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RESEARCH ARTICLE

COMPARATIVE STUDY OF PLASMODIUM LACTATE DEHYDROGENASE (PLDH) LEVELS IN MALARIAL PATIENTS IN COASTAL ANDHRA PRADESH REGION

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ABSTRACT

Despite major control efforts, malaria remains a major public health problem that still causes high mortality rate worldwide especially in Africa and Asia. Accurate and confirmatory diagnosis before treatment initiation is the only way to control the disease. The present study was undertaken to evaluate the diagnostic technique sandwich ELISA for the detection of pLDH (plasmodium lactate dehydrogenase) antigen in proven malaria cases. Microscopic examination of blood smears remains the gold standard for the diagnosis of malaria. However it is labor- intensive and requires the skilled operators.

The differentiation of malarial parasites is based on the antigenic differences between the pLDH isoforms. The pLDH assay was performed on all the samples according to the Qualisa manufacturer's instructions. This study was designed to assess the sensitivity and specificity of pLDH assays in detecting and differentiating between various malarial species compared with microscopy. blood samples were provided by King George Hospital, visakhapatnam for our laboratory for routine diagnosis of malaria were included in this study. From each blood sample, thin films and a Quantitative buffy coat (QBC) were made for microscopy. Thin films were stained with Giemsa. Our data demonstrate that pLDH assay, given its accuracy, rapidity, ease of performance and interpretation can be a useful tool for the detection of malaria. However the PCR test with three primer sets was able to detect as few as four parasites per microliter by gel electrophoresis. The primer sets used in this technique were specified in BLAST analysis. Our comparative study of Microscopy, pLDH antigen ELISA and PCR test showed that the results obtained by PCR were equivalent and superior to those obtained by Microscopy.

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INTRODUCTION

Malaria is a mosquito-borne infectious disease of humans and other animals caused by parasitic protozoan called *Plasmodium*. The disease is transmitted by the biting of mosquitoes, and the symptoms usually begin ten to fifteen days after being bitten. In those who have not been appropriately treated disease may recur months later. The disease is transmitted most commonly by an infected female *Anopheles* mosquito. The mosquito bite introduces the parasites from the mosquito's saliva into a person's blood. The parasites then travel to the liver where they mature and reproduce. Five species of *Plasmodium* can infect and be spread by humans. Most deaths are caused by *P. falciparum* because *P. vivax*, *P. ovale* and *P. malariae* generally cause a milder form of malaria. The species *P. knowlesi* rarely causes disease in humans. It is most commonly caused due to *P. falciparum* and *P. vivax* in India. A distinguishing feature of P. falciparum infections is the ability of infected erythrocytes, especially those containing parasites in the latter half of their life cycle, to adhere to the endothelium (i.e.cytoadherence) of capillaries and post-capillary venules. This process leads to the accumulation of parasites in tissues, a phenomenon known as sequestration, which is so effective that mature, pigmented form of P. falciparumare rarely seen in the peripheral blood. Eventually, erythrocytes containing mature parasites (schizonts) rupture, releasing merozoites and malaria pigment, haemozoin. This malaria pigment is the remnant of the parasite-driven process of haemoglobin degradation and is scavenged and removed by monocytes. The clinical syndromes associated with P. falciparum infections range from asymptomatic parasitaemia to convulsions, coma and death. The mechanisms leading to central nervous system dysfunction in severe malaria have not been elucidated, but one hypothesis suggests that sequestration of parasitized erythrocytes in cerebral microvessels with resultant vascular congestion plays a crucial part in pathogenesis. To gain further insight into this hypothesis, it is necessary to have a standardized approach to assess cerebral tissue with respect to malaria findings.[1,2] Malaria is typically diagnosed by the microscopic examination of blood using blood films, or with

