

A Prospective Observational Study to Compare Diagnostic Accuracy of Dark-Ground Microscopy, Blood Culture, IgM ELISA and PCR in the Diagnosis of Leptospirosis

K. Prakash¹

ABSTRACT

Introduction: Leptospirosis is a zoonotic disease of worldwide distribution. Accurate diagnosis and prompt treatment are essential for the successful management of this potentially life-threatening disease, reducing morbidity and mortality. The current study was conducted with an objective to compare the sensitivity and specificity of the dark-ground microscope, blood culture, IgM ELISA and PCR in the diagnosis of leptospirosis against the gold standard (Culture)

Material and methods: Direct examination of blood was done using dark-ground microscopy, the culture was done by inoculation of the blood sample into the EMJH medium, antibodies against *Leptospira* was demonstrated using Panbio IgM ELISA kit, and antigen products were demonstrated using polymerase reaction (PCR) with primers G1 and G2.

Results: Among the 60 tested samples, 12 were positive by dark-ground microscopy, 22 were grown in culture, 55 were IgM ELISA positive, and 32 were PCR positive.

Conclusion: IgM ELISA can be used as a rapid screening tool due to its high sensitivity. Culture is time-consuming even though its high specificity and hence cannot be used. PCR is currently the gold standard due to a rapid demonstration of leptospiral DNA for early initiation of treatment.

Keywords: Leptospirosis, Dark-ground Microscopy, Culture, IgM ELISA, Polymerase Chain Reaction.

INTRODUCTION

Leptospirosis is one of the most widely prevalent zoonotic diseases globally. It is caused by spirochetes of the genus *Leptospira*.¹ Humans are only accidental hosts. The disease is acquired through contact of abraded skin with the water or soil which is contaminated with infected urine. Once in the soil, the bacteria can survive for prolonged periods if the soil is damp.² In India, outbreaks of leptospirosis occur during monsoon seasons due to flooding occurring because of rainfall. In South-India, most of the suspected cases of leptospirosis occur between June and October.³ Once bacteremia occurs in the human body, the signs and symptoms of illness occur. Leptospirosis can manifest as a subclinical infection to as severe as multi-organ dysfunction that is associated with a high case fatality rate. In advanced stages, liver failure, pulmonary haemorrhage, acute kidney injury and bleeding manifestations may occur. Despite the common prevalence and severity of illness, the diagnosis is often missed by clinicians because of its varied manifestations since almost

90% can present as undifferentiated febrile illnesses.⁴ It is often misdiagnosed as influenza, fever of unknown origin or aseptic meningitis.⁵ Furthermore, it is often thought that leptospirosis is a disease of the rural area than the urban. Due to these limitations, laboratory tests are of paramount importance in the diagnosis of leptospirosis. Lab diagnosis includes methods such as Microscopy, Culture, Serology and Molecular diagnostic tests. Darkfield microscopy can visualise leptospire, but it requires 10⁴ organisms/mL to be visible in microscopy.⁶ IgM ELISA is widely used, but it can give false positive results.⁷ PCR can detect leptospira DNA in the serum and urine samples of patients. But it requires a large amount of DNA in the sample to give a positive result.⁸ This study was done to compare the diagnostic efficacy of various diagnostic methods such as Dark ground microscopy, Blood culture, IgM ELISA and PCR in the diagnosis of leptospirosis.

The current study was conducted with an objective to compare the sensitivity and specificity of the dark-ground microscope, blood culture, IgM ELISA and PCR in the diagnosis of leptospirosis against the gold standard (Culture).

MATERIAL AND METHODS

A cross-sectional study was conducted on 60 patients who were suspected of leptospirosis after obtaining informed written consent from them in the local language. A standardized form was used, to collect information from patients suspected of leptospirosis. Information regarding demographic details (age, sex), clinical features, animal or water contact/exposure, and occupation was obtained. Any suspected patient who has been handling farm animals like cows and buffaloes or handling pet dogs or playing with them was considered to have an animal contact. Any

¹Associate Professor, Department of Microbiology, Government Medical College (old RIMS), Karimnagar, Telanagana. 505001, India

Corresponding author: Dr K. Prakash, Ft No 404, B-Block, 4th Floor, Chalmeda College Quarter's, Chalmeda Anand Rao, Institute Of Medical Sciences, Bommakal, Karimnagar, Telanagana. 505001, India

How to cite this article: K. Prakash. A prospective observational study to compare diagnostic accuracy of dark-ground microscopy, blood culture, IgM ELISA and PCR in the diagnosis of leptospirosis. International Journal of Contemporary Medical Research 2020;7(5):E1-E4.

