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COMPARISON OF FIVE METHODS OF MALARIA DETECTION IN THE OUTPATIENT SETTING

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Abstract. In eastern Africa where 90% of the malaria is due to *Plasmodium falciparum,* the accuracy of malaria diagnosis at the outpatient level is becoming increasingly important due to problems of drug resistance and use of alternative, costly antimalarial drugs. The quantitative buffy coat (QBC®) technique, acridine orange staining with an interference filter system, and the *Para*Sight®-F test have been introduced as alternative methods to conventional microscopy for the diagnosis of malaria. Two hundred thirteen outpatients were tested using these alternative methods and conventional microscopy by five experienced technologists; two were randomly allocated to read the results of each test. Paired results showed the highest level of agreement with the *Para*Sight®-F test (99%), followed by Field stain (92%). The results of the QBC® technique showed the least agreement (73%). Using conventional microscopy as the reference standard, the *Para*Sight®-F test had a sensitivity range of 90–92% and a specificity of 99%, staining with acridine orange had a sensitivity range of 77–96% and a specificity range of 81–98% and the QBC® technique had a sensitivity range of 88–98% and a specificity range of 58–90%. All microscopic tests showed lower sensitivities (as low as 20% using staining with acridine orange) in detecting low parasitemias ($\leq$ 320/μl) than the *Para*Sight®-F test (70%). Due to the high cost of the *Para*Sight®-F test, Field-stained blood films remain the most appropriate method for diagnosis of *P. falciparum* in eastern Africa. The *Para*Sight®-F test may be used in situations where no trained microscopists are available, or where malaria is strongly suspected and the results of microscopy are negative.

Malaria is one of the leading causes of morbidity and mortality in eastern Africa. In malaria-endemic areas, most patients with febrile disease receive antimalarial drug treatment. However, in semiarid zones, the risk of malaria transmission is low and may be subject to seasonal variation. The clinical diagnosis of malaria is usually based on a history or presence of fever; presumptive treatment of fever results in over-administration of antimalarial drugs.[1–3] This practice may result in the erroneous treatment of fever caused by other infections, and may exert a high drug pressure on malaria parasite populations, leading to selection of drug-resistant parasites. Reliable diagnosis of malaria requires laboratory confirmation of the presence of malaria parasites in the blood of a febrile patient. In eastern Africa, where 90% of the malaria is due to *Plasmodium falciparum,* accuracy of malaria diagnosis at the outpatient level is becoming increasingly important due to emerging drug resistance and the use of alternative, costly antimalarial drugs.[4]

Laboratory confirmation of malaria infection requires the availability of a rapid, sensitive, and specific test at an affordable cost. Conventional methods of laboratory diagnosis for malaria use microscopic examination of stained thick blood films. Both Giemsa and Field stains are widely used for staining of blood films.[5,6] However, examination of thick blood films requires technical expertise and the availability of a good-quality microscope (binocular with built-in illumination); it is also time-consuming and of limited sensitivity in the detection of low parasitemias.[7]

Alternative methods of malaria diagnosis appropriate for the outpatient setting have been introduced to overcome the limitations of conventional microscopy. Concentration of malaria parasite–infected red blood cells by centrifugation, coupled with staining with acridine orange and fluorescence microscopy (Quantitative Buffy Coat [QBC®] System; Becton Dickinson, Franklin Lakes, NJ), has been reported to be easier to use, more sensitive, and faster in the detection of parasites.[8] However, this method requires specially prepared, disposable tubes coated with acridine orange and anticoagulants, a microhematocrit centrifuge, and the use of a microscope with an ultraviolet lens. A simpler method of fluorescent microscopy has been introduced by Kawamoto[9] that uses acridine orange and an interference filter placed in a standard microscope. Recently, a rapid manual test, the *Para*Sight®-F test (Becton Dickinson Tropical Disease Diagnostics, Sparks, MD), has been introduced. It requires no instrumentation and can be performed by staff with no previous technical training.[10,11] This test detects histidine-rich protein-2 (HRP-2) produced by *P. falciparum* and secreted into blood,[12] using a test strip impregnated with specific antibodies. The reaction is visualized using detector lipid particles containing a dye.

