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**RESEARCH ARTICLE**

# A COMPARATIVE STUDY OF BLOOD SMEAR, QUANTITATIVE BUFFY COAT AND ANTIGEN DETECTION FOR DIAGNOSIS OF MALARIA

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**ABSTRACT**

Malaria is a major health problem in India, as well as in Karnataka, accounting for sizeable morbidity, mortality and economic loss. Microscopy has been the Gold standard for diagnosis from decades. Recently, many new microscopic and non microscopic rapid diagnostic tests are being widely used. The present study was done to compare the sensitivity, specificity, Positive Predictive Value and Negative Predictive Value of Quantitative Buffy Coat examination and antigen detection test using HRP 2 and pLDH by taking Giemsa stained peripheral smear as standard reference. 150 clinically suspected cases were studied by Giemsa stained peripheral blood smear examination, QBC examination and antigen detection test. Out of 150, 56 (37.3%) cases were positive and 94(62.6%) were negative. Males were predominantly affected. *P. vivax* was the major parasite species causing malaria. 56 (37.3%), 53 (35.3%) and 49 (32.6%) cases were positive by PBS, QBC and antigen detection test respectively. Sensitivity of QBC was high (94.6%) followed by antigen detection (87.5%). QBC missed 3 cases and antigen detection test missed 7 cases which were positive in microscopy. PBS examination still remains the "Gold standard" for diagnosis of malaria. However, QBC and antigen detection test can be used for rapid diagnosis.



## INTRODUCTION

Malaria represents one of the oldest documented protozoan disease, caused by the parasite of genus *Plasmodium* and transmitted by the bite of infected female *Anopheles* mosquitoes. (White *et al.,* 2005; Lynne Shore Garcia, 2001) It is the most important protozoal parasitic disease of humans affecting more than one billion people worldwide and causing between 1 to 3 million deaths each year. Malaria has been eliminated from the United States, Canada, Europe, and Russia; in the late twentieth and early twenty-first centuries. However, its prevalence rose in many parts of the tropics, a threat to non endemic countries and danger to travelers. (White *et al*., 2005; Lynne Shore Garcia, 2001) In 2008, there were an estimated 243 million cases of malaria worldwide. A vast majority, about 85 per cent, were in African region followed by the South-East Asia region (10%) and East Mediterranean (4%). Malaria accounted for an estimated 8,63,000 deaths. (Park, 2004) In India 1.06 million cases of malaria are reported, of which *P. falciparum* cases are 0.53 millions, deaths reported are 519 and annual parasite incidence is 0.88 in 2012. (Kakkilaya, 2009) Lack of proper infrastructure, inability to control the disease in endemic areas, and movement of the population are some important factors responsible for failure to curb malaria. (White *et al*., 2005; Muddaiah and Prakash, 2006) Drug resistance and insecticide resistance has also played a significant role in the occurrence and severity of epidemics. (White *et al*., 2005; Lynne Shore Garcia, 2001) Malaria has re-emerged as a major health problem in India. The most affected states are North-Eastern states, Chhattisgarh, Jharkhand, Madhya Pradesh, Orissa, Andhra Pradesh, Maharashtra, Gujarat, Rajasthan, West Bengal and Karnataka. One of the most important problems in controlling malaria is limited access to effective diagnosis and treatment. (Park, 2004) The clinical presentations of malaria are extremely diverse and can mimic a wide variety of diseases. In some cases, clinical diagnosis of malaria is possible especially in endemic areas. However a definitive diagnosis can be established only on demonstrating malaria parasite or its products in blood. (Lynne Shore Garcia, 2001) Complications of malaria are seen in *P. falciparum* infections, hence prompt and accurate diagnosis is essential for effective management. (White *et al*., 2005; Lynne Shore Garcia, 2001, Park, 2004) Laboratory confirmation of malaria infection requires the availability of a rapid, sensitive and specific test at an affordable cost. Conventional method of laboratory diagnosis are laborious, requires technical expertise and availability of

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a good quality microscope. (Subhash Chandra Parija, 2006; Bhat Sandhya *et al*., 2012) Numerous quick and new techniques for malaria diagnosis have developed, like Quantitative Buffy Coat technique and rapid diagnostic test devices, which have advantages in terms of sensitivity, ease, speed, and make them an important tool in the battle against malaria, especially in endemic areas. (Manjunath P Salmani *et al*., 2011)

Present study was done to compare between the peripheral blood smear examination, Quantitative Buffy Coat examination and antigen detection tests in clinically suspected cases of malaria.

