Journal of Communicable Diseases
Volume 58, Issue 2 - 2026, Pg. No. 9-16
Peer Reviewed & Open Access Journal

Research Article

Comparative Evaluation of Serological and Molecular Techniques for the Diagnosis of Leptospirosis in a Tertiary Care Centre

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DOI: https://doi.org/10.24321/0019.5138.202624

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How to cite this article:
Joshua V, Dhanapaul S, Shanmugam P, Appadurai M. Comparative Evaluation of Serological and Molecular Techniques for the Diagnosis of Leptospirosis in a Tertiary Care Centre. J Commun Dis. 2026;58(2):9-16.

Date of Submission: 2025-10-31
Date of Acceptance: 2026-05-28

ABSTRACT

Introduction: Leptospirosis is a common bacterial infection, passed from animals to humans around the world. It presents with a wide range of symptoms. These can mimic other infections like dengue or typhoid, malaria and influenza. Hence, lab testing is required for diagnostic confirmation and initiation of early treatment which can be lifesaving, in some instances. Often, serological methods like IgM ELISA, rapid Immunochromatography, or molecular methods like PCR are used for laboratory diagnosis. The gold standard test, Microscopic Agglutination Test (MAT), is available only in reference laboratories.

Aim: To compare the diagnostic accuracy and utility of common serological tests in practice for diagnosing leptospirosis, such as Rapid IgM (Immunochromatography), IgM ELISA, real-time PCR, and Microscopic Agglutination Test (MAT).

Methodology: Blood samples were collected from 208 patients presenting with fever, accompanied by headache, myalgia, or conjunctival suffusion, between December 2022 and June 2024 at a medical college hospital. All samples were tested by Rapid Leptospira IgM, Anti-Leptospira IgM ELISA, serovar-specific MAT, and Leptospira Real-time PCR. The diagnostic accuracy of each method was studied by comparing it with MAT, the gold standard reference test.

Results: Among the 208 samples, 46 (22%) were IgM ELISA positive, 30 (14.4%) were MAT positive, 19 (9.33%) were real-time PCR positive, and 5 (2.4%) were positive by rapid IgM. The most prevalent serovar was *Leptospira grippotyphosa* with 50 % positivity. 208 samples tested, when compared against the reference gold standard MAT, IgM ELISA showed a sensitivity of 100% with a specificity of 92% whereas rapid IgM Immunochromatography demonstrated a low sensitivity of 17% with a higher specificity of 100%.

Conclusion: The Leptospira IgM ELISA method can be an effective screening tool for diagnosing leptospirosis, and samples positive by IgM ELISA should be confirmed with MAT or real-time PCR.

Keywords: Leptospirosis, