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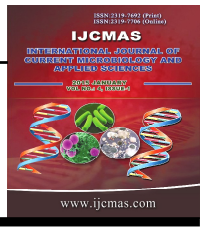

Original Research Article

Role of a Parasite Lactate Dehydrogenase-Based Immunochromatographic Antigen Detection Assay for the Detection of Malarial Parasites in Human Blood Samples

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K e y w o r d s

Lactate dehydrogenase, antigen detection assay, diagnosis of Malaria

A B S T R A C T

Malaria is a serious disease caused by the protozoal parasite *Plasmodium*, and if left untreated, can be fatal. Keeping in mind the seriousness of condition and the current availability of diagnostic modalities, the present study was carried out in a tertiary care hospital in Navi Mumbai with the following aims and objectives 1) To study the comparative efficacy of the peripheral smear examination by Leishman stain and rapid immunological techniques viz. SD Bioline (pLDH) for diagnosis of Malaria. 2) To determine the most optimum diagnostic tool for diagnosis of malarial parasites in terms of cost, technical expertise, lab facilities, rapidity, higher sensitivity and specificity. The present study was conducted in the Microbiology Department of a MGM Medical College & Hospital, Navi Mumbai from July 2012 to June 2013. 339 patients with clinical features s/o Malaria were included in the study. For all the patients in the study group, 1ml of blood was collected by venipuncture aseptically in EDTA bulbs. Thick and thin blood films were prepared with Leishman stain. All the samples were then subjected to antigen detection SD Bioline pLDH (Pf & Pv) Kits. Test was performed as per kit instruction manual and interpreted accordingly. A total of 339 cases were studied, 238 males and 101 females with age ranges from 15 yrs to 72 yrs. Of 339 cases, 138 cases were positive for malarial parasites by gold standard viz. Leishman stained thick smear. Sixty eight cases were infected with *P. falciparum*, 51 with *P. vivax* and 19 had mixed infection. In case of detection of *P. vivax*, performance of SD bioline was 96% and better than conventional staining of thin blood smears examination (92%) by Leishman, stain when compared with gold standard thick smear examination. The logistic, economic and technical factors limit rapid access to microscopic confirmation of malaria in many tropical countries including India. So taking into account of all the factors from our study, we recommend SD Bioline pLDH based antigen detection as a tool for diagnosis of malaria in future. Although no test can completely replace the conventional method of peripheral blood smear examination, these newer diagnostic tests can be used as supplement to microscopic examination of peripheral blood smear where the diagnosis cannot be made on microscopy alone. Also, rapid immunochromatography tests can be used in times of urgency and in areas where facilities of microscopy are not available especially during night times when services of laboratory and experienced Microscopists are not available.

Malaria is the most important parasitic disease of mankind and known since antiquity. The human disease is a protozoan infection of red blood cells transmitted by the bite of a blood feeding female *Anopheline* mosquito (Snow *et al.,* 2005). Prevalence of malaria is around 300 million people worldwide with a global death rate over 1.5 million/year (WHO, 2000; Snow *et al.,* 2005).

Malaria is a serious disease caused by the protozoal parasite *Plasmodium*, and if left untreated, can be fatal. Only four of the known species of *Plasmodium* are able to infect humans: *P. falciparum*, *P. vivax, P. ovale*, and *P. malariae*. The symptoms of malaria include high fevers, chills, rigors, and flu-like illness. Because the symptoms are so familiar, they are often misdiagnosed. The increasing incidence of malaria, the need to identify and treat the additional carriers (reservoirs) and to reduce the chances of transmission has given an impetus for development of simple and rapid methods for the diagnosis of malaria. Conventional Leishman or Giemsa stained peripheral blood smear examination remain the gold standard for the diagnosis of malaria in malaria endemic countries. However it has disadvantages such as it is labor-intensive, time consuming and requires expertise (Mendiratta, 2006).

Numerous rapid techniques like acridine orange (AO) stain, Quantitative Buffy Coat (QBC), detection of soluble histidine rich protein II antigen (HRP2 Ag), pLDH and aldolase enzymes in whole blood have been evaluated to diagnose malaria. Malaria presents a diagnostic challenge to laboratories in most countries. This study attempts to review the current methodology and approach to the diagnosis of malaria in a practical and helpful way for the laboratory and for the physician caring for the patient.