The purpose of this study was to compare five methods of malaria diagnosis: microscopic examination of thick blood films stained with Field stain and Giemsa stain, the QBC® method, an interference filter system for detection of acridine orange–stained malaria parasites, and the *Para*Sight®-F test, for sensitivity, specificity, and appropriateness in the primary health care setting.

MATERIALS AND METHODS

The study was conducted by five experienced laboratory technologists between February and July 1994 in the outpatient clinics of three Ministry of Health facilities in Kenya: two health centers (Matuu in the Machakos District and Sultan Hamud in the Makueni District) and one District Hospital (Machakos). The health facilities lie east of Nairobi between 900 and 1,500 m above sea level, and within 50 km of each other in the same geographic area where malaria is seasonal. Patients attending the outpatient clinics who were referred by the resident clinician for blood slide ex-

amination for initial diagnosis or follow-up of malaria were included in the study. Information including age, sex, antimalarial drugs taken in the previous four weeks was collected from patients' clinical records and/or after a brief interview. Formal oral consent to participate in the study was obtained from adult patients and guardians of young children. Patients less than two years of age were excluded due to the difficulty in obtaining an adequate blood sample. The study was reviewed and approved by the African Medical and Research Foundation.

All five diagnostic tests were carried out on capillary blood samples obtained by fingerprick. A thin blood film was also prepared for parasite species identification if necessary. Each study specimen was given a coded number. An additional thick blood film was prepared and submitted to the health facility laboratory for staining and examination; patients were managed by the clinician according to results of the latter specimen, independent of the study results. One technologist was randomly allocated to collect the blood samples, and another to prepare the specimens for each test. All five technologists participated in the reading of the test results; two technologists were randomly allocated to read the results of each test. The technologists randomly interchanged duties according to a computer-generated scheme. The examiners interpreted the specimens independently and without the knowledge of the results of any other examiner.

Field and Giemsa staining. Field stains A and B and Giemsa stain were prepared according to standard methodologies. Thick films were allowed to air dry before staining. The procedure for Field staining was dipping the film in Field stain A for 4 sec, rinsing in tap water for 5 sec, dipping in Field stain B for 4 sec, followed by rinsing in tap water for 5 sec. For Giemsa staining, thick films were flooded with 5% phosphate-buffered Giemsa, pH 7.2, for 20 min and rinsed in buffer solution. Thick films were examined using a Leitz (Wetzlar, Germany) HM Lux 3 binocular microscope with built-in illumination and a total magnification of 1,000× (10× widefield eyepieces and a 100× oil immersion lens).

Quantitative buffy coat technique. Blood (55–65 μl) was collected into commercially supplied malaria detection tubes (Becton Dickinson) following the manufacturer's instructions; the tubes were centrifuged at 10,000 × *g* for 5 min and examined using a Leitz HM Lux 3 microscope fitted with a Paralens® (Becton Dickinson) UV Microscope Adapter (10× widefield eyepieces and a 60× oil immersion lens).

Acridine orange staining technique. Thin blood films were fixed in 100% methanol and air-dried. A drop of acridine orange stain (100 μg/ml in phosphate-buffered saline, pH 7.0) was placed on a clean coverslip, which was then placed on the film. Films were examined at 400× magnification (10× widefield eyepieces and a 40× dry lens) using an Olympus (Tokyo, Japan) CH-2 binocular microscope fitted with an interference filter above the condenser, a barrier filter in the eyepieces, and a 150 watt halogen light source directed onto a concave mirror.

***Para*Sight®-F test.** This test was performed using commercially supplied test strips and reagents following the manufacturer's instructions. The testing at all three sites was performed by the same technologists using the same equipment and materials. The reagents for all tests were prepared and their quality was tested in advance at the African Medical and Research Foundation Laboratory in Nairobi using known *P. falciparum*-infected blood samples.

Examination and reporting of results. All specimens were examined on-site within 4 hr of collection. Each QBC® tube was examined until parasites were detected, or for a maximum of 5 min. Field-, Giemsa-, and acridine orange–stained smears were reported as negative after examination of the specimen for approximately 5 min, which allowed examination of 100 fields systematically. Positive thick blood films were recorded as rings per 100 white blood cells. The parasite count per microliter of blood was obtained assuming a white blood cell count of 8,000/μl. Positive QBC® and acridine orange test results were graded as few (≤ 2 parasites per field), moderate (3–5 parasites/field), or many (> 5 parasites per field). The *Para*Sight®-F strip results were reported as positive or negative. Field- and Giemsa-stained thick films, thin films, and the *Para*Sight®-F strips were stored for reference.

