A preliminary comparative report of quantitative buffy coat and modified quantitative buffy coat with peripheral blood smear in malaria diagnosis

Manali Kochareka¹, Sougat Sarkar², Debjani Dasgupta³, Umesh Aigal⁴

¹Institute of Science, Mumbai, India, ²Glenmark Ltd, Mumbai, India, ³Department of Biochemistry, Institute of Science, Mumbai, India, ⁴Kasturba hospital for Infectious Diseases, Mumbai, India

The quantitative buffy coat (QBC) technique is a method of diagnosing malarial parasites based on micro-centrifugation, fluorescence, and density gradient of infected red blood cells. The aim of the present study was to modify the QBC technique in order to reduce the cost per test of malaria diagnosis. This was achieved by introducing some modifications to routine QBC wherein REMI centrifuge (cost Rs 19 000/–) and ultra-violet microscope (Rs 115 000) were used instead of parafuge (Rs 108 000) and paralens (Rs 293 625/–). With the above modification, the cost per test for laboratories dealing with high patient load was reduced by 13%, whereas for smaller laboratories with low patient load, the cost per test was reduced by 48%. This is a significant difference in cost. The results of the modified QBC method were compared with the current diagnostic methods: peripheral blood smear (PBS) and routine QBC. Blood samples collected from 96 patients were subjected to the above tests. Considering PBS as the gold standard, routine QBC showed 91% sensitivity and 96% specificity for *Plasmodium vivax*- and 91% sensitivity and 94% specificity for *Plasmodium falciparum*-infected patients. It was seen that the modified QBC technique had 91% sensitivity and 98% specificity for *P. vivax* and 91% sensitivity and 96% specificity for *P. falciparum*. It was concluded that modification of the QBC technique renders it cheaper without compromising the specificity and sensitivity of the method.

Keywords: Malaria, *Plasmodium falciparum*, *Plasmodium vivax*, Quantitative buffy coat, Thick and thin smears

Introduction

Malaria occurs throughout the tropics causing over 100 million cases and over one million deaths are reported every year. Early diagnosis of malaria not only mitigates suffering but also reduces transmission of the parasite in the community. Therefore, precise laboratory diagnosis and species identification is important for optimal management of malaria.³

In a developing country like India where malaria is endemic, a procedure which is sensitive and specific enough to detect malaria parasite in a short span of time will facilitate early diagnosis and timely therapy, thus averting the florid manifestations of malaria, reducing morbidity and mortality.¹⁰

The conventional method used for the diagnosis is the thick-and-thin peripheral blood smears (PBS). Modified quantitative buffy coat (QBC) method is

less expensive than routine QBC and the sample is easier to prepare than microscopy. The availability of competent technician to make appropriate smears for correct interpretation of results is a disadvantage of PBS microscopy which is also a very tedious and time-consuming method.

The time required for the routine QBCTM technique in detecting malarial parasites is 8–15 minutes which is a definite advantage over PBS microscopy which requires more than 60 minutes of sample examination.

The QBC detection limit is approximately five parasites/μl of blood and establishes diagnosis earlier than the PBS microscopy which limits the detection in low parasitaemia cases (average microscopist can detect 50–100 parasites/μl).⁸

QBC technician can be trained in only 3–5 days as compared to a microscopist who requires years of experience before doing the detection optimally. In the QBC method, the staining is easily done by just rolling the QBC tubes unlike in PBS microscopy which involves a tedious method of staining.

Correspondence to: D Dasgupta, Department of Biochemistry, Institute of Science, Madam Cama Road, Churchgate, Mumbai, Maharashtra 400099, India. Email: drdasgupta88@rediffmail.com

However, the main disadvantage of routine QBC is that it is very expensive and modifying the QBC renders it much less expensive.

In view of the above, a method that utilizes the advantages and eliminates the disadvantage of routine QBC would be of greater value as a screening tool. Thus, this study aims at modification of the routine QBC method and further comparing the results of the routine and modified QBC method with the gold standard, i.e. PBS.

