Laboratory diagnosis of malaria by conventional peripheral blood smear examination with Quantitative Buffy Coat (QBC) and Rapid Diagnostic Tests (RDT) - A comparative study

Bhat Sandhya K [1*], Sastry Apurba S [1], Nagaraj E.R. [2], Sharadadevi Mannur [2], Sastry Anand S [3]

[1] MD, DNB; Department of Microbiology, Meenakshi Medical College, Kanchipuram, India
[2] MD; Department of Microbiology, Sri Siddhartha Medical College Hospital and Research Centre, Tumkur, Karnataka, India
[3] MD; Department of Medicine, Karpaga Vinayaga Institute of Medical Sciences, Kanchipuram, India

**** Corresponding Author:*** Dr. Sandhya Bhat K
Assistant Professor, Department of Microbiology
Meenakshi medical college and Research Institute, Kanchipuram, Near Chennai, India
Mobile: 09 444 327 316 / 09 444 327 314 | Email: sandhyabhatk@gmail.com

Abstract

Aim: Rapid diagnosis is prerequisite for effective treatment and reducing mortality and morbidity of malaria. Microscopy has been the Gold standard for malaria diagnosis for decades. Recently, many new rapid diagnostic tests like Quantitative Buffy Coat (QBC) examination and rapid antigen detection methods are being widely used. We made an attempt to compare peripheral smear, QBC and rapid antigen detection methods for the diagnosis of malaria.

Materials and Methods: A total number of 500 samples were collected from patients presenting with classical symptoms of malaria. Thick and thin blood smears were prepared and stained with Leishman's stain. QBC, Histidine Rich Protein-II antigen test and plasmodium Lactate Dehydrogenase tests were done using commercially available kits.

Results: Taking thick smear as gold standard, thin smear had sensitivity, specificity, positive and negative predictive values of 90.9%, 100%, 100% and 98.6% respectively. QBC showed sensitivity, specificity, positive and negative predictive values of 95.45%, 100%, 100% and 99.31% respectively. HRP-II antigen detection showed sensitivity, specificity, positive and negative predictive values of 56.06%, 100%, 100% and 94.20%. pLDH showed sensitivity, specificity, PPV and NPV of 95.45%, 100%, 100% and 99.40% respectively.

Conclusion: In our study, QBC had highest sensitivity followed by pLDH assay. Leishman's stained thick smear is cost effective but requires technical expertise to interpret the results, so if

facilities are available, QBC can be used for routine diagnosis. In places where facilities are not available rapid diagnostic test devices can be used, especially in endemic areas.

Keywords: Malaria, diagnosis, QBC, HRP-II antigen detection, pLDH assay

Introduction

Malaria is the most important parasitic disease of humans, transmitted in 108 countries containing three billion people and causes nearly one million deaths each year. Malaria has been eliminated from the developed countries like United States however, its prevalence rose in many parts of the tropics including India.[1]

According to the WHO South East Asia Regional (SEAR) Office estimates, during 2000-2009, malaria incidence remained between the range 2.16 -2.83 millions and malaria deaths between 3188 - 6978 in SEAR. The proportion of *P. falciparum* being 44 – 60% and more than 70% of these cases being reported from India.[2]

The most important problems in controlling malaria in India are its diverse clinical presentations and limited access to effective diagnosis and treatment.[3] Laboratory confirmation of malaria infection requires the availability of a rapid, sensitive, and specific test at an affordable cost. Over the years many new tests have been developed in an attempt to improve the diagnosis of malaria, but conventional method by smear microscopy remains the gold standard against which all other tests have been evaluated. However, the microscopy requires technical expertise and availability of a good quality microscope.[4]

