Original Research Article

Rapid Diagnosis of Leptospirosis by IgM ELISA in Resource Poor Settings

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ABSTRACT

Background & Objectives: Early and accurate diagnosis of leptospirosis is important for proper and prompt treatment, which is life saving for patients with severe illness. IgM ELISA is particularly useful in making an early diagnosis, since it is positive in the first week of illness, a time when the clinical manifestations may be nonspecific. This study was done to evaluate the prevalence of leptospirosis in Aligarh and to compare the efficacy of IgM ELISA to PCR and MAT as a rapid, sensitive and cost effective diagnostic tool for leptospirosis.
Methods: Patients with acute febrile illness, acute renal failure and acute hepatitis were included in the study. Clinical & epidemiological history was evaluated on the basis of modified Faine's criteria. ELISA, MAT and PCR using G1/G2 primer which amplifies a 285 bp fragment was done for confirmation of diagnosis.
Results: Thirty one (14.9%) patients were found positive for specific anti-leptospira IgM antibodies by ELISA. On comparing the results of IgM ELISA to MAT the sensitivity was 100% while specificity was 87.6%, while it was 100% and 97.3% respectively in relation to PCR. None of the patients had a Faine's score > 25 and 21(67.7%) patients had a score between 20 - 25.
Interpretations &Conclusions: ELISA is a cost-effective, sensitive and specific test that can be used as a first line diagnostic test for early diagnosis of leptospirosis.

Key words: Diagnosis, ELISA, Faine's criteria, Leptospirosis, MAT, PCR.

INTRODUCTION

Leptospirosis is a re-emerging zoonotic disease with clinical manifestations varying from mild clinical illness to severe fulminant disease with multi-organ involvement. Severe manifestations of leptospirosis include Weil's syndrome and severe pulmonary haemorrhage syndrome (SAPH). [1] In India it is endemic in the western & southern parts of the country, [2] but actual prevalence in North India is not known. The reasons for under-reporting of the disease are due to overlapping of clinical symptoms with malaria, dengue, influenza, viral hepatitis, rickettsial infections, typhoid fever, melioidosis and many other illnesses, [3,4] non- specific clinical manifestations; low index of clinical suspicion and non-availability of a reliable, specific, sensitive, cost effective and easy to perform diagnostic test. [3] Early and accurate diagnosis of leptospirosis is important for proper and prompt treatment, which is life saving for patients with severe illness. [5] Hence there is a need for availability of rapid diagnostic modalities to initiate proper and timely management. [6] Leptospira can be isolated from blood and CSF during the leptospiraemic phase (up to eighth day) and from urine during leptospiruric phase (from

2nd to 3rd week of illness), [3] amongst the serological tests, microscopic agglutination test (MAT) is considered the gold standard. It is highly specific and is based on the detection of antibodies against the leptospiral lipo-polysaccharides. [7] A four-fold rise in antibody titres in paired acute and convalescent serum samples is diagnostic. ELISAs are popular for diagnosis of *Leptospira* infection and several assays are available. They can be performed with commercial kits or with antigen produced "in house". A broadly reactive so-called genus-specific antigen is generally used to detect IgM, and sometimes also IgG antibodies. ELISA can detect IgM-class antibody in the early phase of the disease so that current or recent infection may be indicated. Where no antibody is detected or only a low ELISA titre is found, a second serum sample should be examined for sero-conversion or a significant rise in titre. [8]

Recently, polymerase chain reaction (PCR) has proved to be a useful tool to demonstrate leptospires in tissue and body fluids. PCR can be useful also for a rapid diagnosis of leptospirosis particularly in the case of acute phase of the disease in which other diagnostic techniques can give negative results or are time consuming. [3,9]

This study was done to i) evaluate the hospital based prevalence of leptospirosis in patients with acute febrile illness, acute viral hepatitis and acute renal failure, in Aligarh region and ii) to compare the efficacy of IgM ELISA to PCR as a diagnostic tool for leptospirosis.