Material and Methods

Study design

A comparative study evaluating routine QBC versus modified QBC for the diagnosis of malaria in patients suffering from uncomplicated symptomatic malaria due to *Plasmodium falciparum* or *Plasmodium vivax* was conducted after approval from Independent Ethics Committee.

Study subjects and selection criteria: patients, attending General or Medical Outpatient Department, with a history of fever over the past 24 hours suspected to be suffering from malaria in Kasturba Hospital for infectious diseases, Mumbai, India, from May 2010 to May 2011, were screened. Patients of either sex in the age group between 18 and 70 years presenting with symptomatic, uncomplicated malaria confirmed by the presence of either or both of the following criteria were included:

1. blood smears positive for *P. falciparum* or *P. vivax* asexual parasitemia and/or sexual parasites;
2. fever or history of fever within the past 24 hours was included in the present study both as positive and negative patients. Patients with severe clinical manifestations, which require immediate referral, were excluded from the study.

A written informed consent was taken from the patients. The vital parameters such as temperature, pulse, blood pressure, etc. were recorded at the start of the study.

Sample collection: Blood for study purpose was collected by finger prick method.

Two drops of blood were placed on a glass slide and 55–65 µl directly added into each of the two QBC tubes for preparation.

Methods used in this study

Microscopic diagnosis using stained thin and thick PBS

Thick and thin film was prepared on the same slide stained with Giemsa method and examined under oil immersion lens by light microscopy. Asexual- and sexual-stage parasite densities were counted separately per 200 leukocytes and were then expressed in microliters (µl). If more than nine parasites were detected per 200 white blood cells, parasites were counted up to 500 white blood cells. Thick films

were considered negative if no parasites were seen in at least 100 consecutive oil immersion fields.

QBC

Principle: QBC for detecting malaria parasites is based on using specialized capillary tube containing a plastic float. Blood containing capillaries are centrifuged at high speed for 5 minutes. The plastic float which has the same specific gravity as that of the buffy layer automatically gets positioned within the buffy coat layer, between the packed red cells and the plasma. During centrifugation, the components of the buffy coat separate according to their densities, forming discrete bands. Platelets constitute the topmost layer, lymphocytes and monocytes the middle layer, and granulocytes, being the heaviest of the buffy coat cells, concentrate immediately above the packed red cells.

Reagents which coat the QBC test tube promote separation and metachromatic staining of these cells. Examination of the centrifuged blood under a fluorescence microscope, or a light microscope with the QBC ParaLens Advance™ LED fluorescence microscope attachment, readily permits the detection of malaria in the infected cells and plasma. Direct malaria detection by the QBC test method is therefore more rapid than conventional blood-film microscopy.¹

Method: The QBC tube (Becton Dickinson) was filled with blood from the end with EDTA (blue-lined end). The tube was then rolled between the fingers and tilted to mix the blood with the anti-coagulant and orange coated stain.

The tubes were capped at the blue lined end and plastic float was inserted on the other end

Routine QBC: The tubes were centrifuged in parafuge (cost of this centrifuge is Rs 108 000/working on the same principle as Remi 12 C microcentrifuge) at 12 000 rev/min for 5 minutes. The tubes were placed in paraviewer tubes holder. 2–3 drops of immersion oil was added and the buffy coat area of the tube was brought to focus. The microscope stage was moved until the top of the expanded red blood cell layer was in the field of view and the sample was examined.

The interpretation was done using a light microscope fitted with QBC Paralens Advance™ LED fluorescence microscope attachment (QBC Diagnostics Inc., Port Matilda, PA, USA) (costing Rs 293 625) and a × 60 objective of the light microscope (Labomed Ltd, India).

The total examination time to exclude a negative was 7–10 minutes.

Results for the technique were reported in terms of the following: (1) presence or absence of parasite and (2) morphology.

