Newer versus Conventional Methods in the Diagnosis of Malaria : A Comparison

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Abstract

Background: This study attempts to evaluate and compare the efficacy of polymerase chain reaction (PCR) and quantitative buffy coat (QBC) assay with conventional Giemsa stained peripheral blood smear (PBS) examination in the diagnosis of malaria.

Methods: The study was conducted on 50 cases of smear positive malaria (group 1), 50 cases of clinically suspected malaria (group 2) and 15 healthy controls. All were subjected to Giemsa stain slide examination both thick and thin smear, QBC assay and PCR. PBS examination by Giemsa stain was taken as gold standard.

Result: In this study the overall sensitivity and positive predictive value (PPV) of QBC assay in group 1 was 100% and that of PCR was 60% and 100% respectively. In group 2 the sensitivity, specificity, PPV and NPV of QBC assay was 100% and that of PCR was 71%, 100%, 100% and 73% respectively as compared to the gold standard. All the 15 healthy controls were negative by all the three assays showing 100% specificity.

Conclusion: QBC assay was an excellent alternative to the conventional method as it is rapid and less time consuming and can directly demonstrate the parasite. Utility of PCR lies in species-specific diagnosis of falciparum malaria especially when there is a high degree of clinical suspicion and the report is negative by the other two methods.

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Introduction

Malaria continues to be one of the largest contributors of disease burden in terms of death and suffering. WHO data reveal approximately 300-500 million clinical cases of malaria with 1.5-2.7 million deaths annually. It is endemic in 91 countries, with 40% of world population at risk [1].

With the increasing morbidity and mortality resulting from malaria and with a view to reduce the same, it becomes imperative that a rapid and correct diagnosis be made, to initiate prompt treatment in clinically suspected cases. The Giemsa stained peripheral blood smear remains the gold standard. It has the time-honoured advantage of low cost and wide applicability, even in a small laboratory set up. However, it is time consuming and less sensitive particularly at low levels of parasitaemia [2].

Presently the rapid immunochromatographic methods such as those based on the parasite specific lactate dehydrogenase or pan-malarial plasmodium aldolase antigen have taken priority over microscopy due to ease of performance and the rapidity of the results. The major disadvantage of these tests is the variable sensitivity depending on the level of parasitaemia [3]. The

indisputable fact still stays that direct demonstration of the parasite will always remain a gold standard. This study attempts to evaluate quantitative buffy coat (QBC) assay, which is a rapid method of direct demonstration of the parasite and compare it with the gold standard. The study also evaluates the utility of polymerase chain reaction (PCR) in the diagnosis of malaria especially in those cases where there is a high degree of clinical suspicion and the direct demonstration of the parasite by QBC assay and PBS is negative.

Material and Methods

The study was carried out at the Department of Microbiology, Armed Forces Medical College (AFMC), Pune (India) for a period of two years from March 2000 to February 2002. The patients comprising the study group were taken from a group of tertiary care hospitals. Sample size consisted of 100 cases. Group 1 consisted of 50 cases of smear positive malaria and group 2 consisted 50 cases of clinically suspected malaria. PBS and QBC examination was done from a finger prick blood sample and 2 ml of blood was also collected by veni-puncture from the above group in sterilised ethylene diamine tetra acetate (EDTA) vials for PCR. Fifteen blood samples from healthy blood donors were used as negative controls for all the three assays.

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Thick and thin smears were prepared and stained with Giemsa stain. All the smears were air dried, fixed in methanol and stained for 15-30 minutes. Both the smears were examined for a minimum of 200 oil immersion fields before declaring a sample negative.

QBC examination was done simultaneously using QBC tube. Blood sample (55-65 µl) was drawn into the QBC tube (Beckton Dickinson Franklin Lake, N.J.) by the finger prick method. This assay combines an acridine orange coated capillary tube and an internal float that separates layers of white blood cell (WBC) and platelets following centrifugation. The haematocrit tube is centrifuged at 12,000g for 5-7 minutes and examined under the fluorescent microscope. Parasites concentrate to a thin zone between the red blood cell (RBC) and the granulocyte layer of the capillary blood column (Fig. 1). Parasites can be viewed through the capillary tube using special long focal length objective (60 X paralens). The parasite deoxyribose nucleic acid (DNA) takes up bright apple green stain while RBC do not take any stain, thereby enhancing the contrast which makes the parasites easily visible thus resulting in great reduction in examination time.

