Original Article

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Rapid Laboratory Techniques in Diagnosis of Malaria in a Tertiary Care Hospital, Chennai

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Abstract

Introduction: The resurgence of malaria has renewed interest in developing not only preventive measures, but also rapid diagnostic techniques. Several methods have been developed to supplement and replace the conventional microscopic method.

Purpose: To study the efficacy and accuracy of rapid laboratory techniques in the diagnosis of malaria.

Methods: A cross-sectional study of 230 samples of which 190 from clinical malaria cases, 20 samples each from disease and healthy population. 190 samples from clinical malaria cases were tested by peripheral blood smear (PBS) and quantitative buffy coat (QBC). Among 190 samples, 61 were selected by the simple random method and tested by recent diagnostic tests such as immunochromatography, dot-enzyme immunoassay (ELISA) for *Plasmodium falciparum* and polymerase chain reaction (PCR).

Results: All the 190 clinical malaria cases were tested by Jaswant Singh Battacharya stained PBS and QBC. They have shown 29.4% and 57.3% positivity, respectively, for malarial parasite. Out of 61 randomly selected samples, 32 (52.4%) cases (*Plasmodium vivax* 29, *P. falciparum* 3) were positive immunochromatography, whereas 31 (*P. vivax* 28, *P. falciparum* 3) 50.8% cases were positive by PCR assay. Through, dot-ELISA, only three samples were detected of *P. falciparum*. Those samples were also detected by immunochromatography and PCR. None of the control samples were positive for malarial parasite. Through the analysis of all samples, it was observed that predominant species are causing malarial infection to be *P. vivax*.

Conclusion: PBS is a simple and inexpensive test for detection of the malarial parasite, while QBC assay was found to be more sensitive. The recent techniques of immunochromatography, DOT-enzyme immunoassay for *P. falciparum* and PCR are found to be more sensitive, specific, and accurate enough to identify the *Plasmodium* species.

Key words: Gold standard, Immunochromatography, Malaria, Microscopy, Polymerase chain reaction, Rapid techniques

INTRODUCTION

Malaria presents a diagnostic challenge to the medical community worldwide and has always been a major public health concern, probably representing the most important parasitic disease in human.[1] Human malaria is basically a febrile illness caused by species belonging to the genus *Plasmodium* and is transmitted by the bite of infected female *Anopheles* mosquitoes, which breeds in fresh water.[2] The species that infect humans are *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium ovale*, *Plasmodium malariae*, and *Plasmodium knowlesi*. A heavy burden on tropical communities poses a threat to non-endemic countries and a danger to travelers.[3] Malaria remains uncontrolled to date due to various reasons viz. Emergence of drug resistant parasite, pesticide resistant mosquito vector, and non-availability of suitable and effective malarial vaccine.[4] Malaria rapid diagnostic devices (MRDD) have been developed with the hope that they would offer accurate, reliable, rapid, cheap, and easily available alternative to traditional methods of diagnosis.[5] Assay for rapid diagnosis has the potential to enhance diagnostic capabilities in those instances in which skilled microscopy is not readily available.[6] Conventional peripheral blood smear (PBS)

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examination for a demonstration of malarial parasite remains the "gold standard" for diagnosis of malaria.[7] The quantitative buffy coat (QBC), Dot-enzyme immunoassay (ELISA) for *P. falciparum*, immunochromatographic test (ICT), and polymerase chain reaction (PCR) have been evaluated here, particularly to overcome the disadvantages of conventional PBS study. Rapid, accurate diagnosis is fundamental to effective management and control of malaria. Modern methods of malaria diagnosis include fluorescent microscopy, flow cytometry, automated blood cell analysis, serology-antibody detection, molecular methods and laser desorption mass spectrometry, immunochromatographic assays detect malarial antigen Histidine-Rich Protein 2 (HRP-2) and enzymes parasite lactate dehydrogenase (pLDH), aldolase, and PCR. All these new technologies are compared with the accepted "gold standard" method.[6] The present study highlighted the efficacy and accuracy of recent techniques to diagnose malaria, also to meet the need for a reliable diagnostic adjunct to microscopy in diagnosing malaria with a low level of parasitemia.