Among the newer diagnostic modalities, the commonly used methods are Quantitative buffy coat examination (QBC) and Rapid diagnostic tests (RDT) detecting parasitic antigens like histidine-rich protein-2 (HRP 2), plasmodium lactate dehydrogenase (pLDH) and pan-specific aldolase. Though QBC is sensitive but it has drawbacks like expensiveness, imperfect speciation and associated with false positive results can be reported due to artifacts, such as cell debris.[5-6] RDTs are simple, easy to perform and give quick results but it is also expensive, associated with false positive test due to persistent antigenemia, and cross-reactions with auto antibodies such as rheumatoid factor and false negative test in severe malaria, probably due to immune-complex formation, prozone phenomenon and unknown causes.[2,7]

There are not enough data about comparison between malarial diagnostic techniques in and around Tumkur. In the present study we have made an attempt to compare between the peripheral blood smear examination, Quantitative Buffy Coat examination, Histidine Rich Protein-II antigen and plasmodium Lactate Dehydrogenase tests in clinically suspected cases of malaria.

Materials and Methods

The study was conducted in a tertiary care hospital during December 2008 to May 2010. Five hundred patients with clinical suspicion of malaria presenting with pyrexia with chills and rigor or atypical presentations were taken for the study. Leishman stained thick and thin blood smears, QBC, HRP-II antigen detection using paracheck Pf dipstick and pLDH detection using SD Malaria Antigen dipstick were performed.

Peripheral smear preparation

Thick and thin blood smears were prepared and stained with Leishman stain according to the standard guidelines described elsewhere.[2,8] After staining, the smears were examined at x1000 magnification. Atleast100-200 fields, each containing 20 WBCs were examined before thick smear was reported as negative for malaria. The red blood cells in the tail end of the thin smear were examined for the species identification and stages of the parasites.[2,8]

Quantitative Buffy Coat Examination

QBC Malaria capillary tubes of Becton Dickinson Tropical Disease Diagnostics were used in the study. The capillary tube was filled with 55 µl to 65 µl of blood and was centrifuged at rate of 12000g for five minutes .The entire circumference of the Buffy coat area of the tube was screened under UV microscope adaptor with 60x oil immersion objective attached to light microscope. Total examination time to exclude as negative was approximately 2 minutes.[9] The signet ring forms appeared as distinct apple green dots inside the faint RBC, 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.[2,6,10]

Rapid Diagnostic Tests (RDT)

HRP-2 antigen detection was done using *paracheck pf* Dipstick of Orchid Biomedical Systems and pLDH detection was done using SD BIOLINE Malaria antigen test kit of Standard Diagnostics. The kits were all from the same batch and were used before the expiry date and performed according to the instruction manual by the manufacturer.

Paracheck Pf is a rapid self performing, qualitative immunochomatographic test for the detection of *P.falciparum* specific HRP-II antigen in whole blood samples. SD BIOLINE Malaria Antigen Test is also an immunochomatographic test that contains a nitrocellulose membrane strip pre-coated with two polyclonal antibodies as separate lines across the test strip, one line specific to the LDH of *P.falciparum* and the other line pan specific to the LDH of all plasmodium species. The procedure and interpretation of test results of both the rapid tests were carried out according to the manufacture's literature guideline.

Statistical analysis

Descriptive statistical analysis has been carried out. Results on continuous measurements were presented on Mean ± SD (Min-Max) and results on categorical measurements were presented in number (%). Chi-square/ Fisher Exact test has been used to find the significance of study parameters on categorical scale between two groups. 95% Confidence Interval has been computed to find the significant features.[11]

Results

Maximum numbers of cases were seen between the age group of 21-30 years. The male to female ratio was 1.45:1. Fever with chills was the most common symptom (100%) followed by body ache, headache, abdominal pain, nausea, vomiting, jaundice and cough with expectoration. Seasonal variation was observed and there was an increased in the incidence of malaria positive cases from the month of April to June.

Of the 500 cases tested, 13.2% (66 out of 500) of the cases were positive by thick blood smear examination (figure 1), where as thin blood smear examination showed a positivity of 12% (60 out of 500), of which 6.6% were positive for *Plasmodium falciparum*, 4.6% were positive for *Plasmodium vivax* and 0.8% was mixed cases (Figure 2).

