

Original Research Article

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A Prospective Comparative Study of Peripheral Blood Smear, Modified Centrifuged Buffy Coat Smear and Rapid Malaria Antigen Detection Test in Diagnosing Malaria at a Tertiary Care Hospital in Western Maharashtra, India

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Malaria is a life-threatening parasitic disease & is a major public health problem. Considering the prevalence of malaria infection in India, there is an urgent need to look for tests that are simple, inexpensive and can be used to improve diagnosis, so that accurate treatment and management can be done. Present study was undertaken to compare and study the efficacy of peripheral blood smear (PBS), modified centrifuged buffy coat smear (CBCS) & rapid malaria antigen detection test (RDT) in malarial diagnosis. The study included all cases with fever for >24 hours with symptoms suggestive of malaria. Patients who came for follow up visit of an earlier episode of malaria or within 4 weeks of post treatment were excluded. For statistical analysis of results, RDT was used as gold standard. Highest number of cases were detected by RDTs (97.83%) followed by CBCS (91.30%). PBS was found to be a better test to diagnose malaria than RDT. CBCS were found to be of less utility in diagnosing malaria as compared to RDT. CBCS would be especially useful with negative or low parasitic index (PI) which may be missed by the conventional PBS. Hence, present study concludes that, the CBCS method can be adopted in the routine as it just needs an additional centrifugation procedure to the conventional method making PBS.

Introduction

Malaria is a life-threatening parasitic disease, transmitted to people through the bites of infected female *Anopheles* mosquitoes.

According to the latest WHO estimates, released in December 2016, there were 212 million cases of malaria in 2015 and 4,29,000 deaths.^{[1][2][3]} As per World Malaria Report

2016, India contributes for 89% of the total malaria incidence in the South-East Asia Region.^[4]

Infection with malaria parasites may result in symptoms from absent or very mild symptoms to severe disease and even death. Generally, malaria is a curable disease if diagnosed early and treated promptly.

Hence, a laboratory confirmation of the disease will help in the proper management of the causative microbiological agent. An optimal diagnosis of malaria requires the detection and identification of each *Plasmodium* species present in the patient's blood. Microscopic examination of PBSs as stained thick and/or thin blood smears is the standard method for malaria diagnosis, which is easily available and has low cost but its reliability is questionable at low level of parasitaemia.^[5]

Considering the prevalence of malaria infection in India, there is an urgent need to look for tests that are simple, inexpensive and can be used to improve diagnosis, so that accurate treatment and management can be done. Hence, the present study was undertaken to compare and study the efficacy of PBS, modified CBCS and RDTs for malaria diagnosis.

The main aim and objectives of this study includes, to compare peripheral blood smear, modified centrifuged buffy coat smear and rapid malaria antigen detection test in diagnosing Malaria.

Materials and Methods

The present study was prospective study, carried out at department of Microbiology of a tertiary care hospital over a period of one year from September 2016 to August 2017.

Patients presenting clinically with fever with chills and rigors and other symptoms suggestive of malaria for more than 24 hours were included in the study. Patients who came for follow up visit of an earlier episode of malaria or within 4 weeks of post treatment were excluded from the study. In the present study for statistical analysis of results, RDT was used as gold standard. A total number of 767 samples with a clinical suspicion of malaria received in Microbiology department

were included. A detailed history and clinical examination of the study cases were done after taking the consent. 3 ml of blood was collected from each of the patient in an EDTA bulb. Thick and thin smears were prepared, dried, fixed and were stained with field's stain (Figure I & II). The average time spent on screening each slide varied depending on parasite density. Thick smears were reported negative after examination of 200–300 oil immersion fields with no parasite. A thin smear was given negative when no parasites were observed in 200 oil immersion fields.^[6]

Secondly, RDT was performed. It was performed using commercially available antigen detection test kit (Accucare Malaria Pf/Pv Antigen Card Test, Lab care diagnostics (I) Pvt. Ltd, Gujarat, India) detecting *P. falciparum* HRP-2 and *P.vivax* pLDH malaria antigen in human blood as per manufacturer's instructions.