antigen-based rapid diagnostic tests . Methods that use the polymerase chain reaction to detect the parasite's DNA have been developed, but are not widely used in areas where malaria is common due to their cost and complexity. To address the need for a diagnostic test for malaria, we have been developing tests based on the detection of a soluble glycolytic enzyme that is expressed at high levels in the blood-stage parasite, *Plasmodium* lactate dehydrogenase (pLDH).We have found that all four human malarial parasites produce a unique pLDH activity and that this activity follows the level of parasitemia in *in vitro* cultures. We have also found that pLDH activity in patient plasma samples can follow parasitemia measured by microscopy, indicating that pLDH may be a good marker for following active malarial infections. We have recently described an enzymatic assay that can specifically measure pLDH in the presence of human LDH.[3]. Malaria is usually confirmed by the microscopic examination of blood films or by antigen-based rapid diagnostic tests (RDT). The current "gold standard" for malarial detection in most clinical laboratories remains microscopic examination of Giemsa stained thick and thin blood films.[4] for Despite its widespread usage, diagnosis by microscopy suffers from two main drawbacks: levels of the parasite in the blood. The sensitivity of blood films ranges from 75-90% in optimum conditions, to as low as 50%. Commercially available RDTs are often more accurate than blood films at predicting the presence of malaria parasites, but they are widely variable in diagnostic sensitivity and specificity depending on manufacturer, and are unable to tell how many parasites are present. RDTs for malaria could be considered for patients in endemic regions, especially in poor power settings where there is shortage of qualified manpower in Africa. However, there is very little evidence, especially from malaria endemic areas to guide decision makers on sensitivity and specificity of these RDT's. RDT's mainly come in two forms. One is antigen based and normally requires use of hemolysed blood cells while the other is antibody based and normally requires use of extracted serum. Antibodies are better expressed in serum otherwise plasma also could stand in place of serum for antibody based method. ELISA detects the presence of malaria genus specific pLDH released by parasitized blood cells. It is a kind of sandwich ELISA within which an Antigen is sandwiched between two Antibodies. Addition of the substrate will impart color to the reaction and when it is stopped and results are read at 450nm in an ELISA reader. *Plasmodium* infections were identified with a genus-specific primer set, and species differentiation between *Plasmodium falciparum* and *Plasmodium vivax* was analyzed by multiplex PCR. The PCR test with any of the three primer sets was able to detect as few as four parasites per micro liter by gel electrophoresis or by nonisotopic paper hybridization chromatography. The diagnoses obtained by PCR correlated closely with those obtained by Giemsa staining except for two samples observed having mixed *P. falciparum-P. vivax* infections. These were initially missed by microscopic analysis. In comparison with antigen-capture assays for *P. falciparum*, the PCR assays were able to detect three infections that were missed by the Para Sight-F test[]. The PCR test was negative for nine Para Sight-F-positive samples and one ICT Malaria Pf-positive sample, and these were confirmed to be false-positive results. The PCR thus gave no false negative or false-positive results. Patients undergoing antimalarial therapy were also monitored by the PCR

MATERIALS AND METHODS

The suspected malarial blood samples were collected from King George Hospital, Visakhapatnam .The accepted laboratory practice for the diagnosis of malaria is the preparation and microscopic examination of blood films stained with Giemsa, Wright's, or Field's stain. Blood obtained by pricking a finger or earlobe is the ideal sample because the density of developed trophozoites or schizonts is greater in blood from this capillary-rich area. Blood obtained by venipuncture collected in heparin or Sequestrine (EDTA) anti coagulant-coated tubes is acceptable if used shortly after being drawn to prevent alteration in the morphology of white blood cells (WBC) and malaria parasites.

Both thick and thin blood films should be prepared. The thick blood film provides enhanced sensitivity of the blood film technique and is much better than the thin film for detection of low levels of parasitemia and reappearance of circulating parasites during infection recrudescence or relapse. The lysis of the RBC during the staining process can make the process of scanning for parasites more difficult until experience is gained in finding the parasites among the WBC and platelets. In thin blood film, there is fixed monolayer of RBC available in this procedure, the morphological identification of the parasite to the species level is much easier and provides greater specificity than the thick-film examination. The thin blood film is often preferred for routine estimation of the parasitemia because the organisms are easier to see and count ELISA was done by using a specific Qualisa Malaria kit used which is specific for pLDH activity. Its procedure includes about 100µl of antibody reagent was added and diluted with sample diluent in each well .Added 25µl control or whole blood specimen in separate wells .Gently shaken the plates to mix the contents. Applied plate sealer and incubate for 30 min at 37ºC.Washed the contents 6 times with soak time of 30 sec. for each wash. Blot dried.100µl of conjugate in each well was added and incubated for 30 min. at 20-28ºC.Washed six times again with soak time of 30 sec. for each wash. Blot dried. Then 100µl of ready to use substrate was added and incubated in dark for 30 min. 100µl stop solution to stop reaction. Absorbance read at450nm within 30 min. PCR involves Isolated DNA from the test and control samples by using phenol/chloroform method. Suspended the DNA pellet in TE buffer and made up to 50µl.Specially designed primer sets for the confirmation of plasmodium species were added in the blood samples, for P.v and P.f are selected .These primers were suspended in TE buffer, and 10µl of it is taken .Added the dNTPs, 5x buffer, Taq polymerase, and distilled water at appropriate volumes and 50µl of it was taken. The addition of Taq DNA polymerase was withheld until the reaction reaches 95ºc. When the specific gene sequences get amplified, the bands were observed under UV light after the electrophoresis run.[5]

