



COMPARISON BETWEEN QUANTITATIVE BUFFY COAT (QBC) AND OPTIMAL TEST FOR MALARIA DIAGNOSIS AT DMC, LAHERIASARAI, BIHAR

Microbiology

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ABSTRACT

The National Vector Borne Disease Control Programme (NVBDCP) of India reports about 2 million malaria parasite positive cases annually, of which about 50% are *Plasmodium falciparum*. The gold standard for malaria detection is microscopic detection which is tedious and dependent on technical expertise. Therefore tests like Parahit total, Advantage mal card, and QBC have been introduced. In view of the seriousness of the malarial infection and paucity in current availability of diagnostic facilities across India.

KEYWORDS

INTRODUCTION

Malaria is a protozoan disease transmitted by the bite of infected female anopheles mosquitoes. It is the most important parasitic disease of human. According to World Malaria Report (2011) malaria is present in 106 countries. Malaria is reported to cause 250-660 million infections every year and more than a million deaths.

Malaria has now been eliminated from the United States, Canada, Europe and Russia, but despite enormous control efforts, has resurged to many parts of the tropics. Added to this resurgence are the increasing problems of drug resistance of the parasite and insecticide resurgence of the vectors. Occasional local transmission after importation of malaria has occurred recently in several southern and eastern areas of United States and in Europe indicating the continual danger to non malarious countries.

Although there are promising new control and research initiatives, malaria remains today as it has been for centuries, a heavy burden on tropical communities, a threat to non endemic countries and, a danger to travelers.

THE HUMAN MALARIA IS CAUSED BY FOUR SPECIES OF PLASMODIUM NAMED AS –

- *Plasmodium vivax*
- *Plasmodium falciparum*
- *Plasmodium malarie*
- *Plasmodium ovale*

However *P. Knowlesi* is also known to cause human malaria.

Plasmodium falciparum infection occurs principally in tropical areas worldwide, whereas *plasmodium vivax* infection occurs in both tropical and temperate zones. *Plasmodium malarie* also occurs worldwide but to a much lesser extent than either *plasmodium falciparum* or *plasmodium vivax*. *Plasmodium ovale* is the least frequent cause of the malaria, with most cases being acquired in Western Africa, India and South America.

Plasmodium is transmitted by the bite of infected mosquitoes. In the human body, the parasites multiply in the liver and then infect red blood cells, giving rise to the following manifestations –

- Fever with chills
- Headache
- Vomiting
- Fatigue
- Abdominal discomfort
- Muscle ache etc.

If not treated, malaria can quickly become, life threatening by disrupting the blood supply to vital organs. In India, with the

implementation of modified plan of operation (MPO) in 1977, the upsurge of malaria cases dropped down from 6.74 million cases in 1976 to 2.1 million cases in 1984. Since then the epidemiological situation did not show any great improvement.

Since 1997 there is a consistently declining trend in the annual malaria incidence in the country. According to NVBDCP, 2010, total malaria cases in our country 1.49 million, *p. falciparum* cases was 0.77 million (52.12%) with 767 death and *p. vivax* cases was 0.72 million.

Diagnosis of malaria involves identification of malaria parasites or its antigens/products in the blood of the patient.

Although this seems simple, the efficacy of the diagnosis is subject to many factors – The different forms of four malarial species, different states of erythrocytic schizogony; the endemicity of different species, the relationship between the modes of transmission, immunity, parasitemia and the symptoms.

The problems of recurrent malaria, drug resistance, persisting viable or non viable parasitemia and sequestration of the parasites in the deepest tissues and the use of chemoprophylaxis or even presumptive treatment on the basis of clinical diagnosis, can all have a bearing on the identification and interpretation of malarial parasitemia on a diagnostic test.

The diagnosis of malaria is confirmed by blood tests and can be divided into

- Microscopic test
- Non-microscopic test

RAPID DIAGNOSTIC TESTS :

- Para-sight F test
- OptiMAL assay
- The immunochromatographic Test
- Polymerase chain reaction
- Detection of antibodies by radio immuno assay,

immunofluorescence or enzyme immuno assay.

