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Original Research Article

Serological and molecular approaches for leptospirosis at a tertiary care centre in northern India

Saurabh Chaurasia[1], Raj Kumar Kalyan[2]*, Prashant Gupta[2], Kamlesh K. Gupta[3], Chandra Kanta[4], Akanksha Gupta[2]

[1]Department of Microbiology, Era's Lucknow Medical University, Lucknow, Uttar Pradesh, India
[2]Department of Microbiology, [3]Department of Medicine, [4]Department of Paediatric, King George's Medical University, Lucknow, Uttar Pradesh, India

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***Correspondence:**
Dr. Raj Kumar Kalyan,
E-mail: drrkkalyan1973@yahoo.co.in

ABSTRACT

Background: Aims and objectives of the study was to determine prevalence rate of leptospirosis and recognition of common epidemiological situation and clinical manifestations of leptospirosis in patients with pyrexia of unknown origin at a tertiary care centre.
Methods: This was a hospital based prospective observational study. The duration of study was from August 2015 to July 2016. Patients with clinical symptoms of pyrexia of unknown origin attending Gandhi Memorial and Associated Hospital, King George's Medical University Lucknow during the study period were enrolled in this study. We performed the IgM ELISA and PCR for the leptospirosis at our centre and for the Micro Agglutination Test (MAT), we sent the serum samples to regional medical research centre Department of Health Research Ministry of Health and Family Welfare, Govt. of India Port Blair.
Results: A Total of 104 symptomatic patients were recruited. Of total, IgM ELISA for leptospirosis were positive in 25 patients, PCR in 20 patients and MAT shows significant titres in 3 samples. The ROC curve analysis revealed significant diagnostic accuracy of IgM ELISA with 100.00% sensitivity and 78.22% specificity however showed less positive predictive value (12.0%) but high negative predictive value (100.0%). Most common presentation were fever, jaundice and rashes (40.4%) followed by fever and jaundice (29.8%).
Conclusions: Leptospirosis IgM ELISA and PCR showed good detection accuracy. Age, sex, occupation is not significantly associated to the leptospirosis.

Keywords: ELISA, Leptospirosis, Microscopic Agglutination Test, PCR, Pyrexia of unknown origin

INTRODUCTION

Leptospirosis is an apparent or unapparent disease of animals and man.[1] Leptospirosis is the world's most widespread zoonotic infection of global importance. Leptospirosis is now being recognized as a re-emerging infectious disease.[2] The disease has a broad geographical distribution due to the large spectrum of mammalian hosts that harbour and excrete the spirochaete agent from their renal tubules.[3-5] Many animal species, including rodents, are considered natural hosts of the microorganism.[6-9] Natural hosts are disseminating the agent in nature through their urine.[10] It is caused by Leptospira interrogans.[11] Leptospires were first identified as the cause of Weil's disease in Japan, where it was common among coal miners.[12] Most outbreaks in India are reported from the coastal regions of the states. Significant outbreaks have occurred in Orissa, Mumbai and the Andaman Islands.[13-15] The spectrum of disease ranges from subclinical infection to a severe syndrome of

multiorgan dysfunction characterized by headache, fever, myalgia, jaundice, hepatomegaly and convulsions.[4] The true incidence of human leptospirosis in India is not known either because of lack of awareness on the part of the treating physicians or the lack of diagnostic techniques.[15-17]

Measurement of antibodies has emerged as reliable diagnostic test with good specificity and sensitivity achieving a positive serologic test increases with the duration of disease and a good correlation between results of MAT and ELISA.[18,19] IgM detection has repeatedly been shown to be more sensitive than MAT when the first specimen is taken early in the acute phase of the illness.[20] PCR is more specific test than any others available tests for confirm diagnosis.

METHODS

This was a hospital based prospective observational study. The duration of study was one year from August 2015 to July 2016. Patients with pyrexia of unknown origin and symptoms related to leptospirosis and willing to give consent to participate in the study attending Medicine and Paediatric departments of Gandhi Memorial and Associated Hospital, KGMU Lucknow during the study period were enrolled in the study. 5ml of venous blood on day one and second sample was taken on one week apart. Whole blood was collected into a sterile plain vial. Serum was transferred into a clean, dry, labelled eppendorf and stored in refrigerator at 2-8^{0}C. Serum was divided into two equal aliquots. Half of the sample was used for serological testing, other half of the sample was stored in refrigerator for MAT and PCR. Convalescent sera were used for further serological tests. We used novatec leptospira IgM ELISA kit procured from SCIMEDX Corporation USA as per manufacturer's instructions to see the IgM antibodies against the leptospira. PCR was performed using the primer as mentioned in the Table 1.

Table 1: Primers used in study.