QBC examination was positive for 12.8% (64 out of 500) cases of which seven % were positive for *Plasmodium falciparum* (Figure 3), five % were positive for *Plasmodium vivax* and 0.8% were mixed cases. HRP-II antigen detection was positive for 7.4% (37 out of 500) of the cases indicating *Plasmodium falciparum* infection (Figure 4). *Plasmodium vivax* and Mixed infections were not detected by this test. 12.6% (63 out of 500) of the cases were positive by pLDH detection of which 7% were positive for *P. falciparum* (Figure 5), 5.6% were positive for non falciparum species.

Discussion

Malaria is a parasitic infection of global importance and is a major public health problem in India, as well as in Tumkur, Karnataka and accounts for sizeable morbidity, mortality and economic loss. A key to effective management of malaria is prompt and accurate diagnosis. During the last decade, several new rapid diagnostic techniques have been developed and evaluated widely. They aim at prompt and accurate diagnosis of malaria parasite that helps in early start of appropriate antimalarial drug to prevent the complications.[12]

In the present study, maximum numbers of patients (28.2%) were in the age group of 21-30 years which is similar to the other studies conducted elsewhere.[13,14] The male preponderance is because of the outdoor life that the males lead and females in India are usually are better clothed than males.[9,14]

Peripheral bloods smear examination

Microscopic analysis of appropriately stained thick and thin blood smears have been the standard diagnostic technique for identifying malaria for more than a century. Thick smear allows for detection and quantification even in low parasitemia while thin smear helps in speciation of the *Plasmodium* species. This technique is capable of accurate and reliable diagnosis when performed by skilled microscopist using defined protocols. It is relatively inexpensive, sensitive to a threshold of 5-50 parasites/ µl and able to characterize the infecting species and their relative densities.[15-16] However, it has limitations like time consuming, labour intensive and need of a skilled technician. In addition, in patients with *P.falciparum* malaria, sometimes the parasites can be sequestered and are not present in the peripheral blood. However, the blood film is still the only widely available tool against which the newer methods for diagnosis of malaria can be compared.[15,17]

In the present study, the thick blood smear positivity was 13.2%, which is comparable with Ndao et al, Mendiratta DK et al (18.28%) and Parija SC et al. (19.95%).[7,18,19] *P.falciparum* accounted for 54.54% followed by P.vivax in 39.4% cases and mixed infection in 4.54% cases which is in contrast to the various studies.[7,18-19] *P.vivax* has the widest geographic distribution throughout the world. 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 [20].The failure of control measures with regard to the vectors and thus establishment of chain of transmission of malaria has upset the balance of prevalence of the two species *P.vivax* and *P.falciparum*. Due to emergence of drug resistance and establishment of transmission, following failure of eradication programme, *P.falciparum* has shown resurgence.[20] This could be the possibility for increased incidence of *P.falciparum* in our study. Thin blood smear had a sensitivity, specificity, positive and negative predictive values in comparison with thick blood smear examination are consistent with other study by Parija SC et al.[19]

QBC method

The major advantages of QBC method over peripheral smear study are its speed, ease to interpret, simple, highly sensitive and easy to detect *P.falciparum* gametocytes, *P.vivax* schizonts and ability to detect levels of parasitaemia.[9,17] The limitations of QBC method are expensiveness, difficult to speciate the ring forms, false positive results due to Howell- Jolly bodies, artifacts such as cell debris.[5,6,10]