The simplest and surest test is the time honoured peripheral smear study for malaria parasites. None of the other newer tests have surpassed the 'Gold standard' peripheral smear study.

Key interventions to control malaria include – Prompt and effective treatment with Artemisinin based combination therapies.

- Use of insecticidal nets by people at risk.
- Indoor residual spraying with insecticide to control the vector mosquitoes.

AIM OF THE STUDY

The current study is being undertaken for the diagnosis of malaria parasites with comparative study of Quantitative Buffy Coat and Rapid Diagnostic Test.

MATERIALS AND METHODS

The patients for the present study were selected from MOPD, CHOPD, Emergency wards and Indoor Medical wards of Darbhanga Medical College & Hospital, Laheriasarai and study were done at Microbiology Department of DMCH. A total of 120 cases were included in the study suspected to be suffering from malaria. 80 were confirmed cases of malaria in, whom malarial parasites were demonstrated in the peripheral blood smear. 40 cases were negative for malaria by PBS.

METHODS:**HISTORY SHEET:**

A detailed history was taken of every case under the following headings:

Name, Age, Sex, Religion, Socio-Economic status, Occupation, Full address Chief complaints of patient with duration in chronological order:

HISTORY OF PRESENT ILLNESS:

Fever with chills and rigors, Headache, Weight loss, Malaise, Abdominal Pain Weakness, Anorexia, Jaundice, Cough, Diarrhea, Swelling of feet.

PAST HISTORY:

History of malaria in the past, History of malnutrition, History of tuberculosis History of incomplete therapy of initial diseases, Any history of chronic illness in the past, History of diabetes mellitus, History of traveling to an area which is known high endemic area of malaria.

FAMILY HISTORY:

History of malnutrition in family members, History of tuberculosis, History of malaria, History of any other diseases.

PERSONAL HISTORY:

Dietary habits, History of addiction

DRUG HISTORY:**CLINICAL EXAMINATION:****GENERAL EXAMINATION:**

General built, Level of consciousness, Degree of pyrexia (Temperature), Lymphadenopathy, Pallor, Cyanosis, Clubbing, Jaundice, Pedal Edema, Skin-Pigmentation, Tongue – Color/hydration, Pulse, B.P., Respiration rate, J.V.P.

SYSTEMIC EXAMINATION:**ABDOMEN:**

Tenderness, muscle guarding, rigidity, Spleen – size, surface, consistency, tenderness, Liver – size, surface, consistency, tenderness, Any other lump, Ascites.

RESPIRATORY SYSTEM:

Done to find out any evidence of pulmonary tuberculosis, pneumonia or any other pulmonary diseases.

CARDIOVASCULAR SYSTEM:

Other than pulse, B.P. and JVP examination of cardiovascular system was done to find out any evidence of cardiac diseases.

CENTRAL NERVOUS SYSTEM:

Peripheral neuropathy, myopathy, motor neuron disease and to exclude any neurological cause of weight loss.

INVESTIGATIONS:

Total RBC count, TLC and DLC of WBC, Haemoglobin percentage, ESR, Bleeding time and clotting time, Blood sugar, Blood urea and serum creatinine Serum bilirubin, SGOT, SGPT, Platelets count, BT, CT, PT, LDH, Alk-phosphatase, Widal Test, Bl. Culture, Routine examination of urine, Culture and sensitivity of urine, X-ray chest PA view, Thick and thin blood smears for malaria parasites, Quantitative Buffy Coat Test for Malarial parasite, Rapid diagnostic tests (OptiMAL) for malaria parasites.

The splenic size was measured from the 9th costal margin to the tip. The liver was measured in cms from costal margin at midclavicular line to

the edge (WHO, 1982).

NORMAL HEMATOLOGICAL VALUES:

(1) Total leucocytes count – The normal range of the TLC was taken as 4000 to 11,000 cells/cmm of blood.