MATERIALS AND METHODS

The study design was a cross-sectional study.

The study group includes:

1. 190 patients with clinically diagnosed as malaria
2. Patients diagnosed as enteric fever, 20 in number - disease control
3. Apparently healthy individuals, 20 in number - healthy control.

A total of 230 blood samples was collected, of these 190 samples were from clinically diagnosed as malaria patients with symptoms of fever and chills irrespective of age and sex. 20 blood samples each were from patients diagnosed with enteric fever (disease control) and from healthy individuals (healthy control). The criteria for the selection of healthy control were the history of absence of fever for a period of one month prior to the study. Patients already on antimalarial drugs were excluded from the study. 190 samples of clinically diagnosed as malaria and control group were tested by PBS and QBC. 61 random samples were tested by recent methods such as ICT for antigen detection (HRP-2 and pLDH), Dot-ELISA for *P. falciparum* and PCR assay.

The data obtained were analyzed using SPSS statistical software. The proportions were calculated and the diagnostic performance was determined by calculating the test sensitivity, specificity and predictive values. Statistical significance was stated at the 5% level and 95% confidence interval. Ethical clearance for the study was obtained from the Institutional Ethical Committee. Under strict aseptic precautions, thick and thin PBS were obtained by finger prick, and 3-5 ml of blood samples was collected in a pre-sterilzed aliquot with anticoagulant (EDTA) by venipuncture before administration of anti-malarial drug. Samples were transported to the laboratory immediately. Smears were stained by Jaswant Singh Battacharya (JSB) technique. QBC. Assay was also carried out simultaneously. The remaining portion of the samples was aliquoted and stored at −20° for subsequent Dot-ELISA, ICT, and PCR assay.

PBS

Thick and thin PBS were prepared on a clean grease-free glass slide and examined under oil immersion.

QBC Assay

QBC was done using QBC malaria test kits provided by BD (Becton Dickinson) diagnostics. The QBC capillary tube was filled with about 50-60 µl of blood soon after the collection, centrifuged, and examined using paralens in the region between the red blood cell and granulocytes where parasites are most abundant.

ICT (Detection of HRP-2 and Pan Malarial Antigen [PMA])

The samples were tested for Pf HRP-2 and PMA of other species of malaria, according to manufacturer's instruction using the NOW ICT test kit.

Dot-ELISA for *P. faliciparum*

Pf HRP-protein against *P. falciparum* was detected by Dot-ELISA.

PCR

PCR was performed as per the method of Mullis and Falona (1987) and Saiki *et al.* using Qiagen Taq PCR core kit.

All the parameters of the tests were assessed with microscopic detection as the gold standard and recorded (Table 1).

RESULTS

"Of the 190 samples from patients with clinical malaria, 55 were positive for *P. vivax* and 1 was positive for *P. falciparum*

Table 1: Primers specific for *P. falciparum* and *P. vivax*

Primer	Primer sequence	Product size
Pf 1	5' AGA AAT AGA GTA AAA AAC AAT TTA 3'	918 bp
Pf 2	5' GTA ACT ATT CTA GGG GAA CTA 3'	
Pv 1	5' CCG AAT TCA GTC CCA CGT 3'	523 bp
Pv 2	5' GCT TCG GCT TGG AAG TCC 3'	

P. falciparum: Plasmodium falciparum, P. vivax: Plasmodium vivax

by thin PBS. In thick smear, 56 cases (29.4%) were positive for malarial parasite and its 95% confidence interval was 23-36%. Disease and healthy control ($n = 40$) were negative for malarial parasite by JSB staining method. Among the total of 190 clinically suspected cases, QBC assay detected the presence of the malarial parasite in 109 (57.3%) cases and its 95% confidence intervals is 50-64%. The control group was found to be QBC negative.

Table 2 shows the sensitivity of 100%, specificity of 69.5%, positive predictive value (PPV) of 51.3%, and negative predictive value (NPV) of 100% of QBC assay. By comparing the QBC assay and smear method, QBC assay shows higher positivity. Of the 61 cases, 32 (52.4%) were positive for malarial parasite (P.v. 29, P.f. 3) by ICT method and its 95% confidence interval was 40% to 65%. The control group was found to be negative by this technique.

