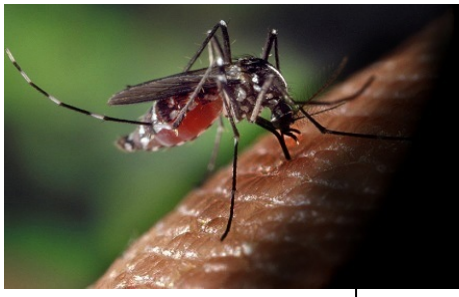

International Journal of Mosquito Research

ISSN: **2348-5906**
CODEN: **IJMRK2**
IJMR 2020; 7(6): 33-39

Received: 15-09-2020
Accepted: 21-10-2020

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Diagnostic performance of rapid diagnostic test (RDT) and enzyme-linked immunosorbent Assay (ELISA) in comparison with microscopy, for Malaria

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Abstract
Introduction: Malarial parasite belongs toprotozoan family causes malaria in vertebrates including human being, which is transmitted by the bite of infected female anopheles mosquitoes considered an important tropical disease in terms of morbidity and mortality. In endemic and resource poor regions it poses diagnostic challenge. The present study was conducted in Malaria patients presented at a tertiary care Centre to evaluate and observe the diagnostic performance of Rapid Diagnostic Test (RDT) and Enzyme-Linked Immunosorbent Assay (ELISA) in comparison with microscopy.
Material and Methods: It was a cross-sectional study conducted for a period of one year in which purposive sample were included from the patients visiting the outpatient department as well as all the those admitted in inpatient departments and intensive care units. Blood samples of 146 clinical suspects of malaria was assessed for RDT/ELISA against microscopy as the gold standard.
Results: Out of146 samples from clinical suspects 73 were positive by microscopy and 73 negative, whereas by ELISA 70 true positive and 68 true negative was found respectively. By Rapid Diagnostic Test (RDT) true positive was found to be 67 and true negative 63.As compared to microscopy, ELISA was found to be 95.9% sensitive, 93.2% specific whereas RDT was found to be 91.8% sensitive and 86.3% specific.
Conclusion: Detection rate among microscopically positive malaria cases was found to be higher for ELISA as compared to RDT. Though RDT had a lower sensitivity as well as specificity as compared to microscopy yet owing to ease of performing the test, rapid outcomes and its ability to differentiate between *falciparum* and *vivax* species, RDT may be recommended as the initial diagnostic tool, however, microscopy for malarial parasite identification is still considered a gold standard method.

Keywords: Microscopy, ELISA, RDT

Introduction
Malaria, which is also called the "King of Diseases", caused by the protozoan parasite belonging to genus Plasmodium, transmitted among humans by hematophagous bite of infected female Anopheles mosquitoes. Four species of the genus *Plasmodium vivax, P. ovale, P. malariae* and *P. falciparum* cause nearly all infections in humans. Reported cases of malarial infestation in the South-East Asia is found to be 2.5 million, India alone contributes about 70% of the total malaria cases. India has reported 1.3 million confirmed malaria cases and 753 attributable deaths in year 2011, but was estimated that exact positive cases and deaths were 10 to 20 times more [1]. In India above 70% of the infections reported are considered to be due to *P. vivax*, 25 to 30% due to *P. falciparum* and 4-8% to mixed infection [2]. A complex life cycle is demonstrated by these parasites, in the invertebrate vector as well as vertebrate hosts, multiply in liver cells, and later invade red blood cells (RBCs), corresponding to the symptomatic period of the disease. Clinical symptoms of malaria includes mainly spells of fever with spikes on every third day, with headaches, malaise, chills, rigor, fatigue, nausea, and anemia where as severe features of disease are organ failure, delirium, consciousness impairment, and convulsions, followed by coma and death [3]. Malarial disease presents a diagnostic challenge in endemic and regions of inadequate resources where diagnosis is mainly