## MATERIALS AND METHODS

The present study was conducted in the Department of Microbiology, at Bangalore Medical College and Research Institute, Bangalore over a period of one year between August 2012 and August 2013. A total number of 150 patients with clinical suspicion of malaria were taken for the study. Giemsa stained thick and thin blood smears, Quantitative Buffy Coat examination, HRP 2 and pLDH detection using SD BIOLINE Malaria Antigen P.f/P.v Test were performed on all the 150 patients.

**Method of collection**

The study population included both out patients and inpatients attending different clinical departments of Victoria and Vani Vilas hospital, BMC &RI presenting clinically with fever, chills and rigor and other symptoms suggestive of malaria. Informed consent was taken from the patient prior to the collection of specimens. Approximately 5 ml of venous blood was collected in Ethylene Diamine Tetra Acetate (EDTA) tube from each patient during the peak of fever and transported to the laboratory and processed immediately.

**Laboratory procedures**

**Peripheral smear preparation**

Thick and thin blood smears were prepared as per the standard method. The smears were stained with Giemsa stain. The smears were examined under oil immersion for presence of parasites in thick film and thin film for species identification. Thick smears were reported negative after examination of 200-300 oil immersion fields with no parasites; a thin smear was given negative when no parasites were observed in 200 oil immersion fields.

**Quantitative Buffy Coat Examination**

QBC Malaria tubes of Parascan malaria testing kit provided by Diagnova, RFCL Ltd were used in the study. The QBC malaria tube was filled with 55 µl to 60 µl of blood from venous sample collected in EDTA. A plastic float was inserted inside the tube and centrifuged at rate of 12000 rpm for 5 minutes.
The centrifuged tube was inserted into the groove of the paraviewer placed on the stage of the microscope. The buffy coat area of the tube was brought into focus. 2-3 drops of fluorescence optical immersion oil was added. Using 60x objective buffy coat layers was observed. The entire circumference of the tube was examined. The parasites will be seen in buffy coat layer and the interface between RBC and WBC regions, wherein parasite stains green (DNA; nucleus) and orange (RNA; cytoplasm).

The presence of malarial parasites was indicated by the distinct bi-coloured signet ring forms of trophozoites in cells near the granulocyte layer. Gametocytes of *P. falciparum* appeared as yellow sickle-shaped bodies. Schizonts of *P. vivax* were recognized by the presence of malaria pigment which appeared dark brown colour.

**Antigen detection using HRP 2 and pLDH**

In the study HRP 2 and pLDH detection was done using SD BIOLINE Malaria Antigen P.f/P.v Test kit of Standard Diagnostics.

**Principle**

SD BIOLINE Malaria Antigen P.f/P.v Test contains a membrane strip, which is pre-coated with two monoclonal antibodies as separate lines across a test strip. One monoclonal antibody (test line P.f) is specific to the HRP 2 of *P. falciparum* and the other monoclonal antibody (test line P.f) is specific to the lactate dehydrogenase of *P. vivax*. Control line coated with goat anti mouse IgG.

The test is based on the capture of parasite antigen from peripheral blood using monoclonal antibodies prepared against a malaria antigen target and conjugated to either a liposome containing gold particles in a mobile phase. A second or third capture monoclonal antibody applied to a strip of nitrocellulose acts as the immobile phase. The migration of the antigen-antibody complex in the mobile phase along the strip enables the labeled antigen to be captured by the monoclonal antibody of the immobile phase, thus producing a visible colored line. Incorporation of a labeled goat antimouse antibody capture ensures that the system is controlled for migration.