MATERIALS AND METHODS

The prospective study was conducted in the department of Microbiology, JNMCH over a period of one year from July 2013 to July 2014. Patients presenting in medicine OPD or admitted in medicine ward with acute febrile illness, acute renal failure or acute viral hepatitis were included in the study. Utilizing clinical, epidemiological, and laboratory parameters modified Faine's criterion was scored and assessed. [10] Kuppuswamy's classification was used to for evaluating the socio-economic status of the patients. [11] The cases were divided into the following three groups on the basis of the clinical history taken based on a predesigned proforma.

Acute febrile illness (AFI): A case of AFI was defined as an individual with history of fever (temperature > 38°C) for 3 days or more.

Acute hepatitis: Any individual presenting with signs and symptoms of acute jaundice (bilirubin > 1mg/dl) was defined as a case of acute hepatitis.

Acute renal failure: A case of acute renal failure (ARF) was defined as any individual with rapid deterioration in kidney function (within 48 hours). Deterioration in kidney function was assessed by rise in serum creatinine (absolute increase in serum creatinine of ≥ 0.3 mg/dl or percentage increase in serum creatinine of $\geq 50\%$) or in those patients who presented with a reduction in urine output (defined as <0.5 ml/kg/hr for more than 6 hours).

An informed consent was taken from patients before their inclusion in the study. The study was approved by the Institute ethical committee, J.N.M.C.H., A.M.U.

Exclusion criteria:

- Patients with obvious clinical signs for diseases such as diarrhoea, pneumonia, UTI, typhoid fever, malaria or established fever of unknown origin (FUO).
- Patients found positive for Hepatitis B, Hepatitis C, Chikungunya, Malaria or Dengue.
- Patients with ARF with a known underlying aetiology were also excluded from the study.

Controls: Controls were taken from blood bank.

Seven ml blood was collected with all aseptic precautions, from patients suspected of leptospirosis for the serological tests and for PCR.

Serological tests: The following serological tests were performed:

i. The lepto immunoglobulin M (IgM) ELISA (PANBIO IgM ELISA, Standard Diagnostics, Korea): ELISA was done weekly for all the serum samples using commercially available kits according to the manufacturer's instructions. The results were interpreted according to manufacturer's instructions, i.e. values < 9 PANBIO ELISA units were considered negative, 9–11equivocal, and >11 positive. For samples showing equivocal results, another blood sample was drawn after a period of 10 days, and the test was repeated.

ii. Microscopic agglutination test (MAT): Samples were sent to the National Reference Laboratory for Leptospirosis, Port Blair, Andaman Islands, and India for MAT. MAT was carried out following standard procedure [12] using live leptospiral reference strains as antigens. The strains belonged to serogroups *Australis, Autumnalis, Ballum, Bataviae, Canicola, Grippotyphosa, Hebdo, Icterohaemorrhagiae, Javanica, Pomona, Pyrogenes* and *Tarassovi*. The criteria for a positive MAT test was a titre of >1:400 in a single sample. Paired sera were not available for repeat test. It could be performed on 15 samples only.

Polymerase chain reaction:

i. **DNA isolation:** DNA was extracted from serum samples using QIAamp DNA blood mini kit (Qiagen, Germany) (50

Amplification of DNA: The amplification of DNA was performed in a total volume of 25 μl. The primers [13] used for PCR were:

G1 5'- CTG AAT CGC TGT ATA AAA GT-3' &
G2 5'- GGA AAA CAA ATG GTC GGA AG-3'