In the present study, one case of *P.vivax* and 2 cases of *P.falciparum* were missed by QBC method. 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.[5] One false positive *P.falciparum* was recorded by QBC method, this can be explained by the fact that certain artifacts in blood like Howell Jolly or platelet fragments might resemble ring forms of *P.falciparum*.[19,21] Similarly, one ring form of *P.vivax* was wrongly identified as *P.falciparum*. Ring forms of *P.vivax* are generally larger than that of *P.falciparum*. As parasitized red blood cells are not visible by QBC, it was not possible to use morphology of infected red cells to

support species differentiation, as it is possible in case of thin blood smear.[22] The sensitivity, specificity, PPV and NPV values of present study are consistent with Gay F et al and Parija SC et al.[19,23]

HRP-2 Antigen detection test

HRP-II antigen detection test is rapid, sensitive and specific, easy to perform, does not require special equipment or training (so useful in rural areas) [2,7]. However, its high cost, inability to detect non falciparum malaria infection & inability to quantify the parasitic load and severity of the infection and unable to monitor the response to treatment are some of its limitations.[7,24]

HRP-II antigen detection test had low sensitivity (56.06%), compared to other studies as it failed to detect three *P.falciparum* cases and all 26 cases of P.vivax. HRP-II antigen is water soluble protein produced by asexual stages and young gametocytes of *P.falciparum*, which is specific to *P.falciparum* infection.[17] All three *P. falciparum* cases, which were negative by HRP-II antigen detection test, had parasitaemia of < 100 parasites/µl. At low levels of parasitaemia the sensitivity decreases. Since most of the individuals with symptomatic *P.falciparum* infections have greater than 60 parasites/ µl blood, the HRP-II antigen detection test will be of particular use in rapid diagnosis of febrile patients.[25] The results of present study regarding specificity, positive and negative predictive values are consistent with Lema OE et al and Mishra MN et al.[21,26]

pLDH test

It is simple dipstick capture assay having advantages similar to HRP II antigen detection test. In addition to that it can detect as all four *Plasmodium* species and can be used to follow the efficacy of drug therapy since it detects an enzyme which is only produced by living parasites, so that parasitological clearance coincides with clinical improvement.[3,15,27] Hence, the use of this test could be very useful in monitoring the drug response Disadvantages of this test- Its high cost, detects but cannot speciate the non falciparum malaria infection & inability to quantify the parasitic load and severity of the infection and low sensitivity when level of parasitaemia is < 100 parasites/ µl.[3,15,27]

One case of *P. falciparum* and two cases of *P. vivax* were missed by pLDH test which can be explained as these patients had only gametocytes of *P.falciparum* on blood smear examination (which produces less pLDH compared to live asexual form) or because of low parasitaemia of <100/ µl or because of Plasmodial gene deletion isolates, which express little or no pLDH antigen or Sequestration of parasites.[3]

Conclusion

There are many considerations to be taken into account when reviewing the methods for laboratory diagnosis of malaria like sensitivity, rapidity, availability and cost. QBC is sensitive

and simple but expensive. Rapid antigen detection kits are useful in field trials and rural area set up but its sensitivity and specificity is debatable at low parasitemia. In places where facilities are not available rapid diagnostic test devices can be used, especially in endemic areas. The present debate on the introduction of tests based on new technology is always welcomed. However, it does not avoid the necessity of reviewing correctly stained thick and thin blood films as the standard operating procedure when malaria is suspected.

Microscopy is simple, rapid, economical, sensitive and specific, hence still remains the gold standard method for malaria diagnosis; however, QBC can be used where trained microscopist is not available, rapid diagnostic test devices can be used, especially for field studies and rural set up.

Conflict of Interest: None declared.

