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Comparative evaluation of various methods of diagnosis of malaria and assessment of hematological parameters in malarial infection: A three year prospective study in tertiary care hospital of western Odisha

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Abstract---Background:Malaria remains a substantial health problem in many parts of world, especially in the tropical developing nations. Management of malaria requires early and accurate diagnosis. The aim of this study was to measure and compare the diagnostic accuracy of different methods of diagnosis of malaria and to study the limitations mostly due to low parasite density. We also evaluate the haematological changes in malaria patients. Material and methods: The study was conducted on blood samples of patients presenting with pyrexia, attending the various Departments of VSS Institute of Medical Science & Research, Burla, Sambalpur. Blood samples were subjected to thick and thin blood smear examination, QBC method and Rapid Diagnostic Tests (RDTs). Haematological parameters including haemoglobin, total leukocyte count, differential count and

total platelet count of malaria positive cases were studied. Results: Out of 186 cases of fever, 105 patients were positive for malaria by thick peripheral blood smears. *Plasmodium falciparum* constitutes 86.66% whereas *P. vivax* and mixed infections constitute 8.57% and 4.77% respectively of total malaria. The sensitivity of QBC test approached 100% with parasite densities above 500/cu.mm. Antigen detection method was found to be highly sensitive (92.70%) for the diagnosis of *P. falciparum* infection. Anemia was found in 62.86% and thrombocytopenia in 64.75% cases. Conclusion: QBC method is an important tool for the diagnosis of malaria particularly in the hospital environment. In the field and in emergency RDTs can be employed as a simple and easy tool. Anemia with thrombocytopenia may be important predictors of malaria infection in acute febrile cases.

Keywords---malaria, QBC, RDT, anemia, thrombocytopenia.

Introduction

Malaria is one of the most wide spread disease in the world. It is endemic in almost all tropical and subtropical countries. In most parts of India, the maximum prevalence is from July to November. An estimated 219 million people were infected globally in 2017[1]. India contributed to about 85% of all malaria cases in the World Health Organisation's South-East Asia Region in 2017; the majority of these cases were reported from tribal dominated rural areas of the country [1,2] India aims to eliminate malaria by 2030, but there exist significant gaps in malaria surveillance, diagnosis, treatment and control. Odisha shares 4.7% of Geographical area and about 3.5% population of our country, one of the highest malaria burden state contributing 45% of total cases[3]. Geographically the whole Western Odisha consists of Rocky Mountains, dense forests, vast water reservoirs and hilly terrains, amidst plains. Malaria takes the lion share amongst all fever cases here. It is well-known that *falciparum* malaria is dangerous and needs aggressive management as its severity causes mortality. Hence, accurate and timely diagnosis is critically important in the management of malaria. Confirmation of diagnosis of malaria has traditionally been done by microscopic demonstration of the malaria parasite on a stained peripheral blood smear of the patient[4]. However, the staining and interpretation processes are time consuming, require expertise and trained health workers[5]. Hence, in the last few years much attention has been focused on QBC (Quantitative Buffy Coat) and RDT by antigen detection methods which are rapid methods and increasingly used these days. Besides many new technique like Loop mediated isothermal amplification (LAMP), Flow cytometry, Polymerase Chain Reaction (PCR), Automated blood cell counters which deserve special mention and are generating new strategies for diagnosis of malaria. LAMP is a new technique which employs the rapid amplification and detection of DNA appears to be easy, sensitive and quick[6]. Flow cytometry based on detection of hemozoin pigment within phagocytes pass through flow-cytometer channel and detect the malaria parasites[7]. PCR, a recent development in the molecular diagnosis of malaria has been proven to be one of the most specific and sensitive diagnostic method[8]. PCR can detect low level of parasitemia, but it is relatively expensive and requires

advanced laboratory equipment and technologically skilled execution, not suitable for remote areas. Automated blood cell counter detects malaria parasites by detecting hemozoin pigment in monocytes[9].

QBC method involves staining parasite DNA in microhematocrit tubes having anticoagulant EDTA and fluorescent dyes e. g. Acridine orange and then detection by epi-fluorescent microscopy. Parasite nuclei fluoresces bright green while cytoplasm appears yellow-orange[10]. Rapid diagnostic tests (RDTs) are based on the principle to detect malaria antigen in blood flowing along a membrane containing specific anti-malaria antibodies. Most product targets Histidine-rich proteins-2(HRP-2) and Pan aldolase or LDH enzyme for *P. Falciparum* and other plasmodium species respectively[11]. RDTs most commonly use in resource-poor setting, field conditions, primary health centres and community health centres due to ease handling. RDTs also has been reported to be cost effective with reduced referrals and patient's duration of hospital stay[12].