The program for amplification included 35 cycles of 94°C (denaturation) for 1 min, 55°C (annealing) for 1 min and 72°C (extension) for 2 min and a final extension step at 72°C for 7 min. The PCR was performed in a final reaction volume of 25 μl containing 5 μl of 10x assay buffer [10 mM Tris HCl (pH 9.0), 1.5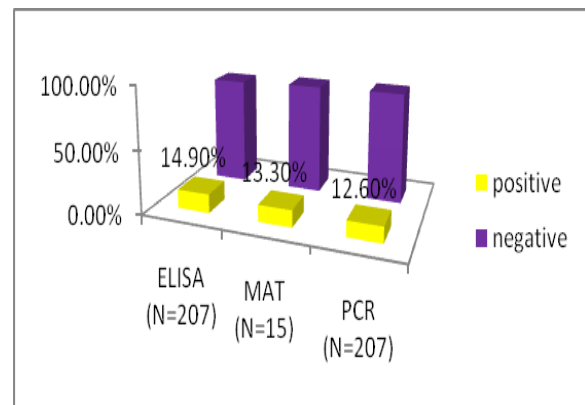 mM MgCl2, 50 mM KCl and 0.01% Gelatin], 200 μM each dNTPs, 20 pM of each primer, 0.5 U of Taq DNA Polymerase, and template DNA (10 μl). The PCR products were loaded in 1% wt/vol agarose gel prepared in TAE (tris base, acetic acid and EDTA- pH 8.0) buffer and detected by ethidium bromide staining after electrophoresis (BioRad, USA).

Amplification of 285 base pair DNA fragment was considered as positive for leptospiral DNA.

Statistical analysis: Statistical analysis was performed with the IBM SPSS Statistics 19. Sensitivity and specificity was calculated by chi square test.

RESULTS

A total of 207 consecutive patients were included in the study on the basis of clinical history and examination, of which 130 (62.8%) were males and the rest 77 (27.2%) were females (Male to female ratio 1.7:1). The patients were uniformly distributed in all age groups. Majority of these patients, 163 (78.7%) had acute febrile illness, 25 (12.1%) were suspected to have ARF and 19 (9.2%) had acute jaundice.

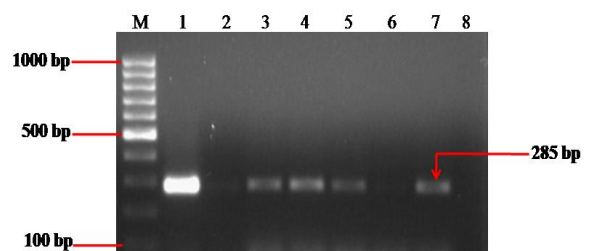**Graph 1: Distribution of Positive and Negative cases of Leptospirosis by ELISA, MAT & PCR**

Figure: Lane M showing DNA ladder, Lane 1 & 8 showing positive & negative control respectively, Lane 3, 4, 5 & 7 showing 285 bp amplified PCR product of Leptospira, while Lane 2 & 6 showing no amplification.

Out of the 207 patients suspected of leptospirosis, 31 (14.9%) were found positive for specific anti-leptospira IgM antibodies by ELISA (PanBio) **(Graph 1).** MAT could be performed in only 15 cases, out of which, only two cases (13.3%) had a significant titre. One of these was positive for the serovar *Hebdo* & one for both *Hebdo* and *Pyrogenes*. On PCR, the 285 bp fragment of the G1 & G2 primer **(Figure 1)** was found in 26 cases (12.6% of clinically suspected cases, 83.9% of ELISA positive). Five cases positive by ELISA were negative by PCR **(Table 1).** On comparing the results of IgM ELISA to PCR the sensitivity was 100% while specificity was 97.3%.
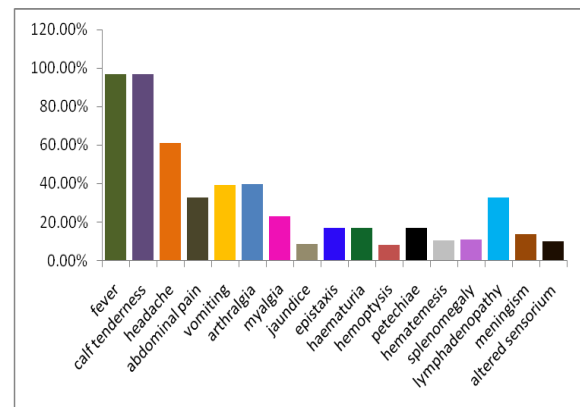
Table 1: Comparison of ELISA To PCR & MAT

