



A NEWER APPROACH FOR THE SERODIAGNOSIS OF LEPTOSPIROSIS OF LEPTOSPIROSIS USING OUTER MEMBRANE PROTEINS OF LEPTOSPIRA INTERROGANS SEROVAR TARASSOVI

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ABSTRACT

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Objective: Leptospirosis in humans occurs throughout the world as an acute infection ranging imperceptible to severe, potentially fatal, renal and liver failure accompanied by haemorrhage and jaundice. In India, according to number of reports, Leptospirosis has been a significant health problem since 1980. This disease mimics influenza, hepatitis, dengue owing to which it often goes undiagnosed due to lack of awareness and diagnostic facilities in most of the laboratories in the country. This study was undertaken to diagnose suspected cases of Leptospirosis by IgM ELISA, DRIDOT and MAT, and also to standardize an in house IgM ELISA using OMP antigens of the *L.tarassovi* the predominant serovar in the study

Materials & Methods: Blood samples were collected from a total of 287 patients (186 male and 101 female) presenting with acute febrile illness and fulfilling the criteria of clinical diagnosis of Leptospirosis. All the samples & healthy controls were subjected to ELISA (IgM serion), dridot, MAT, and *Leptospira* serovar Tarassovi OMP IgM ELISA, and for isolation of *Leptospira*.

Results: IgM antibody could be detected by ELISA, Dridot and OMP ELISA of *L. Tarassovi* from Group I and Group II sera samples. It was observed by MAT that *L.Tarassovi* was the predominant serovar followed by *L.australis*, *L.autumnalis*, *L.sejroe*, *L.pyrogenes*, *L.ballum*, *L.canicola*, *L.icterocopenhageni*, *L.cynopteri*. A total of 5 isolates were recovered from Group I patients.

Conclusion: IgM ELISA using sarcosyl extracted OMP antigens of serovar Tarassovi when compared with MAT the overall sensitivity and specificity was 72% and 81% respectively. The same sera reacted with serovar Australis, serovar Tarassovi, serovar Sejroe, serovar Pyrogenes, serovar Ballum and serovar Icterocopenhageni in MAT.

Key words: Leptospirosis, OMPs, IgM ELISA, *L. tarassovi*, serodiagnosis.

OMP IgM ELISA for diagnosis of Leptospirosis

INTRODUCTION

Leptospirosis is an important zoonotic disease that affects humans on all continents. A variety of infectious diseases that present as undifferentiated febrile syndromes such as malaria, dengue, influenzae as well as haemorrhagic fevers can mimic Leptospirosis. Rapid diagnostic methods are required to diagnose Leptospirosis early in order to institute an appropriate therapy. In 1966 human Leptospirosis was reported in Delhi, North India¹. After a gap of 36 years it was reinvestigated^{2,3}. This study was undertaken to diagnose suspected cases of Leptospirosis by IgM ELISA, Dridot, and MAT, and also to standardize an in house IgM ELISA using OMP antigens of the predominant serovar in the study i.e *L.tarassovi*.

MATERIAL AND METHODS

STUDY GROUP & CONTROL GROUP

Blood samples were collected from a total of 287 patients (186 male / 101 female) presenting with acute febrile illness and fulfilling the criteria of clinical diagnosis of Leptospirosis who were admitted to the Medical and pediatric wards of University College of Medical Sciences, Guru Tegh Bahadur Hospital (UCMS, GTBH), Delhi and All India Institute of Medical Sciences (AIIMS) Hospital, New Delhi. These samples were collected during the period from October 2002 – August 2005 from patients in the age group between 3-75 years. Serum was separated and stored at -20°C. Serum samples from 40 healthy controls were also collected with no history of fever.

2.1. Laboratory Diagnosis

Criteria for confirmatory diagnosis (Group-I): Isolation of *Leptospira* from blood or urine and demonstration of 1:200 titer against different serogroups in microscopic agglutination test is considered as confirmatory evidence. Criteria for Presumptive Diagnosis (Group – II): A suspected

case of Leptospirosis positive by any rapid test (IgM ELISA & Dridot) and MAT in low titers 1:100.

Clinically suspected case of Leptospirosis but serologically negative (ELISA, MAT and Dridot) (Group - III).

Healthy Controls (Group - IV).

Inclusion criteria: An acute febrile episode with headache, generalized body ache and associated with any of the following symptoms and signs, jaundice, oliguria, respiratory symptoms (cough, hemoptysis and breathlessness), haemorrhagic manifestations (hematemesis), bleeding gums, and sub conjunctival haemorrhage), altered sensorium, signs of meningeal irritation and convulsions (as defined by Indian Council of Medical Research, National Symposium/ Workshop (1997).

Acute renal failure cases after exclusion of obstructive uropathy, obstetrical cause, acute glomerulonephritis and fulminant hepatic failure cases were also included.