Malaria causing plasmodium are parasites of blood and hence induce haematological alterations [13]. The anaemia is caused by excess removal of non parasitized erythrocytes in addition to immune destruction of parasitized red cells, and impaired compensation for this loss by bone marrow dysfunction [14, 15].Thrombocytopenia is a common finding in *falciparum* and *vivax* malaria. Thrombocytopenia seem to be occur through peripheral destruction, excessive removal of platelet by spleen pooling as well as platelet consumption by the process of disseminated intravascular coagulopathy (DIC)[16].Immune mediated destruction of circulating platelets has been postulated as a cause of thrombocytopenia seen in malaria infection. Neutrophilic leucocytosis is an important abnormality in patients with severe *falciparum* malaria and is associated with a bad prognosis [17].

Main objective of the study was to compare the Leishman - stain thin smear, QBC and rapid antigen detection methods with the JSB stain thick smear and evaluate their sensitivity in detecting malaria parasites and find out the alterations in haematological parameters and their respective incidences, which could guide the diagnosis of malaria.

Materials and Methods

The present study was done in the department of Pathology and Central Laboratory, VSS Institute of Medical Science & Research, Burla, Sambalpur, Odisha over a period of 3 years from February 2013 to February 2016. The study was undertaken after approval by institutional ethics committee (IEC). Informed consent was obtained from all included patients. About 2 ml venous blood was collected in sterile EDTA vacutainer tube from patients presenting with pyrexia, attending the various Departments. Blood samples were subjected to thick and thin smear examination, antigen detection - as per kit instruction and QBC method. Thick blood smear was stained with Jaswant Singh Bhattacharya (JSB) stain and thin smear was stained with Leishman's stain. The parasite density, expressed as the number of parasites per microL of blood, was calculated by dividing the number of parasites by the number of WBCs counted and then multiplying it by an assumed WBC density of 6000 microL. We graded the parasite

count as 1+, 2+, 3+ and 4+ for <100/microL, 100 – 500/microL, 501 – 1000/microL and >1000/microL respectively. The QBC technique using microhematocrit tubes which have the acridine orange, fluorescence from malaria parasites and detect through an epi-fluorescent microscopy. An attempt was made to estimate the relative quantity of parasites in the specimen using the plus systems as 1+, 2+, 3+ and 4+ for <1 parasite per QBC field, 1-10 parasites per QBC field, 11-100 parasites per QBC field and >100 parasites per QBC field respectively. Malascan Rapid test for malaria *Pf/Pan* Zephyr Biomedical, India was used to diagnose malaria infection. This malaria RDT targets the detection of histidine rich protein-2 (HRP-2) antigen of *plasmodium falciparum* and Pan Specific Aldolase of non *falciparum* species. Hematological parameters of malaria patients, evaluated using SYSMEX XN1000, CBC counter provide data on WBCs, Hb level, Total platelet count, MCV, MCH, RDW and differential count.

Statistical Analysis

The sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) of thin smear, QBC and antigen detection methods were determined using the software statistical package for the social sciences (SPSS) version 2016.

Results

A total of 186 samples of febrile patients were observed over the period of study. Of these a total of 105 samples were positive by microscopy in JSB stained thick smear. Males constitute 68 (64.76%) of malaria cases whereas females constitute 37 (35.24%) of total number of malaria cases. Most of the patients were in the age-group of 26 – 35 years which constitutes 28 (22.66%) of all cases. In almost all the age-groups males constituted more number of cases than females. JSB stained thick smears showed plenty of trophozoites (ring form) of *P.falciparum* [Fig-1a] Thin smears showed sickle shaped gametocyte of *P.falciparum* [Fig-1b]. Leishman stained peripheral smears showed ring form of malaria, many Red cells infected with trophozoites and Schizonts forms of *P vivax* [Fig 1c]. High degree of parasitemia was seen in RBC infected with *P.Falciparum* in QBC. [Fig 1(d)]. *P.falciparum* constitute 91 (86.66%) of the total number of malaria cases whereas *P. vivax* and mixed infections constitute 09 (8.57%) and 05 (4.77%) respectively[]