Interpretation of results of RDT: (Figure III)

- 1) *P. falciparum* Positive reaction - The presence of two color bands indicates a positive result for *P. falciparum*. The Pf HRP-2 present in the sample reacts with the pan anti pLDH conjugate and moves through the test strip where the Pf HRP-2 is captured by the anti-*P. falciparum* specific histidine rich protein-2 (Pf HRP- 2)
- 2) *P.vivax* or other *Plasmodium* species Positive reaction- The presence of two color bands indicates a positive result for *P.vivax* or other *plasmodium* species. The pLDH present in the sample reacts with the pan anti-pLDH conjugate and moves through the test strip where the pLDH is captured by pan specific anti pLDH.
- 3) The presence of three colour bands indicates a positive result for *P. falciparum* and *P.vivax* - The Pf HRP-2 present in the

sample reacts with the Pf HRP-2 conjugate and moves through the test strip where the Pf HRP-2 is captured by the anti-*P. falciparum* specific histidine rich protein-2 (Pf HRP-2). The pLDH present in the sample reacts with the pan anti-pLDH conjugate and moves through the test strip where the pLDH is captured by pan specific anti-pLDH.

4) Negative reaction- The presence of only one band within the result window indicates a negative result.

5) Invalid- test is invalid if the C line does not appear. If this occurs, the test should be repeated using a new cassette.

Thirdly, CBCS were prepared by using 3 ml blood collected in a wide bore 4 ml tube with EDTA which was centrifuged (2000– 3000 rpm for 15 min). The supernatant plasma was separated and layer of buffy coat and equal thickness of RBC layer just below was picked up to prepare smears which were stained by field's stain.

Examination of smears (CBCS)

Thick and thin smears were stained with field stain and were examined under oil immersion for presence of malaria parasite. At least 100 fields screened for not less than 5 minutes.

Morphological forms of malarial species observed by PBS and CBCS were noted along with corresponding Parasitic Index (PI).

PI was estimated by counting 1000 RBCs on thin smear.

Thin smears

The % of infected RBCs is determined by enumerating the number of infected RBCs in relation to the number of uninfected RBCs.

$\text{No. infected RBCs} \div \text{Total No. RBCs counted} \times 100 = \text{Per cent Infected RBCs}$

Multiple-infected RBCs are counted as one.

Gametocytes are not figured in calculations.

The degree of parasitaemia was graded as <2%, 2-10% and >10%

<2% parasitaemia – < 1, 00,000 parasites/ microliter

2-10% parasitaemia – 1, 00,000- 5, 00,000 parasites/ microliter

>10% parasitaemia – >5, 00,000 parasites/ microliter^[19]

Statistical analysis

Statistical analysis was performed with the software package: SPSS statistic 20 for Windows. The significance of difference of proportion of categorical variables among groups was examined by the chi-square test (large samples) and Fischer's exact test (small samples).

The difference in mean was examined by students t test. A value of P of ≤ 0.05 was considered significant for all statistical analyses and is marked with an asterisk.

Results and Discussion

Total 767 samples with a clinical suspicion of malaria were received, during the study period of September 2016 to August 2017. Of 767 cases, 92 patients were diagnosed as cases of malaria on testing positive for *plasmodium* species by any one or more of the diagnostic tests i.e. PS, CBCS and malaria antigen detection test.

Species wise distribution of malaria positive cases (Table 1)

Most commonly found species in both gender was *P.vivax*. The species-wise distribution of malaria cases in both genders was not found to be statistically significant. (P value- 0.800)

Comparison of morphological forms of *Plasmodium* species by microscopy (Table 2)

Various morphological forms of malarial parasite such as ring forms, gametocytes and schizonts are depicted in Figure I, II and III

Comparison of parasite forms in PBS and CBCS (Table 3)

% of various parasite forms observed on PBS and CBCS were not significantly different. (P value- 0.100)

Comparison between PBS, CBCS and RDT (Table 4)

Highest number of cases were detected by RDT (90, 97.83%) followed by CBCS examination (84, 91.30%), and PBS examination (74, 80.43 %), among which 73/92 (79.34%) were positive by all three

tests. 8/92 (8.7%) cases were detected exclusively by RDT while 9/92 (9.78%) cases were detected by both CBCS and RDT. CBCS detected 1 case which was negative by PBS and RDT while another one case was detected by PBS and CBCS.