RESULTS AND DISCUSSION

The results showed that it could be possibly appreciated that the serum method appeared reliable as a specific method of malaria diagnosis. Since microscopy was adopted as the gold standard, all that came out negative with Antigen method still had negative. Quality RDT is a valuable complement to microscopy because it helps to expand the coverage of parasite-based diagnosis to the periphery and minimize exclusively clinical diagnosis.

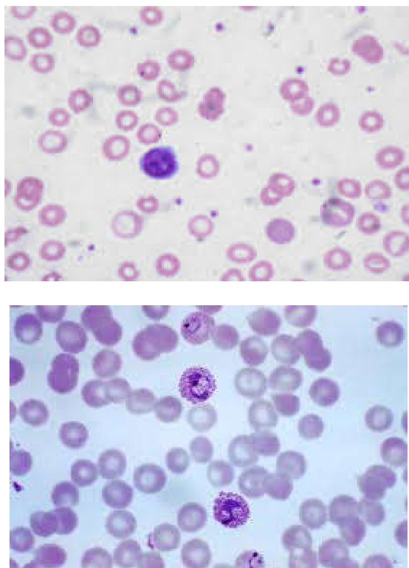

Figure 1 and 2 microscopic examination of Thick and Thin blood films:- The microscopic examination of blood films prepared on a glass slide. Both the pictures contains the effected blood sample which is stained with Giemsa stain and observed under the microscope. The effected cells take up the stain and appear purple color, while the unaffected cells remain colorless.

The cost of improved malaria diagnosis will inevitably increase, whether by investment in microscopy or RDTs or both. However, such investment offers a more promising strategy to deal with increasing costs of therapy driven by drug resistance. Today's multi-million dollar investment in anti-malarial drug development should be accompanied by a parallel commitment to improve diagnostic tools and their availability to those living in malarious areas.

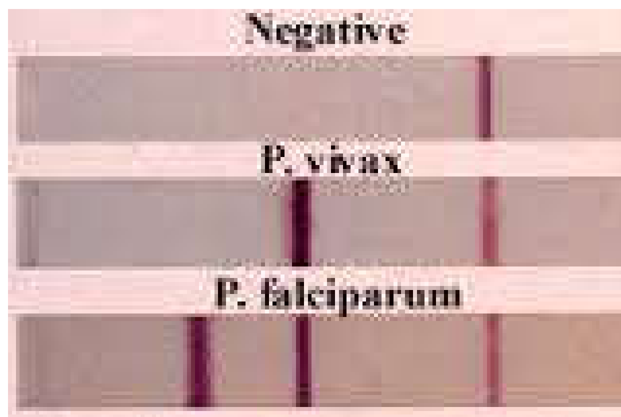

Figure 2 Malaria detection using RDTs:-Malaria RDTs detect specific antigen produced by malarial parasites present in the infected blood samples. Presence of parasites belonging to specific subspecies will form a band on the card. The above picture differentiates the blood sample as p. vivax and p. falciparum.

In the original sample analysis, 30 samples were parasite positive by RDT but negative by microscopy. When these same samples were tested by PCR, 92% of these false-positive samples were true positives as determined by amplification of parasite genes. Plasmodium falciparum single-species infections were detected in 19of 30 samples, and infection with P. vivax was confirmed in a single false-positive non-falciparum case. Mixed species infections were detected in four additional samples. Only four of the false-positive samples were negative by PCR.

