

EFFICACY OF DARK FIELD MICROSCOPY AND IGM-ELISA IN THE DETECTION OF LEPTOSPIROSIS

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ABSTRACT

BACKGROUND

Leptospirosis is one of the most serious zoonotic disease. The spectrum of human disease caused by *Leptospira* is extremely wide, ranging from subclinical infection to a severe syndrome of multi organ infection with high mortality. Dark field microscopy which is considered as a conventional method for detecting *Leptospira* is still being practiced but MAT was considered to be the gold standard. Alternatives to MAT are the IgM dot-ELISA dipstick test (DST), and the indirect haemagglutination assay (IHA).

AIM

To assess and compare the efficacy levels of dark field microscopy and IgM ELISA in comparison with PCR in diagnosing leptospirosis.

MATERIALS AND METHODS

A cross-sectional study was conducted in our hospital during the period of August 2014 – July 2015. All the patients with more than 12 years of age and symptoms suggestive of *Leptospira* were included in the study. All patients were subjected to DFM, and ELISA IgM within 48 hours of admission and on 7th day the patients had undergone PCR. The procedure of testing was followed as per the guidelines given in the kit.

RESULTS

The sensitivity for dark field microscopy in detecting *Leptospira* in comparison with PCR was found to be 92.8% and the specificity was 87%. The positive predictive value was 78.7% and the percentage of false positives was 12.9% whereas for IgM-ELISA the sensitivity, specificity and positive predictive value was 100%, 96.2% and 93.3% and the percentage of false positives is 3.7%. The sensitivity, specificity, PPV and NPV of IgM ELISA was higher when compared to the dark field microscopy and the percentage of false positives was comparatively lesser than the dark field microscopy.

CONCLUSIONS

Leptospirosis being an emerging and usually under diagnosed disease, newer diagnostic tests and rapid case detection particularly during an outbreak becomes the need of the hour and IgM-ELISA would satisfy these things in the detection of *Leptospira*.

KEYWORDS

Leptospirosis, Dark Field Microscopy, IgM ELISA, Diagnosis.

HOW TO CITE THIS ARTICLE: Rajamani SS, Krishnan S, Radhakrishnan S. Efficacy of dark field microscopy and IGM-ELISA in the detection of leptospirosis. *J. Evid. Based Med. Healthc.* 2016; 3(65), 3542-3546. DOI: 10.18410/jebmh/2016/760

INTRODUCTION: Leptospirosis is one of the most serious zoonotic disease leaving behind a major impact with respect to veterinary sciences and public health.¹ The disease is caused by *Leptospira interrogans*. It causes important economic losses in livestock. Although the infection is usually mild and often subclinical among the animals it still leads to

great losses due to abortion, stillbirth, infertility, mastitis, weak progeny, decreased milk production and, with certain *Leptospiral* serovars, death.^{2,3}

The spectrum of human disease caused by *Leptospira* is extremely wide, ranging from subclinical infection to a severe syndrome of multiorgan infection with high mortality.⁴ Icteric leptospirosis with renal failure, was first reported over 100 years ago by Adolf Weil in Heidelberg, Germany.⁵ Human hosts commonly acquire infection through skin abrasions and mucosal surfaces following contact with water or soil contaminated with urine of infected rodents or other mammals. Leptospirosis has a wide range of clinical manifestations, from a simple febrile illness to a severe and potentially fatal illness characterised by

Financial or Other, Competing Interest: None.

Submission 07-07-2016, Peer Review 20-07-2016,

Acceptance 01-08-2016, Published 13-08-2016.