Sensitivity, specificity and validity of PBS and CBCS in comparison to RDT (Table 5)

PBS was found to be a better test to diagnose malaria than RDT. CBCS was found to be of less utility in diagnosing malaria as compared to RDT.

Parasitaemia in microscopy confirmed malaria infections (Table 6)

In PBS, significantly higher % of *P.vivax* cases had parasitic index (PI) <2% as compared to *P. falciparum* and mixed infection.

In CBCS, significantly higher % of *P.vivax* cases had PI <2%, whereas significantly higher % of cases of *P. falciparum* had PI between 2-10%

In RDT, significantly higher % of *P.vivax* cases were detected at PI <2% as compared to *P. falciparum* and mixed infection.

Table.1 Species wise distribution of malaria positive cases

SEX	<i>P.falciparum</i>	<i>P.vivax</i>	Mixed	Total
Female	4 (13.8%)	21 (72.4%)	4 (13.8%)	29 (100%)
Male	7 (11.1%)	49 (77.8%)	7 (11.1%)	63 (100%)
Total	11 (12%)	70 (76%)	11 (12%)	92 (100%)

Table.2 Comparison of morphological forms of *Plasmodium* species by microscopy

Species	<i>P.falciparum</i> (n=11)			<i>P.vivax</i> (n=63)			Mixed (n=10)	
Morphological forms	Rings	Gameto cyte	Rings & Gameto cyte	Rings	Schizonts	Rings & Schizonts	Rings of both	Rings of <i>P.falciparum</i> & schizonts of <i>P.vivax</i>
No of cases	8 (72.73%)	2 (18.18%)	1 (9.09 %)	7 (11.11%)	48 (76.19%)	8 (12.7%)	8 (80%)	2 (20%)

Table.3 Comparison of parasite forms in PBS and CBCS

Species	Parasite form	PBS	CBCS
<i>P. falciparum</i>	Gametocyte of <i>P. falciparum</i>	2 (2.7%)	2 (2.4%)
	Rings and gametocyte of <i>P. falciparum</i>	1 (1.3%)	1 (1.2%)
	Rings of <i>P. falciparum</i>	8 (10.8%)	8 (9.5%)
<i>P.vivax</i>	Rings of <i>P.vivax</i>	5 (6.8%)	7 (8.3%)
	Scizonts of <i>P.vivax</i>	40 (54.1%)	48 (57.1%)
	Rings and scizonts of <i>P.vivax</i>	8 (10.8%)	8 (9.5%)
Mixed	Rings of <i>P. falciparum</i> and vivax	8 (10.8%)	8(9.5%)
	Rings of <i>P. falciparum</i> and scizonts of <i>P.vivax</i>	2 (2.7%)	2 (2.4%)
Total		74 (100.0%)	84 (100.0%)

Table.4 Comparison between PBS, CBCS and Antigen detection test

No.	PBS	CBCS	Antigen test	No. of samples	Interpretation
1	Negative	Negative	Negative	675 (88.01%)	PBS+CBCS+ RDT negative
2	Negative	Positive	Negative	1 (0.13%)	Only CBCS positive
3	Negative	Negative	Positive	8 (1.04%)	Only RDT positive
4	Negative	Positive	Positive	9 (1.17%)	CBCS + RDT positive
5	Positive	Negative	Negative	0	Only PBS positive
6	Positive	Positive	Negative	1 (0.13%)	PBS+CBCS positive
7	Positive	Negative	Positive	0	PBS + RDT positive
8	Positive	Positive	Positive	73 (9.52%)	PBS+CBCS+RDT positive
Total	74 (9.65%)	84 (10.95%)	90 (11.73%)	767 (100%)*	92 (11.99%)

(*) Total no of samples

Table.5 Sensitivity, specificity and validity of PBS and CBCS in comparison to RDT

Test	Sensitivity	Specificity	PPV	NPV
PBS	81.11	99.85	98.65	97.55
CBCS	91.11	99.71	97.62	98.83

Table.6 Parasitaemia in microscopy confirmed malaria infections

PI	PBS (n=74)			CBCS (n=84)			RDT (n=82)		
	P. Falci	P. vivax	Mixed	P. falci	P. vivax	Mixed	P. Falci	P. Vivax	Mixed
< 2%	4	44	6	0	40	3	4	52	6
2-10%	4	9	4	8	23	7	4	9	4
>10%	1	0	0	1	0	0	1	0	0
Gametocytes	2	0	0	2	0	0	2	0	0
Total	11	53	10	11	63	10	11	61	10
Chi square value	23.1			32.5			26.8		
P value	0.001*			<0.001*			<0.001*		

Excludes the 8 smear negative and antigen positive cases.