The blood samples for PCR were centrifuged at 1000 g for five minutes and plasma was discarded and cells were separately stored at -20°C. The stored samples were subsequently thawed and the DNA extraction and PCR assay was carried out as described by Barker and Snounou et al [4,5]. Modifications of this method has also been used by other researchers worldwide and is still the benchmark in the diagnosis of malaria by PCR [6-8]. DNA for positive control was procured from National Center of Cell Sciences (NCCS), Pune.

After parasite DNA extraction, PCR amplification was performed to amplify species-specific sequences of the small

subunit of the 18s ribosomal ribonucleic acid (ssrRNA) of merozoite surface protein of *Plasmodium vivax* and *Plasmodium falciparum* by incubating in the thermocycler for 35 cycles of 95°C for 30 seconds, 50°C for 120 seconds and 72°C for 180 seconds and a final extension temperature of 72°C for 7 minutes. The 5' primer and the 3' primer sequences used for *P. vivax* and *P. falciparum* were 5'-GGGAATTCTACTTGATGGTCTCTC-3', 5'-GAATTCCTTGACATGCGTAAGCG-3' and 5'-GAAGATGCAGTATTGACAGG-3', 5'-GAGTTCTTAATAGTGAACAAG-3' respectively. The amplified product was resolved by 2% agarose gel electrophoresis. The gel was stained with ethidium bromide for visual detection by UV transillumination. A 100 base pair molecular ladder was used. The gel was observed in gel documentation system under 260-280nm ultraviolet (UV) light (Fig. 2).

Sensitivity of QBC assay and PCR was assessed with PBS examination as the gold standard. Specificity was calculated on the basis of the results of the smear negative healthy controls and smear negative patients in group 2. The 95% confidence interval (CI₉₅) was calculated using the modified Wald method to indicate the statistical significance of an estimate.

Results

In group 1, out of the 50 smear positive cases, 35 (70%, (CI₉₅) 56.1-80.9%) were positive for *P. falciparum* and 15 (30%, (CI₉₅) 19-43.8%) were positive for *P. vivax*. The QBC examination results for group 1 was identical to those seen by Giemsa stained PBS as shown in Fig. 3. All the 50 slide positive cases of group 1, the 50 clinically suspected cases of malaria in group 2 and all the 15 samples of healthy blood donors negative by both PBS examination and QBC assay were evaluated by species specific PCR for the diagnosis of *P. falciparum* (PCR-Pf) and *P. vivax* (PCR-Pv). Of the 50 cases of group 1, 30 (60%, (CI₉₅) 46.1-72.4%) were positive by PCR-Pf and no positivity was obtained for PCR-Pv. As compared to Giemsa, PCR was unable to detect 20 slide positive cases (15 of *P. vivax* and 5 of *P. falciparum*) in group 1. All the 15 healthy controls were negative by PCR. In this Group, the results obtained by QBC assay were identical to those obtained by Giemsa. The overall sensitivity and positive

Fig. 1: Microcentrifugation tube with blood column after centrifugation for QBC assay.

Fig. 2: Detection of *P. falciparum* using MSP-1 primers.

Fig. 3 : Comparison of smear positive cases results of group 1 (n=50).

predictive value (PPV) of QBC assay as compared to Giemsa stained PBS was 100% and that of PCR was 60% and 100% respectively. Individually, the sensitivity and PPV of PCR for slide positive *P.falciparum* cases was 85% and 100% respectively (Table1).