Modified QBC technique

In modified QBC, the method was essentially the same as that of routine QBC with changes such as

centrifugation done in locally available RM-12 C REMI™ (REMI Laboratory Instruments, Mumbai, India) microcentrifuge (cost Rs 19 000) instead of ParaFuge (QBC Diagnostics Inc.) (cost Rs 108 000) at 12 000 rev/min for 5 min. The centrifuged tube was placed into the groove of the ParaViewer tube holder and examined under $\times 60$ objective of ultra-violet (UV) microscope (Labomed Ltd) (costing Rs 115 000 instead of ParaLens costing Rs 293 625). Parasite nuclei fluoresces bright green, while cytoplasm appears orange.

The total examination time to exclude a negative was 7–10 minutes. Results for the above technique were reported in terms of the following: (1) presence or absence of parasite and (2) morphology.

Data analysis

Most of the analysis was done by Graphpad Instat version 3.06 and SPSS version 10.0. Sensitivity was also calculated as $TP/(TP + FN) \times 100\%$, specificity as $TN/(TN + FP) \times 100\%$. Sensitivity and specificity were used to calculate the likelihood ratios for a positive test result [$sensitivity/(1 - specificity)$] and a negative test result [$(1 - sensitivity)/specificity$]. All the parameters of routine QBC and modified QBC tests were assessed with PBS microscopy as the gold standard. McNemars Chi-square one-sided test was applied for sensitivity for *P. vivax* and *P. falciparum* for non-inferiority of modified QBC to routine QBC, both compared to PBS as the gold standard.

Results

The median (range) age of the patients diagnosed with Falciparum malaria was 32 (18–54) years and that of vivax malaria was 33 (18–60) years.

The median and SD parasite density at baseline in falciparum group was 1370 ± 1249.57 and in Vivax group, it was 1467.83 ± 929.15 (Table 1).

Patients suspected of malaria were screened for malarial parasites by thick and thin PBS. After screening, 96 patients were enrolled for the study based on the inclusion criteria. Forty-five were positive for malarial parasites by PBS and 51 negative. Twenty-two were infected with *P. vivax* and 23 with *P. falciparum*. In the 45 patients, 35 were male patients and 10 were female patients.

Out of the 51 PBS negative patients, routine QBC were false-positive for *P. falciparum* in three and modified QBC in two (Table 2).

It was found that modified QBC was non-inferior to routine QBC when both were compared with PBS microscopy as gold standard using McNemars Chi-square one-sided test for sensitivity and specificity for *P. vivax* and *P. falciparum*. The sensitivity, specificity, likelihood ratio positive, and likelihood ratio negative of vivax arm and that of falciparum arm have been calculated using 2×2 table with peripheral blood smear as gold standard as shown in Table 3.

One patient who was negative by PBS microscopy on day 1 was found to be positive for *P. falciparum* by both routine QBC and modified QBC. The same slide on day 1 was again reviewed by another experienced microscopist to check for reader error and was found to be negative by PBS microscopy. On day 3, the patient presented again with high-grade fever and was positive by PBS microscopy.

QBC methods failed to pick up two PBS vivax positives. Further routine QBC misdiagnosed two, while modified QBC one patient as false-positive which were negative by PBS microscopy.

The costs of the all three methods over a period of 1 year with large and small sample loads have been computed in Tables 4 and 5. The UV microscope of Labomed and centrifuge of REMI is relatively inexpensive, thus making modified QBC cheaper.

Discussion

The results thus obtained were analyzed and a comparative evaluation was done taking peripheral blood smear as the 'gold standard'. PBS microscopy has historically been the mainstay of malaria diagnosis and continues to be the gold standard.