Primers	Location	Primer sequences
Lig1[20]	632-650	TCA ATC AAAACA AGG GGC T
Lig2[20]	1080-1100	1100 ACT TGC ATT GGA AAT TGA GAG

For PCR DNA was extracted from blood using Qiagen DNA extraction Kit (Gmbh, Hilden, Germany). The Lig1/Lig2 primers were procured from the Thermo Fisher Scientific Company. (GenBank accession numbers AF368236 and AF534640). PCR tests were performed in a GeneAmp 9700 cycler using the following profile: an initial denaturation at 95.8°C for 5min followed by 35 cycles of amplification. Each cycle consisted of denaturation at 95.8°C for 30s, annealing at 48.8°C for 45s, and extension at 72.8°C for 30s. The cycles were followed by a 7 min extension at 72.8°C. The final concentrations of the reagents in 25 ml reaction mixture were as, dNTP, 0.2mM; $MgCl_2$, 3.0mM; primers, 0.68mM and Taq DNA polymerase (1.25 units, Invitrogen). The PCR products were run on 1.5% agarose gels (Invitrogen) and visualized by staining with ethidium bromide.

For MAT we sent the serum samples to regional medical research centre department of health research ministry of health and family welfare, Govt. of India Port Blair, and Andaman and Nicobar Island, India and get the result of MAT with the panel of serovars used (Table 2). Dilution: Doubling dilution (Starting Dilution-Including antigen 1 in 40, excluding antigen 1 in 20).

Table 2: Serovars used in MAT.

Serogroup	Serovar	Strain	Genomospecies
Australis	Australis	Ballico	Leptospirainterrogans
Autumnalis	Bangkinang	Bangkinang 1	Leptospirainterrogans
Canicola	Canicola	Honduterecht iv	Leptospirainterrogans
Grippotyphosa	Grippotyphosa	Moskva v	Leptospirainterrogans
Hebdomadis	Hebdomadis	Hebdomadis	Leptospirainterrogans
Icterohaemorrhagiae	Icterohaemorrhagiae	Rga	Leptospirainterrogans
Icterohaemorrhagiae	Lai	Lai	Leptospirainterrogans
Pomona	Pomona	Pomona	Leptospirainterrogans
Pyrogenes	Pyrogenes	Salinem	Leptospirainterrogans
Sejroe	Hardjo	Hardjoprajitno	Leptospirainterrogans
Bataviae	Bataviae	Swart	Leptospirainterrogans
Tarassovi	Bakeri	Lt-79	Leptospirasantarosai
Cynopteri	Cynopteri	3522 c	Leptospirakirschneri
Javanica	Poi	Poi	Leptospiraborgpetersenii
Djasiman	Djasiman	Djasiman	Leptospirainterrogans

Statistical analysis

Continuous data were summarized as Mean ±SD while discrete (categorical) in number and %. Categorical groups were compared by chi-square (χ^2) test or Fisher's exact test. The diagnostic accuracy (sensitivity and specificity) of ELISA IgM and PCR against MAT (gold standard or definitive) to detect Leptospirosis was assessed using receiver operating characteristics (ROC) curve analysis. A two-tailed (α=2) p value less than 0.05 ($p<0.05$) was considered statistically significant. Analysis were performed on SPSS software (Windows version 17.0). The χ^2 test showed significant difference in detection between the two markers (χ^2=19.97, $p<0.001$) indicting poor detection accuracy of ELISA IgM as compared to MAT (gold standard).

RESULTS

A total of 104 symptomatic patients were enrolled. A demographic details and chief complaints among patients were recorded (Table 3 and 4). A comparative analysis of the tests performed were done (Figure 1). Comparison of leptospirosis detection between IgM ELISA and MAT (gold standard) was also done (Table 5).

Table 3: Demographic characteristics of patients.

Demographic characteristics	No. of patients (n=104) (%)
Age (yrs.): Mean ±SD, range, median	21.32±15.77, 0.5-57.0, 18
0-10	32 (30.8)
10-20	22 (21.2)
20-30	18 (17.3)
30-40	14 (13.5)
40-50	11 (10.6)
50-60	7 (6.7)
Sex	
Female	45 (43.3)
Male	59 (56.7)
Location:	
Rural	49 (47.1)
Urban	55 (52.9)
Occupation:	
Farmer	31 (29.8)
House wife	2 (1.9)
Laborer	17 (16.3)
Store keeper	4 (3.8)
Student	16 (15.4)
Zoo keeper	1 (1.0)
Children	33 (31.7)

MAT result

About 1:40 titre was present against the serovars Grippotyphosa and Lai like in a single sample while Icterohaemorrhagiae and Pomona serovars were present in two different samples.