However in group 2, of the 50 clinically suspected cases of malaria 28 (56%, (CI₉₅) 42.3 -68.8%) were positive for malarial parasites. Of these 20 were positive for *P.falciparum*, 6 were positive for *P.vivax*, and two had a mixed infection by Giemsa stained PBS examination as shown in Fig.4. The QBC assay showed positivity in 30 cases (60%, (CI₉₅) 46.1-72.4%) of which 22 were positive for *P.falciparum*, four for *P.vivax* and four showed mixed infection with both the species (Fig. 4). QBC picked up four new cases, two of *P.falciparum* and two of mixed infections that were negative by Giemsa stained PBS, but failed to pick up two cases of *P.vivax* which were positive by PBS examination. PCR was positive for 20 samples (40%, (CI₉₅) 27.5-57.8%) in this group all for *P.falciparum*, 18 were those that were positive both by Giemsa and QBC assay. PCR failed to pick up two cases of *P.falciparum* that were slide and QBC assay positive but detected two new cases that were negative by both Giemsa and QBC assay. One was a case of cerebral malaria and the other a case of suspected blackwater fever. Sensitivity, specificity, PPV and NPV of QBC assay for individual infections is reflected in Table 1.

All the 15 samples taken from healthy blood donors were negative for malaria by all the three assays. The overall sensitivity, specificity, PPV and NPV of QBC assay and PCR as compared to the gold standard in group 2 is shown in Table 1.

Discussion

The results thus obtained were analysed and a comparative evaluation was done taking peripheral blood smear as the "Gold Standard". Microscopy has historically been the mainstay of malaria diagnosis and continues to be the gold standard. However the main disadvantage is its poor results in case of low parasitaemia. It is difficult to interpret thick blood films when the parasitaemia is less than 500/ μ l of blood [2]. This could be attributed to the lower detection rate of Giemsa stained PBS as it missed two cases of

Fig. 4 : Comparison of clinical cases results of group 2 (n=50).

Table 1

Comparative evaluation of sensitivity, specificity, PPV & NPV of group 1 & 2

	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
QBC-overall (group 1)	100	*	100	*
QBC-overall (group 2)	100	100	100	100
QBC- Pf (group 2)	100	91.6	90	100
QBC- Pv (group 2)	66	100	100	91.6
QBC-mixed (group 2)	100	91.6	50	100
PCR-overall (group 1)	60	*	100	0
PCR-Pf (group 1)	85	*	100	0
PCR (overall group 2)	71.4	100	100	73
PCR-Pf (group 2)	90	91.6	90	91.6

* Specificity and NPV of group 1 cannot be calculated as this group had all smear positive cases.

P.falciparum and two of mixed infection in comparison to QBC assay in group 2 (Fig. 4). Since Giemsa stained PBS was the gold standard, the specificity of QBC-Pf and QBC-mixed came down to 91.6% (Table1). However Giemsa stained PBS had an edge over the other two assays in detecting *P.vivax* infection especially in the study group 2 where the sensitivity of QBC assay for detecting this infection was only 66% as it failed to detect two cases. Thin smear is of great value especially in identification of Plasmodium species but a minimum of 200-300 oil immersion fields should be examined before reporting the smear as negative, thus making it a time consuming assay [9].

The QBC assay is more sensitive and rapid than peripheral blood film examination. This assay concentrates malarial parasites to a small compact zone below the buffy coat layer. This increases the speed and ease of interpretation especially in case of low parasitaemia [9]. Probably this could be the reason that four slide negative cases, one a case of idiopathic thrombocytopenic purpura (ITP), second a case of malaria on quinine therapy both positive for *P.falciparum* and two of mixed infections with massive splenomegaly, were positive by QBC assay. In the present study gametocytes and ring stage of

Fig. 5 : Gametocyte of *P.falciparum* by QBC assay.

Fig. 6 : Ring stage of *P.falciparum* by QBC assay.

P.falciparum and early trophozoite stage of *P.vivax* were detected with ease as seen in Figs. 5-7.

The two samples not detectable by QBC but positive by PBS examination were both of *P.vivax* infection. It is known that the specific gravity of late trophozoites of *P.vivax* are similar to the leucocytic layer of the buffy coat, hence these get obscured in the granulocytic layer following centrifugation [10]. They are difficult to identify as they become compact and lose their amoeboid shape during centrifugation [10,11]. Despite the need of a fluorescent microscope and a higher cost of installation and the expertise needed to detect the parasite, it holds promise as a good alternative to Giemsa stained PBS due to its speed and sensitivity [12].