Out of 105 thick smear positive sample, 84 (80.00%) were positive by thin smear, 97 (92.38%) were positive by antigen detection (RDT) and QBC was positive in 92 (87.61%) sample. 81 samples out of total 186 samples were negative by thick smear. Out of 81 such samples 5 samples were positive by QBC method. Subsequently these 5 samples found to be negative for thick, thin and antigen detection test. Sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), of thin smear, QBC, and RDT methods were compared with thick smear as gold standard. Measure of sensitivity, specificity, PPV, NPV of thin smear was 80, 100, 100, 82.92 respectively, for QBC it was 87.61, 93.82, 94.84, 87.85 respectively and 92.38, 100, 100, 91.75 respectively represent for RDT [Table 1a]. Sensitivity of QBC and RDT with respect to species identification was calculated. *P. faciparum*, *P. vivax* and mixed infection the sensitivity of QBC was 89.01, 77.78, 80.00 respectively [Table 1b]. For RDT the sensitivity of

P. falciparum and non-falciparum was 92.70 and 88.89 respectively [Table 1c]. Mixed infection was taken as *P. falciparum* infection in RDT as differentiation not possible. Antigen detection method was found to be highly sensitive (92.70%) for the diagnosis of *P. falciparum* infection. Evaluation of QBC and RDT test in detection of different levels of parasitaemia was done. The sensitivity of QBC test approached 100% with parasite densities above 500/microL of blood [Table 1d]. The sensitivity of antigen detection test approached 100% with parasite densities above 100/microL of blood [Table 1e].

In this study haematological abnormalities mostly anemia and thrombocytopenia was observed in malaria infection [Fig 2a & Fig 2b]. Leucocytosis observed in 26 (24.76%) cases with neutrophilia in 18 (17.14%) cases. Anemia was observed in 66 (62.86%) cases with 42 (40%) had mild anemia [Table 2a]. 68 (64.75%) cases had thrombocytopenia with 45 (42.86%) had mild degree of thrombocytopenia i. e. TPC between 1 to 1.5 lacs/cu.mm [Table 2b].

Discussion

Most of the malaria cases are reported from rural and tribal areas of India. Tribal population constituting 8.6% of total population account for more than 50% of total malaria cases and about 50% death due to malaria in the country [2]. Malaria diagnosis is challenging task in rural and tribal areas because of poor healthcare infrastructure. The reluctance of these tribal individual to seek medical care further complicate the issue of malaria diagnosis in tribal areas. We have evaluated the sensitivity, specificity, PPV, NPV of QBC and RDT method when JSB stained thick smear was adopted as gold standard.

Out of a total number of 105 positive cases 91 patients (86.66%) had *falciparum* malaria, 9 patients (8.57%) had *vivax* malaria and 5 (4.77%) had mixed infection. This observation reflects the fact that the maximum number of malaria cases came to the hospital are cases of *falciparum* malaria. Since *falciparum* malaria gives rise to various complications which required admission to the hospital, the percentage of *falciparum* malaria cases in this hospital-based study out numbered that of *vivax* malaria. The sensitivity of Leishman-stained thin smear was found to be 80%, specificity and positive predictive value were 100% each. The sensitivity was higher and specificity was comparable to what as reported else [18]. The sensitivity of the QBC has been reported as high as 99.70% by Benito a etal [19] and relatively low sensitivity 78.94% has observed by Parija SC etal [18]. One of the reasons for this low sensitivity could be that as the hospital is present in an endemic region for malaria the levels of parasitemia could have been low. The specificity of the QBC test as reported by previous study was 98% by Parija SC etal & Bosch I etal [18,20]. The sensitivity and specificity of the QBC method for detection of malaria parasite in this study turned out to be 87.60% and 93.82% respectively. There were five cases found to be positive by QBC, which were subsequently found to be negative for thick, thin and antigen detection test. This can be explained by the fact that certain artefacts in blood like Howell Jolly bodies, normoblast or platelet fragments might resemble the ring forms of malariaparasites. The sensitivity of the QBC test increases significantly with a rise in the level of parasitemia. It is seen that with parasite density of less than 100/microL of blood the sensitivity of the QBC test is 68.14%, with parasite

density of 100 – 500/microL of blood it is 96.15% and it is 100% with parasite densities above 500/microL of blood. Antigen detection method for diagnosis of malaria had a sensitivity, specificity and PPV of 92.38%, 100% and 100% respectively. This test was based on detection of HRP-2 and aldolase with the help of monoclonal antibodies. The sensitivity obtained for this kit-based procedure was close to those observed on similar principle[21,22].The low sensitivity could be attributed to low parasitemia levels as observed by study who observed 75% sensitivity at parasitemia<100/ μ L[23]. The specificity was comparable to other observers using the tests based on similar principle[24,25].The sensitivity of the antigen detection test increases significantly with a rise in the level of parasitemia. It is seen that with parasite density of less than 100/microL of blood the sensitivity of the RDT is 77.14%, with parasite density above 100/microL of blood it was 100%. So the test was found to be user friendly and interpretation was more objective as compared to smear and QBC.