Made only on the basis of clinical signs and symptoms, although inaccurate as symptoms are considered to be very nonspecific and often overlap those of other febrile illnesses, therefore role of laboratory diagnosis is important to support clinical diagnosis." Gold standard" laboratory procedure for the diagnosis of malaria is microscopic examination of Giemsa stained thick blood smears [4]. Reliability on microscopy of blood smear is always under question, as it requires technical expertise, in case of variable and low parasitemia, sequestration of parasites in later stages of development in P. *falciparum* malaria [5]. Diagnostic limitation soften affects medical care and increases case fatality due to malaria, but can be cured if diagnosed as early as possible. In order to overcome these limitations many new diagnostic approaches has been developed that includes fluorescent staining of smear (Acridine orange), dipstick antigen detection of histidine rich protein-2 (HRP-2) and parasite LDH, Plasmodium aldolase, PCR and automated blood cell analysis [6]. The antigen capture tests are available as rapid test kits, uses two parasitic antigens histidine rich protein-2 (HRP-2) released from *P. falciparum* parasitized erythrocytes and lactate dehydrogenase (pLDH antigen) released from all four Plasmodium parasitized erythrocytes, includes all the necessary reagents, extensive training to perform the test or on equipment or to interpret their results is not required.

(Shiff *et al.*, 1993; Faiz *et al.*, 2000) [7, 8] reported that HRP-2 based test results found persistent antigenemia in a number of patients as late as 7-28 days after the smears became negative, suggesting that positivity could be detected even in those patients who became aparasitemic by microscopy by the time they were got admission in a hospital of tertiary level.

The Enzyme-Linked Immunosorbent Assay (ELISA) has been also found to be quick, sensitive and specific method for the detection of Plasmodium *sps*. This method has mass detection capability, therefore used for screening large number of samples. Antigen detection ELISA have shown high sensitivity and specificity [9]. As a fact, infectious level of *Plasmodium* species can be low upto 10-20 parasites in immune-compromised recipients [10], a highly sensitive technique is preferred to confirm the diagnosis.

In tertiary setting like large teaching hospital in endemic area, unique problems are encountered in diagnosis of malaria. Critically ill patient reach these centers building enormous pressure on clinician for presumptive therapy even before samples for smear examination are collected and also these Patients may have possibly received partial presumptive therapy before, hence smear may be negative.

Hence, keeping in mind the seriousness of the disease and need for early diagnosis and the limitations of currently available diagnostic facilities across India, this particular study was undertaken to evaluate effectiveness of Rapid screening methods like ELISA and Rapid Detection Tests (RDT) (pLDH and Pf-HRP 2) with the gold-standard diagnostic laboratory based microscopic method to find the most feasible and reasonable technique, in diagnosis of malaria.

Materials and Methods

Settings

The study was conducted at Department of Microbiology, Era's Lucknow Medical College and Hospital, Era University, Lucknow, a tertiary care teaching hospital with state-of-the-art infrastructure and caters to the semi urban and rural population of Lucknow primarily from deprived sections of Lucknow and neighboring districts.

Duration of Study: One year.

Type of Study: Cross-sectional study.

Sampling Technique: Purposive sampling.

Selection of subjects

The study population was comprised of patients of all age groups and gender with history of fever for 2-3 days duration associated with any of the following symptoms: chills and rigor, splenomegaly, hepatomegaly, headache, and abdominal discomfort, visiting the outpatient department and those admitted in various wards of the hospital including surgery, orthopedics, internal medicine, obstetrics and gynecology, and pediatrics wards, as well as emergency and intensive care units. Those on antimalarial therapy were excluded.

Sample size estimation

The sample size was estimated by keeping a targeted accuracy of 95% for RDT / ELISA assessments against microscopy as the gold standard. The following formula was used for this purpose:

$$N = z^2 s_n(1- s_n) / e^2$$

Where
s_n= 85% or 0.85(sensitivity of RDT/ELISA)
z=1.96 at 95% confidence and 80% power
Error in sensitivity (e) = 10% or 0.10
Sample size comes out to be: 73
For a targeted sensitivity of 95% - a sample size of 73 true positive cases was required.
For a targeted specificity of 95% - a sample size of 73 true negative cases was required.
Thus the experiment was done on a total of 146 specimen

Sample collection

5 ml blood was obtained aseptically by 5 ml disposable syringe from antecubital vein. (Venipuncture) from all clinically suspected patients. Out of the 5 ml, 2ml of venous blood was kept in a sterile vial (containing potassium EDTA) for preparation of thick and thin smears and for performing RDT, the remaining 3 ml was used for serological examination (ELISA).