In our study, Leptospirosis IgM ELISA and PCR showed good detection accuracy, as compared to MAT (gold standard), with a significant difference ($p< 0.001$). The ROC curve analysis revealed significant diagnostic accuracy of ELISA IgM (AUC=0.891, Z=3.14, p=0.002) with 100.00% sensitivity (95% CI=30.5-100.0) and 78.22% specificity (95% CI=68.9-85.8) however showed less positive predictive value (12.0%) but high negative predictive value (100.0%). Similarly, PCR also showed significant diagnostic (AUC=0.916, Z=3.73, $p<0.001$) with 100.0% sensitivity (95% CI= 30.5-100.0) and 83.17% specificity (95% CI=74.4-89.9) but with less positive predictive value (15.0%) and high negative predictive value (100.0%).

Table 4: Distribution of chief complaints among patients.

Chief complaints	No. of patients (n=104) (%)
Fever	3 (2.9)
Fever/Abdominal pain	4 (3.8)
Fever/Altered sensorium/Seizures	1 (1.0)
Fever/Chills & Rigors	9 (8.7)
Fever/Jaundice	31 (29.8)
Fever/Jaundice/Altered sensorium	1 (1.0)
Fever/Jaundice/Rashes	42 (40.4)
Fever/Rashes	11 (10.6)
Fever/Seizures	1 (1.0)
Fever/Vomiting	1 (1.0)

Table 5: Comparison of leptospirosis detection between ELISA IgM and MAT (gold standard).

Outcome	MAT (n=104) (%)	ELISA IGM (n=104) (%)	χ^2 value	P Value
Absent	101 (97.1)	79 (76.0)	19.97	<0.001
Present	3 (2.9)	25 (24.0)		

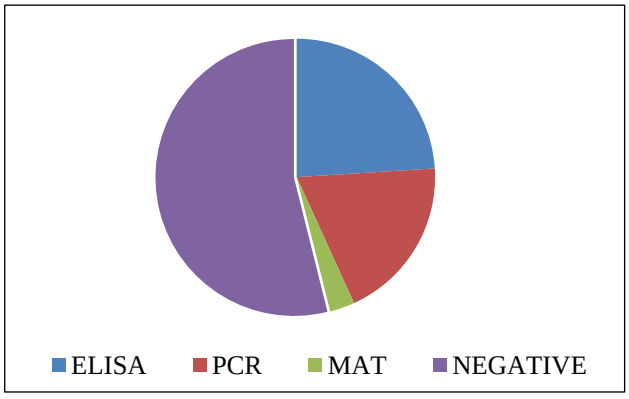

Figure 1: Positivity of leptospirosis by various tests.

DISCUSSION

Out of 104 cases leptospirosis were positive in 25 (24%) cases by IgM ELISA, 20 (19.2%) by PCR and 3 (2.9%) by MAT. The overall seropositivity for leptospirosis was 24.0% per cent in our study. In a Study done by Chaudhary R et al, out of the 1453 patients, 391 (26.90%) were positive serologically by IgM ELISA, MAT was positive in 50 of 138 (36.23%), and PCR from blood was positive in10 cases out of 115 (8.7%).[21] IgM ELISA finding were close to our finding, while in our study only 3(12%) cases were MAT positive out of 25 IgM ELISA positive cases and PCR positivity was higher in our study (19.2%).

A study of Zakeriet S et al, PCR identified 60 (50.4%) of 119 cases of leptospirosis in Mazandaran Province, and serologic analysis identified 35 (29.4%) of 119 cases.[22] The serological prevalence is slightly high in this study as compare to our study. But PCR positivity was very high in this study as compare to our study. In a study done by Chaudhary R et al, at Delhi shows, out of 75 cases 32 patients (42.6%) had a positive ELISA test for Leptospira IgM antibody. The results of MAT were positive in 21 (65.6%) of the 32 ELISA-positive serum samples. MAT data can give a general impression about which serogroups are present within a population. In a study of Angnani R et al from Nagpur (Maharashtra), the prevalence of leptospirosis in patients being investigated for Pyrexia of unknown origin (PUO) was 35%.[23] It is very high than our study and In another study of Sumathi G et al, from Chennai , the year-wise prevalence of leptospirosis in 2004, 2005 and 2006 were 14.7, 24.9 and 32.3 per cent, respectively.[24] These studies support that prevalence of leptospirosis is more in southern India.

A limitation of PCR-based diagnosis of leptospirosis is the inability of most PCR assays to identify the infecting serovar. While this is not significant for individual patient management, the identity of the serovar has significant epidemiological and public health value.

CONCLUSION

Leptospirosis IgM ELISA and PCR showed good detection accuracy. Age, sex, occupation was not significantly associated to the leptospirosis.

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