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DOI: 10.18410/jebmh/2016/760

acute kidney injury, liver derangement, pulmonary haemorrhage, bleeding, and cardiac involvement. In most clinical settings, there is limited availability of specific diagnostic tests, and treating physicians often rely on clinical features to make a probable diagnosis of leptospirosis. This is indeed a problem in areas of high incidence of other infections with similar clinical picture, such as dengue, rickettsial infection, malaria and Hantavirus infections.⁶

Leptospirosis being a highly fatal disease with lot of complications, early diagnosis and treatment plays a very important role. *Leptospira* is usually diagnosed through serological method. The various serological test available to detect the condition are microscopic agglutination test (MAT), detection of organism DNA by polymerase chain reaction (PCR), isolation of the organism through culture methods, or detection of antibodies to the organism.⁷ Though isolation of organism from culture would be more specific it needs expertise and takes a lot of time. In clinical settings, MAT is considered as an alternative as it has a very high sensitivity and specificity. However, the MAT is a complex test that requires a large panel of live-cell suspensions to provide adequate coverage of the antigenic diversity represented in a given testing area. Moreover, antibody levels detectable by MAT usually do not appear before day 6 or 7 after development of symptoms; they usually peak by the fourth week, but detectable titres may persist for year.^{8,9}

Dark field microscopy which is considered as a conventional method for detecting *Leptospira* is still being practiced. The major drawback of this method is it lacks sensitivity and the number of false negatives was found to be very high. Several alternatives to the MAT which is now currently in practice and made available commercially are Immunoglobulin M (IgM) Enzyme-Linked Immunosorbent Assay (ELISA), an IgM dipstick assay (LDS), an IgM dot-ELISA dipstick test (DST), and the indirect haemagglutination assay (IHA). Among all these available tests, few studies had shown IgM ELISA with high sensitivity and specificity.^{10,11}

AIM: To assess and compare the efficacy levels of dark field microscopy and IgM ELISA in comparison with PCR in diagnosing leptospirosis.

METHODOLOGY: A cross-sectional study was conducted in RMMC Hospital, Chidambaram during the period of Feb 2011 – Jan 2012. All the patients with more than 12 years of age and with any of the following symptom were included in the study.

- History of fever > 5 days with any one of the following
- Headache/myalgia
- Conjunctival suffusion
- Jaundice
- Oliguria/anuria/elevated RFT
- GI symptoms
- Rash
- Pulmonary symptoms such as cough breathlessness, haemoptysis
- Haemorrhage

- Cardiac arrhythmia
- Meningeal irritation

All patients were subjected to DFM, and ELISA IgM within 48 hours of admission and on 7th day the patients had undergone PCR.

DFM: 5 mL of centrifuged blood was taken and to it approximately 10 µL of plasma was added on a thin microscopic slide and cover slip was applied. It was examined under dark field microscope with low power and high power (X 200 and X 400) for identifying the *Leptospira* and if it is not seen then the plasma was further centrifuged at 3000-4000 g for 20 min. and after carefully removing the supernatant, a drop of the sediment was examined under the microscope as mentioned above.

IgM ELISA: The ELISA was carried out as per the manufacturer's instruction. ELISA kit was obtained from Serion-Virion ELISA (classic *Leptospira* IgM). Serum antibodies of the IgM class, when present, combine with *Leptospira* antigen attached to the polystyrene surface of the Microwell test strips. Residual serum is removed by washing and peroxidase conjugated antihuman IgG, IgA, IgM is added. The microwells are washed and substrate system, para-nitrophenyl-phosphate is added. The substrate is hydrolysed by enzyme, and chromogen changes to yellow coloured. Case and control sera (10 µL) were diluted 1:100 and tested according to the manufacturer's instruction. The result is read with a dual wavelength spectrophotometer at 405 nm and a background of 620 nm. The colour intensity is directly related to the concentration of *Leptospira* IgM antibodies in the test sample. Each set of tests is run with a positive control, negative control and cut-off calibrator in duplicate. The test is valid when the absorbance reading of the above meets the specification of the Serion ELISA instruction. The results were interpreted according to the manufacturer's recommendation. Specimens having an absorbent ratio greater than that of cut-off calibrator were defined as positive.

Calculation for Serion ELISA classic *Leptospira* IgM:

- Serion units of <15 gives a negative result interpreted as no evidence of recent infection.
- A Serion unit of 15-20 is a low positive or borderline result and may suggest a recent infection.
- Serion units of >20 is a positive result suggestive of a recent or current infection.