2.2. Maintenance of *Leptospira* serovars in Ellinghausen, Mc.Cullough and Johnson Harris (EMJH) Medium

Leptospira interrogans serovar Icterocopenhageni, *L.autumnalis*, *L.australis*, *L.cynopteri*, *L.javanica*, *L.tarassovi*, *L.grippotyphosa*, *L.pomona*, *L.canicola*, *L.ballum*, *L.pyrogenes*, *L.hebdomadis*, *L.sejroe* were procured from Regional Medical Research Centre (RMRC), Portblair, and Indian Veterinary Research Institute (IVRI), Izatnagar and maintained in EMJH medium at 28°C in BOD incubator by repeated subcultures at 15-20 days of intervals.⁴

2.3. Enzyme linked immunosorbent assay (ELISA)

ELISA was performed according to the manufacturer's instruction. Evaluation of the test was done by SERION ELISA classic *Leptospira* (Institute Virion, Serion GmbH, Wurzhurg Germany).

2.4. Lepto Tek Dridot

According to the manufacturer's instruction.⁵

2.5. Microscopic Agglutination Test (MAT)

MAT was performed according to WHO guidelines.⁶

2.6. Preparation of outer membrane protein (OMPs) from *Leptospira interrogans* serovar Tarassovi

Leptospire were cultured at 28°C in 500ml EMJH medium to logarithmic phase of growth, harvested by centrifugation at 40,000g for 20min at 4°C, and washed twice in 0.01M phosphate buffered saline (pH 7.2) (PBS). The pellet was suspended in 10mM HEPES (N-2 hydroxy ethyl piperazine-N-2 ethanesulfonic) buffer (pH 7.4). The cells were disrupted by sonication at 60μ for 15sec each four times while interrupting by a 5 sec pause and cellular debris was removed by centrifugation at 1700g for 10min. The supernatant fluid was centrifuged at 100,000g for 60min at 4°C. The pellet was resuspended in 2ml of 1% sodium Lauryl sarcosinate detergent prepared in 10 mM Hepes buffer pH 7.4 and incubated at room temperature overnight. The sarcosyl insoluble fraction was sedimented by centrifugation at 100,000g for 60min at 4°C and suspended in 100μl of distilled water. Both the fractions were stored at -20°C till further use.⁷

2.7. Determination of protein concentration by Lowry method.

Using protein estimation kit (Bangalore genei) Protein concentration was estimated by Lowry method⁸.

2.8. IgM Enzyme linked immunosorbent assay using Sarcosyl extracted OMPs of *L.interrogans* serovar Tarassovi. (*L.tarassovi* OMP IgM ELISA)

Forty-one patient's sera samples from Group I, 40 sera each from the rest of 3 Groups (II-IV) were taken in duplicates to detect the IgM leptospiral antibodies to *L.tarassovi* OMPs. Flat bottomed polystyrene microtiter plate (Nunc) was coated with *L.tarassovi* OMPs i.e 1.5ng/well, suspended in 0.05M sodium carbonate (pH 9.6) and kept overnight at 4°C. The plates were washed thrice with PBS 0.05% Tween 20 (PBST).

The nonspecific binding sites were blocked by adding 300μl of 10% skimmed milk powder in PBS, and was incubated at 37°C for 2hrs at room temperature. Plates were washed as in the previous step.

100μl of each patient serum diluted to 1:100 was added in duplicate to the wells of the plate. Healthy control, positive and negative control sera samples were also included in duplicate and incubated at 37°C for 2hrs. After four washes with PBS-T, 1:5000 dilution of Antihuman IgM goat antibodies conjugated to horse radish peroxidase (sigma) was added to each well and incubated for 1 hr at 37°C. Plates were washed with PBS-T and incubated with 100μl/well with orthophenyline diamine substrate buffer and 0.03% [vol/vol] H₂O₂ for 20min in dark at room temperature. The reaction was stopped by adding 25μl of 2N H₂SO₄ and the absorbance was read at 492nm.

RESULTS

IgM antibody could be detected by ELISA, Dridot and OMP of *L.tarrasovi* from only Group I and Group II sera samples. It was observed by MAT that *L.tarassovi* was the predominant serovar. A total of 5 isolates were recovered from Group I patients.

IgM ELISA using sarcosyl extracted OMP fractions of *L.interrogans* serovar Tarassovi when compared with MAT the overall sensitivity and specificity of the OMP IgM ELISA was 72% and 81% respectively.

DISCUSSION

The rapid diagnostic methods are required to diagnose Leptospirosis in order to institute an appropriate therapy. A total of 287 serum samples were from hospitalized patients and also 40 control sera were subjected to ELISA, MAT, Dridot and *L. tarassovi* OMP IgM ELISA. Serological evidence based on IgM antibodies becomes detectable during the first week of illness^{9,10}.