Anaemia is an inevitable consequence of malaria infection and its degree is related to the density of parasitemia and to the presence of severe manifestations. Haemoglobin values encountered in *Plasmodium falciparum* infections are significantly lower than in *vivax* malaria due to greater proportion of parasitized erythrocytes, shortening of life span of RBC's as well as due to ineffective erythropoiesis. It is difficult to attribute the cause of anaemia solely due to malaria, because of associated Iron, folic acid and other nutritional deficiencies in the endemic areas. We have found anaemia in 62.86% and thrombocytopenia in 64.75% of malaria cases . This incidence was comparable with other author's observations[26,27].Leucopenia was frequently seen in malaria infected patients which was confirmed by studies that have demonstrated leucopenia[28]. and contrast with other study that had demonstrate leucocytosis [17].We have found leucocytosis and leucopenia in 24.76% and 5.71% respectively of malaria patients in our study. Malaria has a significant impact on haematological profile,most marked being thrombocytopenia and anaemia. Out of the infection types prevalent in our part of world *falciparum* malaria causes maximum drop in platelet count and haemoglobin levels.

Limitations

Comparative evaluation with newer techniques like LAMP, Flow cytometry, PCR &Automated Blood cell counter could not be done as the facility was not available during the study period.

Conclusions

From present study it can be concluded that *Plasmodium falciparum* was the predominant species detected in the patients of malaria. Though the QBC method has a few limitations like high cost and relative insensitivity when used in field conditions, the speed and ease of method and its excellent overall sensitivity, particularly in the hospital environment, makes it an important tool for the diagnosis of malaria. Situation, where adequate back up is not available and in emergency simpler and easy to use antigen detection technique, can be employed. Peripheral smear is ideal for species identification, parasite counting and confirmation of malaria. Malaria parasites exhibit important changes in many

haematological parameters, most commonly changed parameters were anemia and thrombocytopenia and it may be important predictors of malaria infection in acute febrile cases.

Conflicts of interest: Authors have no conflicts of interest to declare.

Acknowledgment

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Legends of figures

Fig.1a Thick Blood film (dehaemoglobinised) showing trophozoites (ring form) of *Plasmodium falciparum* (JSB x500)

Fig.1b Thin Blood film showing sickle shaped gametocytes of *Plasmodium falciparum* (Leishman x1000)

Fig.1c Thin Blood film showing young amoeboid trophozoites and early schizonts of *Plasmodium vivax* (Leishman x1000)

Fig.1d QBC film showing trophozoites of *Plasmodium falciparum*

Fig.2a Severity of anemia (%) in malaria patients

Fig.2b Severity of thrombocytopenia (%) in malaria patients

Table 1a .Comparison of diagnostic test when peripheral thick blood smear was adopted as gold standard

Methods of diagnosis of Malaria	Sensitivity(%)	Specificity (%)	PPV(%)	NPV(%)
Thinsmear	80	100	100	82.92
QBC	87.61	93.82	94.84	87.85
RDT	92.38	100	100	91.75

Table 1b. Sensitivity of QBC for species identification

Species of Malaria	No of cases	No of QBC +VE	Sensitivity(%)
P. falciparum	91	81	89.01
P.Vivax	09	07	77.78
Mixed infection (Pf+Pv)	05	04	80.00B

Table 1c. Sensitivity of RDT For species identification

Species of Malaria	No of cases	No of RDT +VE	Sensitivity(%)
P. falciparum	96	89	92.70
Non Falciparum	09	08	88.89B