The urgency and importance of obtaining results quickly from the examination of blood samples from patients with suspected acute malaria render some of the more sensitive methods like PCR, DNA Probe assay for malaria diagnosis impractical for routine laboratory use, although they may be considered reference procedures (Mendiratta, 2006; Mendiratta, 2006; Parija, 2009). So, in the present study an attempt is made to evaluate and compare Parasitic LDH based immunochromatographic antigen assay with the Leishman stained thick and thin blood smears which is considered as Gold standard.

Keeping in mind the seriousness of condition and the current availability of diagnostic modalities, the present study was carried out in a tertiary care hospital in Navi Mumbai with the following aims and objectives.

1) To study the comparative efficacy of the peripheral smear examination by Leishman stain and rapid immunologically techniques viz; SD Bioline (pLDH) for diagnosis of Malaria.

2) To determine the most optimum diagnostic tool for diagnosis of malarial parasites in terms of cost, technical expertise, lab facilities, rapidity, higher sensitivity and specificity.

Materials and Methods

Methodology

The present study was conducted in the Microbiology Department of a tertiary Care Hospital from July 2012 to June 2013. The study was cleared by Institutional ethics committee and 339 patients were included in the study (Table 1).

Criteria for enrolling patients

Inclusion criteria

Patients with clinical features s/o Malaria which includes

- history of fever within the preceding 48 hrs,
- chills and rigors, or
- fever at the time of presentation or
- Fever of unknown origin, with or without splenomegaly, systemic complications or CNS manifestations.

Exclusion criteria

- Those patients treated for malaria in last 2 weeks.
- Those patients with confirmed diagnosis of infections like typhoid, pneumonia, urinary tract infection, septicemia, dengue, or leptospirosis were ruled out from the study.

Sample collection

For all the patients in the study group, 1ml of blood is collected by venepuncture aseptically in EDTA bulbs. Ethics committee and Research committee of the institution had approved the study

Conventional staining methods:

Sample preparation (thin film and thick film)

For diagnosis of malaria, thick and thin smears are prepared from blood collected in EDTA. Thick and thin blood films are prepared on slides for Leishman stain. Thick smear helps in rapid diagnosis, even when the parasitaemia is low. Thin smear is preferred for determination of species and morphological details of the parasite. Thin film also provides information regarding erythrocyte morphology, leukocytes, and platelets. The thick smear and thin smear was stained with Leishman stain.

Methods of counting malarial parasites in thick blood films

Examination of the thick film

- At least 100 good fields are screened before a slide can be pronounced negative.

- Determination of parasites/µl of blood is done by enumerating the number of parasites in relation to a standard number of leukocytes/µl (8000). Initially smear is examined for the presence of parasites and species identification. Tally counter is used one for counting leucocytes and other for parasites. After counting 200 leucocytes number of parasites is recorded. Then a simple mathematical formula multiplying the number of parasites by 8000 then dividing this figure by 200 (no. of leucocytes counted) is used.

Parasites counted/ no. of leucocytes x 8000 = parasites/µl of blood.

In this study, 200 leucocytes are counted, thus the formula:

No. of parasites x 40

Examination of thin film: examine for at least l00 fields to determine whether the blood film is positive or negative for malaria.

Rapid Immunological techniques: All the samples are then subjected to antigen detection using SD Bioline (Pf & Pv) Kits. Test was performed as per kit instruction manual and interpreted accordingly.

SD Malaria antigen

Test explanation & Principle

The SD Malaria antigen test contains a membrane strip, which is precoated with two polyclonal antibodies as two separate lines across a test strip. One polyclonal antibodies (test line Pf) are specific to lactate dehydrogenase of *P. falciparum* and the other polyclonal antibodies (test line P.v/ pan) are Pan specific to the lactate dehydrogenase of *Plasmodium* species.

All the results of conventional staining methods and rapid immunological techniques are compared and evaluated for the sensitivity and specificity for the diagnosis of malaria.

Results and Discussion

Of the 339 patients, 68 (20%) were positive for falciparum malaria by Leishman stained thick blood smear, 60(18%) by Leishman stained thick & thin blood smear, and 57(17%) by SD Bioline antigen Kit.

Of the 339 patients, 51(15%) were positive for vivax malaria by Gold Standard Leishman stained thick blood smear, 47 (13.8%) by Leishman stained thin blood smear, and 49 (14.4%) by SD Bioline antigen Kit. Thin smear by Leishman failed to detect 4 (7%) of the positives by Gold standard, while SD Bioline failed to detect 2 (3.9%) cases.