DOI: <http://dx.doi.org/10.21276/ijcmr.2020.7.5.9>



icteric sample or sample exhibiting haemolysis, lipaemia, or microbial growth was excluded from the study. Direct examination of blood was done using dark-ground microscopy, the culture was done by inoculation of the blood sample into the Ellinghausen-McCullough-Johnson-Harris (EMJH) medium, antibodies against *Leptospira* was demonstrated using Panbio IgM ELISA kit. ELISA was done with Lepto-M *Leptospira* Microwell Serum ELISA kit. The test procedure was performed according to the protocol provided along with the kit. The results were interpreted according to the manufacturer's instructions. Negative and positive controls were kept with each test run. Cut-off was calculated and reporting of results was done as positive, negative and equivocal as per the manufacturer's guidelines provided along with the kit. After 2-3 weeks, a second blood sample was collected from equivocal cases and once again tested by ELISA for IgM antibodies. Those giving positive result were defined as laboratory confirmed leptospirosis. For Dark Field Microscopy, 5-ml blood samples were collected in EDTA vial. The sample was transferred to a sterile test tube and was centrifuged at 1000 rpm for 15 min. After this, 10 µl of Buffy coat or plasma was transferred to a new, clean slide and a cover slip was placed over it and the preparation was examined under dark field microscope (Zeiss). Then the plasma was spun at 3000-4000 rpm for 20 min, at the end of which the supernatant was discarded and wet preparation was done with a drop of sediment, which was examined under a dark field microscope. The sample was reported negative if no spirochete was observed after screening of approximately 100 fields in each of the preparations. Antigen products were demonstrated using polymerase reaction (PCR) with primers G1 and G2. Direct Polymerase Chain Reaction (PCR) on specimens enables rapid and direct diagnosis, at least in the early and convalescent stages of infection. The reaction detects leptospiral DNA in the specimen, down to extremely small amounts equivalent to the DNA content of about 10 leptospire or less. A limitation of PCR-based diagnosis of leptospirosis is the inability of most PCR assays to identify the infecting serovar. Leptospiral genomic DNA was extracted from suspected human serum samples. The DNA was air-dried, dissolved in TE buffer (10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA), and kept at -20°C until use. The DNA was quantified by agarose gel electrophoresis and spectrophotometrically by calculating the A 260 /A 280 ratios and the A 260 values to determine protein impurities and DNA concentrations. *Leptospira* DNA was amplified by using the primers. These primers amplified all pathogenic and non-pathogenic *Leptospira* species.^{4, 8}

List of primers used:

Primer	Targeted genes	Annealing temperature (°C)	Product size	Sequences	Sources
G1	<i>secY</i>	55	285 bp	5'-CTGAATCGCTGTA TAAAAGT 3'	Murgia R et al ⁹
G2				5'-GGAAAACAAAT GGTCGGAAG 3'	

RESULTS

The study population included a total of 60 subjects with 63% males and 37% females. Among the study population, 11 individuals (18%) belonged to the paediatric age group of fewer than 18 years, and the remaining 42 individuals (82%) were adults. The age distribution of the study subjects based on gender is given in table 1.

Majority (38%) of the study subjects were farmers. There were 17% each working as poultry workers or in animal rearing. The proportion of subjects working in sewers, gardening and sedentary jobs respectively was 8% in each category. Only 4% were involved in the veterinary occupation. With regards to contact with infected animals, almost 47% of the study population had a history of contact with dogs. 23% had a history of contact with cattle. Only 14% had a history of contact with rodents. Among the rest, history of contact with cats and hens were present among 12% and 4% of the study population respectively.

With regards to water being the source of infection, 36% of the study population had a history of contact with a public source of water. History of contact with ponds or sewage was present among 18% each of the study population. 21% had a history of contact with canal water. Only 7% had a history of contact with river water.

With regards to clinical signs, fever, conjunctival congestion and nausea were present in all the study subjects. All except 2 individuals, had headache and jaundice at presentation. The next most common symptom was abdominal pain present among 92% of the study population. Vomiting and oliguria were present among 67% and 42% respectively. The proportion of subjects with skin rashes, cough, hemoptysis and breathlessness respectively was 8% under each symptom. The proportion of subjects with Pedal edema, neck stiffness, altered sensorium and seizures respectively were present in 3% each. The clinical signs found among the study population are summarised in table 2. As inferred from table 2, pallor and icterus are the predominant clinical signs found among the majority (96%) of the study population. Only half of the study population (50%) had Hypochondrial tenderness. Hepatomegaly and splenomegaly were present among 67% and 33% respectively.

The comparison of different diagnostic tests for diagnosis of leptospirosis is summarised in table 3. As seen in table 3, the highest number of positive results are obtained with IgM ELISA, followed by PCR and Culture. Dark ground microscopy was positive among only one-fifth (20%).

PCR is taken as the gold standard and the diagnostic accuracy of dark ground microscopy, blood culture, IgM ELISA are expressed in terms of sensitivity and specificity, positive predictive value and negative predictive value. The results

are summarised in tables 4 and 5.

As seen in Table 5, Dark ground microscopy has high specificity and positive predictive value but low sensitivity. Hence it does not have much utility as a screening tool in the

Age in year	Male	Female
0-10 yrs	3(5%)	0(0%)
10-20 yrs	5(8%)	3(5%)
20-30 yrs	7(12%)	2(3%)
30-40 yrs	8(13%)	2(3%)
40-50 yrs	10(17%)	12(20%)
>50 yrs	5(8%)	3(5%)
Total	38(63%)	22(37%)

Table-1: Age and sex distribution of subjects in the study

Clinical signs	Frequency (%)
Pallor	58(96%)
Icterus	58(96%)
Lymphadenopathy	10(17%)
Edema	2(3%)
Purpura	5(8%)
Hypochondrium tenderness	30(50%)
Hepatomegaly	40(67%)
Splenomegaly	20(33%)
Meningeal signs	2(3%)

Table-2: Clinical signs among the study population.