**The test was done according to manufacturers instructions. Interpretation of the test result was done as below:**

1. Negative result:
The presence of one colour band (C-control line) within the result window indicates a negative result.
2. Positive result:
*P. falciparum* positive: The presence of two colour band (P.f test line and C control line) within result window, no matter which band appears first, indicates *P. falciparum* positive result.
*P. vivax* positive: The presence of two colour band (P.v test line and C control line) within result window, no matter which band appears first, indicates *P. vivax* positive result.
Mixed infection: The presence of three colour band (P.f, P.v test line and C control line) within result window, no matter which band appears first, indicates mixed infection.
3. Invalid result
If the control band fails to appear within the result window, the result is considered invalid.

## RESULTS AND ANALYSIS

The tables and figures illustrate the results in detail with a brief explanation about the contents of each table provided after it.

Of the 150 suspected cases of malaria, 56 (37.3%) cases were positive and 94(62.6%) were negative.

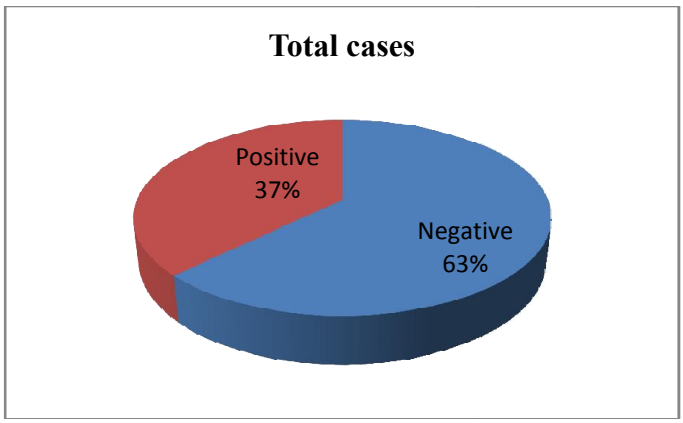


**Fig. 1. Graphic representation of total cases**

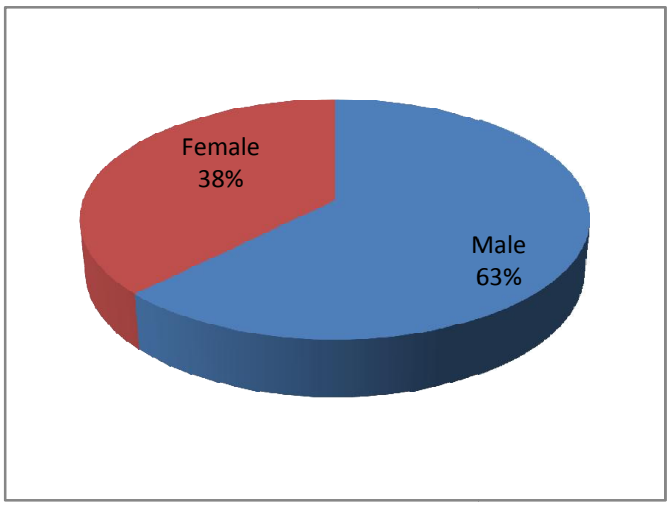


**Fig. 2. Graphic representation of sex distribution of positive cases**

98(65%) were males and 52(35%) were females. Among which, 35 (62.5%) and 21 (37.5%) were positive respectively indicating males were affected more than females. The male to female ratio was 1.66 : 1. Maximum number of cases were seen between the age group of 21-30 years (33.9%) followed by 31-40 years(19.6%). Mean age is 32.6 years. Standard deviation is 14.7 year.

Fever with chills was the most commonest symptom (100%) followed by body ache (79.3%), headache(55.3%), abdominal pain(40.6%) and jaundice (3.5%) 91% of patients had rise in temperature, 62% of the patients had pallor, 1.7% had icterus, 9.3% of patients had splenomegaly and 3.5% had hepatomegaly.

The maximum number of positive cases were found in the month of June (21.4%) followed by July (16.1%) and August (14.2%) corresponding to rainy season.