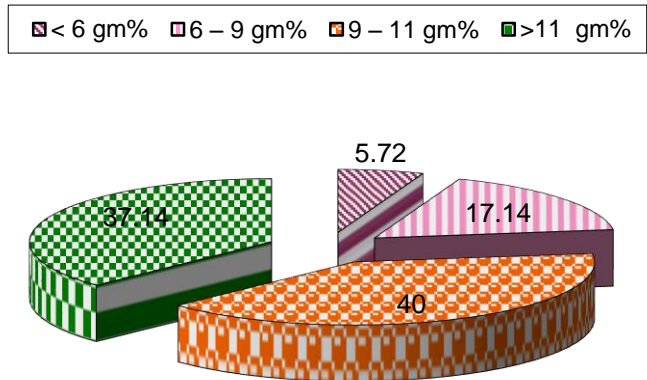
Table 1d. Sensitivity of QBC in different levels of Parasitemia

Parasite Density	Smear +ve cases	QBC +VE cases	Sensitivity(%)
<100/ μ L	35	24	68.57
100 - 500/ μ L	52	50	96.15
501 - 1000/ μ L	10	10	100
>1000/ μ L	08	08	100

Table 1e. Sensitivity of RDTs in different levels of Parasitemia

Parasite Density	Smear +ve cases	RDT +VE cases	Sensitivity(%)
<100/ μ L	35	27	77.14
100 - 500/ μ L	52	52	100
501 - 1000/ μ L	10	10	100
>1000/ μ L	08	08	100

FIG.2a Severity of Anemia (%) in Malaria



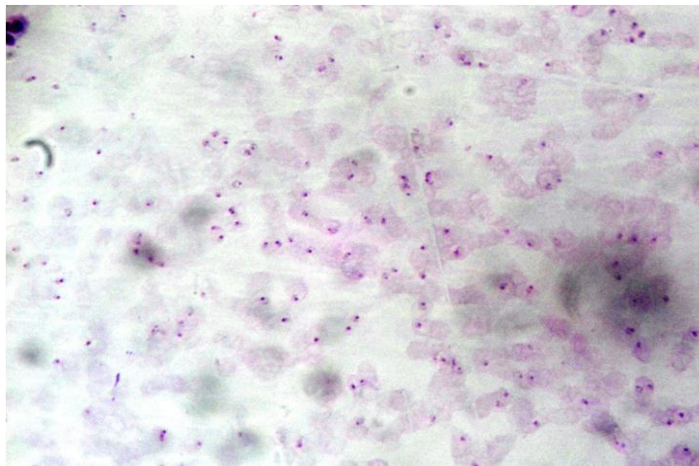
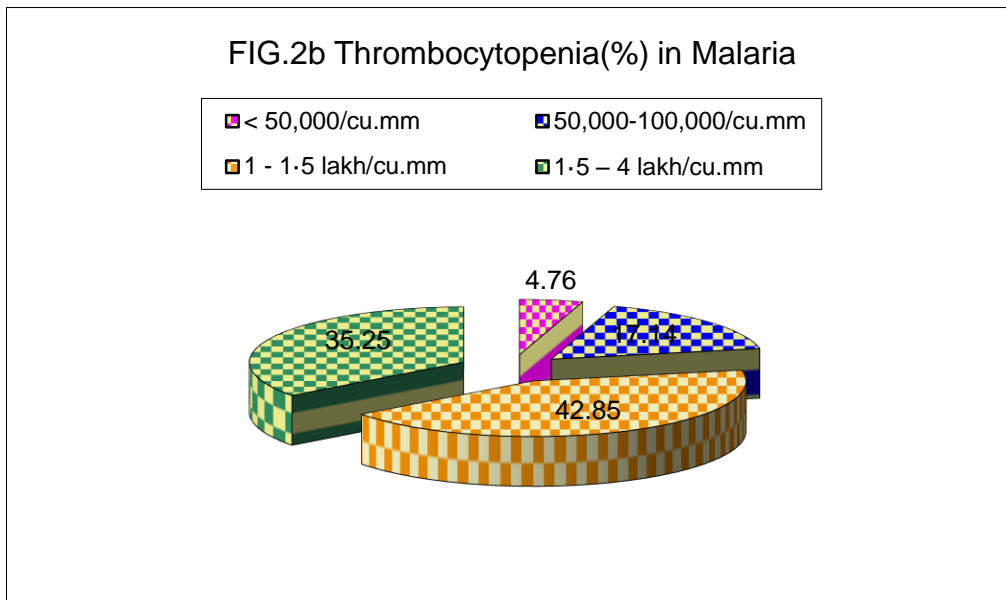


Fig 1(a)Thick blood film (dehaemoglobinised) showing trophozoites (ringform)of *Plasmodium falciparum*. (JSB, X500)

