Comparison of utility of Rapid versus Serological testing in laboratory diagnosis of Malaria
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ABSTRACT:

Background
Malaria is an endemic infectious disease in India caused by the parasite, Plasmodium, which infects Red Blood Cells. Human malaria is caused by four different species of Plasmodium: P. falciparum, P. malariae, P. ovale and P. vivax. In infected blood, various stages of Plasmodium development may be seen, such as ring form, trophozoite, schizont or gametocyte. There are several known for laboratory methods for diagnosis of Malaria- Microscopic diagnosis, Antigen detection by rapid diagnostic tests, Molecular diagnosis (detection of parasite nucleic acids via PCR) and serological tests such as IFA and ELISA, that detect antibodies against malaria parasites. Microscopy of Giemsa stained thick and thin films remains the gold standard for Malaria diagnosis.

A major issue in controlling the morbidity and mortality caused by malaria is the limited access to effective diagnosis and treatment in endemic areas. The most widely used routine method of microscopy needs laboratory infrastructure and expertise and is labour intensive. Therefore, development of rapid, sensitive and specific diagnostic tests to diagnose malaria is of paramount importance. With this aim, several studies have been done to compare different diagnostic tests available for Malaria diagnosis. Rapid and effective diagnostic tests in Malaria will also reduce or eliminate the need for empirical therapy.

Objectives: In this study, the rapid antigen detection tests and microscopy are compared with serological tests in 48 febrile patients.

Test results and conclusion
Light microscopy with Leishman’s stain showed 100% sensitivity and specificity for Malaria. Rapid Card tests showed a high specificity but lower sensitivity compared to microscopic technique. A significant number of positive malaria cases had a high Eosinophil count.

Abbreviations used:
IFA-Indirect immunofluorescence; ELISA-Enzyme-linked immunosorbent assay; PCR-Polymerase chain reaction

BACKGROUND:
Malaria is the most important parasitic disease of humans, transmitted by mosquitoes. Developing nations have limited resources that lead to inadequate Malaria diagnosis. In wealthy countries, poor familiarity with Malaria may lead to clinical and laboratory misdiagnosis.

The causative parasite, Plasmodium, infects red blood cells. Malaria can be caused by 4 species of Plasmodia- vivax, falciparum, ovale and malariae. Female hematophagous Anopheles culicifacies and Anopheles fluviatilis mosquitoes are the natural vectors because they are susceptible to Malarial parasites, repeatedly bite humans with alternation of hosts. Man is the intermediate host. Mosquitoes are the definitive host of the parasite. Sexual cycle (sporogony) of the parasite takes place in the mosquito.

The gametocytes, male (microgametocytes) and female (macrogametocytes), are ingested by an Anopheles mosquito during a blood meal. The parasites’ multiplication in the mosquito is known as the sporogonic cycle. While in the mosquito’s stomach, the microgametes penetrate the macrogametes generating zygotes. The zygotes in turn become motile and elongated (ookinetes), which invade the midgut wall of the mosquito where they develop into oocysts. The oocysts grow, rupture, and release sporozoites, which make their way to the mosquito’s salivary glands. Inoculation of the sporozoites into a new human host perpetuates the malaria life cycle.

In the human, sporozoites first enter the blood stream and migrate to the liver. They infect liver cells, where they multiply into merozoites, rupture liver cells and escape back into the blood stream. Then, the merozoites infect red blood cells, where they develop into ring forms, then trophozoites (a feeding stage), then schizonts (a reproductive stage) and then back into merozoites. Sexual forms called gametocytes are also produced, which, if taken up by the mosquito, will infect the insect and continue the life cycle.

Symptoms of Malaria:
Infection with malaria parasites may result in a wide variety of symptoms, ranging from absent or very mild symptoms to severe disease and even death. Malaria disease can be categorized as uncomplicated or severe (complicated). In general, malaria is a curable disease if diagnosed and treated promptly and correctly.
All the clinical symptoms associated with malaria are caused by the asexual erythrocytic or blood stage parasites. Fever is the key clinical manifestation. When the parasite develops in the erythrocyte, numerous known and unknown waste substances such as hemozoin pigment and other toxic factors accumulate in the infected red blood cell. These are dumped into the bloodstream when the infected cells lyse and release invasive merozoites. The hemozoin and other toxic factors such as glucose phosphate isomerase (GPI) stimulate macrophages and other cells to produce cytokines such as Interleukin (IL)-1 and Tumour necrosis factor (TNF)alpha. Overproduction of IL-1 and TNF induces fever, chills and other symptoms associated with Malaria.

Plasmodium falciparum-infected erythrocytes, particularly those with mature trophozoites, adhere to the vascular endothelium of venular blood vessel walls and do not freely circulate in the blood. When this sequestration of infected erythrocytes occurs in the vessels of the brain it is believed to be a factor in causing the severe disease syndrome known as cerebral malaria, which is associated with high mortality.