Table-1 Serological Analysis

S.No.	Diagnostic Tests	Group I(n=41)	Group II (n=92)	Group III (n=154)	Group IV (n=40)
1	ELISA(IgM) SERION	30 (73.0%)	51 (55.0%)	0 (0%)	0 (0%)
2	Dridot	18 (43.9%)	42 (45.7%)	0 (0%)	0 (0%)
3	MAT	36 (87.6%)	27 (29.3%)	0 (0%)	0 (0%)
4	Isolation EMJH	5 (12.0%)	0 (0%)	0 (0%)	0 (0%)
5	<i>L.tarassovi</i> OMP IgM ELISA	29/41 (70.7%)	19/40 (20.7%)	0 (0%)	0 (0%)

Table-2. Sensitivity and specificity of the IgM ELISA (Serion) and *L.tarassovi* OMP IgM ELISA vs. MAT.

Diagnostic Tests	MAT (+)	MAT (-)	Sensitivity	Specificity	PPV	NPV
ELISA IgM Serion (+)	32	50	50.8%	81.1%	39.0%	87.4%
ELISA IgM Serion (-)	31	215				
OMP IgM ELISA (+)	37	20	72.5%	81.8%	64.9%	86.5%
OMP IgM ELISA (-)	14	90				

An immunoglobulin IgM enzyme linked immunosorbent assay (ELISA) is often used as an alternative test in routine diagnostic laboratories¹¹. IgM antibodies to *Leptospira* were detected by ELISA i.e 30/41 (73%) and 51/92 (55%) of Group I and Group II respectively, IgM antibodies could not be detected in any of the sample from Group III and Group IV, (Table 1). Leptospirosis shows extreme variability of its clinical features which range from mild anicteric illness to a severe life threatening disease with jaundice or renal failure or bleeding. Thus the relationship between clinical criteria and IgM ELISA the most commonly performed test is of

vital importance. Results of Dridot were similar in Group I and Group II with positivity rate approaching 45%, (Table 1).

Microscopic agglutination test is the reference test for the diagnosis and detects antibodies at serovar level. Both IgM and IgG class antibodies are detected by MAT¹². In Leptospirosis antibodies usually appear within 5-7 days, after the onset of symptoms and in significant proportions of patients, antibodies persist in detectable quantities for many months¹³.

The information about circulating serovars was lacking so a panel of serovars recommended were used as antigens in MAT to detect the infecting

serovar and also 1:200 was taken as the cut off titer¹⁴. *L.tarassovi* was the predominant serovar followed by *L.australis*, *L.autumnalis*, *L.sejroe*, *L.pyrogenes*, *L.ballum*, *L.canicola*, *L.icterocopenhageni*. Growth in EMJH culture was observed in 5 blood samples. Isolation rate in this study was not very high which could be due to prior intake of antibiotics before hospital admission. Leptospiral antibodies could be demonstrated in 3/5 of the culture positive. All these patients had fulminant infection, liver failure or thrombocytopenia. The cases of thrombocytopenia were admitted as suspected cases of Dengue fever but tested negative for it. Similar type of presentation of Leptospirosis was reported in Dengue outbreak in Bangladesh¹⁵.

Since IgM ELISA Kits were expensive an effort was made to standardize an in house IgM ELISA using the extracted OMP antigens of the predominant serovar i.e. *L.tarassovi* in this study. IgM antibody to OMP could be detected in 29/41 (70.7%), 19/40 (20.7%) by in house *L.tarassovi* OMP IgM ELISA, (Table I).

Using the result of MAT as a gold standard, over all sensitivity and specificity of the ELISA (Serion Virion) was 50.8%, 81.1% whereas *L.Tarassovi* OMP IgM ELISA was 72.5%, 81.1% respectively (Table-2).

It is, because of the partially purified sarcosyl OMP enriched fractions were used in *L.tarassovi* OMP IgM ELISA where as the whole cell crude antigens were used in the IgM ELISA (Serion Virion). Certain proteins of *Leptospira* have found to be immunogenic during natural infection in humans¹⁶. Therefore, in the present study, *Leptospira* proteins which are recognized during infection can be potentially useful for sero diagnosis and vaccination studies.

CONCLUSION

Serological evidence of Leptospirosis was found in Group I and Group II patients by MAT using a battery of antigens. *Leptospira* were recovered from Group I patients during the rainy season from clinically

suspected cases of dengue. Due to similar clinical presentation leptospirosis may be missed in suspected dengue outbreak therefore leptospirosis should be ruled out in such cases. The sera which reacted with serovar Australis, serovar Tarassovi, serovar Sejroe, serovar Pyrogenes, serovar canicola, serovar Ballum, serovar Icterocopenhageni in MAT were tested positive in serovar Tarassovi OMP IgM ELISA. This assay may be suitable for rapid screening of Leptospirosis where Laboratory facilities for MAT are not available.

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