(2) DIFFERENTIAL LEUCOCYTE COUNT –

Normal range of DLC is as follows –

Neutrophils	- 40-75%
Lymphocytes	- 20-45%
Monocytes	- 2-10%
Eosinophils	- 1-6%
Basophils	- 0-1%

(3) HEMOGLOBIN ESTIMATION –

Hemoglobin estimation was done by Sahli's method.

Normal value in

Male	- 13-16 gm/dl
Female	- 11.5-15.5 gm/dl

(4) RBC COUNT –

Normal value - 4.5-5.5 million/cmm of blood

QUANTITATIVE BUFFY COAT TEST:

The QBC Test, developed by Becton and Dickenson Inc., is a new method for identifying the malarial parasites in the peripheral blood. It involves staining of the centrifuged and compressed red cell layer with acridine orange and its examination under uv light source. It is fast, easy and claimed to be more sensitive than the traditional thick smear examination.

METHODS:

The QBC tube is a high-precision glass haematocrit tube, precoated internally with acridine orange stain and potassium oxalate. It is filled with 55-65 microlitres of blood from the finger, ear or heel puncture. A clear plastic closure is then attached. A precisely made cylindrical float, designed to be suspended in the packed red blood cells is inserted. The tube is centrifuged at 12,000 rpm for 5 minutes. The components of the buffy coat separate according to their densities, forming discrete bands. Because the float occupies 90% of the internal lumen of the tube, the leucocyte and the thrombocyte cell band widths and the top most area of red cells are enlarged to 10 times normal. The QBC tube is placed on the tube holder and examined directly under fluorescent microscope or by using a standard white light microscope equipped with the uv microscope adapter, an epi-illuminated microscope objective. Fluorescing parasites are then observed at the red blood cells/white blood cells interface.

The key feature of the method is centrifugation and thereby concentration of the red blood cells in a predictable area of the QBC tube, making detection easy and fast. Red cells containing plasmodia are less dense than normal ones and concentrate just below the leucocytes, at the top of the erythrocyte column. The float forces all the surrounding red cells into the 40 micron space between its outside circumference and the inside of the tube. Since the parasites contain DNA which takes up the acridine orange stain, they appear as bright specks of light among the non-fluorescing red cells. Virtually all of the parasites found in the 60 µl of blood can be visualized by rotating the tube under the microscope. A negative test can be reported within one minute and positive result within minutes.

PRECAUTIONS:

- For in vitro diagnostic use only.
- All samples and kits used should be handled cautiously as if they contain infectious agents. Established precautions against microbiological hazards should be observed while performing all procedures and the standard procedures for proper disposal of used samples and used tubes.
- Protective clothing, eye protection and disposable gloves should be used while performing the assay. Hands should be washed thoroughly when finished.

STORAGE:

The test tube is designed to be stored at room temperature (20-28°C) for the duration of its shelf life. Exposure to temperature over 30°C can impair the performance of the test and should be minimized. The tube should not be frozen. It should be prevented from humidity.

TEST PROCEDURE :

Approximately 50 ml of whole blood was taken into a capillary tube coated with acridine orange and fitted with a cap. A plastic float was inserted inside the tube and then spun in the QBC microhaemocrit centrifuge at 12000 rpm for 5 minutes. The tube was then mounted on a small plastic holder and examined through an ordinary light microscope with customized fluorescence.

PRINCIPLE :

It is based on the fact that on centrifugation at high speed, the whole blood separates into plasma Buffy coat and packed red cell layer. The float gets buoyed by the packed blood cells and is automatically positioned within the buffy coat layer.

Blood cells in the buffy coat layer separate according to their densities forming visible discrete bands. Platelets remaining at the top, lymphocytes and monocytes within the middle layer and granulocyte at the bottom.

Due to acridine orange, the malarial parasite stains Green (DNA; Nucleus) and Orange (RNA; Cytoplasm). The tube is examined in the region between the red blood cells and granulocytes and among the granulocytes and mononuclear cell layer, where parasites are most abundant.

An attempt was made to estimate the relative quantities of parasites in the specimens, using the plus system.

+ [+1] - 1 parasite per QBC field
++ [+2] - 2-10 parasites per QBC field
+++ [+3] - 11-100 parasites per QBC field
++++ [+4] - >100 parasites per QBC field.