Keeping Giemsa-stained PBS as gold standard, the sensitivity and specificity of modified QBC were marginally better than those of routine QBC method with respect to falciparum and vivax malaria (Table 2 and 3). The sensitivity of QBC has been reported to

Table 2 Comparison of peripheral blood smears examination with routine QBC and modified QBC for malaria parasite detection

Blood Smear	QBC		Modified QBC	
<i>P. vivax</i>	Positive	Negative	Positive	Negative
Positive-22	20	2	20	2
Negative-51	2	49	1	50
Total-73	22	51	21	52
<i>P. falciparum</i>	Positive	Negative	Positive	Negative
Positive- 23	21	2	21	2
Negative-51	3	48	2	49
Total-74	24	50	23	51

Table 1 Baseline characteristics of included patients

Parameters	Falciparum arm	Vivax arm	Negative arm
Age (mean $\pm$ SD), years	32.04 $\pm$ 10.16	33.30 $\pm$ 11.71	28.72 $\pm$ 10.36
Asexual parasite density (mean $\pm$ SD)	1370 $\pm$ 1249.57	1467.83 $\pm$ 929.15	0
Gametocyte density (mean $\pm$ SD)	234 $\pm$ 536.96	1945.73 $\pm$ 1075.42	0
Duration of fever (mean $\pm$ SD), days	3.45 $\pm$ 1.55	2.86 $\pm$ 0.94	1.9 $\pm$ 0.64

be as high as 90% by Bimala *et al.*,⁴ 96% by Bhandari *et al.*,³ and 99.7% by Benito *et al.*² Our results are consistent with these studies.

This study also indicated that Giemsa-stained PBS had an edge over routine QBC and modified QBC techniques. QBC methods failed to pick up two PBS vivax positives as the specific gravity of late trophozoites of *P. vivax* is similar to the leucocytic layer of the buffy coat causing the parasites to probably get obscured in the granulocytic layer following centrifugation. They also become difficult to identify as they get compacted and lose their amoeboid shape during centrifugation.^{7,9} Also routine and modified QBC failed to pick up two PBS falciparum positives of the same patients. They had low parasitaemia of 32 and 80/μl. This may be due to difficulty in identifying parasites by the first-time users and also young rings of *P. falciparum* may be missed by staining with acridine orange. This has been reported when the parasite concentration is low.⁶ Despite this, the specificities of the routine and modified QBC test in vivax arm were 96% and 98%, respectively, while those of falciparum arm were 94% and 96%, respectively, which indicates that it is a good diagnostic test.

Further routine QBC misdiagnosed two, while modified QBC one patient as false-positive which were negative by microscopy in vivax arm and routine QBC detected three and modified QBC two as false-positives for *P. falciparum* which were negative by microscopy. This 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.⁶

Thin smear is of great value especially in identification and differentiation of *Plasmodium* species, despite a need for a minimum of 200–300 oil immersion fields to be examined before reporting the smear as negative, thus making it a highly time-consuming and tedious technique.

The QBC technique was found to be more rapid than peripheral blood film examination as centrifugation causes the malarial parasites to concentrate to a small compact zone below the buffy coat layer. This increases the speed and ease of interpretation especially in the case of low parasitaemia.⁵

Results are possible in just 8 (minimum time) to 15 minutes (maximum time) which is a fraction of the time required for of thick film methods. Training time

Table 3 Comparison of routine QBC with modified QBC (95% confidence interval) considering PBS microscopy as gold standard [sensitivity, specificity, LR (+), and LR (–)]

Parameters	Routine QBC	Routine QBC	Modified QBC	Modified QBC
	Vivax arm	Falciparum arm	Vivax arm	Falciparum arm
	N=22	N=23	N=22	N=23
Sensitivity (%)	91 (75–98)	91 (76–98)	91 (76–95)	91 (76–98)
Specificity (%)	96 (89–99)	94 (87–97)	98 (92–99.9)	96 (89–99)
Likelihood ratio positive	23	16	46	23
Likelihood ratio negative	0.094	0.092	0.092	0.090

Note: The McNemars Chi-square one-sided test applied for sensitivity for *P. vivax* and *P. falciparum* for non-inferiority of modified QBC to routine QBC, both compared to PBS as the gold standard. It is not found to be significant. The *P* value equals 1.00.