Fig.1 *Plasmodium falciparum* –A. RBCs showing ring forms (trophozoite) and banana shaped gametocytes. B. Accole form of *P. falciparum*

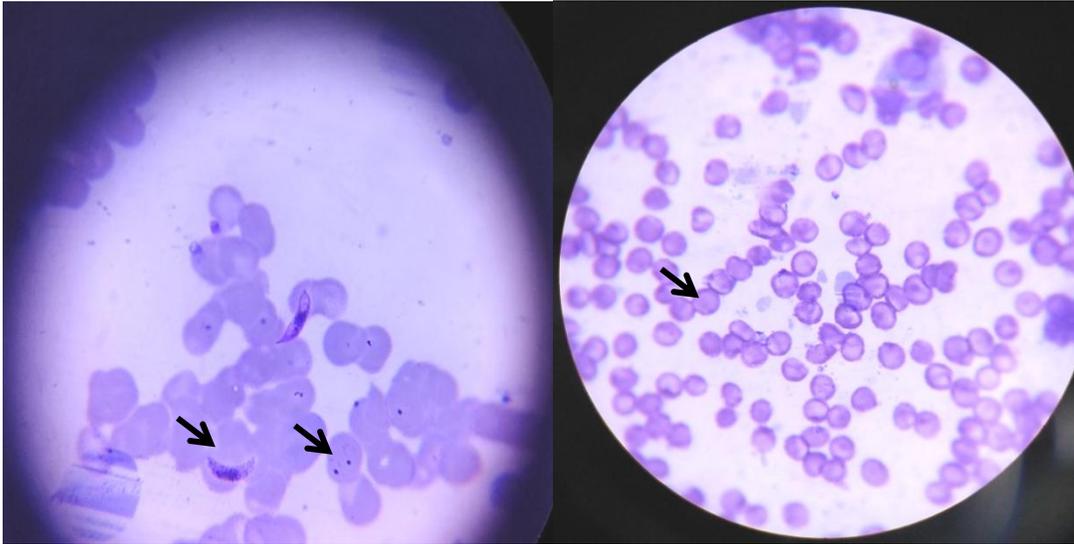


Fig.2 *Plasmodium vivax*- A. Infected RBCs are larger and showing amoeboid shaped schizonts containing merozoites. B. RBCs showing ring forms having thick cytoplasm with a single large chromatin dot