	PCR (N=207)		MAT (N=15)	
	Positive	Negative	Reactive	Non-reactive
Reactive by ELISA (N=31)	26(83.6)	5 (16.4)	2(13.3)	13(86.6%)
Non-reactive by ELISA (N=176)	-	176(100%)	Not done	

Table 2: Distribution of Patients On The Basis Of Faine's Categories and Socio-Economic Background

Faine's score (part A+B)	ELISA reactive					ELISA non-reactive				
	Kuppuswamy scale					Kuppuswamy scale				
	0-5	5-10	11-15	16-25	26-29	0-5	5-10	11-15	16-25	26-29
0-10 (-ve)	-	-	-	1	-	47	15	3	8	7
11-20 (-ve)	4	5	-	-	-	50	26	3	8	5
21-25 Presumptive	14	4	3	-	-					
>25 confirmed	-	-	-	-	-					

Out of the 31 patients found positive by *Leptospira* serology, 25 (80.6%) were males and 6 (19.4%) were females. Majority were from the rural background (73.5%) and belonged to low 18(58.1%) to upper-lower 9(29%) socioeconomic group (as per Kuppuswamy index). Approximately 80% patients had animal contact history (rat, cattle, dog, sheep etc.) **(Table 2).** Amongst the seropositive patients, 24(77.4%) had acute febrile illness, 5(16.2%) had ARF and 2(6.4%) had acute jaundice. Fever (96.7%), calf tenderness (96.7%) and headache (61.3%) were the most common presenting complaints of majority of patients included in the study. Abdominal pain (32.7%), vomiting (39.1%), arthralgia (39.7%), myalgia (22.8%), jaundice (8.8 %), bleeding manifestations (27%) in the form of epistaxis, haematuria, hemoptysis, hematemesis & petechial rashes were other common complaints **(Graph 2).** A significant number of patients had splenomegaly (42%). Lymphadenopathy, meningism, altered sensorium was noted in few patients. Fever and calf tenderness were the most consistent symptoms in the sero-positive patients. None of the patients had a Faine's score > 25 and 21(67.7%) patients had a score between 20 - 25.

Graph 2: Clinical presentation of patients with leptospirosis

DISCUSSION

Leptospirosis in India, is a grossly under reported disease, probably due to the lack of diagnostic modalities and lack of awareness of the disease among physicians. Though social, environmental and occupational factors offer ideal conditions for successful transmission of leptospirosis, the disease was considered infrequent in occurrence until early 1980, after which it has been reported sporadically or as epidemics from different parts of the

country. [14] Prevalence in India has been reported in a wide range from around 60% in South India [15] to nearly 30% in North India. [2] There is no accurate estimate of the problem of leptospirosis in non-endemic areas like North India. [16] This study was conducted to assess the prevalence of leptospirosis in Aligarh region and to evaluate the efficacy of IgM ELISA as first line diagnostic test for leptospirosis. To the best of our knowledge, this is the first study in Aligarh region to compare different serological test for diagnosing leptospirosis.

Majority of the patients included in the study were males (63%) while around 37% were females. This may be due to the reason that males are more exposed to the outdoor environments, which are most of the time wet moist fields, giving higher chances of exposure to rodents and other animals, particularly in rural areas from which majority of our patients come from. The higher prevalence in males is a universal phenomenon [17] and is generally attributed to their more frequent outdoor activities and with the age group that are most active in agricultural activities. Nearly 80% of our patients had given a history of animal contact.