PCR: Efficacy of primers capable of amplifying conserved outer membrane protein (OMP) genes i.e., LipL21 and LipL32 of *Leptospira* strains was tested for rapid and early diagnosis of the leptospirosis using a polymerase chain reaction (PCR). These OMP genes were found to be conserved in various *Leptospira* serovars viz., Canicola, Pomona, Icterohaemorrhagiae, Pyrogenes, Sejoe, Grippotyphosa, Ballum and Tarassovi as PCR products of 561 bp and 756 bp were obtained by PCR employing UpL21 and lipL32 based primers, respectively, in all these serovars.

Absence of such amplicons in DNA extracted from Pasteurella, Campylobacter and Brucella confirmed the specificity of the primers.

The PCR was conducted using a 25 µL reaction mixture that consisted of 2.5 µL of 10 x PCR buffer, 1 µL of 10 mM dNTP mix, 1.5 µL of 25 mM MgCl₂, 1.0 U of Taq DNA polymerase, 1 µL (25 pmol) each of forward and reverse primer and 50 ng of template DNA. LipL21 and lipL32 specific PCR was performed with the following conditions: initial denaturation at 94°C for 5 min., followed by 30 cycles of denaturation at 94°C for 1 min., annealing at 55°C for 45 sec., extension at 72°C for 30 sec. and a final extension at 72°C for 6 min.

All the data were entered and analysed using SPSS version 17. The sensitivity, specificity, positive predictive value, negative predictive value and the percentage of false positives and false negatives were calculated for dark field microscopy and IgM ELISA in comparison with PCR.

RESULTS: The age and sex wise distribution of the study population was shown in table 1. The minimum age was 21 and the maximum age was 72 years. Majority of the study samples were in the age group of 30–50 years with a mean age of 39.4 years and 33.2 years among the males and females respectively. The male: female ratio was 1.7:1. The most common clinical manifestation which was found among the study subjects was fever of more than five days and myalgia. Conjunctival suffusion and jaundice was found among 75.6% and 32% of the subjects respectively (table 2).

The efficacy of dark field microscopy was assessed in comparison with PCR, which is considered as gold standard. Of all the 82 samples, PCR had shown positive for Leptospira in 28 samples, DFM had shown positive for 33 and IgM ELISA had shown positive for 30 patients. The sensitivity for dark field microscopy in detecting Leptospira in comparison with PCR was found to be 92.8% and the specificity was 87%. The positive predictive value was 78.7% and the percentage of false positives was 12.9% (table 3). Similarly the efficacy of IgM ELISA in comparison with PCR was shown in table 4. It is seen from the table that the sensitivity, specificity, PPV and NPV were higher when compared to the dark field microscopy and the percentage of false positives was comparatively lesser than the dark field microscopy. Table 5 shows the efficacy of dark field microscopy in comparison with IgM ELISA, where the sensitivity and specificity had improved and the percentage of false positives had come down.

Age Group	Male	Female	Total
20 – 30	5(9.6%)	3(10%)	8(9.7%)
31 – 40	18(34.6%)	13(43.3%)	31(37.8%)
41 – 50	12(23%)	9(30%)	21(25.6%)
51 – 60	7(13.4%)	4(13.3%)	11(13.4%)
61 – 70	8(15.3%)	1(3.3%)	9(10.9%)
>70	2(3.8%)	0	2(2.4%)
Total	52(100%)	30(100%)	82(100%)
Mean±SD	39.43±3.26	33.21±2.46	

Table 1: Age and Sex wise Distribution of the Study Population

Clinical manifestation	Frequency (n=82)	Percentage
History of fever > 5 days	82	100%
Headache/myalgia	82	100%
Conjunctival suffusion	62	75.6%
Jaundice	27	32%
Oliguria	16	19.5%
GI Symptoms	34	41.4%
Rash	29	35.3%
Pulmonary symptoms like breathlessness and cough	24	29.2%

Table 2: Clinical Manifestations reported among the Study Subjects

Sensitivity	92.8%
Specificity	87%
Positive predictive value	78.7%
Negative predictive value	95.9%
Percentage of false positives	12.9%
Percentage of false negatives	7.1%