Reference standard. The results of the Field- and Giemsa-stained thick films were used as the reference standard in this study. When examiners disagreed on the results of the same film or where the Field and Giemsa stain results disagreed in the field, both Field- and Giemsa-stained films were re-examined later at the African Medical and Research Foundation Laboratory by two technologists using a blinded technique, and for a longer period of time (10 min). The presence of parasites were confirmed by a third examiner. When paired films were not available (two Field-stained films washed off during staining and four Giemsa-stained films were subsequently lost), the available film was rechecked and the result was used. In view of a previous report of the high sensitivity of the QBC® technique,[8] all discrepant results between positive QBC® and Field- and/or Giemsa-stained films were rechecked and confirmed. The only results that were not confirmed were those in which there was universal agreement between both examiners on the results of all five tests in the field. Agreement on the results of Field- and Giemsa-stained films by the examiners in the field and/or at the African Medical and Research Foundation Laboratory was used as the gold standard.

Calculations. Sensitivity was calculated as TP/(TP + FN) × 100%, specificity as TN/(TN + FP) × 100%, positive predictive value (PPV) as TP/(TP + FP) × 100%, and negative predictive value (NPV) as TN/(TN + FN) × 100%, where TP = true positive, TN = true negative, FP = false positive, and FN = false negative.

In the field, discrepant results were obtained when one of the two examiners reported an incorrect result. As a measure of the possible outcomes with only one examiner, ranges of sensitivity, specificity, and predictive values were calculated. The lowest values were calculated by considering that the discrepant field results, at worst, may all have been wrongly reported, while the highest values were obtained on considering that these results, at best, were correctly reported. Calculations were similarly performed for samples with high and low (≤ 320/μl) parasitemias.

RESULTS

Two hundred thirteen patients (134 females and 79 males) entered the study; 83 patients were from the Matuu Health

TABLE 1

Agreement between two examiners working in field conditions and the comparison of each test performance in the field with the reference standard*

Test results	Gold standard			Agreement (%)	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
	Postive	Negative	Total					
Giemsa stain								
Both positive	48	1	49					
Both negative	1	137	138					
Discrepant	3	23	26					
Total	52	161	213	88	92–98	85–99	67–98	97–99
Field stain								
Both positive	44	0	44					
Both negative	1	150	151					
Discrepant	6	10	16					
Total	51	160	211	92	86–98	94–100	81–100	96–99
AO								
Both positive	40	4	44					
Both negative	3	129	132					
Discrepant	9	27	36					
Total	52	160	212	83	77–96	81–98	60–92	91–98
QBC®								
Both positive	44	16	60					
Both negative	1	92	93					
Discrepant	5	51	56					
Total	50	159	209	73	88–98	58–90	40–68	94–99
Para*Sight*®-F								
Both positive	47	2	49					
Both negative	4	158	162					
Discrepant	1	0	1					
Total	52	160	212	99	90–92	99	96	97–98

* PPV = positive predictive value; NPV = negative predictive value; AO = acridine orange; QBC = quantitative buffy coat.

Center, 55 from the Sultan Hamud Health Center, and 75 from the Machakos District Hospital. Thirty eight (18%) patients were 2–5 years old, 32 (15%) were 6–10 years old, 30 (14%) were 11–15 years old, 49 (23%) were 16–20 years old, 30 (14%) were 21–30 years old, and 34 (16%) were 31–80 years old. Forty-three (20%) patients indicated they had taken some antimalarial treatment in the past four weeks: 23 (55%) had taken chloroquine, 12 (27%) had taken quinine, four (9%) had taken pyrimethamine/sulfametopyrazine, and four (9%) did not know the type of antimalarial treatment they had taken.

All positive films from study patients showed *P. falciparum* except for one (*P. ovale*). No mixed infections were detected. Two (0.9%) of the Field-stained blood films washed off the slide during staining and there was insufficient material for interpretation. Four (1.9%) of the QBC® tubes could not be examined: three tubes cracked and one tube spilled during centrifugation. The breakages occurred during the first day of the study due to improper technique. The results of one acridine orange–stained blood film and one *Para*Sight®-F strip were recorded by only one examiner in the field.