Laboratory diagnosis:

1. Microscopic diagnosis: Malaria parasites can be identified by examining under the microscope a drop of the patient's blood, spread out as a "blood smear" on a microscope slide. Prior to examination, the specimen is stained (most often with the Giemsa stain) to give the parasites a distinctive appearance. This technique remains the gold standard for laboratory confirmation of malaria. However, it depends on the quality of the reagents, of the microscope, and on the experience of the laboratory technician.

2. Antigen detection-rapid: Various test kits are available to detect antigens derived from malaria parasites. Such immunologic ("Immunochromatographic") tests most often use a dipstick or cassette format, and provide results in 2-15 minutes. These "Rapid Diagnostic Tests" (RDTs) offer a useful alternative to microscopy in situations where reliable microscopic diagnosis is not available. Malaria RDTs are currently used in some clinical settings and programs. However, before malaria RDTs can be widely adopted, several issues remain to be addressed, including improving their accuracy; lowering their cost; and ensuring their adequate performance under adverse field conditions.

3. Molecular diagnosis: Parasite nucleic acids are detected using polymerase chain reaction (PCR). Although this technique may be slightly more sensitive than smear microscopy, it is of limited utility for the diagnosis of acutely ill patients in the standard healthcare setting. PCR results are often not available quickly enough to be of value in establishing the diagnosis of malaria infection. PCR is most useful for confirming the species of malarial parasite after the diagnosis has been established by either smear microscopy or RDT.

4. Serology: Serology detects antibodies against malaria parasites, using either indirect immunofluorescence (IFA) or enzyme-linked immunosorbant assay (ELISA). Serology does not detect current infection but rather measures past exposure.

5. Drug resistance tests: done to assess the susceptibility of parasites to antimalarial compounds.

Two main laboratory methods are available:

**In vitro tests:** The parasites are grown in culture in the presence of increasing concentrations of drugs; the drug concentration that inhibits parasite growth is used as endpoint. Molecular characterization: Molecular markers assessed by PCR or gene sequencing also allow the prediction, to some degree, of resistance to some drugs. Center for Disease Control, USA, recommends that all cases of malaria diagnosed in the United States should be evaluated for evidence of drug resistance.
This study aims to compare RDT, microscopic methods and ELISA in the diagnosis of Malaria.

AIMS AND OBJECTIVES:
1. Compare sensitivity and specificity of Malaria testing by microscopic (thin film/thick film and QBC) methods and serology (ELISA)
2. Study cases that are clinically suggestive of Malaria but negative by laboratory testing

INCLUSION CRITERIA:
- Fever of unknown origin
- Empirically NOT given anti-malarial therapy

TESTS DONE IN OUR STUDY:
1. Total White Blood cell (WBC) count
2. Differential count
3. Leishman stained thin blood film
4. QBC method
5. Rapid card test
6. ELISA

PLACE OF STUDY:
The Department of Immunology at the Tamil Nadu Dr MGR Medical University, Chennai

PERIOD OF STUDY: January 2014 through December 2016

METHOD:

Design: Qualitative descriptive study

Method: Blood samples of 48 patients with Pyrexia of unknown origin were collected. These were patients from two local community hospitals in urban Chennai, India.

1. Total WBC count and Differential Count:

Materials required:
- Syringe with needles
- Disposable gloves
- Surgical spirit
- EDTA tubes
- Auto-analyzer
- Distilled water
- Tissue paper

Procedure:
- 2 ml of blood was collected from the patients using a syringe
- The whole blood was then stored in the tubes
- The tubes were placed in the auto-analyzer machine
- Appropriate setting was done in the auto-analyzer for Total Count mode
- The results displayed on the auto-analyzer were noted.
- By setting appropriate mode for differential count, the readings were noted, expressed as percentages, indicating the composition of various WBCs in the blood like Neutrophils, Eosinophils, Lymphocytes, Monocytes and Basophils

2. LEISHMAN STAIN-Thin smear:

Materials required:
- Microscope slides, Leishman stain, distilled water, DPX mount oil, tissue paper, micropipette and tips, disposable gloves

Procedure:
- A drop of whole blood was placed on one corner of a clean slide
- Then, using another slide, a thin smear of the blood was prepared.
- Leishman stain was poured over the smear and left for a few minutes
- Excess stain was removed using distilled water and air-dried
- The slide was then viewed under 100X (oil immersion) using compound microscope
- Malarial parasites in the red blood cells in various stages were detected

3. QBC (Quantitative buffy coat):

Materials required:
- QBC tube, plastic closure, cylindrical float, QBC centrifuge, QBC reader and fluorescent microscope