DRAWBACKS :

Identification of plasmodium species, ring stages of *P.falciparum* and *P.vivax* are difficult to distinguish by the QBC. This problem is particularly important in endemic areas where *P. falciparum* coexists with *P.vivax*, under that condition individuals with positive QBC should be further evaluated by Giemsa stained blood smear in order to confirm infecting species of Plasmodium parasites.

RAPID DIAGNOSTIC TEST (OPTIMAL) :**PRINCIPLE AND METHOD :**

OptiMAL, a rapid diagnostic test for malaria parasite detection is developed by Flow Inc., Portland Oreg. OptiMAL is a rapid (10 minutes) malaria detection test which utilizes a dipstick coated with monoclonal antibodies against the intracellular metabolic enzyme, parasite lactate dehydrogenase (pLDH). Differentiation of malarial parasites is based on antigenic differences between the pLDH isoforms. To detect antigens RDTs indicate infection by the use of immunochromatography (Lateral Flow of an antigen or antibody) protein produced by a person in response to an antigen in a filter paper resulting in a colour change. Some RDTs detect only one species of malaria and some detect one or more species. Since pLDH is produced only by live plasmodium parasites, this test has the ability to differentiate live from dead organism.

One drop of whole blood was mixed with 2 drops of lysis buffer A, which disrupts the red blood cells and releases the plasmodium lactate dehydrogenase. The specimens were then allowed to migrate to the top of the pLDH strip. After 8 minutes, the strips were placed in washing Buffer B, which clears the haemoglobin from the strip.

Interpretation of the results :

Interpretation of the test results was performed immediately. A negative control sample taken from an individual, who had not been exposed to malaria for 3 years, was included with each batch tested. In the pLDH assay, there are 2 diagnostic zones of reactions containing different antibodies.

The first diagnostic zones contain a monospecific antibody that recognizes only plasmodium falciparum, if it is present. The second diagnostic zone contains a pan specific antibody immediately above the

first zone. This monoclonal antibody recognizes the pLDH isoforms of *P.vivax*. A third reaction zone containing a pan specific monoclonal antibody is present at the top of the test strip and serves as a positive control for the assay. The test can be completed in 10-15 minutes.

PRECAUTIONS :

- (i) For in vitro diagnostic use only.
- (ii) All specimens and kits used should be handled safely as they may contain infectious agents.
- (iii) Established precautions against microbiological hazards should be observed while performing all procedures and for proper disposal of samples and kits should be followed.
- (iv) Protective clothing, eye protection and disposable gloves should be used while performing the test.
- (v) Hands should be washed thoroughly when finished.

STORAGE :

The sealed pouch should be stored in the least humid and coolest place. Storage site should be clean and as dry as possible. The strips should not be frozen. Test should be performed within one hour after removal from pouch.

COLLECTION OF BLOOD :

Whole blood should be tested with this test strip. Test should be performed as soon as possible after collection.

Kit Contents :

- (i) Individual pouched test strip
- (ii) 2 vials of Buffer solution

LIMITATIONS :

- (i) This test only indicates the presence of antibodies to test strip in patients with malaria, and should not be used as the sole criteria for the diagnosis of malaria.
- (ii) If the result is negative and clinical symptoms persist, additional follow up testing is recommended.
- (iii) A false positive result may occur, further testing is advised especially in asymptomatic cases.
- (iv) Persons with advanced HIV infection or other immunocompromised diseases frequently have low or undetectable antibodies.

STEPS FOR PLASMODIUM SPECIES IDENTIFICATION WITH QBC :**STEP-1**

- Examine the red blood cell layer at the granulocyte/RBC interface.
- Look for the presence of ameboid forms and multiple maturation stages of *P. vivax* and for early ring forms of *P.falciparum*.
- Look for banana shaped gametocytes of *P.falciparum*.