Table 3: Efficacy of Dark Field Microscopy in Detecting Leptospira in Comparison with PCR

Sensitivity	100%
Specificity	96.2%
Positive predictive value	93.3%
Negative predictive value	100%
Percentage of false positives	3.7%
Percentage of false negatives	0%

Table 4: Efficacy of IgM ELISA in Detecting Leptospira in Comparison with PCR

Sensitivity	100%
Specificity	94.2%
Positive predictive value	90.9%
Negative predictive value	100%
Percentage of false positives	5.7%
Percentage of false negatives	0%

Table 5: Efficacy of Dark Field Microscopy in Detecting Leptospira in Comparison with IgM ELISA

DISCUSSIONS: Leptospirosis is an acute febrile disease, being considered as an emerging or re-emerging disease in the tropical and subtropical regions. Leptospirosis is frequently underdiagnosed, because of the non-specific symptoms early in the disease and the difficulty of performing the culture. Leptospirosis is one of the diseases with high economic impact, so its diagnosis and sero-surveillance are very important for any control program. Many tests have been widely used in the field of screening for leptospirosis. The present study was designed to

compare the efficacy of IgM-ELISA and dark field microscopy in the diagnosis of leptospirosis.

In the present study, majority of the study population were in the age group between 20–40 years and the males were comparatively more in number than the females and the similar type of results were also shown by a study done by Tanwi Panwala in the region of Gujarat.¹² The most common clinical manifestation presented in our patients were fever, headache, myalgia and conjunctival suffusion which was in par with the study done by Jaiswal in Bihar.¹³ In the present study, the sensitivity of dark field microscopy was found to be 92% and the specificity was 87% when compared with PCR, which was much higher when compared to the study done by Kanchan Sharma¹⁴ where the sensitivity and specificity was reported to be only 62%. The reason for low sensitivity and specificity in those studies is because the comparison was done with MAT and the serological test was taken in patients with symptoms of more than 15 days.

The current study had shown the sensitivity, specificity, positive predictive value and negative predictive value for IgM ELISA in comparison with PCR as 100%, 96.2%, 100% and 93.3% respectively. Various studies have shown sensitivities ranging from 68 to 100% reported for various ELISAs.^{15,16,1,17} There are several possible explanations for the variability in screening test sensitivity observed between studies and the selection of the control population would have made the difference.¹⁸ Lijmer et al¹⁹ report that studies using a diseased population and a separate control group significantly overestimate the diagnostic performance of screening tests compared to studies using a single clinical population. In all screening tests for Leptospirosis diagnosis, antigen should be broadly reactive with different infecting *Leptospira* serovars. The characteristics of the Leptospiral antigen differ from place to place. So, usually the screening test should be able to detect the antibodies produced against the site-specific *Leptospira* serovars. Hence, the laboratories are in need to validate the performance of screening tests in those particular settings in whichever they are to be used. Sometimes, Leptospirosis patients might have co-infection or cross reactive antibodies of other diseases like syphilis, dengue, malaria, relapsing fever, Lyme's disease, legionellosis. These diseases were not analysed in this study, but numbers of these disease agents have been reported by other investigators to cross react in leptospirosis serologic assay.^{20,16,21}

In the present study, the sensitivity, specificity, positive and negative predictive value of dark field microscopy had improved when it was compared with IgM ELISA and a similar type of result was shown by a study done by Chandrasekaran et al²² and he also suggested that considering the cost effectiveness and the early diagnosis dark field microscopy can be used as a tool in the diagnosis of leptospirosis.

CONCLUSION: The present study suggests that IgM-ELISA can be very good cost effective investigative tool with high efficacy in diagnosing leptospirosis. Though PCR and MAT

are being considered as the gold standard tests, the cost factor and unavailability of the technical expertise would be major drawbacks. Leptospirosis being an emerging and usually underdiagnosed disease, newer diagnostic tests and rapid case detection particularly during an outbreak becomes the need of the hour, and IgM-ELISA would satisfy these things in the detection of *Leptospira*.

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