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Indian Journal of Microbiology Research

Journal homepage: www.ijmronline.org

Original Research Article

Diagnosis of leptospirosis among paediatric population by different serological and molecular methods in a tertiary care centre in South India

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Abstract

Background: Leptospirosis is an emerging public health problem globally. The clinical spectrum of illness in Leptospirosis is extremely wide ranging from undifferentiated febrile illness to severe multisystem diseases. Although children experience frequent exposure to surface waters and animals, studies on paediatric leptospirosis are very scanty, perhaps due to low index of suspicion. This study was conducted to determine the incidence of leptospirosis in clinically suspected paediatric patients by various serological and molecular methods.
Materials and Methods: The study comprised 150 paediatric patients who presented with clinical signs and symptoms of Leptospirosis. Blood samples were taken, serum separated and processed for serological and molecular tests including macroscopic slide agglutination test (MSAT), Microscopic agglutination test (MAT), IgM ELISA and Polymerase chain reaction (PCR).
Results: Among the 150 clinical suspected cases, 96.66% presented with fever, followed by myalgia 93.33% and headache90.6%. With regard to clinical signs hepatomegaly 58.66%, was the most common followed by muscle tenderness 57.3%, jaundice 54.3%, and conjunctival suffusion 48.6%. Among the 150 samples 32(21.33%) were MSAT positive, 20(13.3%) were IgM ELISA positive and 29 (19.33%) were MAT positive. The most prevalent serovar was Leptospira Pomona with 31.03% positivity. Out of 32 MSAT positive samples 4 were positive by PCR. Serological tests showed higher positivity than PCR in this study.
Conclusion: This study suggests the incorporation of both the serological and molecular methods for early diagnosis of paediatric leptospirosis, which is indispensable for the timely management and better outcome of the patient.

Keywords: Leptospirosis, Macroscopic slide agglutination test, Microscopic agglutination test, Polymerase chain reaction.

Received: 16-11-2024; **Accepted:** 30-01-2025; **Available Online**: 29-03-2025

1. Introduction

Leptospirosis is an endemic, zoonotic disease of public health importance in Chennai throughout the year and especially during monsoon.[1] It is caused by spirochetes of the genus Leptospira.[2] The disease is acquired through contact of abraded skin with the water or soil which is contaminated with infected urine. Hence humans are accidental hosts. The bacteria can survive for prolonged periods if the soil is damp.[3] Leptospirosis is an emerging public health problem globally. An international survey conducted by the International Leptospirosis Society reported ≥350,000 cases of severe leptospirosis annually.[4] Although children experience frequent exposure to surface waters and animals, studies on paediatric leptospirosis are very scanty, perhaps due to low index of suspicion.[3]

The clinical spectrum of illness in Leptospirosis is extremely wide ranging from undifferentiated febrile illness to severe multisystem diseases. The symptoms often mimic many other diseases like dengue, viral hepatitis, meningitis, influenza and viral haemorrhagic fevers. This extreme variation in clinical presentation mimicking other diseases is partly responsible for the under diagnosis and misdiagnosis of the disease.[5]

In recent years, number of paediatric cases of leptospirosis is apparently increasing in Chennai.[6] Although

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https://doi.org/10.18231/j.ijmr.2025.010

dark ground microscopy was thought to be an alternate for the early diagnosis of leptospirosis, where facilities for performing microscopic agglutination test (MAT)and Enzyme-linked immunosorbent assay (ELISA) are not available, this technique has been shown to be unreliable with significant loss of sensitivity and specificity.[7] Therefore, the definitive diagnosis of leptospirosis depends on serological tests.[8] The availability of species-specific primers made polymerase chain reaction (PCR)-based diagnosis more feasible and popular with high levels of sensitivity and specificity.[9,10]

The current study was designed to compare MSAT, MAT, ELISA and PCR based investigations for laboratory detection of leptospirosis in clinically suspected paediatric patients.

2. Materials and Methods

2.1. Inclusion criteria

Patients in the age group of 0-17 years of both gender with clinical evidence of leptospirosis (compatible clinical syndrome with any combination of fever, chills, myalgia, jaundice, conjunctival suffusion, renal failure) were included in the study.

2.2. Exclusion criteria

Adults ≥18 years age and patients with Malignant and autoimmune disorders were excluded from the study.

2.3. Blood sample collection

Under sterile aseptic precaution 2ml of venous blood was collected in purple vacutainer tube for Haematological and biochemical parameters and 3 ml of venous blood was collected in a sterile red vacutainer tube as per standard operative procedure. Serum was separated and stored at -70° C for serological and Molecular testing.