**Table 1. Showing detection of malarial species by pbs, qbc and antigen detection**

| Methods | Number of positive cases | *P. vivax* | *P. falciparum* | Mixed (*P. vivax* + *P. falciparum*) |
|---|---|---|---|---|
| Peripheral blood smear | 56 | 31(55.3%) | 20(35.7%) | 5(8.9%) |
| QBC | 53 | 30(56.6) | 18(33.9) | 5(9.4) |
| Antigen detection using HRP 2 and pLDH | 49 | 29(59.2%) | 17(34.7) | 3(6.1) |

Of the 150 cases tested, 56 (37.3%), 53 (35.3%) and 49 (32.6%) cases were positive by PBS, QBC and antigen detection test respectively.

**Table 2. Showing positive cases by combination of various methods**

| Combination of various methods | Number of samples and percentage |
|---|---|
| PBS positive + QBC positive | 53 (35.3%) |
| PBS positive + antigen detection test positive | 49 (32.6) |
| PBS positive + QBC positive + antigen detection test positive | 48 (32%) |
| PBS positive + QBC positive + antigen detection test negative | 5 (3.3%) |
| PBS positive + QBC negative + antigen detection test positive | 1 (0.6%) |
| PBS positive + QBC negative and antigen detection test negative | 2 (1.3%) |

All the QBC and antigen detection test positive samples were positive by peripheral smear. 53 (35.3%) cases were positive by both peripheral smear and QBC, 49 (32.6%) cases were positive by both peripheral smear and antigen detection test and 48 (32%)were positive by all three methods. 5(3.3%) cases were positive by both PBS and QBC but negative on antigen detection test. 1 (0.6%) case was positive by both PBS and antigen detection test but negative on QBC. 2 (1.3%) cases were positive by PBS but negative by both QBC and antigen detection test

Peripheral blood smear was considered as gold standard for statistical analysis. When QBC and antigen detection were considered individually, QBC was more sensitive than antigen detection method and are equally specific. Of various combinations, combination of peripheral smear and QBC was more sensitive.

## DISCUSSION

Malaria is a parasitic infection of global importance and is a major public health problem in India, as well as in Karnataka state, accounting for sizeable morbidity, mortality and economic loss. [8] Rapid detection and effective treatment is a pre-requisite for reducing the morbidity and mortality due to malaria. (Manjunath P Salmani *et al*., 2011, Parija *et al*., 2009) Newer techniques like QBC and antigen detection tests are rapid, simple and easy to interpret. (Parija, *et al*, 2009). In our study, the total number of males outnumbered females with the ratio of 1.66 : 1. This finding co related with Durand *et al.* and Bhat Sandhya *et al*.

