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DIAGNOSIS OF LEPTOSPIROSIS BY POLYMERASE CHAIN REACTION

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ABSTRACT

Leptospirosis is an important emerging zoonotic infection in developing regions of the world. Laboratory testing for leptospiral infections is important both for diagnosis and management of the patients. The conventional laboratory methods for diagnosis have many shortcomings. The availability of molecular techniques like Polymerase Chain Reaction (PCR) as diagnostic tool has improved speed and accuracy of detection. In this study serum samples from 100 patients were tested by the microscopic agglutination test MAT, IgM ELISA and PCR. The clinical symptoms mainly observed among the patients were fever (98%), jaundice and headache (78%), myalgia (77%). Among the 33 cases of leptospirosis diagnosed by MAT, 29 (87.9%) serum samples were positive by IgM ELISA and 4 (13%) were PCR positive. By considering MAT as the standard test, the sensitivity and specificity of IgM ELISA was 43% and 76%, respectively. A relevant finding in our study was the number of positive cases verified by PCR 4/28(14.3%) and IgM ELISA 11/28(39.3%) among the 28 unconfirmed cases by MAT, demonstrating the value of PCR in the diagnosis of human leptospirosis.

KEYWORDS

Leptospirosis, diagnosis, Polymerase Chain reaction, 16SrRNA

INTRODUCTION

Leptospirosis is one of the most widespread zoonoses in the world [1]. *Leptospira* is a highly invasive bacterium, capable of infecting a broad range of mammalian hosts. Transmission occurs either through direct contact with an infected animal or through indirect contact with soil or water contaminated with urine from a host with chronic renal infection. Symptoms and severity of leptospirosis vary greatly from mild, flu-like illnesses to fatal hemorrhagic forms with severe involvement of vital organs such as the liver, lung

and kidney conventionally called 'Weil's disease'.[2] Thus, the term 'great imposter' has been applied to this spirochetal infection. Clinical diagnosis is inaccurate, especially in the tropical regions where other similar acute febrile illnesses are common. Leptospirosis may be confused with malaria, viral hepatitis, influenza, dengue fever, rickettsial infections, typhoid fever, melioidosis and others[3] .Hence early and accurate diagnosis of leptospirosis is important for proper and prompt treatment, which is life saving for patients with severe illness. Direct demonstration of *Leptospira* in preparations from specimens by dark field microscopy has been hampered due to lack of specificity.[4] Isolation of the pathogen is labour intensive and time consuming. Leptospires require complex culture media and they have a long generation time. Hence, culture is not a method of early diagnosis. Current diagnostic methods for leptospirosis usually depend upon demonstration of serum antibodies [5]. The most common serological test is the microscopic agglutination test (MAT), but it is time-consuming and lacks sensitivity [6].

Several studies found that the polymerase chain reaction (PCR) method has come into increasing use for diagnosing infectious disease caused by slowly growing or fastidious microorganisms[7].Numerous PCR-based techniques such as random amplified polymorphic DNA, PCR followed by restriction analysis or hybridization have been developed and evaluated based on typing of leptospiral reference strains [8, 9, 10, 11].

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 [8, 9].

The general consensus is that the PCR amplification of 16SrRNA gene can serve as an alternative to the currently used serological methods for identifying bacterial pathogens.

This study was designed with the aim to detect leptospiral DNA by PCR amplification of 16SrRNA in serum of patients with clinical diagnosis of leptospirosis and to compare the results with that of microscopic agglutination test (MAT) and IgM ELISA.

MATERIALS AND METHODS:

Blood samples were sent to the Microbiology Department from 100 patients with clinical suspicion of leptospirosis attending our hospital during January 2007 to February 2008. 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 100 patients other causes of prolonged fever were ruled out by doing tests like Widal test for typhoid, standard tube agglutination test for brucellosis and HBsAg screening for hepatitis B. Sera were separated from all these samples and were tested by IgM ELISA, MAT and PCR.

IgM ELISA: It was performed using the IVD LEPTOSPIRA IgM Microwell ELISA Test (IVD Research Inc, Carlsbad, CA92010 USA, as 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: It was performed as per standard procedure as per Cole et al [12].

Leptospiral strains used:

The leptospiral serovars used included the following serovars in the species

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)
Semaranga (Patoc I)

were used. All the strains were obtained from National Leptospirosis Reference Centre, Regional Medical Research Centre (WHO 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^{0}C in screw capped test tubes.

Hyperimmune serum was raised against each of the 12 serovars in duplicate healthy rabbits [13]. This was used as positive control while performing MAT.

Preparation of antigens: 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^{0}C for 5-7 days. It 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 3200 were prepared. Twenty five microliter 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 6400. The highest serum dilution showing approximately 50% agglutinated leptospires or reduction in 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 [14].

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.

Polymerase Chain Reaction (PCR):

Reference strains tested by PCR method:
All the serovars that were used for the MAT in this study were tested by PCR.

DNA isolation: DNA was extracted from serum samples using guanidine isothiocynate method as reported by Boom et al. [15].

PCR: For PCR, pathogenic serovar specific primers of the 16S rRNA gene were used, as per Hookey et al [16].