Sample Processing

Preparation of Thin and Thick Blood Smear

Thin blood smear was prepared as soon as possible by placing the smooth edge of spreader (glass slide) in a drop of blood, keeping the angle between the slide and spreader to 45^0, and then smearing the blood on the slide till "tails" was formed followed by air dry and thick Blood Smear is prepared by a thick drop of blood on a slide which is moved in circular motion with the corner of another slide, to form a smear and allowed to air-dry [11]. The smear then placed in distilled water in glass cylinder for 5 to 10 minutes for de-haemoglobinization. Both the smears are then covered with Leishman stain for 2 minutes and diluted twice its volume of buffered distilled water. After 15- 20 minutes, washed with

buffered distilled water and dried and observed under oil-immersion objective [11].

Detection of Antigen by RDT

The SD Bioline malaria antigen test cassette has a membrane test strip, pre-coated with one monoclonal antibody and one polyclonal antibody as two separate lines across it. In one line, monoclonal antibodies specific to HRP-2 released from *P. falciparum* parasitized red blood cells and in the other polyclonal antibodies to lactate dehydrogenase common to all four species (*P. falciparum, P. vivax, P. malariae and P. ovale*) is to be used. A 5µl disposable specimen loop dipped into the blood and placed into the round sample well after which 4 drops assay diluents is added in the square assay diluent well.

Interpretation for RDT: lt was interpreted within 20-30 minutes.

Negative Result: The presence of one color band ("C" control line) within the result window.

Positive Result: The presence of two color bands ("*P.f*" Test line and "C" Control line)in the result window, indicated positive result for *P. falciparum* and presence of two color bands ("*Pan*" Test line and "C" Control line) within the result window, indicated other *Plasmodium* species (*P.v, P.m, P.o*) positivity whereas mixed infection with *P.f* and *P.v* (or *P.m, P.o*), the presence of three color bands ("*P.f*", "*Pan*" Test lines and "C" Control line) within the result window, indicated mixed infection.

Enzyme Linked Immunosorbent Assay (ELISA)

For ELISA, ErbaLisa® PAN (LDH) MALARIA Assay was performed, a fast and sensitive enzyme immunoassay for the qualitative determination of Detection of pLDH Antigen of all four species of Plasmodium *(P. vivax, P. falciparum, P. ovale, P. malariae)* in Human Whole Blood.

Principle of the Assay

Micro titer strip wells are pre-coated with monoclonal antibodies to LDH of Plasmodium species which binds to corresponding antigens of specimen. Based on Sandwich method, after washing of wells in order to remove the unbound test material, horseradish peroxidase (HRP) labeled anti-human IgG and IgM conjugate is added which binds to the captured Plasmodium-specific antigen. The Antigen-antibody complex which is formed by the bound conjugate can be seen by adding Tetra methyl benzidine (TMB) substrate which express a blue colored product intensity of which is proportional to the amount of Plasmodium-specific antigen present. At the end of reaction after addition of sulfuric acid a yellow color is produced, the absorbance of which is read at 450 nm using the ELISA micro well plate reader.

Calculation of Results

The cut-off is the mean absorbance value of the Cut-off control determinations.

Interpretation of Results

Samples were considered REACTIVE if the absorbance value was equal or greater than the cut-off value and NON-reactive with an absorbance value was less than the pre-fixed cut-off value.

Statistical Analysis

Data collected was subjected to analysis using Statistical Package for Social Sciences version 15.0. Data was represented as mean±SD and frequencies and proportions (%) specificity, sensitivity, positive predictive, negative predictive and accuracy value of different diagnostic tests were calculated against the gold standard.

Results

Total of 146 patients were taken into this present study, as they fulfilled the inclusion criteria and gave consent for the study. According to the present study incorporated in Table 1. Microscopic detection rates were 50% owing to purposive sampling. In the specimen used for assessment, the detection rate of ELISA was 51.4% whereas detection rate of RDT was 52.7%, thus indicating obvious presence of false positive detection. Microscopically as well as through RDT, *P. vivax* was the dominating pathogen and *P. falciparum* was seen in 9 (12.3%) of cases in both the techniques. For ELISA, no such discrimination could be made.