Among the total 61 cases, only 3 cases were positive by Dot-ELISA for *P. falciparum* (DRDE). The control group was negative by this technique. Dot-ELISA for *P. vivax* was not done. Hence, this method was not included in the comparative study with PBS. 31 (51.8%) out of 61 cases were positive for malarial parasite (*P. vivax* 28, *P. falciparum* 3) by PCR technique and its 95% confidential interval was 38-63%. Control group was negative by PCR method. The sensitivity, specificity, PPV, and NPV of PCR assay were calculated against the gold standard of PBS (Tables 3-5).

Out of 190 cases tested with different techniques in detecting malarial parasite, the predominant species identified was *P. vivax* (Table 6).

Table 2: Comparison between JSB stained PBS and QBC

QBC assay	JSB stained PBS		Total
	Positive	Negative	
Positive	56	53	109
Negative	0	121	121
Total	56	174	230

McNemar test $P<0.0001$, Kappa agreement value 0.526. JSB: Jaswant Singh Battacharya, QBC: Quantitative buffy coat, PBS: Peripheral blood smear

Table 3: Comparison between JSB stained PBS and ICT

ICT	JSB Stained PBS		Total
	Positive	Negative	
Positive	20	12	32
Negative	1	68	69
Total	21	80	101

McNemar test $P=0.003$, Kappa agreement value 0.672, sensitivity 95.2%, specificity 85%, PPV 62.5%, and NPV 98.5%. ICT: Immunochromatographic test, JSB: Jaswant Singh Battacharya, PBS: Peripheral blood smear, PPV: Positive predictive value, NPV: Negative predictive value

DISCUSSION

The resurgence of malaria has renewed interest in developing not only preventive measures, but also rapid diagnostic techniques. Several methods have been developed to supplement and replace the conventional microscopic method. The most promising new malaria diagnostics are the QBC. Assay, Assay for detection of antigen HRP-2 and PMA by ICT, only HRP-2 by Dot-ELISA for *P. falciparum* (DRDE) and detection of specific nucleic acid sequences (P.f. 918 bp, P.v., 523 bp) by PCR. In this study, 190 clinically diagnosed as malaria patients were tested by PBS and QBC assay for presence of *P. vivax* and *P. falciparum* in blood. Out of 190 samples, 61 were selected by the simple random method and tested with recent techniques such as ICT, Dot-ELISA for *P. falciparum* and PCR. The control group was also tested with PBS, QBC, ICT, and Dot-ELISA for *P. falciparum* and PCR assay. The results obtained were as follows. Romanowsky stains (Giemsa's, Leishman's, Fields', and JSB) still appear superior in species identification. In the present study, malarial parasite was detected in 56 cases (P.v. 55, P.f. 1) by PBS

Table 4: Comparison between PCR technique and JSB stained PBS

PCR	JSB stained PBS		Total
	Positive	Negative	
Positive	19	12	31
Negative	2	68	70
Total	21	80	101

McNemar test $P=0.013$, Kappa agreement value 0.584, sensitivity 90.5%, specificity 85%, PPV 61.3%, NPV 97.1%. JSB: Jaswant Singh Battacharya, PBS: Peripheral blood smear, PPV: Positive predictive value, NPV: Negative predictive value, PCR: Polymerase chain reaction

Table 5: Evaluation of recent techniques in diagnosis of malaria

Test	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
QBC	100	65.5	51.3	100
ICT	95.2	85	62.5	98.5
PCR	90.5	85	61.3	97.1

PPV: Positive predictive value, NPV: Negative predictive value, PCR: Polymerase chain reaction, ICT: Immunochromatographic test, QBC: Quantitative buffy coat

Table 6: Positivity of malarial parasite by various techniques

Test	Number of sample	Positive (%)	*P. vivax*	*P. falciparum*
PBS	190	29.4	55	1
QBC	190	57.3	-	-
ICT	61	52.4	29	3
PCR	61	50.8	28	3
Dot-ELISA for P.f.	61	-	-	3