The samples were tested for leptospirosis by macroscopic slide agglutination test (MSAT), Microscopic agglutination test (MAT), IgM ELISA and polymerase chain reaction (PCR) at the Leptospira research cell of our Institute.

MSAT (In house) is a slide agglutination test using formalin treated antigen of locally prevalent pathogenic serovars along with Patoc 1 strain. A sterile 12 welled cavity slide was taken, and 7 µl of phosphate buffer solution was added. Then 13 µl of prepared pooled antigen was added to the depression of the slide. Then 6 µl of suspected patient's serum sample was added to the respective wells. Appropriate positive, negative, and Antigen controls were included. Slide was placed in a rotator and allowed to rotate for about 8mins at 180rpm. Then the slide was viewed macroscopically for presence of an agglutination.

2.4. Interpretation

Clumps of agglutination with complete clearing of leptospiral antigen reported as 4+ obvious agglutination but partial clearing of suspension reported as 3+

50% agglutination reported as 2+

25% agglutination reported as 1+

No agglutination reported as negative

*Agglutination of ≥ 2+ is considered as positive

MAT (In house) a gold standard test for diagnosis of leptospirosis was performed by doubling dilution of anti-sera in a micro titre plate. An equal volume of antigen was added to all the dilution and allowed to react for 2hrs at room temperature. The degree of agglutination and end point titre value is determined by examining a drop of the mixture by dark field microscope. The highest dilution of serum which showed 50% agglutination was taken as end titre for that particular antigen. An initial titre of 1:80 or four fold rise in titre was considered significant. A panel of six serovars were used including Pomona, Autumnalis, Semaranga, Australis, Bataviae and Sejroe.

ELISA was done using IgM ELISA Kit (Panbio) as per the manufacturers instruction. Real-time PCR was done using Leptospira Real-time PCR Kit (Helini) two sets of primers (G1, G2 and B641, B651) were used which enabled the amplification of target DNA fragment from leptospiral species.

The Leptospira real-time PCR kit is an in vitro nucleic acid amplification kit for the detection of Leptospira genus specific DNA. It contains reagents and enzymes for the specific amplification of the conserved region of the Leptospira genome (Outer membrane protein) and for the direct detection of the specific amplicon in FAM channel. In addition, it contains an internal control amplification system to identify possible PCR inhibition. The use of two sets of primers (G1, G2 and B641, B651) enabled the amplification of target DNA fragment from leptospiral species.

3. Results

This study was conducted over a period of 1 year in 150 paediatric patients of 0-17 year's age group, who presented with clinical signs and symptoms of Leptospirosis. Among 150 cases, 82 (54.66%) were male children and 68 (45.33%) were female children. Among the age group, majority 58.66% were in the age group of 13-17 years, 29.33% in 6-12 years and 12% in 0-5 years. Most of the cases presented with fever followed by myalgia and headache. Jaundice, muscle tenderness and conjunctival suffusion were the predominant signs (**Table 1**). Out of 150 clinically suspected cases 21.33% were MSAT positive. Out of 32 MSAT positive cases, 29 were MAT positive which contributes around 19.33% positivity (**Table 2**). Both MSAT and MAT showed

high positivity during 9-20 days of illness (**Table 3**). In this study, the panel of serovars used for testing were *L. Pomona, L. Autumnalis, L. Semaranga, L. Australis, L. Betaviae, L. Seiroe* (**Table 4**). Of this *L. Pomona* was found to be the predominant serovar. Sensitivity and specificity of MSAT and IgM ELISA was 100%, 97.58% and 62.5%, 100% respectively (**Table 5**). The percentage of agreement was found to be 98% for MSAT and for IgM ELISA 94% when compared with reference method MAT (**Table 6**). Out of 32 serologically positive samples tested for PCR, positivity was found to be 12.5%.

Table 1: Symptoms and sign of clinically suspected cases of leptospirosis (n=150)

Symptoms	Total numbers	Percentage % n=150
Fever	145	96.66
Myalgia	140	93.33
Headache	136	90.6
Chills and rigor	85	56.6
Jaundice	82	54.3
Conjunctival suffusion	73	48.6
Abdominal pain	70	46.6
Nausea	65	43.3
Vomiting	48	32
Muscle tenderness	86	57.3
Maculopapaular rash	40	26.66
Lymphadenopathy	49	32.6
Hepatomegaly	88	58.66
Splenomegaly	44	29.33

Table 2: Serological tests (n=150)

Test	Positivity	Percentage
MSAT	32	21.33%
MAT	29	19.33%
IgM ELISA	20	13.33%

Table 3: MAT and MSAT (During different phases of leptospirosis)

Febrile period	MSAT positive (n=32)	MAT positive (n=29)
Early 3–5 days	7 (21.87%)	6 (21.87%)
Late 9–20 days	25 (78.12%)	23 79.31%)

Table 4: Distribution of serovars (MAT)

Serovar	Total Number
L. Pomona	9
L. Autumnalis	8
L. Semaranga	5
L. Australis	3
L. Bataviae	2
L. Sejroe	2

Table 5: Sensitivity and specificity of Serological tests with reference to MAT

Test	Sensitivity	Specificity	Positive Predictive Value	Negative Predictive Value
MSAT	100%	97.58%	90.63%	100%
IgM ELISA	62.5%	100%	100%	90.80%

Table 6: Comparison of MSAT and IgM ELISA with reference to MAT

Parameters	MSAT	IgM ELISA
Positives	32	20
kappa	0.938	0.782
P value	<0.001	<0.001
Percentage of Agreement	98%	94%

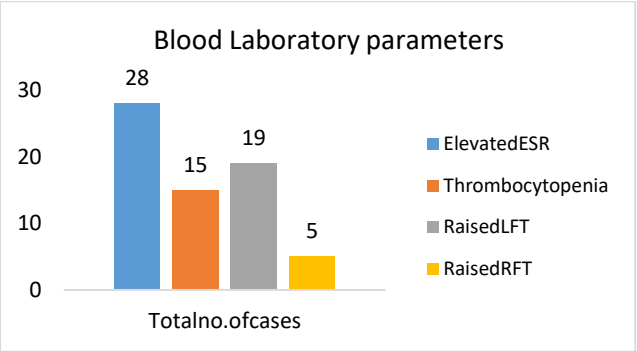

Figure 1:

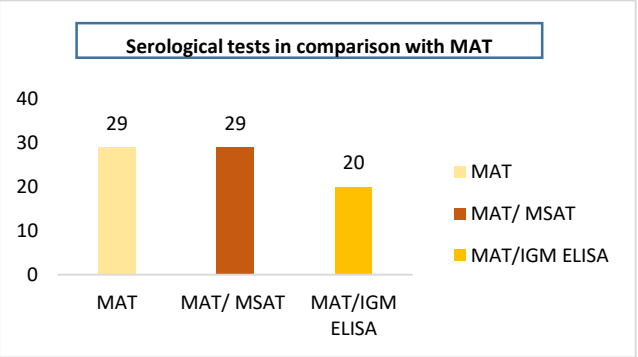

Figure 2: Analysis of the serological test with MAT as gold standard

4. Discussion

Leptospirosis is an emerging infectious disease of public health importance, especially in the endemic areas of our country. Leptospirosis in paediatric age group is underdiagnosed or misdiagnosed because of varied clinical presentation and mimicking other diseases.

In the present study (**Table 1**) among the 150 clinically suspected cases, 145 (96.66%) presented with symptoms of

fever, myalgia in 140 (93.33%) cases and headache in 130 (90.6%) cases. Among the clinical signs, jaundice 82(54.3%), conjunctival suffusion 73 (48.6%), abdominal pain 70 (46.6%), muscle tenderness 86 (57.3%) were seen. This correlates with a study conducted by Gupta N et al, in which fever was present in 97%, and conjunctival suffusion was present in 35% of cases. Haemoptysis, gastrointestinal bleeding, and haematuria were present in 5%, 5% and 12% of patients, respectively.[12] In a study conducted by Bal et al,[13] the common clinical features included were fever (100%), headache (75%), myalgia (55%), arthralgia (45%) and vomiting (39%). In a study conducted by Basu et al in 2015, conjunctival congestion, jaundice and muscle tenderness were the classical signs encountered.[14] Gastrointestinal symptoms like abdominal pain 70 (46.6%), nausea 65 (43.3%) and vomiting 48 (32%) were encountered in Leptospirosis. This correlates with study conducted by Rani et al in 2016, in which the commonest symptom was fever (89.7%) followed by gastrointestinal symptoms including abdominal pain, vomiting and diarrhoea.[15]

In the present study (**Figure 1**) considering the laboratory parameters, ESR was elevated in 31 cases (98.2%), thrombocytopenia in 15 cases (46.8%), LFT raised in 19 (59.37%) cases, RFT raised in 5 (15.62%) cases which reflects the severity of the disease. Similarly, in a study conducted by Threeswaran et al, thirty percent of leptospirosis patients had thrombocytopenia (17% in mild and 36% in severe).[18]