Table 1: Positivity rate for different diagnostic methods (N=146)

Method	No.	%
Microscopy	73	50.0
Pf	9	12.3
Pv	64	43.84
ELISA	75	51.4
RDT	77	52.7
Pf	9	12.3
Pv	68	46.586

As compared to microscopy which had 73 positive and 73 negative specimen, ELISA had 70 true positive and 68 true negative specimen, thus it had 5 false positive and 3 false negative specimen whereas RDT had 67 true positive and 63 true negative and 6 false negative and 10 false positive specimen. On comparing the two methods, no significant difference in false negativity and false positivity rate was observed (p=0.302 and 0.173).

As compared to microscopy, ELISA was found to be 95.9% sensitive, 93.2% specific whereas RDT was found to be 91.8% sensitive and 86.3% specific. The positive and negative predictive values of ELISA were 93.3% and 95.8% respectively whereas the same were 87.0% and 91.3% for RDT. The detection rates against microscopy have been evaluated in Table 2.

Table 2: Sensitivity & Specificity for different tests as compared to Microscopy

Test	TP	FN	TN	FP	Sens	Spec	PPV	NPV
ELISA	70	3	68	5	95.9	93.2	93.3	95.8
RDT	67	6	63	10	91.8	86.3	87.0	91.3
χ^2	1.066		1.858					
P	0.302		0.173					

Accuracy of ELISA = 138/146 = 94.52%
Accuracy of RDT= 131/146 = 89.7%
χ^2 for accuracy =2.313; p=0.128 (NS)

Discussion

This study was carried out to select a reliable laboratory

technique for diagnosis malaria for selecting the correct treatment and thus reducing mortality and morbidity in patients admitted with clinical symptoms suggestive of malaria in a tertiary care hospital in Lucknow. Clinical symptoms of malaria are nonspecific very similar to those of typhoid, gastroenteritis, pneumonia, meningitis and encephalitis.

Diagnosis of critically ill patients coming to tertiary healthcare centers, pose great pressure on clinician to start immediate presumptive therapy, even before sample is dispatched to laboratory. Some patients coming to hospital have taken partial treatment, leading negative smears. Therefore rapid and precise diagnosis of malaria is important for good clinical practice to prevent patients from harmful effects of potentially toxic intravenous drugs.A negative diagnosis might lead to a more active search for alternative diagnosis. For this purpose a purposive sampling technique was done in which 73 microscopy positive and 73 microscopy negative sample were evaluated for diagnostic testing through RDT and ELISA.

In present study, overall detection rate for ELISA was 51.4% whereas for RDT it was 52.7%. As the present study used a purposive sampling design in which 50% were microscopy positive and 50% microscopy negative cases, thus overall detection rate more than 50% indicated a definitive false positive pattern for both the diagnostic techniques. Similar to our study, Bouyou Akotet *et al.* (2014) [12] also observed a higher detection rate than microscopy in their assessment of SD Bioline malaria Ag-Pf/Pan RDT test. A false positivity is indicative of reduction in specificity. However, the actual efficacy of the two methods could only be evaluated with their respective performance against microscopy. In their study Bouyou Akotet *et al.* (2014) [12] also faced a similar situation.

In present study, as compared to microscopy, ELISA had a sensitivity, specificity, PPV and NPV of 95.9%, 93.2%, 93.3% and 95.8% respectively whereas as compared to microscopy, RDT had a sensitivity, specificity, PPV and NPV of 91.8%, 86.3%, 87.0% and 91.3% respectively. These findings indicated that RDT as compared to ELISA, despite having a higher detection rate in suspected cases had a lower sensitivity as well as specificity which might be attributed to a higher number of both false positive as well as false negative cases in this technique as compared to that for ELISA. However, this difference was not significant statistically. With respect to accuracy too, as compared to microscopy, the accuracy rates for ELISA and RDT were 94.5% and 89.7% respectively. Though ELISA had a better accuracy as compared to RDT yet the difference between two techniques was not significant statistically. Leaving aside the statistical consideration, and focusing on clinical relevance in view of the severe consequences of false negative rates, ELISA definitely had an edge over RDT as observed in present study. An overview of diagnostic efficacy of RDT and ELISA in different studies is being shown in Table D1 below:

Table D1: Diagnostic Efficacy of RDT and ELISA in different studies

S. N	Author (Year)	No. of samples	Kit used	Sensitivity	Specificity
ELISA					
1.	Noedl *et al.* (2006) [9]	700	HRP2-based ELISA	98.8	100
2.	Doderer *et al.* (2007) [13]	95 malaria patients + 2152 blood donors	DiaMed ELISA	84.2	84.2
3.	Oh *et al.* (2008) [14]	826	DiaMed ELISA	53.0	94.0
4.	Thongdee *et al.* (2014) [15]	-	NovaLisa test kit	71.4% *Pf* 100% *Pv* 100% Mixed 89% Overall	100% *Pf* 98.7% *Pv* 95.0 Mixed 91.6% Overall
5.	Kang *et al.* (2015) [16]	1163	HRP-2 based ELISA	99.6%	99.7%
6.	Present study (2015)	146	ErbaLisa®Pan (LDH) Malaria	95.9%	93.2%
Rapid Detection Test (RDT)					
1.	Chaijaroenkul *et al.* (2011) [17]		Malaria Antigen Pf/Pan™	98.6% *Pf* 99.0% *Pv*	98.8%
			Malaria Ag-Pf™	97.6% *Pf* 98.6% *Pv*	93.3%
			Malaria Ag-Pv™		98.8%
2.	Ojurongbe *et al.* (2013) [18]	217*		62.3%	87.4%
3.	Kosack *et al.* (2013) [19]	2585	SD Bioline Malaria Ag P.f/Pan 05FK60	90.2% *Pf* 79.4% *Pv/Pm*	98.5% *Pf* 98.7% *Pf/Pm*
4.	Sharma *et al.* (2013) [20]	188	SD Bioline malaria Antigen test	95.83%	85.42%
			Advantage Mal card	91.67%	85.42%
5.	Bouyou Akotet *et al.* (2014) [21]	215	SD Bioline malaria Ag- Pf/Pan	96.8%	89.3%
6.	Djalle *et al.* (2014) [22]	436	Paracheck™-Pf	85.9%	86.6%
			SD Bioline malaria Ag-Pf	92.3%	82.2%
			SD Bioline malaria Ag-Pf/pan	92.3%	81.2%
7.	Present Study (2015)	146	SD Bioline malaria Ag-Pf/pan	91.8%	86.3%

*Used composite reference instead of microscopy as gold standard

An overview of Table D1 above shows that almost all the studies have found ELISA to be more sensitive as well as specific, generally above 90% for ELISA except for Oh *et al.* (2008)[14] who reported a dismally low clinical sensitivity for ELISA (53%). An overview of Table D1 shows and confirms our view that RDT has a lower sensitivity as well as specificity, which in turn affects its accuracy.

With respect to diagnosis of the particular *Plasmodium*

species, in present study, majority of cases (64/73; 87.7%) were *P. vivax* and remaining 9 (12.3%) were *P. falciparum.* Although, in Indo-Gangetic plains *P.vivax* infection is more common as compared to *P. falciparum*, however, some studies have reported a higher prevalence of *P. falciparum* as compared to *P. vivax* in tertiary care settings [23, 24]. False positivity rate for *P. vivax* and *P. falciparum* was evaluated for RDT where kit used had a differentiating ability and was found that all 10 false positive cases detected by RDT were *P. vivax* whereas in case of low positive cases detected that of *P. falciparum* (only 9), no comment could be made on this aspect. So the rapid detection kit used in this study had an egde over the ELISA on this aspect. With respect to high accuracy of RDT in detection of *P. falciparum* the findings of the present study echoed the findings of Kosack *et al.* (2013) [19] who also observed higher accuracy of RDT in diagnosis of *P. falciparum* as compared to *P. vivax.*

Although both the techniques had high sensitivity as well specificity, however, ELISA had an overall high sensitivity as well as specificity but given the rapid detection ability and high efficiency in detection of *P. falciparum*, the RDT's usefulness can also not be denied.