PCR: Polymerase chain reaction, ICT: Immunochromatographic test, QBC: Quantitative buffy coat, PBS: Peripheral blood smear, *P. falciparum: Plasmodium falciparum, P. vivax: Plasmodium vivax*, ELISA: Enzyme immunoassay

(29.4%). Various studies from different areas have reported different positivity rate among the studied population 3.07%, 10.5%, 20%, 31%, and 42.1%, respectively.[8-12] This could be due to multiple factors including training and skills, maintenance, slide preparation techniques, workload, condition of microscope, and quality of essential laboratory supplies. This variability combined with the risk of untreated malaria in the face of safe, inexpensive therapy in the past led clinicians to treat febrile patients without regard to the laboratory results.[13-15] In our study, two blood samples which were detected positive for *P. falciparum* through ICT, Dot-ELISA and PCR assay, were found to be negative in the blood smear examination. This may be explained through the fact that *P. falciparum* can sometimes sequester and may not be present in circulating blood. Stained PBS examination is simple and inexpensive. Parasite stages and species could be identified accurately. The main disadvantages are it takes more time and requires skilled personnel. It may also give poor results with low parasitemia.[5,16] Still the staining procedures are used for screening purpose. QBC method is based on fluorescent staining of the blood cells and parasites. In the present study, QBC assay detected 57.3% cases out of 190 clinically suspected cases, showed 100% sensitivity, 65.5% specificity, PPV 51.3%, and NPV 100%. Moreover, detected more number of positive cases, i.e., 109 (57.3%) than peripheral smear 56 (29.4%) which is consistent with Singh *et al.*,[17] Pinto *et al.*,[9] and Krishna *et al.*[8] Compared to peripheral smear, QBC was found to be 100% sensitive as it was able to detect additional 53 cases which were negative on peripheral smear. All the blood samples which were negative by QBC were also negative by peripheral smear. This is in agreement with Bhandari *et al.*[18] who had 100% sensitivity with QBC. QBC is of great importance in peripheral smear negative cases and should be preferably used as a final diagnostic test and not as a screening test or first line investigation considering its high cost and tend to report false positives.[18] Compared to Leishman stained thick and thin film, sensitivity of QBC assay varies from 97% to 100%. The QBC assay would be ideal to supplement the stained blood film in both clinical and epidemiological studies. The limiting factors for QBC assay are the cost of the microscope, special accessories, and need for adequate training and expertise.[7] The QBC tubes do not remain readable for more than a few days and hence are not suitable for record purposes.[8] The important factor of false positive impression of malarial parasite in QBC system should be considered here. In our study, 8 cases of QBC positive were found negative by PBS, ICT, and PCR. This could be Howell – Jolly bodies, artifacts such as cell debris and bacterial contamination may possibly give false positive results.[8,19] One of the serological method, ICT based on the use of HRP-2 antigen to detect *P. falciparum* infection and a PMA to detect *P. vivax* and infection with other species of malaria. In the present study, we evaluated the performance of ICT Malaria P.f/P.v, P.m and P.o., and Dot-ELISA for *P. falciparum* (DRDE) based on HRP-2 antigen. Only 32 cases (P.v. 29, P.f. 3) were positive by Now ICT method (52.4%) out of 61 cases; showed the sensitivity of 95.2% and specificity of 85% with positive and negative predictive values of 62.5% and 98.5%, respectively. This is in accordance with findings of Tjitra *et al.* by ICT malaria.[20] Antigen detection test was superior to peripheral smear in our study, especially for malignant tertian malaria as it could detect 2 Pf cases which were negative by peripheral smear. This could be due to the persistence of HRP-2 following clearance of *P. falciparum* or due to sequestration *P. falciparum*. The study by Forney *et al.*[12] also reported a sensitivity of 87% and specificity of 87% for *P. vivax* and sensitivity of 100% and specificity of 93% for *P. falciparum* by the parasight F+V assay. The sensitivity of the test increases with increase in parasite density and it is in relation to the observations by Iqbal *et al.*[11] and Rajendran *et al.*[21] Only 3 cases of *P. falciparum* were detected by Dot-ELISA (DRDE) which were also detected by ICT and PCR assay. Thereby indicating 100% efficiency in detection of *P. falciparum* cases. We should consider the merits and demerits of ICT compared to the "gold standard." ICT assay is rapid and no labor-intensive.[22] It could be a useful adjunct to blood film microscopy. Moreover, it might permit a reduction in the duration of hospitalization and give an early warning of treatment failure.[23] Furthermore advantage of ICT assay is speciation and can also be used to indicate drug resistant infection.[24] The demerits of ICT assay are false positivity due to persistence of HRP-2 and PLDH antigenemia after antimalarial therapy.[25] Factors that may contribute to these diverse findings include test kit storage conditions in the field, inadequate adherence to the test protocol, or levels of parasitemia below the detection limit.[10] In our study, one case of *P. vivax* detected by PBS and QBC were not detected by ICT. This may be due to insufficient enzyme production, which occurs during an early malarial infection or the patient's blood sample contained parasites at a concentration below the detection level.[26] There are many published studies showing the improved sensitivity and specificity of PCR-based assays over microscopic and immunochromatographic diagnosis of malaria.[27] In the present study, we also demonstrated the performance of PCR assay, which detected malarial parasite in 31 (50%) cases (28 P.v. 3 P.f.) sensitivity of 90.5% and specificity of 85%, PPV of 61.3%, and NPV of 97.5% correlates well the studies of Barman *et al.*, Kathy *et al.*, and Long *et al.*[19,27,28] In our study 2 cases, of both JSB stained PBS and QBC assay positive, were revealed negative by PCR assay (False negative). The false negative result in PCR could be due to the failure of amplification of target DNA. The failure to amplify the target amplicon could be due to a low copy number of the target sequence to the primer.