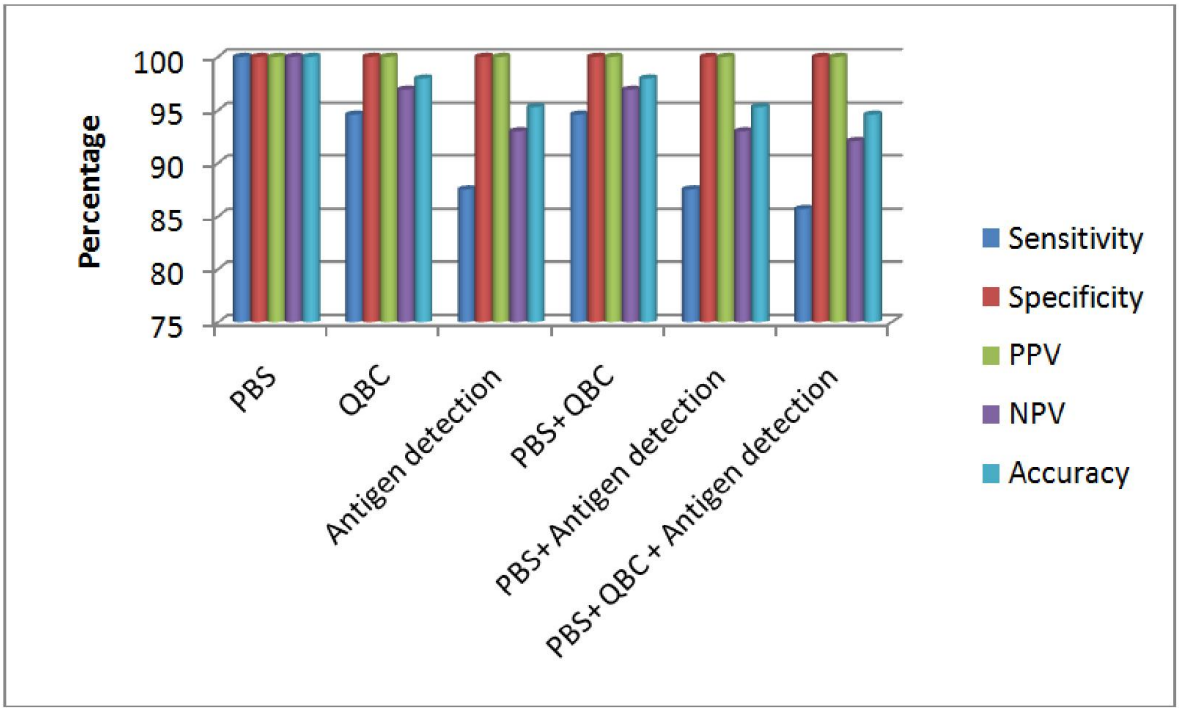


**Figure 3. Statistical analysis of Quantitative buffy coat examination, HRP 2 and pLDH detection by SD BIOLINE malaria antigen P.f/P.v test and various combination in relation to peripheral blood smear examination**

The incidence of malaria was more in men than in women because of the outdoor life they lead. Further, females in India are usually better clothed than males (Park, 2004).

The age distribution showed maximum number of patients (33.9%) between 21-30 years which correlates with Muddaiah M *et al* and Bhat Sandhya *et al* reflecting active working group, because this is the group which is exposed to the mosquito bites especially in the fields and outdoors. Fever with chills was the most common symptom followed by body ache, headache, abdominal pain and jaundice. Pallor was seen in 35 (62%) cases and icterus in 1(1.7%) case This finding co related with Bhat Sandhya *et al*. Maximum cases were seen from June to August, which is similar to the study conducted by Muddaiah *et al*. Malaria is a seasonal disease. It has been observed that the incidence of malaria increases with the onset of the monsoon (June to October), when the water logging helps mosquito breeding and thus transmission of the disease. (Park, 2004)

Of the 56 positive cases, 31 (55.3%) were positive for *Plasmodium vivax,* 20 (35.7%) were positive for *Plasmodium falciparum* which is consistent with the study conducted by Muddaiah *et al*. and 5 (8.9%) were mixed cases which is consistent with the study conducted by Manjunath P Salmani *et al*. In our study, *P. vivax* was the major parasite type causing malaria. In India, about 70% of the infections are reported to be due to *P. vivax*, 25-30% due to *P. falciparum* and 4-8% due to mixed infection. *P. malariae* has a restricted distribution and is said to be responsible for less than 1% of the infections in India (Park, 2004).

Of the 150 cases tested, 53 (35.3%) cases were positive by QBC examination and 97(64.7%) were negative.

Of the 53 positive cases, 30 (56.6%) were positive for *P. vivax,* 18 (33.9%) were positive for *P. falciparum*, and 5 (9.4%) were positive for both *P. vivax* and *P. falciparum*. In the present study, one case of *P. vivax* and 2 cases of *P. falciparum* were missed by QBC method. All the missed cases were asexual stages of *P. vivax* and *P. falciparum*. Younger ring forms of parasites may be missed by staining with acridine orange, and this problem has been reported serious when the parasite concentration is low (Bhat Sandhya *et al*., 2012).

The sensitivity, specificity, PPV and NPV values of our study by QBC method were 94.6%, 100%, 100% and 96.9% respectively. Sensitivity correlates with the study conducted by Bhandari *et al.* and Bhat Sandhya *et al*. Specificity and PPV co relates with the study conducted by Fatima Shujatullah *et al*. Datta *et al.* and Bhat Sandhya *et al*. NPV co relates with the study conducted by Bhandari *et al*. Parija *et al*. and Jafar Ebrahim *et al*. Of the 150 cases tested, 49 (32.6%) cases were positive by antigen detection by SD BIOLINE Malaria Antigen P.f/P.v test, 101(67.4%) cases were negative for malarial parasite. Of the 49(100%) positive cases, 29(59.2%) were positive for *Plasmodium vivax,* 17(34.7%) were positive for *Plasmodium falciparum* and 3(6.1%) were positive for mixed infection.