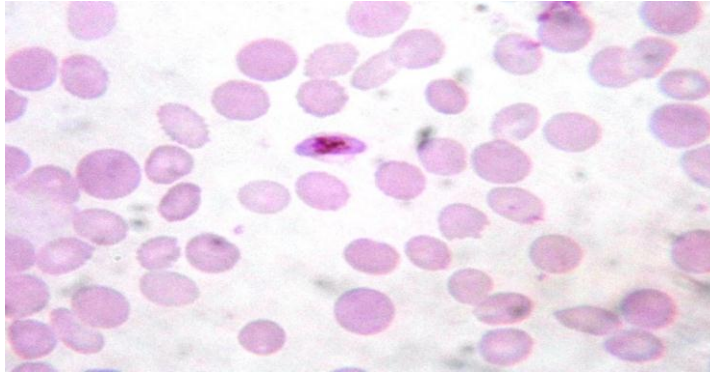


Fig 1(b)Thin blood film showing sickle shaped gametocyte of *Plasmodium falciparum* (Leishman, X500)

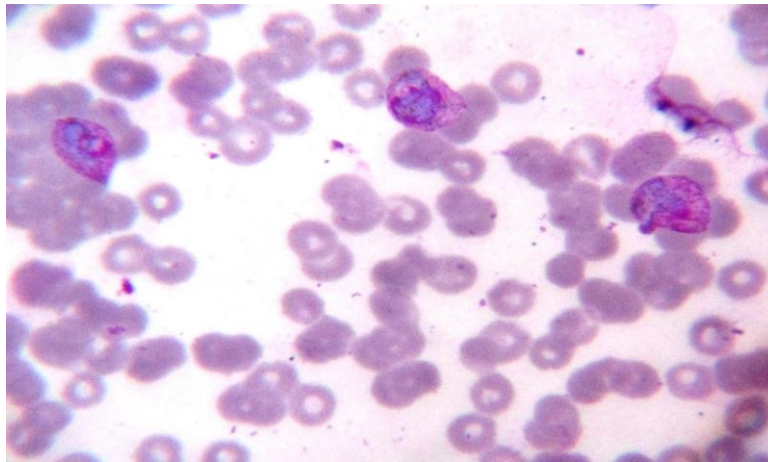


Fig 1(c)Thin blood film showing young ameboid trophozoite and early schizonts of *Plasmodium vivax*

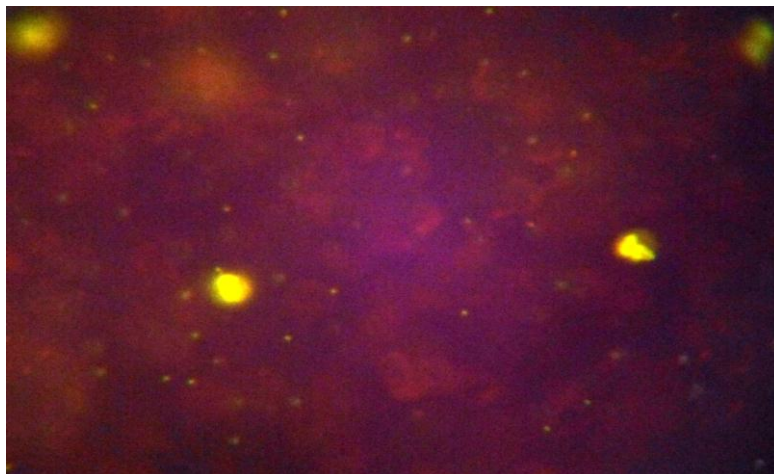


Fig 1(d)QBC film showing trophozoites of *Plasmodium falciparum*.

Table 2a. Severity of anaemia in malaria

Degree of anaemia (Hb%)	No. of cases			percentage
	<i>Pf</i>	<i>Pv</i>	<i>Pf+Pv</i>	
< 6 gm%	06	00	00	05.72
6 – 9 gm%	15	02	01	17.14
9 – 11 gm%	38	03	01	40.00
>11 gm%	33	04	02	37.14

Table 2a. Relationship Of Platelet Count With Malaria

Degree of thrombocytopenia (TPC)	No. of cases			percentage
	<i>Pf</i>	<i>Pv</i>	<i>Pf+Pv</i>	
< 50,000/cu.mm	05	00	00	04.76
50,000-100,000/cu.mm	17	01	00	17.14
1 - 1.5 lakh/cu.mm	40	04	01	42.85
1.5 – 4 lakh/cu.mm	32	03	02	35.25

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