There were 19 positive cases for mixed malarial parasite infection. PLDH SD Bioline under study gave same results for mixed infection as compared to gold standard.

Parasitic index was calculated for all the positive malaria cases by gold standard Leishman stained thick smear. The parasitemias ranged from 80 to 9600 parasites/µl. Sensitivity of all the rapid Leishman thin staining method was 100% at parasitemias > SD Bioline Antigen detection method had a detection threshold of 280parasites/µl for *P. vivax* and 200 parasites/µl. The detection limit for *P. vivax* was 200 parasites/µl.

Data analysis

Sensitivity, specificity, positive predictive value, negative predictive value were calculated for each method by comparing the proportion of positive and negative results for each method with the gold standard Leishman stained thick blood smear examination.

Prevalence = No. of positives by Leishman stained thick blood smear examination/ total no. of samples examined.

Sensitivity = No. of matching Positives by both Leishman stained thick smear examination (Gold std) and the method to be evaluated/number of positives by Leishman stained thick blood smear examination or TP/TP+FN. This is the number of true Positives.

Specificity = No. of matching negatives by both Leishman stained thick smear examination (Gold std) and the method to be evaluated/no. of positives by Leishman stained thick blood smear examination. OR TN/TN=FN. This is the number of true negatives.

Positive Predictive Value (PPV) = sensitivity x (1-Prevalence)/ (sensitivity x prevalence) + (1- specificity) x (1-prevalence) OR TP/ TP + FP

Negative Predictive Value (NPV) = specificity x (1-Prevalence)/ specificity x (1-prevalence) + (1- sensitivity) x prevalence OR TN/FN+TN

The concordance of tests was calculated as follows:

Concordance = No. of matching Positives and negatives by both Leishman stained thick smear examination(Gold std) and the method to be evaluated/ total no. of samples examined OR TP+ TN/ Total no. of samples examined.

The values obtained are multiplied by 100 and reported as percentage.

Where, TP-True positive, TN – True negative

With reference to the data shown in Table 3:

Prevalence of malaria = 40%

For mixed malarial parasite infection, results by all the methods were same as compared to the gold standard.

The majority of cases of malaria worldwide are treated on the basis of clinical diagnosis and microscopy. Several studies have shown that the ability to diagnose malaria by blood film examination alone is about 75% for P.*falciparum* (White, 1996). Rapid detection and effective treatment of malaria is a prerequisite in reducing the morbidity and mortality due to the disease. Leishman stained thick smear examination, which is the corner stone in the laboratory diagnosis of malaria, has undergone little improvement since its inception. In the present study, we included 339 patients attending hospital over a period of one year for different complaints suggestive of malaria. We evaluated and compared five different techniques of rapid detection of malarial parasites with the Gold standard Leishman stained thick smear.

Type of malaria

In India 40% of cases are as result of *P.Vivax* malarial infection. 44.3% are due to *P. falciparum* malarial infection. 10–15% is due to mixed malarial infection (WHO, 2000). In this study, *P. falciparum* contributed to 49% of the total study group, *P. Vivax* contributed to 37% of the total study group and mixed malarial infection was responsible for the remaining of 14% of the study group (Table 2).

Mendiratta *et al.* (2006) studied 443 cases with maximum number of *P. falciparum* cases. Thus our study compares well with WHO report and other similar studies regarding the increased incidence of *P. falciparum*.

In the present study, while comparing the SD Bioline pLDH for *P. falciparum* with gold standard Leishman stained thick blood smear microscopy was 83 %. For detection of *P. vivax*, SD Bioline (96%) detected highest no. of cases followed by thin peripheral smear microscopy by Leishman stain (92%). our results were in agreement with other studies (Parija, 2009; White, 1996; Chakraborthy 2007). In our study there were no false positive results by any of the methods suggesting 100% specificity.

In the present study the results obtained by thin smear examination by Leishman stain had lower sensitivity of 88% for *P. falciparum* and 92 % for *P. vivax* (Figs. 1–

2). It was found that at lower parasitemia, the sensitivity dropped considerably. Sensitivity was 100% at parasitemia level 240parasites/µl or more. Thick smear provides enhanced sensitivity of blood film technique and is much better than the thin film for detection of low levels of parasitemia and relapse or recrudescence (Hunt-Cooke *et al.*, 1999). Warhurst and Williams reported that examination of thin blood films is only 1/10 as sensitive as examination of thick blood films for the quantification of malarial parasites, although morphological identification of the *Plasmodium* species present is much easier using thin films. It is highly recommended that both thick and thin films be prepared and examined each time of blood film examination for parasites is requested (Warhurst and Williams, 1996) (Tables 4 & 5).

