Usefulness of dark field microscopy (DFM), IGM ELISA and microscopic agglutination test (MAT) for the early diagnosis of acute leptospirosis

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Abstract
Introduction: Early diagnosis of leptospirosis could indicate adequate and specific antibiotic treatment. Early treatment could prevent later complications involving liver, kidney, brain and eyes. The purpose of this study was to evaluate role of dark field Microscopy, IgM ELISA and Microagglutination test for the diagnosis of acute leptospirosis.
Methodology: A total of 81 blood samples from the clinically suspected cases were collected and was investigated for DFM, IgM ELISA and MAT. For DFM blood was added to sodium oxalate solution (1%) in phosphate buffer P^H 8.0. A part of the blood samples were added to plane tube without anticoagulant, it was allowed to clot and sera were separated for performing MAT and ELISA.
Results: The sensitivity of DFM, IgM ELISA and MAT was observed as 55.55% (45/81), 65.43% (53/81), 53.08% (43 /81) respectively. It was also observed that DFM and ELISA were more sensitive in cases of 1to 7 days of infection. Sensitivity of DFM declined with more than 7 days of infection. Sensitivity of ELISA and MAT increased in cases of more than 7 days of infection.
Conclusion: DFM and IgM ELISA can be used for the diagnosis of acute leptospirosis. A standard dark field microscope with special condenser is needed for DFM. ELISA reader, multichannel pipette and commercial ELISA kits are required to perform ELISA. MAT can be performed in a centre where standard facilities as per the guidelines are available.

Keywords: DFM, Leptospira, ELISA, MAT.

Introduction
Leptospirosis is a zoonoses caused by pathogenic species of the bacterium *Leptospira*. It is an increasingly known cause of acute febrile diseases in the tropical and sub-tropical parts of the world.[1,2] The clinical manifestations of this disease may vary from a mild self limited pyretic illness to a severe and life threatening illness characterized by jaundice, renal failure, thrombocytopenia, and hemorrhage. Early in illness, leptospirosis is often indistinguishable from other common causes of acute febrile illnesses with similar clinical presentations- e.g. dengue, malaria, scrub typhus, typhoid and others.[3] Early diagnosis plays a major role to treat the patient with appropriate antibiotics for the acute lerptospirosis.[4] However the non specific presentation of this disease provides hindrance for its early diagnosis. There are number of diagnostic test kits available commercially, these tests mainly detects specific antibody against the pathogenic Leptospira. Treatment with appropriate antibiotics based on these tests is always questionable as their accuracy is low.[5]

We have conducted a prospective study to assess the role of Dark field microscopy (DFM), IgM ELISA and Microscopic agglutination test (MAT) for early diagnosis of acute leptospirosis.

Materials and Methods
Study Design and Population
The study was conducted between the years 2017 to 2018. A total of 81 blood samples were collected from the patients having the history of fever, severe headache, vomiting, myalgias, conjunctival suffusion, jaundice, stiff neck, stomach pain breathlessness. 50 blood samples were also collected from healthy donors as control cases. The protocol was approved by Institutional Ethical Committee. Patients showing positive test for dengue, malaria, hepatitis and enteric fever were excluded from this study. After taking verbal informed consent, blood samples (5ml each) were collected aseptically in to two sterile vials, one vial contain 500 µl of 1% sodium oxalate solution as anticoagulant and the other one was a dry plane tube. The former sample with anticoagulant was used for dark field microscopy and the other one was used for serodiagnosis by MAT and ELISA. Data were collected as age, sex, occupation and exposure history of the patient.

Dark Field Examination
The blood samples with 1% sodium oxalate solution were centrifuged at about 3000 rpm for 5 minute to sediment the unwanted cellular materials. 10 µl of plasma from supernatant was placed on a slide of thickness 1mm. A cover slip was put on the drop and pressed to form a thin film without air bubbles. We used binocular dark field microscope with the high power objective and oil immersion objective. The slide was scanned thoroughly to visualize the leptospires. The number of leptospires seen in 100 HPF (high power field) was calculated by simple counting a as *Leptospira* positive per HPF or per 100 high power fields depending upon the concentration. The report of *Leptospira* negative was considered after screening of 100 HPFs.