5’ CTCTGGCGGGCGCGTCTTAAA 3’
5’ TTCACCGCTACACCTGGAA 3’

The amplification of DNA was performed in a total volume of 25µl.Each 25µl PCR reaction contained 2.5 mM MgCl2, 200 µM dNTPs, 50 mM KCl, 10 mM tris-HCl, 1%Triton X-100, 1 unit of *Taq* DNA polymerase (Genei), 5 pmoles of primers, and 30 ng of genomic DNA. A blank control tube containing no added DNA was run with every set of reaction mixtures to serve as negative control. DNA extracted from serum samples artificially spiked with *Leptospira interrogans* serovar Icterohaemmoragiae served as the positive control for the PCR assays.

The PCR profile was as follows: Denaturation of DNA at 94ºC for 1min, annealing at 55ºC for 1-2 mins and extension for 2mins. Final extension of amplified product was at 72ºC for 10mins.A total of 35 cycles were carried out. After the cycles were completed, the final extension of the amplified product was done at 72ºC for 10 min. The products were analyzed on 1 % agarose gel containing ethidium bromide and viewed under UV illumination and documented.

Specificity of PCR: To determine the specificity of the PCR assay, the ability of the specific pair of primers to amplify the DNAs of all the reference strains was tested. DNA extracted from other

bacteria like *Staphylococcus aureus, Klebsiella pneumoniae, Pseudomonas aeruginosa, Salmonella typhi, Salmonella paratyphi A, and Escherichia coli* were also tested by PCR.

RESULTS:

PCR specificity: All the leptospires amplified products of 16SrRNA. No amplification products of other bacteria were detected using 16SrRNA primers.

Out of the 100 serum samples tested, 18 (18%) were found to be positive by PCR i.e. they amplified the 631 bp fragment of the 16SrRNA gene.(Fig 1). PCR alone was positive in four cases, while in three cases PCR was positive along with MAT and IgM ELISA. Most patients were adult males and the clinical symptoms mainly observed among the patients were fever (98%), jaundice and headache (78%), myalgia (77%). Among the 33 cases of leptospirosis diagnosed by MAT, 29 (87.9%) serum samples were positive by IgM ELISA (Table 1) and 4 (13%) were PCR positive (Table 2). Among the 43 cases positive by ELISA only, 14 (34.14%) samples showed positive amplification by PCR. The sensitivity and specificity of PCR when compared to MAT was 11% and 95% respectively. The reason for the lower sensitivity could be because of the phase of the disease in which the patients' samples were sent. As compared to MAT, ELISA showed a sensitivity of 43% and specificity of 76%. The comparison of results of the three tests reveals that a combination of MAT and ELISA had the highest sensitivity confirming infection in as many as 61 patients. However we had 4 sera from patients with clinical suspicion of leptospirosis negative by both MAT and ELISA but were picked up by PCR

.

TABLE 1
Comparison of results of MAT and IgM ELISA

Serological test	MAT positive	MAT negative	Total
IgM ELISA positive	12	17	29
IgM ELISA negative	16	55	71
Total	28	72	100

Sensitivity = 43%,Specificity=76%

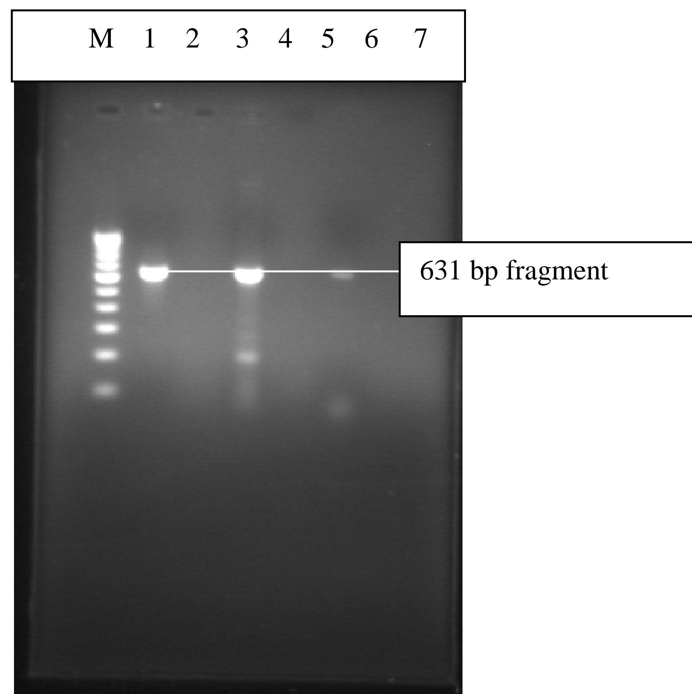
TABLE 2
Comparison of MAT and PCR results

Serological test	PCR positive	PCR negative	Total
MAT positive	2	16	18
MAT negative	4	78	82
Total	6	94	100

Sensitivity= 11%,Specificity=95%

Figure 1

PCR amplification of the 16SrRNA gene.



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 organism responds readily to antibiotics like crystalline penicillin, doxycycline and third generation cephalosporins. The PCR targeting 16S rRNA gene can be a useful tool for the rapid diagnosis of leptospirosis detecting specifically many pathogenic leptospira[17]. In this study a relevant finding was the number of positive cases verified by PCR and IgM ELISA (14) i.e. 50% among the 28 unconfirmed cases by MAT, demonstrating the value of PCR in the early diagnosis of human leptospirosis.

CONCLUSION

Though MAT is the gold standard for diagnosis of leptospirosis, the need for paired serum samples and delayed antibody response, do not contribute much to the early diagnosis. IgM ELISA is a good alternative and can be used along with MAT. So to conclude, PCR alone may not be the ideal test for leptospirosis but in conjunction with the MAT & ELISA it helps to detect more number of cases.

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