The recommended method and current gold standard used for the routine laboratory diagnosis of malaria is the microscopic examination of stained thick blood films. In the most capable hands, this method can be expected to detect 50 parasites/µl (0.001% parasitemia) and to identify to the species level 98% of all parasites seen. In our study parasitemia level of 80parasites/µl or more were detected by thick smear microscopy. This procedure is recognized as difficult and time-consuming, requiring considerable training to obtain the necessary skills.

Immunochromatographic dipsticks offer the possibility of more rapid, nonmicroscopic methods for malaria diagnosis, thereby saving on training and time. These tests are easy to perform and require little training to interpret the results. On June 13, 2007, the U.S. Food and Drug Administration (FDA) approved the first RDT for use in the United States. This RDT is approved for use by hospital and commercial laboratories, not by individual clinicians or by patients themselves. It is recommended that all RDTs are followed-up with microscopy to confirm the results and if positive, to quantify the proportion of red blood cells that are infected (Craig and Sharp, 1997).

RDT currently on the market are easy to use; most are in cassette format with single-application areas for the blood sample and clearing buffer. Most evaluation trials have included temperature and time stability for at least 1 year at 40°C.

The new generation of RDT offers a realistic practical chance to move the diagnosis of malaria away from the laboratory and nearer to the patient. The added assurance that life-threatening parasitemia with *P. falciparum* will not be missed is welcome, particularly for inexperienced laboratory staff during night calls. The ability to detect the majority of the non-*falciparum* malaria cases also makes these tests ideally suited as major backup procedures for malaria diagnosis.

There are many considerations to be taken into account when reviewing the methods for laboratory diagnosis of malaria (Table 6), not the least of which is the important factors of availability and cost. The present debate on the introduction of tests based on new technology is 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 or of replacing a current training program for the identification of the *Plasmodium* species and for detection of parasitemia below the present threshold of detection by RDT.

Table.1 Sex distribution of clinically suspected patients of malaria (n=339)

	MALES No. (%)	**FEMALES No. (%)**	**TOTAL**
Clinically suspected patients	238 (70%)	101(30%)	339

Table.2 Age group Leishman stained thick blood smear positive cases for malaria

			P. falciparum	*P. vivax*	Mix	Total
Age group	15-25 yrs	Count	11	13	5	29
		% within age group	37.90%	44.80%	17.20%	100.00%
	25-50 yrs	Count	53	31	14	98
		% within age group	53.5%	31.2%	14.3%	100.00%
	50-75 yrs	Count	4	7	0	11
		% within age group	36.40%	63.60%	0.00%	100.00%
Total		Count	68	51	19	138
		% within age group	49.2%	37%	13.8%	100.00%

Table.3 Comparison of conventional Leishman stained thick smear examination with other rapid staining methods and rapid immunological techniques for the diagnosis of malarial parasites

	Leishman (gold std)	Leishman stain	JSB stain	SD bioline
	Thick Smear	Thin Smear	Thin Smear	
Negative	201	213	213	214
PF	68	60	60	57
PV	51	47	47	49
MIX	19	19	19	19
Total	339	339	339	339

Table.4 Comparison of sensitivity and specificity of rapid methods for *P. falciparum* with Leishman stained thick smear

	Leishman	SD bioline
	Thin smear	
Sensitivity	88.2 %	83.8%
Specificity	100%	100%
PPV	100 %	100%
NPV	97%	96.2
test efficiency (Concordance of tests)	97.6%	96.7

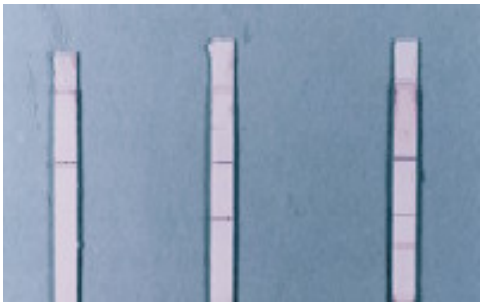
Table.5 Comparison of sensitivity and specificity of rapid methods for *P. vivax* with Leishman stained thick smear

	Leishman	SD bioline
	Thin Smear	
Sensitivity	92.0 %	96.0%
Specificity	100%	100%
PPV	100 %	100%
NPV	98.6%	99.3
Test efficiency (Concordance of tests)	98.8%	99.4