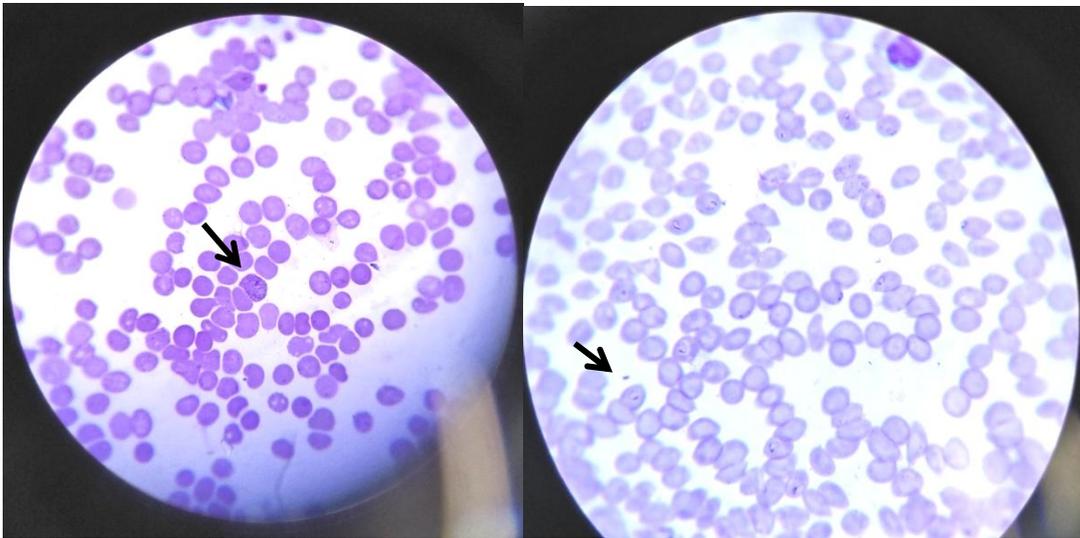


Table.7 Comparison of results of present study with other studies

Author	Year	Place	Observation
Karlekar et al[8]	2012	Gadchiroli	<i>P.vivax</i> 33.3% <i>P.falciparum</i> 66.6%
S Mohanty et al^[17]	2013	New delhi	<i>P.vivax</i> 53.3% <i>P.falciparum</i> 45.1% Mixed 1.91%
Chaya A K et al^[18]	2014	Mumbai	<i>P.vivax</i> 71% <i>P.falciparum</i> 25% Mixed 3.8%
Gurjeet singh et al^[10]	2015	Navi Mumbai	<i>P.vivax</i> 54.76% <i>P.falciparum</i> 17.8% Mixed 27.44%
Ruby Naz et al^[15]	2016	Haryana	<i>P.vivax</i> 73.9% <i>P.falciparum</i> 24.6% Mixed 1.91%
Present study	2017	Mumbai	<i>P.vivax</i> 76.1% <i>P.falciparum</i> 11.95% Mixed 11.95%

Fig.3 Rapid malaria antigen detection test (RDT)



23: Positive for *P. falciparum*
10: Positive for *P. vivax*
4: Positive for both *P. falciparum* and *P. vivax* (Mixed infection)
1: Negative test

Even a century after the discovery of malaria transmission by mosquitoes in India by Sir Ronald Ross in 1897, malaria continues to be one of India's leading public health problems [7]

Most of the studies are correlating with present study [10,14,15] except study conducted by Karlekar *et al* [8]. In this study, the % positivity of *P. falciparum* (66.6%) infection was higher than *P. vivax* (33.3%). Their study was conducted during the period of peak transmission as per their geographical location, which could be the probable reason for the higher percentage of positivity of

cases during November-December. Besides, *P. falciparum* could also be the predominant species prevalent in this area. Comparison of results of present study with other studies is depicted in Table 7.

Earliest symptoms of malaria are very non-specific; hence can lead to overtreatment of malaria or non-treatment of other diseases in malaria-endemic areas. Early and accurate diagnosis of malaria is essential, not only for rapid and effective disease management but also for decreasing community transmission of the disease. In this situation, laboratory

diagnosis can help to confirm malaria positive cases.

WHO recommends prompt malaria diagnosis either by microscopy or RDTs in all patients with suspected malaria before treatment is administered.

The gold standard for diagnosis of malaria is the microscopic examination of PBS, but its reliability is questionable at a low level of parasitaemia. RDTs have variable sensitivities and specificities. Microscopy is used as it can not only detect the species of parasite but also can give the PI indicating the severity of an infection. The other rapid modality in malaria diagnosis is fluorescent staining of qualitative buffy coat (QBC), but this technique is expensive.

The present study used a modified technique for diagnosis of malaria incorporating a centrifugation enhanced step into the conventional method of smear preparation by using the CBCS. In the present study, RDT was used as gold standard. PBS and CBCS follow the same principle of direct detection of a parasite. Hence, these two methods were compared with RDT which utilizes a different principle for detection of malaria infection.

Table 1 shows increase in malaria cases due to *P.vivax* infection 70/92 (76%) compared to *P.falciparum* 11/92 (12%). This correlates with the most of the studies conducted as discussed previously.