Table 1 shows the agreement between two examiners working under field conditions and the comparison of each test performance in the field with the reference standard. Table 2 shows the agreement between two examiners and the range of sensitivities for samples with high parasite counts and low parasite counts ($\leq$ 4 rings/100 white blood cells equivalent to $\leq$ 320/μl).

DISCUSSION

The study was performed under conditions usually encountered in outpatient clinics in Kenya where patients wait

TABLE 2

Agreement between two examiners and the range of sensitivities for samples with high parasite counts (HP) and low parasite counts (LP)*

Test	Agreement (%)			Sensitivity (%)	
	HP	LP	*P*	HP	LP
Giemsa stain	98 (n = 42)	70 (n = 10)	$0.5 > P > 0.2$	97–100	60–90
Field stain	95 (n = 42)	67 (n = 9)	$0.5 > P > 0.2$	95–100	56–89
AO	95 (n = 42)	30 (n = 10)	<0.001	93–97	20–90
QBC®	98 (n = 40)	60 (n = 10)	$0.02 > P > 0.01$	98–100	50–90
Para*Sight*®-F	98 (n = 42)	100 (n = 10)	>0.5	95–98	70

* AO = acridine orange; QBC = quantitative buffy coat.

for the results of tests before receiving treatment. By using experienced technologists and the recommended equipment and techniques, we attempted to evaluate the performance of each test under field conditions. Staining with Giemsa takes longer and is more suitable for batch staining. Field staining is a faster and convenient method for patients who visit the laboratory individually. The main disadvantage with Field staining was the occasional washing off of the film during staining. During routine diagnostic work, these tests are repeated. Thin films can be stained using the reverse Field stain technique.[6]

The acridine orange technique was not preferred by the examiners because the lamp used became excessively hot and the light was uncomfortable to the eyes. The heat problem can be overcome by using a cold lamp (these were not available in Nairobi) or a specially adapted light source or microscope, which are now commercially available. There was uneven staining using the acridine orange method, making some areas shine brighter than others. Good quality thin smears and clean, dust-free slides, and coverslips are essential for accurate diagnosis using this method.[13] The examiners had difficulty in counting parasites against white blood cells using the acridine orange method due to the uneven distribution of white blood cells.

Problems of breakage of QBC® tubes during centrifugation have been noted in other studies.[13–16] In addition, in this study the examiners reported that the shadow of the fiber optic cable and the vibration of the lamp fan interfered with vision. There was difficulty in recognizing parasites using this technique; other structures that fluoresce, e.g., Howell-Jolly bodies (nuclear remnants), may have been mistaken for parasites. Furthermore, quantifying parasitemias using this method was not possible. Young rings of *P. falciparum* may be missed by staining with acridine orange, and this problem has been reported to be serious when the parasite concentration is low.[5] In addition, in a small laboratory with only one microscope, where numbers of different samples are being examined, microscope attachments are unnecessarily cumbersome.

This is the first study examining discrepancies between examiners under field conditions using five methods of malaria diagnosis. Surprisingly, where two results in all five tests were available, the examiners agreed on only 35 (67%) positive specimens and 65 (40%) negative specimens. Discrepant results within and/or between tests were reported in 105 (49%) specimens. The fact that drops of blood from the same individual used for preparing each test are, strictly speaking, different samples cannot be excluded as a possible cause of discrepant results between tests.

There are few studies that have enumerated the discrepancies between experienced examiners in the interpretation of the results of a single test under field conditions. Wongsrichanalai and others[13] noted a range in sensitivity for *P. falciparum* of 66.7–91.7% with the QBC® technique between two examiners. Both Rickman and others[8] and Wongsrichanalai and others[13] observed discrepancies in the reading of Giemsa-stained thick films when a positive result was recorded by at least one examiner. In this study, agreement between examiners was higher in specimens with high parasite counts ($> 320/\mu l$) in all tests. Despite the few specimens with low parasite counts, the difference in agreement between the results of the specimens with high and low counts was statistically significant for the acridine orange ($P < 0.001$) and QBC® ($0.02 > P > 0.01$) methods.

Ten Field- and 23-Giemsa stained films read as positive by one examiner and one Giemsa-stained film reported as positive by both examiners in the field (but negative by Field stain) were confirmed as negative on rechecking. Similar observations of positive results by Giemsa staining that were changed to negative on re-examination have been reported.[17,18] This may occur purely as a result of chance for films with very low parasitemias ($< 32/\mu l$).[7,17] Alternatively, it is possible that the staining of some parasites may fade with time.

