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Direct acridine orange fluorescence examination of blood slides compared to current techniques for malaria diagnosis

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Abstract

The renewed interest in the use of fluorochromes for malaria diagnosis prompted us to evaluate the acridine orange fluorescence technique on blood slides, and to compare it with established techniques using thick and thin blood films and the QBC™ malaria test, using the Giemsa-stained thick film technique as our standard method for comparison. We compared 123 positively diagnosed cases and 120 negative cases. For primary samples (day 0), the sensitivity of the thin blood film fluorescence acridine orange technique (AO) was 96·4%, and its specificity was 95·1%. In cases of imported malaria, with a prevalence rate of 16·2%, the positive predictive value was 79·2% and the negative predictive value 99·3%. Sensitivity of AO was significantly higher than that of Giemsa-stained thin blood films for parasitaemias <5000/μL. The potential of AO for species diagnosis of *Plasmodium* was 85·2%, using Giemsa-stained thin films as the reference technique. Where QBC™ imposes a cost limitation, especially in developing countries, despite its high performance, the AO diagnostic technique is a valuable alternative, because of its simplicity, almost negligible cost, and its diagnostic reliability. The method may also have potential value in the diagnosis of other microbiological diseases.

Keywords: malaria, diagnosis, acridine orange fluorescence

Introduction

In parasitology, the use of fluorochromes such as acridine orange for diagnosis was first applied to blood parasites by AMBROISE THOMAS *et al.* (1965).

In 1970, SODEMAN described for the first time the staining of thick blood films by acridine orange. A few years later, the World Health Organization Malaria Working Group proposed acridine orange staining for the identification of malaria parasites (SHUTE & SODEMAN, 1973). The technique has been evaluated and validated under experimental conditions and during field surveys (RICHARDS *et al.*, 1969; JANIS *et al.*, 1971).

WARDLAW & LEVINE (1983) described a diagnostic technique for blood parasites, combining concentration by centrifugation and the staining of nucleic acids by acridine orange, known as the QBC™ malaria test. This very rapid technique, with high sensitivity and specificity, does not need highly qualified personnel; however, it is expensive because of the high cost of the special capillary tubes. It gives an erroneous specific diagnosis in 10–30% of cases, and does not permit the estimation of parasite density (WONGSRICHANALAI *et al.*, 1991; GAY *et al.*, 1994).

The use of acridine orange and thin blood films was recently again proposed by KAWAMOTO (1991), and the technique has been evaluated in Indonesia and Kenya (SYAFRUDDIN *et al.*, 1992; LOWE *et al.*, 1996).

Our principal objective was to assess the validity of the thin film acridine orange fluorescence technique (AO) compared with the 3 available techniques, Giemsa-stained thick and thin blood films and the QBC™ malaria test, and to investigate its diagnostic value, staining quality, and the time needed for the examination of the slides.

Materials and methods

This study was done among travellers returning to France from malaria endemic areas. Blood samples were collected at the Service de Parasitologie of the Groupe Hospitalier Pitié-Salpêtrière in Paris over a period of 6 months, including primary (day 0) samples and follow-up samples from patients under treatment.

Thick and thin blood films were made with 2 μL of blood stained by a rapid trichrome technique, equivalent to Giemsa's stain, consisting of an eosin and methylene blue quick dip stain (RAL 555). In parallel, for the QBC™ malaria test, 60 μL of blood were aspirated in capillary tubes coated with anticoagulant and acridine orange and processed as described (WARDLAW & LEVINE, 1983).

For the AO technique, a thin blood film was prepared from a fingerprick or from 2 μL of blood plus heparin (or ethylenediaminetetraacetic acid). After drying rapidly in air, the film was fixed with methanol and again allowed to dry. It was then covered for one minute with acridine orange solution (see below), rinsed in water, and covered with a cover-slip for examination. The cover-slip was then removed and the slide rehydrated with the acridine orange solution before re-examination.

The acridine orange solution was prepared from a stock 1/200 solution, which was diluted with phosphate-buffered saline (pH 7·2) to a final dilution of 1/25000 (20 μg of stain per mL). Bacterial growth was prevented by the addition of a few drops of sodium azide. Stability of the solution is improved by storage for a month or more at +4°C in the dark; clarity and pH stability are essential to obtain the green staining of the deoxyribonucleic acid and red-orange staining of the cytoplasmic ribonucleic acid, which permits rapid and reliable recognition of the malaria parasites.

The films were examined using a standard fluorescence microscope with halogen illumination (Wratten®) lamp or a mercury vapour 'daylight' lamp (Osram®) attachment, or an ordinary light microscope fitted with a Paralens™ epi-illumination system.

The thick and thin blood films and QBC™ samples were prepared in parallel but examined 'blind'. If no parasite was seen on a Giemsa-stained thick film or by QBC™, the sample was defined as negative. If parasites were seen by one or both of these techniques, the sample was deemed to be positive. For this study, we preferentially selected blood samples which were positive by thick film and/or QBC™ examinations but negative by thin film examination, thereby choosing samples with low parasitaemia. AO preparations were examined 'blind' by an experienced microscopist, for a maximum period of 5 min.