Table.6 Comparative evaluation of different methods based on following parameters for diagnosis of malaria

	Parameters	**Leishman thick smear Stain**	**Leishman thin smear stain**	**SD Bioline kit**
1	Sensitivity	80parasites/µl	240parasites/µl	200parasites/µl
2	Specificity	All species	All species	*P. falicparum* and *P. vivax*
3	Cost	Rs 5/test	Rs5/test	Rs 60/ test
4	Technical expertise	Highly skilled	Highly skilled	minimal skills
5	Equipment	lab, microscope, electricity	Lab, microscope, electricity	Kit only, No special equipment
6	Time for result	45-60mins	30-45mins	5 -15mins
7	Subjectivity Variation	High	High	Low

Figure.1 Interpretation of the test



1. Negative 2.*P. vivax* 3. *P. falciparum*

Figure.2 Ring forms of *P. falciparum* on Leishman stained thin smear

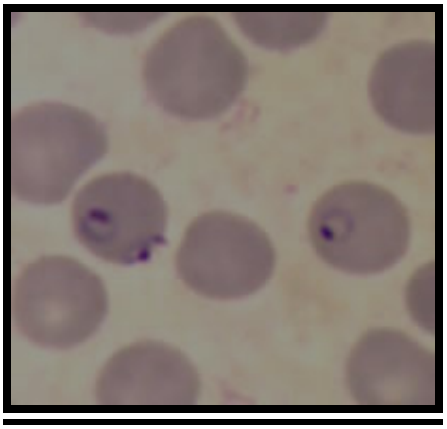


Figure.3 Leishman stained smear showing Schizonts of *P. vivax*

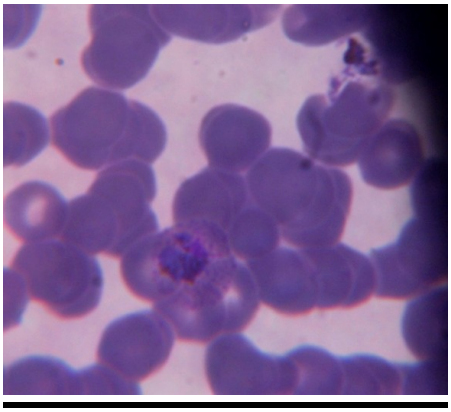


Figure.4 Leishman stained smear showing ring forms of *P. vivax*

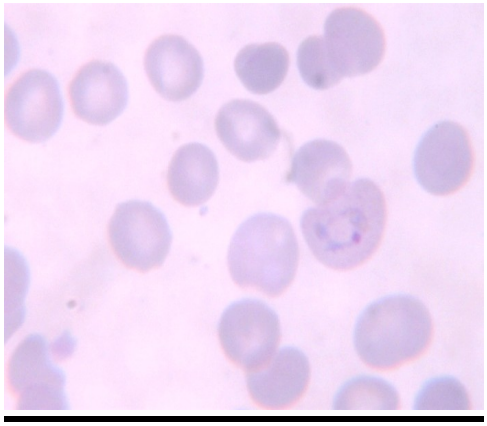
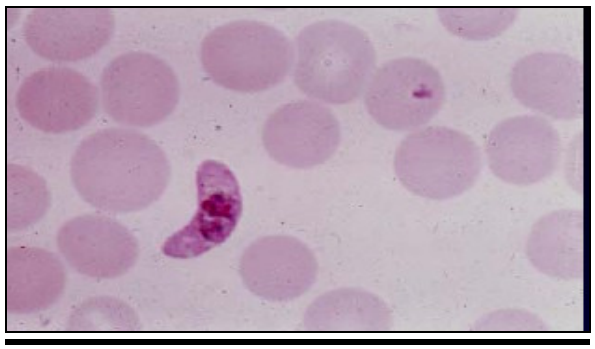


Figure.5 Leishman stained smear showing gametocyte forms of *P. falciparum*



The logistic, economic and technical factors limit rapid access to microscopic confirmation of malaria in many tropical countries including India. So taking into account of all the factors from our study (Table 6), we consider pLDH detection kit as a tool for diagnosis of malaria in future. Although no test can replace the conventional method of peripheral blood smear examination, these newer diagnostic tests can be used as supplement to microscopic examination of peripheral blood smear where the diagnosis cannot be made on microscopy alone. These tests can be used in times of urgency and in areas where facilities of microscopy are not available especially during night times when services of laboratory and experienced microscopists are not available.

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