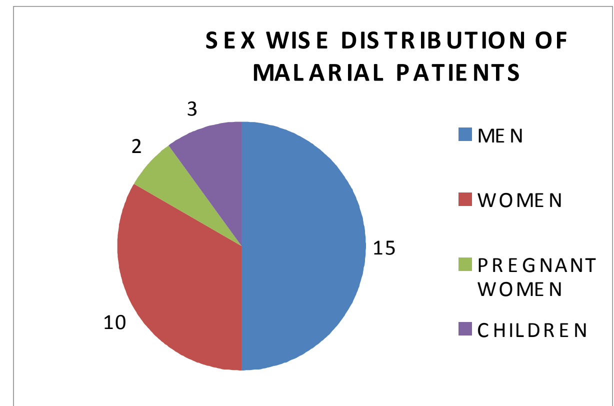

Figure 3 pictorial diagram showing the sex wise distribution of malarial patients

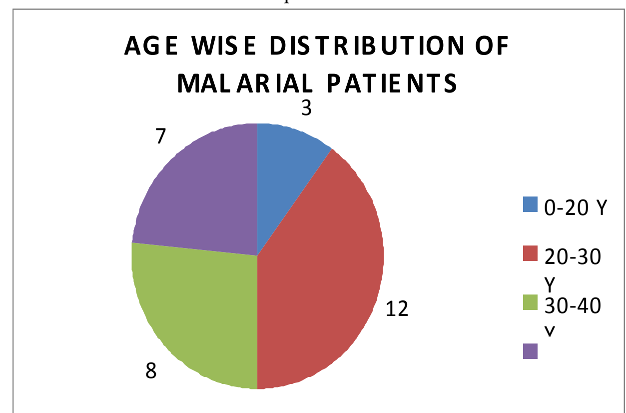

Figure 4 pictorial diagram showing the age wise distribution of malarial patients

In ELISA, which was done to measure the levels of plasmodium lactate dehydrogenase (pLDH) the affected samples show elevated levels of pLDH than that of the normal samples. The difference among both of them was calculated by means of the absorbance value which was compared with that of the standard cutoff value shows that the affected sample has increased amount of the enzyme.

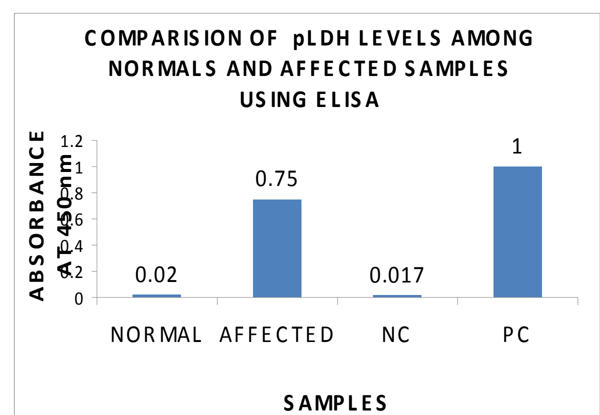

Figure 5 Microwell stripes are coated with monoclonal anti-pLDH antibodies. Samples along with positive and negative controls are added in the coated wells and incubated simultaneously with biotinylated antibody. The wells are washed to remove unbound components. The presence of biotinylated antibodies is detected by adding streptavidin-HRP conjugate. After washing wells to remove unbound enzyme. Substrate is added. The reaction is stopped after specific time with acid and absorbance is determined for each well at 450nm with an ELISA reader. The cutoff value is calculated by given formula and absorbance are compared with the cutoff value. Any sample more than this value is reactive

The samples with high absorbance value than the standard cut off value are reactive, while the samples with less absorbance values are non reactive. The results for Plasmodium falciparum *were clearly superior to expert microscopy alone, particularly mi*xed infections.