Of the 150 cases, 32 (21.33%) were MSAT positive, 29(19.33%) were MAT positive and 20(13.3%) were IgM ELISA positive (**Table 2**). In a study conducted by Narayanan et al,[16] among 134 children who presented with clinical signs and symptoms of Leptospirosis 35(26.1%) were MSAT positive. MSAT is a simple, rapid, reliable screening test for diagnosis of leptospirosis during an outbreak in endemic regions.[14] According to Alia et al, out of the 50 clinically suspected patient's samples, 19 were confirmed positive for leptospirosis by rapid tests, out of which six (12%) were confirmed positive by MAT.[21] IgM ELISA was positive in 20 cases (13.3%) which correlates with a study conducted by Lancy DJ et al in which 27 were positive among 373 cases (7.2%).[11] Most of the IgM ELISA positive cases were MSAT grading 3+.[24] In the present study, **Table 3** shows 78.125% MSAT positivity during >9 days of infection. MSAT positivity was higher during the second week of illness than during the first week. In Leptospirosis, initially there will be Leptospiremia, and the immune phase of illness starts after 4 days and last still 30 days of illness. This phase is characterized by resolution of the symptoms and appearance of antibodies. The MAT agglutination titer begins to rise by the end of the 2nd week and peaks in 3rd to 4th week. Hence most cases turned MAT positive during the 3rd week. (>15 days). In our study, MAT showed 79.31% positivity during >15 days of illness. This substantiates the high positivity of MSAT and MAT during the late phase of illness. According to Jaiswal et al, MSAT, MAT, IgM ELISA showed higher positivity of 72% during the late phase (9-30 days) of illness.[19]

The present study (**Table 4**) shows the predominant serovar *L. Pomona* followed by *L. Autumnalis*, *L. Semaranga*. In a study conducted by Dubeyetal,[22] the common serovars identified were *L. Pomona*,, *L. Australis*, *L. Grippotyposa*, *L. Hardjo* and *L. Autumnalis*. However, in a study conducted by S. Shivakumar *L. Autumnalis*, *L. Australis* and *L. Icterohemorrhagiae* were the common serovars identified.[23]

IgM ELISA is a simple test to detect the current infection. Combined with MAT, IgM ELISA test serves as a good diagnostic tool for early detection of the illness. In this study MSAT and IgM ELISA were compared with MAT as gold standard test. (**Table 5**) MSAT showed 100% sensitivity and 97.58% specificity whereas IgM ELISA was 100%specific and 62.5% sensitive. In this study, out of 29 MAT positive cases, 20 were IgM positive. The detection rate by IgM ELISA is around 62.5%, which correlates with the study conducted by Kumar et al, in which the detection rate by IgM ELISA was 65.43%.[19]

The interrater reliability between MAT and MSAT (**Table 6**) is significant with Kappa value of 0.938, p value <.001 and with IgM ELISA Kappa value is 0.782 and p value <.001. This establishes MSAT as a first alternate test and IgM ELISA as a second alternate test in resource limited setting with high prevalent population.

In the current study among the 32 samples, 4 were PCR positive which is about 12.5%. According to Lancy DJ et al in her study, 111 samples were sent to National institute of virology and 9 were positive (8.1%).[11] However, in a study conducted by Phillip et al in 2020, out of 165 cases 38% were positive by PCR. In many other studies PCR showed a high positivity with good sensitivity and specificity.[24]

PCR is positive only during the early phase of Leptospiraemia. Since our hospital setting is a tertiary care / referral centre most of the cases presented during late phase of clinical illness. Initial management would have been done in a peripheral Hospital and referred here for further management and hence the positivity of PCR is less in this study when compared with other clinical studies.

5. Conclusion

The study emphasises the importance of diagnosing the leptospirosis among the paediatric population. As children experience frequent exposure to surface waters and animals, diagnosis of paediatric leptospirosis a multisystem disease with varying presentation is challenging, due to the low index of suspicion. This study highlights the utilisation of simple and rapid test like MSAT, supplemented with IgM ELISA for screening of Leptospirosis among the clinically suspected cases, followed by confirmation with MAT. Serological tests

are the mainstay of confirmatory diagnosis of Leptospirosis. PCR is a diagnostic tool for the early diagnosis of the disease with increased sensitivity and specificity.

6. Ethics Approval

Institutional ethics committee of Madras Medical College (EC Reg. No. ECR/270/Inst./TN/2013).

7. Source of Funding

None.

8. Conflict of Interest

None.

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Cite this article: Subhan TB, Rajasekar K, Sundaram V, Damodharan V, Narayanan R. Diagnosis of leptospirosis among paediatric population by different serological and molecular methods in a tertiary care centre in South India. *Indian J Microbiol Res.* 2025;12(1):71–75.