Enzyme Linked Immunosorbent Assay (ELISA)
Leptospira IgM antibody was detected in the serum using PANBIO *Leptospira* IgM ELISA kit (Panbio-Alere, Australia). SELISA procedure was followed as per the instructions provide in the kits. Optical density was recorded in an ELISA reader by using 405 nm filters.

Microscopic Agglutination Test (MAT)

Microscopic Agglutination Testing (MAT) was done using the following antigens (serogroup followed by serovar in parentheses): serogroup Andamana (serovar Andamana), Australis (Australis), Bataviae (Bataviae), Canicola (Canicola), Cynopteri (Cynopteri), Djasiman (Djasiman), Grippotyphosa (Grippotyphosa), Icterohaemorrhagiae (Icterohaemorrhagiae), Pomona (Pomona) and Sejroe (Hardjo). Sera were screened at a dilution of 1:100 and positive sera were titrated to endpoint using standard methods. This was performed by mixing the test serum with a culture of leptospires and then evaluating the degree of agglutination using dark-field microscope. The end-point was evaluated for serum as per the CDC guidelines.

Statistical Analysis

Categorical variables were summarized by percentages. χ^2 tests performed for trend of ordinal variable.

The sensitivity of DFM and PANBIO IgM ELISA in this study was found as 55.55% and 65.43% respectively. We have also observed that DFM sensitivity was higher when the patient sample was collected during acute phase of infection of 1-7 days. Sensitivity of DFM decreased during convalescent phase of infection of 8-14 days and ≥15 days. Sensitivity of PANBIO IgM ELISA was higher when the sample was collected during convalescent phase while it was lower during acute phase (Table 1 and 2).

The DFM sensitivity was recorded as 55.55% while MAT sensitivity was observed as 53.08%. It was found that DFM sensitivity was higher when the patient sample was collected during acute phase of infection of 1-7 days. Sensitivity of DFM decreased during convalescent phase of infection of 8-14 days and ≥15 days. Sensitivity of MAT was higher when the sample was collected from the patient having infection of ≥15days, while it decreased during acute phase (Table 3 and 4).

Results

Table 1: Results of DFM based on different case categories using single blood samples

Clinical category	Total No of cases	DFM + IgM ELISA -	DFM + IgM ELISA +	DFM - IgM ELISA +	DFM - IgM ELISA -
Pyrexia, headache, myalgia, vomiting, hepatomegaly	40	5	16	9	10
Pyrexia, headache jaundice with abnormal LFT	25	3	12	6	4
Pyrexia, headache altered sensorium, conjunctival suffusion	12	2	4	4	2
Pyrexia, headache, Abnormal renal function test with glomerulonephritis	4	1	2	0	1
Total	**81**	**11**	**34**	**19**	**17**

P value = 0.12 by Chi-Square test

Table 2: Sensitivity patterns of DFM and PANBIO IgM ELISA in relation with duration of infection

Duration of infection (days)	Total No of cases	DFM +/IgM ELISA -	DFM +/IgM ELISA+	DFM -/IgM ELISA+	DFM -/IgM ELISA -	DFM Sensitivity (%)	ELISA Sensitivity (%)
1-7	39	7	17	5	10	61.53	56.41
8-14	25	3	10	8	4	52.00	72.00
≥15	17	1	7	6	3	47.05	76.41

+ Positive, - Negative

Table 3: Results of DFM and MAT by using single blood sample based on case categories

Clinical category	Total No of cases	DFM + MAT -	DFM + MAT +	DFM - MAT +	DFM - MAT -
Pyrexia, headache, myalgia, vomiting, hepatomegaly	40	7	16	6	11
Pyrexia, headache jaundice with abnormal LFT	25	5	8	7	5
Pyrexia, headache altered sensorium, conjunctival suffusion	12	4	3	1	4
Pyrexia, headache, Abnormal renal function test with glomerulonephritis	4	0	2	0	2
Total	81	16	29	14	21

Table 4: Sensitivity patterns of DFM and MAT in relation with duration of infection