The McNemars Chi-square one-sided test applied for specificity for *P. vivax* and *P. falciparum* for non-inferiority of modified QBC to routine QBC both compared to PBS as the gold standard. It is not found to be significant. *P* values for *P. vivax* and *P. falciparum* are 0.93 and 0.91, respectively.

Table 4 Comparison of cost per test of PBS microscopy, routine QBC, and modified QBC in a malaria clinic with a large patient load

	Microscopy	QBC	Modified QBC
Chemicals			
Chemicals: total Giemsa staining	Rs 1.80	...	...
Immersion oil for microscopy	Rs 0.65/–	Rs 1.29/–	Rs 1.29/–
Glass slides/QBC tubes/lancets	Rs 1.60	Rs 113	Rs 113
One time equipment purchase	45 000/–	401 625/–	1,34000/–
	(light microscope)	(paralens + parafuge)	(UV microscope + centrifuge)
Cost of equipment per test	Rs 2.5	Rs 22.81	Rs 7.44
Labour salary	Rs 12	Rs 0.79	Rs 0.79
Total cost	Rs 18.55	Rs 137.89	Rs 122.52

Note: Cost has been calculated for a government laboratory where patient load is extremely high due to negligible consultation charges. The patient load is 18 000 patients per year. The one time equipment cost has also been considered for calculation per test.

Table 5 Comparison of cost per test of PBS microscopy, routine QBC, and modified QBC in smaller pathological laboratories with a lower patient load

	Microscopy	QBC	Modified QBC
Chemicals			
Chemicals: total Giemsa staining	Rs 1.80	...	...
Immersion oil for microscopy	Rs 0.65/-	Rs 1.29/-	Rs 1.29/-
Glass slides/QBC tubes/lancets	Rs 1.60	Rs 113	Rs 113
One time equipment purchase	45 000/- (Light microscope)	401 625/- (paralens + parafuge)	134 000/- (UV microscope + centrifuge)
Cost of equipment per test	Rs 36.35	Rs 324.41	Rs 108.24
Labour salary	Rs 96.93	Rs 77.66	Rs 77.66
Total cost	Rs 137.33	Rs 516.36	Rs 300.19

Note: Cost has been calculated for a pathological laboratory where patient load is low. The patient load is 1238 patients per year. The one time equipment cost has also been considered for calculation per test.

is also reduced, as users with 3–5 days of training can produce results comparable to an experienced microscopist using thick film. Centrifugation makes parasites easier to locate within the tube. The reason why one patient who was negative by PBS microscopy on day 1 was found to be positive for *P. falciparum* by both routine QBC and modified QBC may be due to the fact that routine QBC and modified QBC test concentrates 55–65 µl blood samples, allowing users to quickly and easily see a far greater number of parasites than Giemsa test. Modified QBC holds promise as a good alternative to Giemsa-stained PBS due to its speed, sensitivity, and specificity as routine QBC especially where the patient load is extremely high.

Considering the cost per test over a 1-year study (Table 4) in a malaria clinic with a large patient load, the cost difference of routine QBC and modified QBC was found to be 13%. However, in the smaller laboratories with a lower patient load, the cost difference of QBC and modified QBC method becomes significant (48%) (Table 5). It was found that the modified QBC is more cost-effective as compared to the routine QBC method.

The sensitivity and specificity of modified QBC are also not compromised.

Although PBS examination is associated with lower costs in a resource poor and endemic country like India, the expert microscopists are now rare and difficult to find. PBS is also highly time-consuming. Given the above consideration, modified QBC method is more rapid than PBS microscopy. It is non-inferior and as sensitive as routine QBC. It is also more cost-effective than routine QBC. Therefore modified QBC could be the method of choice for laboratory setting in India.

In modified QBC technique, experience and expertise of microscopist do not effect the investigation critically and the cost is reduced relative to

routine QBC. This decrease in cost could make a difference in a developing country like India which has a large sample load and lesser financial capacity.

The present pilot study would give the lead for further studies for evaluation of modified QBC in malaria clinics.

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