References

1. Fauci AS, Kasper DL, Longo DL, Braunwald E, Hauser SL, Jameson JL, et al. Malaria. In: Osler W, editor. Harrison's principles of internal medicine. 17 ed. New York: McGraw Hill; 2008: 1280-93.
2. Kakkilaya BS. Malaria Site- All about Malaria. [Monograph on the internet] India; 2009Available from: URL: http://www.malariasite.com/
3. Malik S, Khan S, Das A, Samantaray JC. Plasmodium lactate dehydrogenase assay to detect malarial parasites. Natl Med J India. 2004 Sep;17(5):237-9.
4. Krudsood S, Wilairatana P, Mason DP, Treeprasertsuk S, Singhasivanon P, Looareesuwan S. Hidden Plasmodium falciparum infections. Southeast Asian J Trop Med Public Health. 1999 Dec;30(4):623-4.
5. Rickman LS, Long GW, Oberst R, Cabanban A, Sangalang R, Smith JI, et al. Rapid diagnosis of malaria by acridine orange staining of centrifuged parasites. Lancet. 1989 Jan 14;1(8629):68-71.
6. Krishna B, Deshpande A. Comparison between conventional and QBC methods for diagnosis of malaria. Indian J Pathol Microbiol. 2003 Jul; 46(3):517-20.
7. Mendiratta D, Bhutada K, Narang R, Narang P. Evaluation of different methods for diagnosis of P. falciparum malaria. Indian J Med Microbiol. 2006 Jan;24(1):49-51.
8. Chakraborthy P. Plasmodium and Babesia. Textbook of medical parasitology. 1st ed. Kolkata: New Central Book Agency (P) Ltd; 2004: 83-104.
9. Estacio RH, Edwin ER, Cresswell S, Coronel RF, Alora AT. The Quantitative Buffy Coat technique (QBC) in early diagnosis of malaria: The Santo tomas University Hospital experience. Phil J Microbiol Infect Dis. 1993;22(2):56-9.
10. Pinto MJ, Rodrigues SR, Desouza R, Verenkar MP. Usefulness of quantitative buffy coat blood parasite detection system in diagnosis of malaria. Indian J Med Microbiol. 2001 Oct;19(4):219-21.
11. Rao PSS, Richard J. An Introduction to Biostatistics-A manual for students in health sciences.New Delhi: Prentice hall of India. 2004: 86-160.

12. Kumar KR, Sudarshan KS. Clinical evaluation of a rapid diagnostic kit (paracheck Pf) for diagnosis of *Plasmodium falciparum* in Karnataka state of India. Indian J Prev Soc Med. 2004;35(1):10-4.
13. Muddaiah M, Prakash PS. A study of clinical profile of malaria in a tertiary referral centre in South Canara. J Vector Borne Dis. 2006 Mar;43(1):29-33.
14. Durand F, Crassous B, Fricker-Hidalgo H, Carpentier F, Brion JP, Grillot R, et al. Performance of the Now Malaria rapid diagnostic test with returned travellers: a 2-year retrospective study in a French teaching hospital. Clin Microbiol Infect. 2005 Nov;11(11):903-7.
15. Chayani N, Das B, Sur M, Bajoria S. Comparison of parasite lactate dehydrogenase based immunochromatographic antigen detection assay (optimal) with microscopy for detection of malaria parasites. Indian J Med Microbiol. 2004 Apr;22(2):104-6.
16. Playford EG, Walker J. Evaluation of the ICT malaria P.f/P.v and the OptiMal rapid diagnostic tests for malaria in febrile returned travellers. J Clin Microbiol. 2002 Nov;40(11):4166-71.
17. Moody A. Rapid diagnostic tests for malaria parasites. Clin Microbiol Rev. 2002 Jan;15(1):66-78.
18. Ndao M, Bandyayera E, Kokoskin E, Gyorkos TW, MacLean JD, Ward BJ. Comparison of blood smear, antigen detection, and nested-PCR methods for screening refugees from regions where malaria is endemic after a malaria outbreak in Quebec, Canada. J Clin Microbiol. 2004 Jun;42(6):2694-700.
19. Parija S, Dhodapkar R, Elangovan S, Chaya D. A comparative study of blood smear, QBC and antigen detection for diagnosis of malaria. Indian J Pathol Microbiol. 2009 Apr;52(2):200-2.
20. Park K. Arthropod-borne infections. Textbook of preventive and social medicine. 15 ed. Jabalpur: Banarsidas Bhanot. 1997: 188-201.
21. Lema O, Carter J, Nagelkerke N, Wangai M, Kitenge P, Gikunda S, et al. Comparison of five methods of malaria detection in the outpatient setting. Am J Trop Med Hyg. 1999 Feb;60(2):177-82.
22. Uguen C, Rabodonirina M. Parasight-F rapid manual diagnostic test of Plasmodium falciparum infection. Bulletin of World Health Organization. 1995;73(5):643-9.
23. Gay F, Traore B, Zanoni J, Danis M, Gentilini M. Evaluation of the QBC system for the diagnosis of malaria. Sante. 1994;4(4):289-97.
24. Iqbal J, Sher A, Rab A. Plasmodium falciparum histidine-rich protein 2-based immunocapture diagnostic assay for malaria: cross-reactivity with rheumatoid factors. J Clin Microbiol. 2000 Mar;38(3):1184-6.
25. Beadle C, Long GW, Weiss WR, McElroy PD, Maret SM, Oloo AJ, et al. Diagnosis of malaria by detection of Plasmodium falciparum HRP-2 antigen with a rapid dipstick antigen-capture assay. Lancet. 1994 Mar 5;343(8897):564-8.
26. Mishra MN, Misra RN. Immunochromatographic Methods in Malaria Diagnosis. MJAFI. 2007;63:127-9.
27. Jelinek T, Grobusch MP, Schwenke S, Steidl S, Sonnenburg FV, Nothdurft HD, et al. Sensitivity and Specificity of Dipstick Tests for Rapid Diagnosis of Malaria in Nonimmune Travelers. Journal of Clinical Microbiology. 1999; 37(3):721-3.