Procedure:
- QBC tube coated internally with Acridine Orange stain and Potassium Oxalate was filled with 55-65µl of blood
- A clear plastic closure was then attached
- A precisely made cylindrical float, designed to be suspended in the packed red blood cells was inserted
- The tube was then centrifuged at 12,000 rpm for 5 minutes
- The components of the buffy coat separate according to their densities, forming discrete bands
- The QBC tube was then placed on the tube holder and examined using a standard white light microscope equipped with the UV microscope adaptor, and epi-illuminated microscope objective
- Fluorescing parasite were observed at the red blood cell/ white blood cell interface

4. SERUM SEPARATION:

Materials required:
- Centrifuge tubes, micropipette, tips and disposable gloves

Procedure:
- The whole blood collected was taken in a centrifuge tube
- It was then centrifuged at 4000 rpm for 3 minutes
- The supernatant (serum) was then transferred to a fresh Eppendorf tube using a micropipette and stored at -2ºC

5. RAPID CARD TEST:

Materials required:
- Dia-Med Opti-Mal-IT test device (card) , one pipette dropper per test device, serum sample and diluents buffer

Procedure:
- The frozen sample and the test components were brought to room temperature.
- Frozen serum sample was mixed thoroughly and thawed well prior to assay.
- The card (test device) was then placed on a clean flat surface.
- One drop (20µl) of specimen from serum sample was then transferred with pipette dropper to the sample well and allowed to soak in.
- 3 drops (90µl) of diluents buffer was added into the sample window immediately.
- The test result was read within 5-20 minutes. Colour bands were then visualized.

6. ELISA:

Materials required:
- Micropipettes (0-25µl, 50-200µl and 0-500µl), ELISA reader, absorbent pad, incubator, disposable gloves and measuring cylinder (10 ml)
Standard Diagnostic kit (Qualisa) components include Monoclonal anti-pLDH antibody coated plate, enzyme conjugate (streptavidin-HRP conjugate(50X)), conjugate diluents, Goat-anti mouse serum (positive control), Bovine serum albumin (negative control), TMB and hydrogen peroxide substrate, sample diluents, stopping solution, washing solution (20X concentrations), adhesive strips and black cover.

Procedure:

a. Frozen serum samples were brought to the room temperature by thawing.

b. All reagents and ELISA kit was brought to the room temperature.

c. Required number of strips was taken arranged on the plate and closed immediately.

d. 100µl of sample diluents containing diluted antibody reagent was added in each well.

e. 25µl control was added. 2 negative controls and 3 positive controls were added in the respective wells.

f. 25µl samples were then added in each well.

g. Plate was then gently shaken to mix the contents and plate sealer was placed on its top and it incubated for 30 minutes at 37°C.

h. 350µl of diluted wash buffer was added in each well giving 30sec soak time for each wash and aspirated for 6 times and blot dried.

i. 100µl of diluted conjugate was added in each well and incubated for 30 min at room temperature (20-28°C).

j. 350µl of diluted wash buffer was added in each well giving 30sec soak time for each wash and aspirated for 6 times and blot dried.

k. 100µl of substrate was then added in each well and incubated at room temperature (20-28°C) away from light for 30 minutes.

l. 100µl of stop solution was then added in each well added in each well to stop the reaction.

m. Absorbance was then read at 450nm with 600 – 700nm as reference within 30 minutes of stopping the reaction.

Calculation of cut off value: 1 blank, 2 negative controls (NCs), 3 positive controls (PCs).

\[ \text{Mean} = \frac{0 + 0.010}{2} = 0.005 \]

\[ \text{Cut off} = \text{mean NCs} + 0.1 = 0.005 + 0.1 = 0.105 \]

**RESULTS:**

### Total and Differential White cell count:

<table>
<thead>
<tr>
<th>Test</th>
<th>Normal Reference range</th>
<th>Level in Malaria positive patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total WBC Count</td>
<td>4,000-10,000 /µl</td>
<td>• Elevated in only 8%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Normal in 92%</td>
</tr>
<tr>
<td>Neutrophil Count</td>
<td>50-62%</td>
<td>• Elevated in 77</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Normal in 23%</td>
</tr>
<tr>
<td>Lymphocyte Count</td>
<td>25-40%</td>
<td>100% had normal levels</td>
</tr>
<tr>
<td>Eosinophil Count</td>
<td>0-3%</td>
<td>• Elevated in 62%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Normal in 38%</td>
</tr>
</tbody>
</table>

**Leishman Stained thin blood film:**

Leishman stain for 48 blood samples were performed and viewed under light microscopy under 100X. 27% were found to have malarial parasite *Plasmodium vivax* with different diagnostic stages like schizonts, merozoites and gametocytes. Rest 73% was devoid of malarial parasites indicating negative result to gold standard technique.