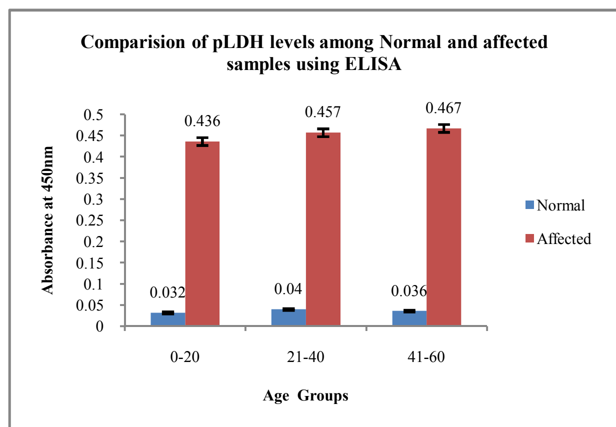

Figure 6 pLDH antibodies were estimated using ELISA in different age groups.

Microscopy combined with ELISA reaches sensitivity and specificity similar to PCR- adjusted microscopy for the diagnosis of Plasmodium falciparum *while being considesrably less expensive and faster. We conclude that EL;ISA serves as an excellent tool to augment microscopy as the gold standard for* Plasmodium falciparum *diagnosis in research settings and should be further evaluated for screening in blood banks.* PCR is the highly specific diagnostic measure for malaria. PCR analysis for detection of the Plasmodium genus and species determination were run as independent assays. With the genus-specific primers L1 and L2, a 100% detection rate for the presence of Plasmodium infection was achieved. With the L1 and L2 primers, a 595-bp PCR product was obtained. Amplification with the species-specific primers gave rise in 16 samples to a 422-bp PCR product which correlates to the P. falciparum primer set Pf1-Pf2. In the other 34 samples, a 332-bp PCR product was observed and this product correlates to the P. vivax primer set Pv1-Pv2.

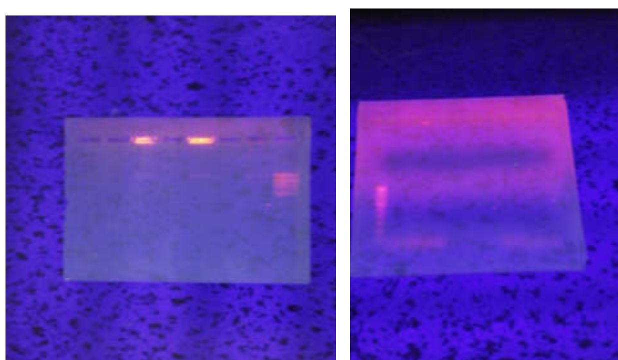

Figure 7.1 and 7.3 Detection of multiplex PCR products with species-specific primers:-PCR is the rapid procedure for the diagnosis of malaria infections directly from blood samples by DNA amplification is evaluated. the PCR test with any of the 3 primer sets was able to detect as few as 4 parasites per micro litre by gel electrophoresis .The first picture confirms the presence of DNA isolated from the infected samples In the second picture shows that PCR detects p.vivax and p. falciparum subspecies.

In the course of these diagnostic tests, two samples were observed to have both the 422-bp and the 332-bp fragments. These two blood samples were from the two patients with mixed infections, which were initially misdiagnosed as only P. falciparum infections by light microscopy. Paper chromatography hybridization detection of the multiplex PCR products obtained with the species-specific primers was performed with probes designed to detect the two plasmodial species fixed on nitrocellulose strips. Thus, colorimetric detection was able to differentiate the two species in the same PCR.

Even though the technology of microscopy is simple and straightforward, making and interpreting malaria smears requires adequate training and experience. The diagnostic advantages of microscopy are that it permits definitive identification of infecting species as well as mixed infections; can be used to determine the magnitude parasitemia; can be used for serial examinations to monitor the efficacy of therapy; requires little laboratory infrastructure; and is comparatively inexpensive. Microscopic slide examination does have diagnostic disadvantages, including it does not detect very low parasitemias; errors in interpretation are most common with either very low or very high parasitemias (for which accurate diagnosis is very important); mixed infections are often missed; and it is not as useful in areas without endemic malaria because of the inability of persons reading smears to remain sufficiently competent to make accurate and reproducible diagnoses[6-10].

CONCLUSION

P LDH is a marker enzyme in the detection of malaria by using various diagnostic methods such as ELISA and PCR. However the PCR test was able to detect as low as four parasites per microlitre of blood. Our comparative study of microscopy, RDT, ELISA and PCR test showed the results obtained by PCR were superior to those obtained by microscopy.

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