The range of sensitivities of the QBC®, acridine orange, and *Para*Sight®-F tests are consistent with those of other studies.[8,14–16,19,20] Reduced sensitivities with low parasitemias have been noted for the QBC® test[13,15,16] and for the *Para*Sight®-F test.[20] In our study, at least one examiner reported using the Giemsa, Field and QBC® tests correctly with all true positive specimens with high parasite counts. In all tests, false-negative results obtained by both examiners were reported in specimens with low parasitemias ($\leq 320/\mu l$). The sensitivity of stained thick blood films can be increased by reading more fields.[5,7,17,21] In this study, errors were probably due to a shortage of time in field conditions since there was no disagreement with parasite recognition during re-examination. Negative results in the QBC® with samples with high parasite counts have also been reported.[16]

Unexplained false-negative results in the *Para*Sight®-F test have been reported in another study.[20] The threshold of detection of *P. falciparum* using this test is estimated to be 25–50/μl,[22,23] although negative results have been reported with high *P. falciparum* parasitemias.[10,20] In our study, one positive sample was due to the presence of *P. ovale.* If one considers that this test was designed to detect only *P. falciparum,* and one excludes this sample, the sensitivity of this test may be as high as 94%. However, focal areas with a high prevalence of other species of *Plasmodium* exist in eastern Africa, e.g., *P. malariae* occurs at a frequency of greater than 25% in parts of central Tanzania,[24] thus warranting local appraisal before introducing this test.

The high false-positivity rate of the QBC® test has been suggested as a measure of the superior sensitivity of this test, but this cannot be confirmed without further studies such as molecular testing[23] or careful follow-up of patients.[25] Positive results on the *Para*Sight®-F test, which cannot be verified microscopically, have been suggested to be due to circulating *P. falciparum* HRP-2 antigen following treatment [10,20] or from sequestered parasites.[22] Individuals participating in an experimental study were found to be positive by a dipstick assay even before challenge with infected mosquitoes.[20] Recently, false-positive results have been reported with *P. vivax* infections when the *Para*Sight®-F test was evaluated by comparison with the polymerase chain reaction.[23] In this study, two patients who were antigen positive but parasite negative by microscopy reported no prior use of antimalarial drugs, although verbal patient information may be of questionable accuracy.[26]

The overall sensitivity and specificity of Field and Giemsa stain methods may have been overestimated because the

same tests were used to define the reference standard. Despite this, these tests had a potentially low sensitivity, in common with the other microscopic tests, in detecting low parasitemias, while the *Para*Sight®-F test appeared to be more sensitive. This test also exhibited the highest PPV and NPV (96–98%). This study demonstrates that duplicate testing using blood smears can be a powerful field tool to evaluate the relative merits of new techniques developed for malaria detection.

If the agreement between two examiners is taken as a measure of the ease of interpretation of a test result, then the *Para*Sight®-F test was the easiest to interpret, and the QBC® test the most difficult. Ease of use, convenience in the clinical setting, and low cost are important considerations for the selection of diagnostic laboratory tests. A good quality microscope with a built-in power illumination and technical expertise are essential for good-quality microscopy. The sites selected for the study had electricity; however, 12 volt DC battery-powered microscopes are used successfully in areas of eastern Africa without electricity. Lowe and others[16] estimated the cost of reagents and consumable supplies per test as $0.06 for the Giemsa and acridine orange staining methods and $0.83 for the QBC® test. In addition, the acridine orange and QBC® methods require special equipment (estimated to be as high as $5,250 for the QBC® test). Currently, we estimate the cost of the QBC® or *Para*Sight®-F reagents at $2.25 per test, while Field stain costs less than $0.02 per test. The Field, Giemsa, and acridine orange staining methods are quantitative, but staining with Giemsa takes longer to perform. On the basis of sensitivity, specificity, convenience and cost, the Field-stained thick blood film remains the appropriate method for diagnosis of *P. falciparum* in health facilities with laboratories in most endemic areas of eastern Africa. The *Para*Sight®-F test may be used where there are no trained or experienced microscopists, in hypoendemic areas, and for patients in whom malaria is strongly suspected and the results of conventional microscopy are negative.

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