice and experienced microscopist showed no significant differences, and this method required less practice to perform the test as well as to identify the parasite.

It is possible that the technique would be equally valuable in the diagnosis of other parasitic diseases (*e.g.*, trypanosomiasis, filariasis, *etc.*), and not only with blood samples but also with other biological specimens (*e.g.*, stool, bone marrow, cerebrospinal fluid) and even in microbiology as previously reported for tubercle bacilli [2, 9]. The AO method proved to be more economical than the Giemsa staining. Additional costs for the AO method included about $15 for the halogen bulb and $60 for the interference filters [10].

The MDM microscope we used was conveniently operable by using a standard AC power supply or a 12-V DC car battery. It is also easily convertible to a conventional biological microscope by replacing the barrier filters with an ordinary blue filter.

In conclusion, because of its simplicity and rapidity, the acridine orange fluorescence technique using an MDM-ESL series microscope compared favorably with Giemsa staining and, with the exception of species differentiation, would be an appropriate method for both clinical and epidemiological studies.

References

1. Kawamoto F: Rapid diagnosis of malaria by fluorescence microscopy with light microscope and interference filter. Lancet (1991) **337**, 200–202.
2. Kong HH and Chung DI: Comparison of acridine orange and Giemsa stains for malaria diagnosis. Korean J Parasitol (1995) **33**, 391–394.
3. Sodeman TM: The use of fluorochromes for the detection of malaria parasites. Am J Trop Med Hyg (1970) **19**, 40–42.
4. Syafruddin, Kamimura K, Hasegawa H, Toma T, Miyagi I, Kawamoto F, Nainggolan IJ, Tumewu-Wagey M, Mandagi-Waworuntu H and Kapoojos FX: Epidemiological study of malaria in North Sulawesi, Indonesia, by fluorescence and Giemsa staining. Jpn J Med Sci Biol (1992) **45**, 175–184.
5. Lowe BS, Jeffa NK, New L, Pedersen C, Engbaek K and Marsh K: Acridine orange fluorescence techniques as alternatives to traditional Giemsa staining for the diagnosis of malaria in developing countries. Trans Soc Trop Med Hyg (1996) **90**, 34–36.
6. Gay F, Traore B, Zanoni J, Danis M and Fribourg-Blanc A: Direct acridine orange fluorescence examination of blood slides compared to current techniques for malaria diagnosis. Trans Soc Trop Med Hyg (1996) **90**, 516–518.
7. Wongsrichanalai C, Chuanak N, Webster HK and Timasarn K: Rapid test for malaria diagnosis. Lancet (1992) **339**, 1176.
8. Agabani HM, el Hag IA, el Toum IA, Satti M and el Hassan AM: Fluorescence microscopy using a light microscope fitted with an interference filter for the diagnosis of malaria. Trans Soc Trop Med Hyg (1994) **88**, 61.
9. Shute GT and Sodeman TM: Identification of malaria parasites by fluorescence microscopy and acridine orange staining. Bull World Health Organ (1973) **48**, 591–596.
10. Craig MH and Sharp BL: Comparative evaluation of four techniques for the diagnosis of *Plasmodium falciparum* infections. Trans Soc Trop Med Hyg (1997) **91**, 279–282.