Comparison of Peripheral smear examination (PBS), Centrifuged buffy coat examination (CBCS) and Rapid malaria antigen detection test (RDT)

On analyzing the different morphological forms of the *Plasmodium* species in Table 2&3 in *P. falciparum* infections, the ring

form was predominantly observed followed by gametocyte of *P. falciparum*. Most of the development of *P. falciparum* takes place in the capillaries of the internal organs. Hence, only the ring forms will be circulating in the peripheral blood, whereas in *P.vivax* infections all the stages of erythrocyte schizogony take place in the peripheral blood. Hence, both schizonts and rings of *P.vivax* are predominantly seen in the present study.

Compared with traditional PBS examination (Table 4), CBCS detected 10 more cases as malaria positive. The PBS failed to detect the malaria infection in 18 samples. However, the use of CBCS leads to the demonstration of the parasite in 10 of these 18 samples which are not picked by PBS, thereby providing the good correlation between antigen test and direct demonstration of a parasite. The addition of centrifugation to the conventional smear technique to the present study has helped to identify 10 more cases of malaria which otherwise would have been missed if an only conventional method of PBS had been used. Eight cases could not be detected by microscopy but could be detected by antigen test alone. On comparing other studies, Shamim Akhtar *et al*^[6] has reported 6 more cases(49%) as CBCS positive compared with PBS (44%). Similarly, in another study where centrifugation enhanced heparinized capillary tubes used for smear preparation and examination found that out of 100 patients the modified centrifuged buffy coat detected 7 more samples as malaria positive compared with conventional PBS. The addition of centrifugation to the conventional smear technique improved sensitivity from 86.79% to nearly 100% in this case.

The third study by Shujatullah *et al*^[16] noted that out of 50 patients diagnosed as cerebral malaria, 28 (56%) were positive by PBS for *P. falciparum*, whereas QBC and Parasite- F

(antigen detection test) was positive in 47 (94%) and 46 (92%) patients, respectively.

On comparing sensitivity, specificity and validity of PBS and CBCS in Table 5, it was observed that PBS had sensitivity of 81.11%, specificity 99.85%, NPV 97.55% and PPV 98.65%; while CBCS had sensitivity of 91.11%, specificity 99.71%, NPV 98.83% and PPV 97.62%. It was observed that CBCS had a 10% higher sensitivity compared to PBS in detecting the parasite. At the same time, a lower specificity 1.03% was observed in CBCS, probably because during the centrifugation process the morphology of the parasites may have got disturbed thus not allowing for proper speciation. All the three tests detected malaria infection equally as showed in Table 6. At PI of 2-10%, PBS and RDT detected same number of cases of *P.falciparum*, *P.vivax* and mixed infections. At PI of <2%, PBS and RDT detected the same number of cases of *P.falciparum* and mixed infection. However, RDT failed to detect 2 cases of *P.vivax* which were positive by microscopy probably because both these cases had a low PI.

Four cases of *P.falciparum*, 14 cases of *P.vivax* and 3 cases of mixed infection detected at PI of <2% by PBS could not be detected by CBCS method. As seen in Table 6, it is apparently seen that PBS has been able to detect more number of cases at lower PI compared to CBCS. It is not completely true. The centrifugation process used in the CBCS technique has concentrated the parasites and hence all though 44 cases of *P.vivax* showed a PI of <2% by PBS and only 30 of the 44 cases showed a PI < 2% in CBCS. The balanced 14 cases which had a PI of < 2% in PBS and not CBCS have actually attained PI of 2-10% in CBCS because of the centrifugation process. An additional 10 cases of *P.vivax* with PI < 2% was detected only by CBCS, not PBS. Hence, a total of 23

cases were seen to be having a PI of 2-10% by CBCS method compared to 9 cases seen in PBS. Similarly, 4 cases of *P.falciparum* and 3 cases of mixed infection which had a PI of < 2% in PBS have actually got detected by CBCS with 2-10% PI.

In conclusion, it was observed that CBCS had a higher sensitivity compared to PBS in detecting the parasite. At the same time, a lower specificity was observed in CBCS, probably because during the centrifugation process the morphology of the parasites may have got disturbed thus not allowing for proper speciation.

CBCS examination helps in concentrating the parasites to give an apparent higher parasitic index. This method would be especially useful with negative or low parasitic index which may be missed by the conventional peripheral smear method. Hence, the CBCS method can be adopted in the routine as it just needs an additional centrifugation procedure to the conventional method making peripheral smear.

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