Results

The AO technique gave 5 positive results with specimens that were negative by the 3 other techniques, and therefore were considered as false positives (Table 1). The 16 false negative results corresponded to samples with low parasitaemias (1–5 parasites/μL); 12 were post-treatment follow-up samples. The overall results re-

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Table 1. Comparison of acridine orange fluorescence examination of thin blood films and standard Giemsa-stained thick blood film techniques for diagnosis of malaria

	Giemsa staining	
	+	−
Acridine orange		
+	107	5
−	16	115
Total	123[a]	120

[a]Including 111 primary and 12 follow-up blood samples.

vealed a sensitivity of 87% and a specificity of 95·8% for the AO technique. However, if only the 111 primary (not follow-up) samples were considered, the sensitivity rose to 96·4%.

Comparing the examination of ordinary thin blood films with the use of thick films and QBC™, the thin film examination had a sensitivity of 65·9% and a specificity of 100% for all samples, and a sensitivity of 72% if only the primary samples were considered.

These results allowed us to compare 2 methods which are technically almost identical, but use different staining procedures: Giemsa staining for thin blood films and direct fluorescence for AO. The small reduction in specificity with direct fluorescence is largely compensated for by a slight increase in sensitivity.

AO had a higher sensitivity than Giemsa-stained thin films at the lowest parasitaemias (Table 2). However, the

Table 2. Sensitivity of acridine orange fluorescence and Giemsa-staining of thin blood films according to malaria parasitaemia, compared with standard Giemsa-stained thick blood film technique

		Sensitivity		
Parasites/ μL	No. of samples	Acridine orange	Giemsa	P^a
>25000	15	100%	100%	–
5000–25000	39	100%	100%	–
1000–5000	41	70·7%	41·5%	<0·008
<1000	28	50%	14·3%	<0·004

[a]Significance of difference between sensitivity of acridine orange and Giemsa's stain techniques.

morphology of the parasites could not be precisely visualized by AO. The ability to diagnose correctly the species of *Plasmodium* by AO was 85·2%, if compared with Giemsa-stained thin films as the reference technique.

The prevalence rate of malaria during this study, estimated from the number of positive results among the 959 samples examined by the QBC™ or Giemsa-stained thick blood film techniques, was 16·2%. This allowed us to calculate the predictive values (Table 3).

The total time taken by the AO method was 7 min, 2 min staining time and 5 min for slide examination.

Table 3. Comparison of acridine orange fluorescence examination of thin blood films and Giemsa-stained thin blood films for malaria diagnosis with standard Giemsa-stained thick blood film technique[a]

	Acridine orange	Giemsa
Sensitivity (%)	96·4	72·9
Specificity (%)	95·1	100·0
Predictive value		
Positive (%)	79·2	100·0
Negative (%)	99·3	95·0

[a]Primary samples only.

Staining unfixed blood films with acridine orange resulted in very strong fluorescence, but the difficulty of distinguishing the blood cells diminished the sensitivity of this procedure.

We also tried staining thick blood films with acridine orange. The results obtained were interpretable, but we often noticed the presence of background fluorescence resistant to the rinsing step. After several trials, we abandoned this technique.

Discussion

Our results demonstrated the higher sensitivity and greater rapidity of AO compared to Giemsa-stained thin blood films, confirming the results of other field studies (SODEMAN, 1970; SYAFRUDDIN *et al.*, 1992). We have taken into account the primary (day 0) results of AO in assessing its diagnostic ability, rather than the post-treatment follow-up results. However, the technique necessitates a fluorescence system, which can pose logistic problems for field investigations. That is why KAWAMOTO (1991) proposed the use of daylight-illuminated microscopes fitted with interference filters, which could have significant advantages for field use (except for species differentiation) (LOWE *et al.*, 1996). We were working with imported malaria cases which generally have low parasitaemias, and the consequence of our adopted method was to select samples with low parasitaemias since we chose slides with discordant results in the thin film and QBC™ examinations. We found a direct correlation between sensitivity and parasitaemia, so higher sensitivity could be expected in practice, especially in endemic areas where parasitaemias are usually higher. If the examination time were increased to 15–20 min, as is usual for blood film examination, the sensitivity would further increase. The AO technique is not, however, satisfactory for specific diagnosis, as the precise morphology of the red cell is not clearly visible. Consumable supplies for AO are inexpensive compared to the QBC™ tubes, but QBC™ has the advantages of being highly sensitive and more rapid.

The comparison of AO with Giemsa staining of thin blood films demonstrated the usefulness of fluorochromes in the direct diagnosis of malaria. It is possible that the technique would be equally valuable in the diagnosis of other parasitic diseases (e.g., trypanosomiasis, filiariasis, 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 (SODEMAN, 1970; SHUTE & SODEMAN, 1973).

Dedication

This paper is posthumously dedicated to Dr André Fribourg-Blanc who initiated the use of this technique for malaria diagnosis with the ultimate goal of developing the use of fluorochromes for microbiological diagnostic techniques in the future. (He diagnosed his own resurgence of pulmonary tuberculosis in the 1950s using an acridine orange technique after a negative examination for the presence of acid-fast bacilli.)

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