Table 1: Comparison of Thin blood smear examination, QBC, HRP-II and pLDH antigen test taking Thick blood smear examination as gold standard

Tests	Sensitivity	Specificity	PPV	NPV	Accuracy	P value
Thin blood smear examination	90.91	100.00	100.00	98.64	98.60	<0.001**
QBC examination	96.92	99.77	98.43	99.54	99.60	<0.001**
HRP -II antigen detection (paracheck Pf)	56.06	100.00	100.00	93.74	94.20	<0.001**
pLDH antigen detection (SD BIOLINE)	95.45	100.00	100.00	99.31	99.40	<0.001**

PPV- Positive predictive value,
NPV- Negative predictive value
** Statistically significant

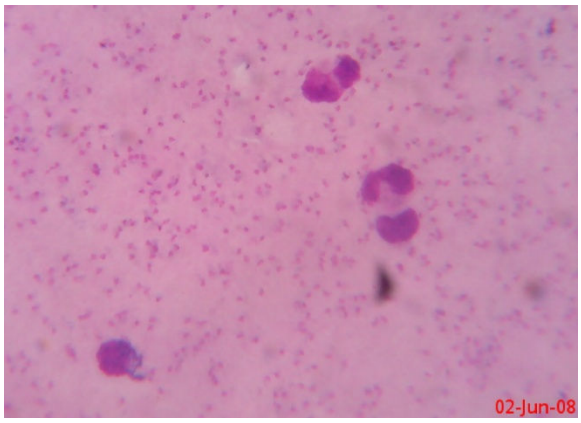

Figure 1: *P.vivax* ring forms in Leishman stained thick blood smear (100X)

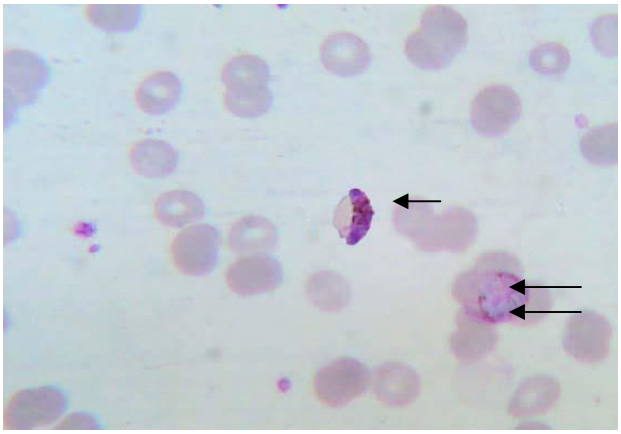

Figure 2: *P.falciparum* gametocyte (single arrow) and schizont of *P.vivax* (double arrow) in Leishman stained thin blood smear (100X) in a mixed case