It is claimed that PCR is capable of detecting a parasitaemia of less than 0.00002% when used in the best possible conditions and theoretically capable of detecting a single parasite in 20 l of blood sample which is hundred times better than a thick film [6], but in reality it is seldom achieved [2,13]. This was seen in this study with an overall sensitivity of 60% in group 1 and 71% in group 2 and 64 to 82.35% in other studies as well [13]. This is due to the presence of inhibitors of PCR. The nature of these inhibitors is poorly understood, but they

Fig. 7 : Ring stage of *P.vivax* by QBC assay and Giemsa.

consist of both extrinsic and intrinsic factors. Intrinsic factors include intracellular substances such as porphyrin ring of haem, which is thought to bind to enzyme Taq polymerase. The extrinsic factors include anticoagulants such as heparin and chelating agents like EDTA, which cause degradation of target DNA. The inhibitory concentration of heparin in PCR is 0.5 U/ml, which is a hundred fold less than the concentration normally used to prevent blood coagulation [2]. Though PCR is labour intensive and requires high level of expertise and standardisation, it holds promise as a good alternative in a clinical setting where there is a high degree of clinical suspicion and all the commonly diagnostic results are inconclusive as was seen in this study. Though PCR was negative for all *P.vivax* infections both in group 1 and 2, its sensitivity for *P.falciparum* in group 1 and 2 were 85% and 90% respectively (Table1). PCR was able to pick up two cases in group 2 that were negative by both PBS examination and QBC assay. The two cases were of complicated malaria, one clinically diagnosed as cerebral malaria and the other blackwater fever. In view of a high degree of clinical suspicion and 100% specificity of PCR, as was evident from the control group, the proposition of false positivity in these two cases was ruled out. Moreover, though a high mortality rate is associated with these complications one of them

survived with early initiation of quinine therapy after the clinical diagnosis was confirmed by PCR. Excessive haemolysis of RBCs and sequestration of the malarial parasites into the capillaries of the internal organs in falciparum malaria, often interfere with the direct detection of parasite both by microscopy and QBC assay [9,14]. This could be the reason why Giemsa stained PBS examination, though considered the gold standard in the diagnosis of malaria, could not pick up these two cases.

PCR was negative for *P.vivax* infection in both group 1 and 2. The common reasons of a negative PCR often seen in other studies are inaccessibility of target DNA due to non-annealing of primers possibly due to strain variation by geographic difference in parasite populations, loss of target DNA during extraction and interference during amplification due to presence of inhibitors in the samples [15]. Probably it could also be due to stochastic sampling variations.

PBS examination is the universal choice especially in a resource poor setting and an endemic country like ours. It also has an advantage of better species differentiation than the other two assays. QBC assay with its high sensitivity, ease and speed of detection can be a good alternative to smear examination and can be the test of choice in tertiary centres with a good sample load. Utility of PCR lies only in species-specific diagnosis of falciparum malaria. With the availability of in vitro diagnostic (IVD) approved commercial PCR kits for detection of malaria and the advent of Real time PCR platform, the sensitivity of this assay has remarkably increased but the cost per test and turnaround time limits its use as a routine diagnostic procedure. It should be used in addition to microscopy to improve the diagnostic algorithm in selected situations like unresolved cases with high clinical suspicion and help expedite the initiation of a definitive treatment and reduce the complications associated with them.

Limitations of this study are small sample size and tests not being validated in actual field conditions. To elicit validity of the various tests (sensitivity, specificity, PPV and NPV), the diagnostic tests should be run in actual outpatients in hospital and not limited to only smear positive cases as has been done in group I in the study. It is hoped that the present pilot study would give the lead for further studies for evaluation of QBC in actual field conditions and with larger sample sizes.

Conflicts of Interest

None identified

Intellectual Contribution of Authors

Study Concept : Col Y Chandra

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