Test	No of positives	Percentage
Dark ground microscopy (DGM)	12	20%
Culture	22	37%
IgM ELISA	55	92%
PCR	32	53%

Table-3: Comparison of positive results of dark-ground microscopy, blood culture, IgM ELISA and PCR

Parameters	Positive	Negative	Total
DGM			
Positive	12	0	12
Negative	20	28	48
Blood Culture			
Positive	22	0	22
Negative	10	28	38
IgM ELISA			
Positive	30	25	55
Negative	3	3	5

Table-4: Comparison of dark-ground microscopy (DGM), blood culture, IgM ELISA with PCR

Test	Sensitivity	Specificity	Positive predictive value	Negative predictive value
DGM	37.50%	100%	100%	58.33%
Blood culture	68.75%	100%	100%	73.68%
IgM ELISA	90.91%	10.71%	54.54%	60%

Table-5: Diagnostic accuracy of dark-ground microscopy (DGM), blood culture, IgM ELISA

diagnosis of leptospirosis since it might miss many cases. On the other hand, blood culture has a high specificity and better sensitivity and negative predictive value compared to dark ground microscopy. But it is time-consuming, hence initiation of treatment cannot be delayed pending lab results. IgM ELISA has a high sensitivity, but low specificity, positive and negative predictive values. Hence it might be a good screening tool, but PCR is needed for the confirmation of the diagnosis of leptospirosis.

DISCUSSION

The source of infection in this study was either through direct contact with animals and poultry or due to their occupation such as farming. This is in concurrence with the review by Levett et al¹⁰ where the authors mention that farmers, veterinarians and abattoir workers are at risk for infection with leptospirosis through direct contact. Indirect modes of transmission can occur in sewage workers or canal workers. Also, similar to the present study, other studies^{11, 12} have also reported outbreaks of leptospirosis after recreational exposure to water such as swimming, exposure to public water sources, ponds, and canals. With regards to the clinical features of the patients, 96% had icterus in the present study, and all had a fever and conjunctival suffusion. This proportion of icterus in concurrence with the study by Edwards et al¹³ where 95% had jaundice. But a lesser proportion of the study population had a fever (76%) and conjunctival suffusion (54%) when compared to the present study.

The extent of respiratory involvement is different among various studies on leptospirosis. In the current study around 8% of the study population had respiratory symptoms. In the study by Yersin et al¹⁴, 12% of the study population had hemoptysis, and pulmonary infiltrates on chest X-ray which is higher compared to the present study.

In the current study, the sensitivity and specificity of IgM ELISA were 90.91% and 10.71% respectively. In the study by Tanganuchitcharnchai et al¹⁵, the sensitivity and specificity of IgM ELISA were 95% and 41.4% respectively. The sensitivity is comparable to the present study while the specificity is much higher compared to the present study. In the study by Desakorn et al¹⁶, the sensitivity of panbio IgM ELISA was 52.3% which is lower than the present study while specificity was 66.4% which is much higher than the present study. The positive predictive value of IgM ELISA was 54.54% in the current study which was higher than the 8.7% PPV reported in the study by Bhatia et al.¹⁷

In the present study, the sensitivity and specificity of dark ground microscopy were 37.5% and 100% respectively. In the study by Sharma and Kalawat et al¹⁸, the sensitivity of dark ground microscopy was 60.5% which was higher compared to the present study. In the study by Vijayachari et al¹⁸, background microscopy had a sensitivity of 40.2% which was comparable to the present study. The specificity of 61.5% was much lower compared to 100% in the present study. In the present study, the PPV and NPV were 100% and 58.33% respectively. This is higher than the 55.2% PPV and 46.6% NPV reported in the study by Vijayachari

et al¹⁹ (2001). In the present study, culture has the highest rates of specificity, but according to various studies⁴, culture methods are cumbersome and hence cannot be used to initiate rapid treatment. The most rapid screening method for the diagnosis of leptospirosis is IgM ELISA. Dark ground microscopy can also serve as a useful tool for the early diagnosis of leptospirosis. Culture has a high specificity, but since it is time-consuming, it cannot be used for rapid assessment of patients for initiating treatment. DNA Methods such as PCR are the current gold standard in the diagnosis of leptospirosis. The genetic material from leptospira organisms can be rapidly demonstrated in the blood samples using PCR and hence it can be used to assess patients rapidly for early initiation of treatment.

ACKNOWLEDGEMENTS

We acknowledge the technical support in data entry, analysis and manuscript editing by “Evidencian Research Associates”

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Source of Support: Nil; **Conflict of Interest:** None

Submitted: 14-03-2020; **Accepted:** 30-04-2020; **Published:** 14-05-2020