Two cases positive for *Plasmodium vivax* by peripheral blood smear were not detected in antigen detection test. One mixed infection which was diagnosed as positive by peripheral blood smear was detected as pure *P. falciparum* by antigen detection. *Plasmodium vivax* was not detected in these cases. This false negative results could be because of low parasitaemia, plasmodial gene deletion isolates, which express little or no pLDH antigen. (Bhat Sandhya *et al*., 2012) Three cases positive for *Plasmodium falciparum* by peripheral blood smear

were not detected in antigen detection test and one case of mixed infection by peripheral blood smear examination was detected as pure *Plasmodium vivax* by antigen detection method. *Plasmodium falciparum* was not detected. This may be due to insufficient enzyme production which occurs during early malarial infection or the patient's blood sample contained parasites at concentration below the detection level. Occasional false negative results may be caused by deletion or mutation of the HRP-2 gene. It has been suggested that anti-HRP-2 antibodies in humans may explain why some tests were negative despite significant parasitemia. (Bhat Sandhya *et al*., 2012) However there was 100% agreement between blood film results and antigen detection test results for the other 48 cases.

The sensitivity of *Plasmodium vivax* is 94.4% which is consistent with Ameh *et al*. and Jessica Maltha *et al*. The specificity of *Plasmodium vivax* is 100% which is consistent with Sun Xiaodong *et al*. The sensitivity of *Plasmodium falciparum* is 91.6% which is consistent with Sun Xiaodong *et al*. The specificity of *Plasmodium falciparum* is 100% which is consistent with Rubayet Elahi *et al* and Sun Xiaodong *et al*. The overall sensitivity of *Plasmodium vivax* and *Plasmodium falciparum* is 87.5% which is consistent with Sun Xiaodong *et al*. The overall specificity of *Plasmodium vivax* and *Plasmodium falciparum* is 100% which is consistent with Sun Xiaodong *et al* and Jafar Ebrahim *et al*. In the present study, sensitivity, specificity and positive predictive value of combination of peripheral smear and QBC were 94.6%, 100% and 100% respectively which correlates with Mitesh N. Suthar *et al*. NPV of 96.9% is consistent with Bhat Sandhya *et al*.
In the present study, combination of peripheral smear and antigen detection showed sensitivity, specificity and positive predictive value of 87.5%, 100% and 100% respectively which is consistent with Sun Xiaodong *et al*. NPV of 93% is consistent with Mitesh N. Suthar *et al*.

In the present study, combination of peripheral smear, QBC and antigen detection showed sensitivity, specificity, positive and negative predictive value of 85.7%, 100%, 100% and 92.1% respectively which correlated with Jafar Ebrahim *et al*.

**Conclusion**

Malaria remains a major cause of life threatening parasitic infection in the world, which presents a diagnostic challenge to laboratories. Rapid detection and effective treatment is a pre requisite for reducing the morbidity and mortality due to malaria.
In the present study 150 clinically suspected cases of malaria were studied. Of the 150 cases tested , 37.3% cases were positive and 62.6% were negative. It is found to affect comparatively the younger adult population and showed male preponderance. Maximum cases were seen in rainy season and *P. vivax* was the major parasite type causing malaria.

In the present study, Giemsa stained peripheral smear was considered as gold standard against which QBC and antigen detection test were compared. When QBC and antigen detection were considered individually, QBC was more sensitive than antigen detection method and are equally specific. Of various combinations, combination of peripheral smear and QBC was more sensitive, but QBC missed 3 cases and antigen detection test missed 7 cases which were positive in microscopy.

Microscopy is simple, economical, sensitive, specific and therapeutic decision of malaria depends on parasite growth stages hence peripheral smear examination still remains the "Gold standard" method for diagnosis of malaria. However, QBC can be used to screen a large number of samples in health care facilities with all expertise and antigen detection test can be used in places where specialized laboratories or even microscopy are unavailable and when urgent diagnosis is needed by a clinician, without the delay associated with the laboratory.

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