STEP-2

- Examine the red blood cell layer to a depth of 10-15 fields from the granulocyte/RBC interface.
- Look for the presence of ring forms. *P.vivax* rings and *P.falciparum* rings are differentiated based on relative size of cytoplasmic ring to chromatin dot. *P. vivax* rings are larger. In mixed infections only one type of ring predominates and quite frequently it is the *P. falciparum* ring.

STEP-3

- Examine the granulocyte area at the green lympho-monocyte and orange granulocyte interface.
- Look for schizonts and gametocytes of *P.vivax* and gametocytes of *P. falciparum*. The pigment of *P.vivax* is often scattered and fine pigment of *P. falciparum* gametocytes is often coarse and rice-grain shaped.

STEP-4

- Examine the lympho-monocyte layer.
- Look for the presence of malaria pigment among the leukocytes as well as for the presence of schizonts and gametocytes of *P. vivax*.
- The presence of schizonts or gametocytes in this layer further confirms the identity of *P. vivax*.

DECISION TABLE FOR SPECIES IDENTIFICATION USING THE QBC MALARIA TEST

	Area of QBC tube	<i>P. falciparum</i> forms	<i>P. vivax</i> forms
1.	Red blood cell and granulocyte interface	Gametocytes: green, banana shaped with brownish pigment. Pigment is scattered, coarse, rice grain-shaped. Trophozoites: small, symmetrical rings	Trophozoites: large ameboid rings- only one per RBC. Rings may concentrate within the granulocyte layer.

2.	Uppermost red blood cell layer	Trophozoites: small, symmetrical rings-one or more per RBC (when the RBC cell wall is visible).	Trophozoites: Large ameboid rings – only one per RBC (when the RBC cell wall is visible).
3.	Granulocyte and lymphocyte/monocyte interface	Gametocytes:green, banana shaped when mature, with brownish pigment. Pigment is scattered, coarse, rice grain shaped.	Schizonts: Oval, round; scattered, five pigment surrounded by developing merozoites. Gametocytes:Round, green with pigment.
4.	Lymphocyte/monocytes	None	Schizonts:Oval, round, scattered, fine pigment surrounded by developing merozoites.

Note : While almost all schizonts and gametocytes will be found as described. Occasionally they will be located in the uppermost RBC layer.

RESULTS :

The purpose of the study was to compare QBC test and OptiMAL test as a diagnostic method for malaria detection. The study included 120 patients of different age, sex and with different manifestations.

Females showed a higher preponderance over males in a ratio of 8:7. Young adults were most commonly affected. 40 out of 120 i.e., 33.33% were in the age group of 21-30 years. This was followed by 26.67% in 2-10 years. Among various features of malaria, fever was seen in almost all 120 cases (100%), whereas fever with chills and rigors was seen in 72 cases (60%). Splenomegaly and hepatomegaly were seen in 32 cases (26.67%) and 16 (13.33%) cases respectively. QBC tube test was found positive in 76 out of 120 cases i.e. (63.33%). 4 cases were reported false positive and 4 cases were false negative. While OptiMAL test was found positive in 68 cases out of 120 cases (56.66%), 4 cases were false positive and 12 cases were false negative. Sensitivity of QBC test was 95% whereas sensitivity of OptiMAL was 85%. Specificity of QBC and OptiMAL was 90% for both. Percentage of false positive in cases of QBC and OptiMAL was 10% for both the test. Percentage of false negative of QBC and OptiMAL was 5% and 15% respectively. Predictive value of positive test for QBC and OptiMAL was 95% and 94.4% respectively. Predictive value of negative test for QBC and OptiMAL was 90% and 75% respectively.

CONCLUSION :

From the above study it can be concluded that both QBC and OptiMAL tests are extremely safe, simple, rapid, accurate and minimally invasive with a very high sensitivity and specificity.

Both QBC and OptiMAL test have a few advantages over peripheral blood smear.

- Both are simple tests. OptiMAL test has an added advantage that it can be used in field conditions.
- Extremely simple to perform especially when OptiMAL test is conducted.
- Result can be obtained rapidly.
- User friendly.

All these advantages leads to rapid diagnosis of malaria. Hence, early treatment modalities can be initiated leading to early recovery and the well being of patients.

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