Duration of infection (days)	Total No of cases	DFM + MAT -	DFM + MAT +	DFM - MAT +	DFM - MAT -	DFM Sensitivity (%)	MAT sensitivity (%)
1-7	39	17	7	5	10	61.53	30.76
8-14	25	3	8	10	4	44.00	72.00
≥15	17	1	5	8	3	35.29	76.47

+ Positive, - Negative

The sensitivity of PANBIO IgM ELISA was found as 65.43%. It was observed that PANBIO IgM ELISA sensitivity was higher when the patient sample was collected during acute phase of infection of 1-7 days. Sensitivity of PANBIO IgM ELISA decreased during convalescent phase of infection of 8-14 days and ≥15 days. Sensitivity of MAT was higher when the sample was collected from the patient having infection of ≥15 days, while it decreased during acute phase (Table 5 and 6).

Table 5: Results of IgM ELISA and MAT by using single blood sample based on case categories

Clinical category	Total No of cases	IgM ELISA + MAT -	IgM ELISA + MAT +	IgM ELISA - MAT +	IgM ELISA - MAT -
Pyrexia, headache, myalgia, vomiting, hepatomegaly	40	16	10	8	6
Pyrexia, headache jaundice with abnormal LFT,	25	7	8	7	3
Pyrexia, headache altered sensorium, conjunctival suffusion	12	4	4	4	0
Pyrexia, headache, Abnormal renal function test with glomerulonephritis	4	2	2	0	0
Total	81	29	24	19	9

Table 6: Sensitivity patterns of IgM ELISA and MAT in relation with duration of infection

Duration of infection (days)	Total No of cases	IgM ELISA+ MAT -	IgM ELISA + MAT +	IgM ELISA - MAT +	IgM ELISA - MAT -	IgM ELISA Sensitivity (%)	MAT Sensitivity (%)
1-7	39	17	11	7	4	71.79	46.15
8-14	25	8	9	6	2	68.00	60.00
≥15	17	4	5	6	2	52.94	64.70

+ Positive, - Negative

Discussion

Leptospires are seen in blood or other body fluids during the early phase of the infection therefore it is very important for the healthcare personals to identify it early so that treatment of the patient is not hampered. We should endorse a method, which can identify this disease rapidly with high accuracy. IgM antibody to the leptospira specific antigen starts appearing in the blood during the early phase of infection (2-12 days of infection) and reaches to detectable level at the end of the first week of the illness. In the present study dark field microscopy and ELISA using commercially available kit (PanBio Leptospira IgM ELISA) is nearly equal suitable test for the acute cases of leptospirosis. Earlier study by Chandrasekaran et al has found a high sensitivity of dark field microscopy when the blood samples were collected fresh in 1% sodium oxalate solution. They have found a correlation of 64.70% between DFM and *Leptospira* IgM antibody ELISA.[6] Another study by Vijayachari et al have found a sensitivity of 40.2%, specificity of 61.5%, and positive predictive value of 55.2% and negative predictive value of 46.6% of dark field microscopy for the early diagnosis of leptospirosis which is not in concordance with the present study.[7]

While comparing the results of ELISA and MAT we have found that the sensitivity PANBIO IgM ELISA 65.43% and MAT has a sensitivity of 53.08% when the single blood samples were collected from the patient during early phase of infection. It was also found that PANBIO IgM ELISA sensitivity was higher when the patient sample was collected during acute phase of infection of 1-7 days. Sensitivity of PANBIO IgM ELISA decreased during convalescent phase of infection of 8-14 days and ≥15 days. Sensitivity of MAT was higher when the sample was collected from the patient having infection of ≥15 days, while it decreased during acute phase. Several earlier studies has also shown that IgM ELISA is more sensitive than MAT when the first specimen is taken early in the acute phase of the illness.[8,9]

Conclusion

Our study concludes that DFM and IgM ELISA are very useful for the diagnosis of acute cases of leptospirosis. However IgM ELISA has been found to be more sensitive than DFM and MAT. DFM has good sensitivity in comparison with ELISA and MAT when the samples are collected during early phase of incubation. MAT has limited role as far as early diagnosis is concern. Future comparative Studies will have greater statistical and discriminatory power to consider the utility of individual test for the early diagnosis of leptospirosis.

Conflict of Interest: None.

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