False positive results in PCR could also be due to carryover of parasite – DNA during sample processing or lower sensitivity of the designed primer and PCR method itself.[19] Despite the advantages of PCR, it is unlikely to be useful outside of well-equipped laboratories where a reliable source of electricity and expensive equipment are not available. These limitations exclude PCR from consideration as a field – ready, rapid diagnostic test for malaria.[29] Unfortunately, conventional PCR assays are technically demanding and time-consuming. Moreover, they are prone to carryover contamination during the manipulation of post-amplification products.[30] In the present study, the predominant species identified was *P vivax*. Our findings are close to Jivabhai *et al*.[31] who reported *P. vivax* 61.41% and *P. falciparum* 38.56%, but different from Karlekar *et al*.[32] who reported *P. vivax* 33.8% and *P. falciparum* 66.6% Idris *et al*.[33] from Pakistan reported prevalence of 72.47% for *P. vivax*, 24.1% *P. falciparum*, and 3.44% mixed species, which is similar to our findings. The difference in prevalence of *P. vivax* and *P. falciparum* in different areas can be due to the presence of endemicity of particular type and higher relapses in vivax type.

CONCLUSION

In the present study, we compared peripheral smear a known "gold standard" with a QBC, antigen detection assay, and PCR assay. The QBC is advantageous where workload is high, but it is costly and gives a false positive report. Antigen detection test is a useful device when microscopy is not available, and immediate clinical diagnosis is required, especially for *P. falciparum* cases which may develop cerebral complications. But it gives false positive results even after treatment. PBS is the simple and inexpensive test for detection of the malarial parasite, and the QBC assay was found to be more sensitive. The recent techniques of ICT, Dot-ELISA for *P. falciparum*, and PCR assay are found to be more sensitive, specific, and accurate enough to identify the *Plasmodium* species. In future, MRDDs will play an increasing role, where reliable microscopy has been frequently poor. The new generation of non-microscopic immune chromatography assay offers a practical chance to move the diagnosis of malaria away from the laboratory and nearer to the patient. New rapid, non-microscopy methods for the diagnosis of malaria that complement or support microscopy of blood films would be of great use in the early diagnosis and treatment of patients with malaria and in epidemiological studies.

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