We divided our patients into three groups on the basis of clinical manifestations. More than 2/3rd of the patients had acute febrile illness, nearly 12.1% were suspected to have ARF and the remaining 9.2% had acute jaundice. Other studies from North India had also highlighted the importance of leptospirosis in FUO cases. [18] Apart from fever and calf tenderness, headache, arthralgia, myalgia, jaundice, bleeding manifestations, neurological symptoms etc were also present in varying proportions in these patients. Other authors had also reported fever, calf tenderness and headache as the common manifestation of leptospirosis. [19] These protean and non-specific presentations of leptospirosis have often led to misdiagnosis, especially in malaria, dengue and viral hepatitis endemic regions of South Asia, South America and the Caribbean. [20]

On the basis of serological test results i.e., IgM ELISA the prevalence of leptospirosis in our region was noted as 31 (14.9%). None of these patients had a Faine's score of >25, while 21 had a score between 20 – 25 (presumptive). Majority of these patients belonged to low and upper-lower socio-economic groups. High prevalence in the low socio-economic people may be due the reason that they are more exposed to the open fields and other risk factors of leptospirosis as compared to the people belonging to the high social status.

Fifteen ELISA positive sera were tested by MAT. MAT is a specific test and is considered the gold standard for the diagnosis of leptospirosis. Another advantage is that serovar identification can be done by MAT but it is too labour intensive and there is risk of handling the live leptospires. In our study, only two samples (13.3%) were positive on MAT, one for *Hebdo* and the other for *Hebdo* and *Pyrogenes*. Due to the low titre these were not considered significant. The low MAT titres in our study may be because majority of the samples were collected during the first 3-4 days of illness. MAT become positive after 7-10 days of illness, peak at 3-4 weeks, and may persist at high levels for many years. To make a diagnosis, a fourfold or greater rise in titre between acute- and convalescent-phase serum specimens must be documented. In cases with strong clinical evidence of leptospire infection, a single antibody titre of 1:200-1:800 (depending on whether the case occurs in a low- or high endemic area) in the MAT is required. [21]

DNA-based techniques have been proposed as alternative methods of diagnosis and identification of leptospires. [21] Gravekamp et al [13] proposed the use of a set of primers (G1 & G2) that enabled the amplification of target DNA fragment from leptospiral species. Amplification results in generation of a PCR product of 285 bp and could detect even 1-10 leptospires per ml. In

our study, the 16SrRNA gene was found in 26 cases (12.6% of clinically suspected cases, 83.9% of ELISA positive). PCR is an efficient tool for early diagnosis of leptospirosis during acute phase of the disease, especially when the clinical symptoms are confusing. The most important advantages of PCR are its sensitivity, specificity and rapidity through which the disease can be diagnosed. However, the major drawbacks of this technique are its high operational cost and unavailability of facilities in common diagnostic laboratories.

IgM ELISA is particularly useful in making an early diagnosis, since it is positive as early as 2 days into illness, a time when the clinical manifestations may be nonspecific, and it is extremely sensitive and specific (93%). [22] On comparing to PCR, the sensitivity and specificity of ELISA was found to be 100% and 97.3% respectively. In a similar study, Chaudhary et al (2013) reported that MAT was positive in just 36% of ELISA positive cases, while PCR using urine specimen can detect leptospira in ELISA negative cases. Sensitivity of PCR of urine specimen was higher as compared to blood, since the leptospires get concentrated in the convoluted tubules of kidney and are excreted in urine. [23] However, in our study PCR was done only on blood samples.

CONCLUSIONS

On the basis of our observations we suggest that leptospirosis should be considered as a differential diagnosis in cases of acute febrile illness. In a developing country like India, IgM ELISA can be considered as a cost effective, sensitive and specific diagnostic test for screening leptospirosis.

Limitations: MAT could be performed in only 15 samples.

The criteria for a positive MAT test was a titre of >1:400 in a single sample'. These criteria would have low sensitivity as only a small proportion of true cases would have MAT titres in this range in the sample taken during the acute phase.

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