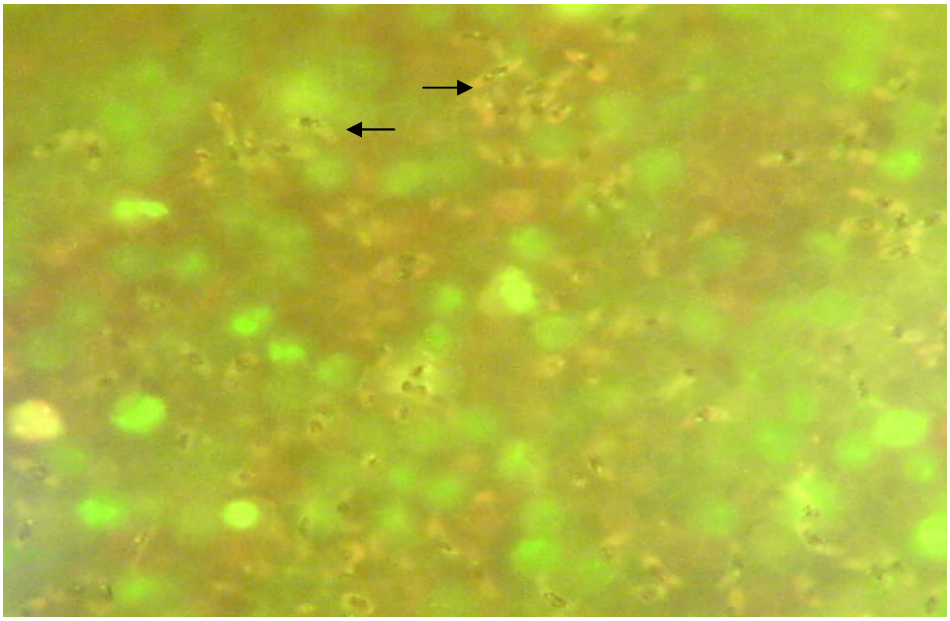

Figure 3: *P.falciparum gametocytes* (arrow) in Quantitative Buffy Coat examination

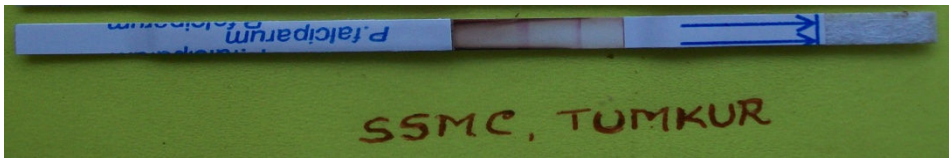

Figure 4: Paracheck Pf dipstick positive for *P.falciparum*

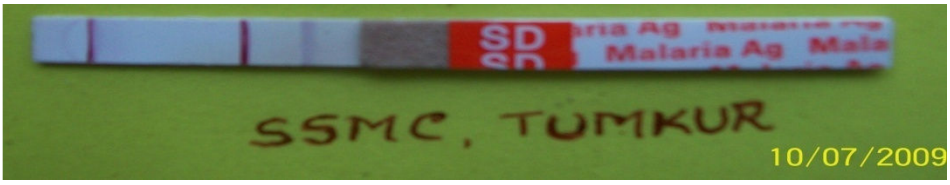

Figure 5: SD BIOLINE Malaria Antigen dipstick positive for *P.falciparum*