Original Article

Clinical and serological evaluation of leptospirosis in Puducherry, India

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Abstract

Background: Leptospirosis is a zoonotic disease with a worldwide distribution. There is a paucity of available data about prevalence of this disease in Pondicherry. Our aim was to investigate the seropositivity rate of leptospirosis in suspected cases and also to identify the predominant serogroups present by performing Microscopic Agglutination Test (MAT). The other aim of this study was to compare the results of a commercially available IgM ELISA with that of MAT.

Methodology: A total of 110 blood samples from patients suspected of leptospirosis were sent for diagnosis. These samples were subjected to IgM ELISA and the microscopic agglutination test (MAT). MAT was done using a panel of 12 *Leptospira* serovars.

Results: MAT analysis of the 110 samples showed 40 (36%) to be positive. Antibodies were predominantly seen against serogroup *Leptospira* Icterohemorrhagiae (27%), followed by Pomona (17%), and Pyrogenes (12%). IgM ELISA done on these samples showed a positivity of 37% compared to MAT.

Conclusion: This study reveals that the MAT test can be standardized in a diagnostic laboratory and used in conjunction with an IgM ELISA.

Key words: IgM ELISA; leptospirosis; MAT

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Introduction

Leptospirosis, a zooanthroponosis of worldwide distribution, is an acute febrile illness, the severity of which can vary from mild to rapidly fatal. The causative bacteria, *Leptospira* species, are responsible for a wide spectrum of clinical symptoms and the disease is often misdiagnosed [1,2], particularly in tropical countries. Because of its variety of clinical symptoms, the diagnosis is based on laboratory tests rather than on clinical symptoms alone [3].

Leptospirosis has been under-diagnosed and under-reported in India due to the lack of awareness of the disease, inadequate epidemiological data, and unavailability of appropriate laboratory diagnostic facilities in most parts of the country [4]. Timely diagnosis is essential since antibiotic therapy provides greatest benefit when initiated early in the course of the disease [5]. The diagnosis of leptospirosis is usually based on the demonstration of antibodies by serological tests such as the microscopic agglutination test (MAT) and enzyme-linked immunosorbent assay (ELISA). MAT is still the cornerstone of leptospirosis diagnosis in spite of its disadvantages. IgM ELISA has been employed as a useful alternative. It is a reliable test and when used in combination with MAT gives good results in diagnosis [6].

Leptospirosis has been reported to be endemic in several parts in South India such as Kerala, Tamil Nadu, Pondicherry and Andamans. In a countrywide study conducted by the National Reference Centre, Regional Medical Research Centre (ICMR), Port Blair, during the period 2000–2001, a seropositivity rate ranging from 0 to 46.8 % among all cases of fever was observed from various parts of India [7]. The positivity rate was highest in South India at 25.6%, followed by 8.3%, 3.5%, 3.1% and 3.3% in northern, western, eastern and central India respectively [8]. The data originating from seroprevalence studies of leptospirosis in Madras indicate a gradual increase over the years from 18% (1983) to 71% (1996) [4,9,10]. A seroprevalence rate of more than 55% was observed in the general population of North Andamans, an endemic area for leptospirosis [11].

In a previous study in 1995 at Jawaharlal Institute of Post graduate Medical Education and Research

(JIPMER), indirect haemagglutination assay (IHA) test was used to demonstrate anti-leptospiral antibodies in suspected cases of leptospirosis. MAT was also done using *Leptospira biflexa* Patoc 1 strain. Both these tests make use of a group specific antigen and hence could not give information about the prevalent serogroups [12]; therefore, the present study was conducted with the objective of estimating the prevalence of antileptospiral antibodies among suspected cases of leptospirosis attending the hospital by performing a commercially available IgM ELISA and MAT. MAT was performed using a battery of different serovars, which would provide information about the prevalent serovars and also the current seropositivity rate of leptospirosis in this area. The results obtained by both the tests were then compared.

Materials and methods

Blood Samples

Blood samples collected from 110 patients with suspicion of leptospirosis attending JIPMER hospital during January 2005 to February 2006 were sent to our department for diagnosis. Faine's criteria were used for suspecting leptospirosis, wherein patients with fever, headache, jaundice, cough and breathlessness, subconjunctival suffusion, signs of meningeal irritation, and convulsions were included. In these 110 patients other causes of prolonged fever were ruled out by doing Widal test for typhoid, standard tube agglutination test for brucellosis and HBsAg screening for hepatitis B.

Patients were divided into two groups: 1. Anicteric cases (n = 62) and 2. Icteric cases (n = 48).

Blood samples from 30 healthy controls (voluntary blood donors) were also included.

Serum from all 140 samples (110 suspected cases and 30 healthy controls) was separated and tested both by MAT and IgM ELISA.

IgM ELISA

IgM ELISA was performed using the IVD LEPTOSPIRA IgM Microwell ELISA Test (IVD Research Inc, Carlsbad, CA92010 USA) per the manufacturer's instructions. The absorbance of positive and negative control serum provided in the kit was used for calculations. A negative result was defined as an absorbance of 0.0-0.3 optical density (OD) units, an equivocal result as 0.5 to ≤ 1 OD units, and a positive result as > 1.0 OD units.

MAT

MAT was performed per standard procedure as described by Cole and colleagues [13].

Bacterial strains

The *Leptospira* serovars included the following:

1. *Leptospira interrogans*: Australis (strain Ballico) Autumnalis (strain Bankinang) Bataviae (strain Swart) Canicola (strain Hond Utrecht IV) Hebdomadis (strain Hebdomadis) Icterohemorrhagiae (strain RGA) Pomona (strain Pomona) Pyrogenes (strain Salinem)

2. *Leptospira kirschneri*: Grippotyphosa (strain Moskva V)

3. *Leptospira borgpetersenii*: Javanica (strain Poi) Tarassovi (strain tarassovi)

4. *Leptospira biflexa*: Semaranga (Patoc I)

All the strains were obtained from the National Leptospirosis Reference Centre, Regional Medical Research Centre (World Health Organization collaborating centre for diagnosis in leptospirosis, ICMR) in Port Blair, Andaman and Nicobar islands.

These serovars were maintained in semisolid 0.1% EMJH agar by using *Leptospira* medium base (Hi-Media) supplemented with 10% enrichment (Tween 80 and Bovine serum albumin) at 28-30°C in screw-capped test tubes.

Hyperimmune serum was raised against each of the 12 serovars in duplicate healthy rabbits [14]. This was used as positive control while performing MAT.

Preparation of antigens

A sample (0.5 ml) of each representative strain from the panel of 12 serovars was inoculated into 10 ml of liquid EMJH medium. A loopful of culture was checked under dark field microscopy to confirm absence of contamination and clumps and presence of viable leptospires. Incubation was done at 30°C for five to seven days. The culture was diluted to MacFarlands 1 for use as antigen (approximately 2-3x 10^8 leptospires/ml).

Procedure

Doubling dilutions from 1 in 50 to 1 in 3,200 were prepared. Twenty-five microliters of the specific serovar was added to all the wells. One of the wells included only the antigen without addition of antibody and served as the antigen control. The final dilutions after adding the antigen were from 1 in 100 to 1 in 6,400. The highest serum dilution showing approximately 50% agglutinated leptospires or a reduction in the number of leptospiral cells as compared to the antigen control was taken as end

point titer. A titer of 1 in 100 or more was considered significant [15].

Patients whose sera were positive by IgM ELISA and had a MAT titer of 1 ≥ 100 or MAT alone with a titre of 1 ≥ 100 were considered as confirmed cases of leptospirosis.

Results

Serum samples from the 30 healthy controls were tested by IgM ELISA and MAT. None of these samples was positive by IgM ELISA and all these samples tested by MAT had a titre of less than 100.

Sixty-six (60%) sera out of the 110 were positive for antileptospiral antibodies either by MAT or ELISA. These 66 positive cases included 44 males and 22 females indicating a male preponderance. The maximum numbers of affected individuals were between 15 and 44 years of age and the positivity rate was highest between 25 and 65 years of age.

Forty of the 110 (36%) suspected cases showed anti-leptospiral antibodies by MAT (Table 1) with the Icterohemorrhagiae (13/40, 32% samples) as a predominant serogroup followed by Pomona (19%) and Pyrogenes (13%). The other serovars observed were Grippotyphosa (10%), Hebdomadis (8%), Autumnalis (5%), Australis (5%), Bataviae (5%), and Javanica (3%). A titer of 100 or more was considered to be significant. (Table 2)

Twenty-seven (67.5%) samples showed antibody titer between 1 in 400 to 1in 800. Table 2 shows the distribution of titers among the MAT positive samples. It is noteworthy that the highest antibody titer was recorded against serovar Grippotyphosa, 1 in 6,400.

IgM ELISA was also performed on the 110 serum samples and 41 (37%) were found to be positive. The comparison of results of IgM ELISA and MAT is shown in Table 1. Forty serum samples out of the 110 (36%) reacted positively by MAT with a titre of ≥ 100 and a positive IgM ELISA or MAT alone positive with a titre of ≥ 100; consequently, these patients were considered as cases of leptospirosis per the diagnostic criteria stated earlier. In 15 (13.6%) cases both MAT and IgM ELISA were positive. The sensitivity of IgM ELISA when compared to MAT was 37.5%. In 26 patients in this study, IgM ELISA was positive but the diagnosis could not be confirmed by MAT. The probable reason for this is that IgM ELISA is a genus specific test and detects IgM antibodies early in the disease. MAT is a serogroup/serovar specific test and false negativity can occur in the early course of the disease since microscopic agglutinating antibodies usually appear in detectable levels only at the end of the first week. Anicteric cases accounted for 56.6% of cases. On the other hand, jaundice was a presenting feature in 48 (43%) of cases and complications involving liver, kidney and brain were seen in them. The number of adults affected was higher as compared to children in this study. Renal and meningeal involvement was observed more often in adults than in children.(Table 3). Seropositivity per the diagnostic criteria among the 62 anicteric cases was (25/62) 40.32% and out of 48 icteric cases 31.25% positivity was seen.

Table 4 shows the relative results of the two serological tests in the two groups of patients. In both the groups IgM ELISA gave more positivity as compared to MAT. On their own the positivity of IgM ELISA was 41/110 (37%) and MAT 40/110 (36%). But the combined efficacy of both these tests was 66/110 (60%).

Discussion

Leptospirosis is a common cause of acute febrile illness in southern India. Early diagnosis is essential. If untreated the illness can progress rapidly and mortality rates are high in severe cases. It is therefore important to differentiate leptospirosis from other causes of acute febrile illnesses. The present study found that 60% of cases had serological evidence of leptospirosis, which is definitely higher than the previous report from this centre in 1995 [12], highlighting the increasing incidence of leptospirosis in this part of the country. This increase may also be explained by the fact that the IHA test used then has been replaced by the more sensitive and specific IgM ELISA and MAT tests. Another factor could be the increased awareness of the disease among the clinicians, which in turn leads to early diagnosis and appropriate treatment of the patients.

The reason for high prevalence of leptospirosis in this area may also be due to the overuse of fertilizers commonly used for agriculture, which makes the pH of the water and soil alkaline, thereby allowing *Leptospira* to survive for a longer time and thus facilitating its transmission [16]. It is reasonable now to believe that leptospirosis is a continuing problem in this part of the country. The preponderance of cases in males between 15 and 44 years of age shows that this disease is common in the working population who are most likely to be exposed to this organism. Since males are more involved in outdoor activities they are at more risk of acquiring the infection.

Table 1. Comparison of results of MAT and IgM ELISA.

Serological test	MAT positive	MAT negative	Total
IgM ELISA positive	15	26	41
IgM ELISA negative	25	44	69
Total	40	70	110

Sensitivity of IgM ELISA: 15/40 (37.5%) Positive Predictive value: 15/41 (36.5%)
Specificity of IgM ELISA: 44/70 (62.8%) Negative Predictive value: 44/69 (63.76%)

Table 2. Details of MAT titers against different serovars.

Titer	Ictero-hemorrhagiae	Pomona	Pyrogenes	Grippo-typhosa	Hebdo-madis	Autum-nalis	Aus-tralis	Bataviae	Javanica
1:100	1	0	0	0	0	0	0	0	0
1:200	1	1	2	1	1	0	1	0	1
1:400	6	5	1	1	1	1	1	1	0
1:800	3	1	2	1	1	1	0	1	0
1:1600	1	1	0	0	0	0	0	0	0
1:3200	2	0	0	0	0	0	0	0	0
1:6400	0	0	0	1	0	0	0	0	0
Total	13	8	5	4	3	2	2	2	1

Table 3. Different clinical manifestations of the suspected cases (n = 110).

Clinical category	Number of cases Adults (n = 89)	Children (n = 21)
1. Fever; headache, myalgia, breathlessness, cough.	53	9
2. Fever; jaundice	27	11
3. Fever; jaundice, renal disease	7	1
4. Fever; jaundice, altered sensorium, convulsions.	2	0
Total	**89**	**21**

Table 4. Results of IgM ELISA and MAT among the two groups.

Group	Total No.	IgM+ MAT-	IgM+ MAT+	IgM- MAT+	IgM- MAT-
1. Anicteric cases	62	16	5	20	21
2. Icteric cases	48	10	10	5	23
	110	26	15	25	44

The overall seropositivity of leptospirosis by MAT was 36.3%. Out of the 40 MAT positive samples, the serovars which were predominantly observed in this area were Icterohemorrhagiae (32%), as was also reported in Mumbai [17] and Pune [18], followed by Pomona (19%) and Pyrogenes (13%) respectively. The highest titer was seen against serovar Grippotyphosa at 1:6400.

Grippotyphosa was implicated as the predominant serogroup in Andamans and Kerela [19]. Ratnam *et al*. have reported Autumnalis in Chennai, Cumbum and Tirunenveli, Panama in Madurai, and Icterohemorrhagiae in Bodi [20].

Detection of IgM antibodies by ELISA is now widely used in diagnosis of leptospirosis, which is more sensitive than the MAT and gives positive results earlier in the acute phase of the disease, which will help in timely treatment. In this study, IgM ELISA showed overall positivity of 37%. The individual positivity rates of both the tests were less than their combined efficacy. MAT remains the gold standard test for diagnosis of leptospirosis. MAT done in this study enabled us to know the prevalent serogroups and icterohaemorrhagiae emerged as the predominant serovar. As shown in this study, this test is feasible and can be standardized and used in routine diagnostic centres. In our opinion a combination of IgM ELISA and MAT offered the most reliable laboratory strategy for confirmation of leptospirosis.

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