Conclusion

The findings suggested in present study, *P. vivax* was the dominating malarial parasite. The detection rate among microscopically positive cases was higher for ELISA as compared to RDT yet the difference between two techniques was not significant. Though RDT had a lower sensitivity as well as specificity as compared to microscopy yet owing to ease of performing the test, rapid outcomes and its ability to differentiate between *falciparum* and *vivax* types, RDT may be recommended as the initial diagnostic tool, however, microscopy still remains the gold standard for malarial parasite diagnosis

Fig 1: SD Bioline Rapid Test (RDT) for Malaria

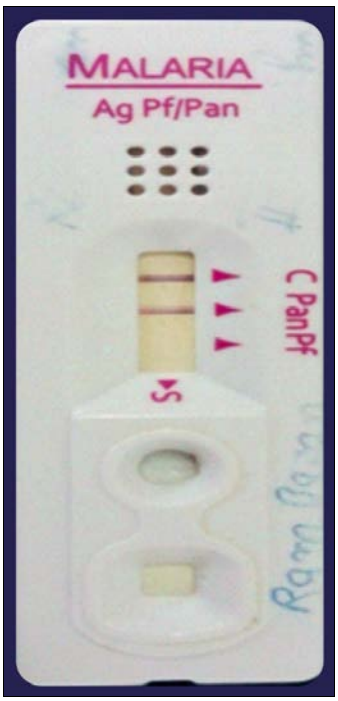

Fig 2: Sample positive for *Plasmodium vivax*

Fig 3: Transasia ErbaLisa®ELISA kit for Malaria (pan LDH)

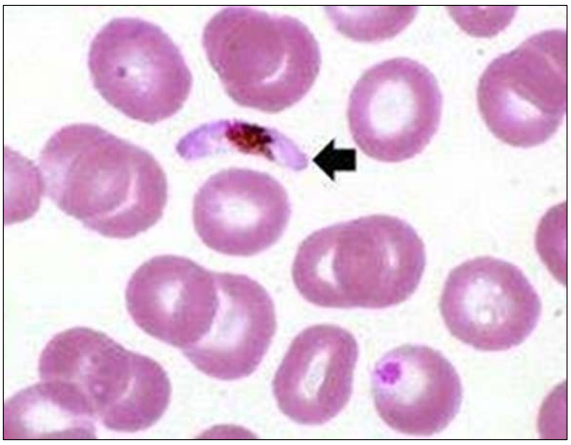

Fig 4: Crescent shaped gametocytes of Plasmodium *falciparum*

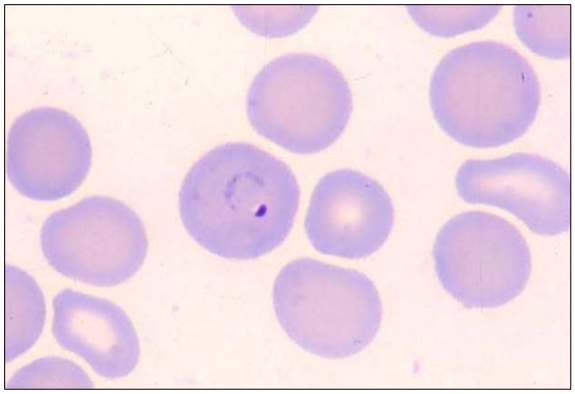

Fig 5: Ring stage of *Plasmodium vivax.*

Work attributed to: Era's Lucknow Medical College and Hospital, Era University, Lucknow, Uttar Pradesh, India

Author's contribution: FH-Concept, coordination, design of the study and interpreted the results, SA- Statistically analyzed and interpreted; reviewed the literature and manuscript preparation; AA- statistically analyzed and interpreted, preparation of manuscript and technical revision of the manuscript; MS- supervised the study, wrote discussion part and gave final verdict for publication.

Acknowledgement: The authors take this opportunity to thank the management and administration of Era's Lucknow Medical College and Hospital, Era University, Lucknow, Uttar Pradesh, India for the support provided to complete this project.

Conflict of interest: None

Source of funding: Era's Lucknow Medical College and Hospital, Era University, Lucknow, Uttar Pradesh, India.

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