**QBC method:**

QBC test were performed for all the 48 blood samples. The QBC tube containing the centrifuged whole blood was detected by QBC reader which showed red blood cells containing Plasmodia (less dense than normal ones) concentrated just below the leukocytes, at the top of the erythrocyte column. Since the parasites contain DNA, the acridine orange stain was taken up by it and appeared as bright specks of light among the non-fluorescing red cells. All the parasites in the 60 µl of blood was visualized by rotating the tube under the microscope and counted. QBC positive was reported by fluorescence of the parasite and by counting the parasite under fluorescent microscope. No fluorescence in the red cells indicated the negative result. 25% was found to be positive and 75% was found to be negative.

**Rapid Card Test:**

Card test was performed for all the 48 serum samples and results were read within 20 minutes after addition of samples in the sample window. A colour band appeared in the right section of the result window which indicated the control band. The right section of the result window indicated the test results and this band were called as test band. Negative result was indicated by the presence of only one band (i.e., the control band) within the result window. Positive result was indicated by the presence of two colour bands (one control band and one test band) within the result window. 20% were found be positive and rest 80% were found to be negative by card test.

**ELISA Method:**

ELISA for the detection of Malaria specific antigen (pLDH) in 48 serum samples was performed. 6% were found to be positive and rest 94% was found to be negative by ELISA test. Samples with absorbance value less than the cut-off value were considered non-reactive and were considered negative for malaria. Samples with absorbance value equal to or greater than cut-off value were considered reactive and positive for malaria.

**CHART 1: Comparison of the diagnostic tools**

<table>
<thead>
<tr>
<th>An Initiative of The Tamil Nadu Dr. M.G.R. Medical University</th>
</tr>
</thead>
<tbody>
<tr>
<td>University Journal of Medicine and Medical Specialities</td>
</tr>
</tbody>
</table>
CALCULATION OF SENSITIVITY AND SPECIFICITY

**TABLE A: CARD TEST-SENSITIVITY and SPECIFICITY:**

<table>
<thead>
<tr>
<th>Positive result</th>
<th>Malaria present</th>
<th>Malaria absent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive result</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>Negative result</td>
<td>3</td>
<td>35</td>
</tr>
</tbody>
</table>

Sensitivity= 10/13=0.77; Specificity= 35/35=1
PPV= 10/10=1; NPV= 35/35=0.92

**TABLE B: QBC- SENSITIVITY and SPECIFICITY:**

<table>
<thead>
<tr>
<th>Positive result</th>
<th>Malaria present</th>
<th>Malaria absent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive result</td>
<td>12</td>
<td>0</td>
</tr>
<tr>
<td>Negative result</td>
<td>1</td>
<td>35</td>
</tr>
</tbody>
</table>

Sensitivity=12/13=0.9; Specificity=35/35=1; PPV=12/12=1; NPV=35/36=0.97

**TABLE C: LEISHAN’S STAIN- SENSITIVITY and SPECIFICITY:**

<table>
<thead>
<tr>
<th>Positive result</th>
<th>Malaria present</th>
<th>Malaria absent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive result</td>
<td>13</td>
<td>0</td>
</tr>
<tr>
<td>Negative result</td>
<td>0</td>
<td>35</td>
</tr>
</tbody>
</table>

Sensitivity=13/13=1; Specificity=35/35=1; PPV=13/13=1; NPV=35/35=1

**TABLE D: ELISA- SENSITIVITY and SPECIFICITY:**

<table>
<thead>
<tr>
<th>Positive result</th>
<th>Malaria present</th>
<th>Malaria absent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive result</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Negative result</td>
<td>11</td>
<td>35</td>
</tr>
</tbody>
</table>

Sensitivity=2/13=0.15; Specificity=35/36=0.97; PPV=2/3=0.67; NPV=35/46=0.76

**Conclusions:**

- Microscopic method using Leishman’s stain showed highest sensitivity and specificity
- QBC method also showed a high sensitivity and specificity in malaria diagnosis
- Rapid card test showed a high specificity and positive predictive value but lower sensitivity and negative predictive values
- ELISA for the detection of Malaria specific antigen (pLDH) showed a high specificity but very low sensitivity and low positive and negative predictive values
- A significant proportion (62%) of malaria positive patients had a high Eosinophil count. Therefore, we recommend testing for White cell Count along with Differential cell counts in febrile patients suspected to have Malaria.

**Discussion:**

Though thin film microscopy using Leishman’s is most sensitive and specific, it requires time, laboratory infrastructure, trained personnel and is labour intensive. QBC method is almost as sensitive and specific in malaria diagnosis, faster and less labour intensive.

**References:**

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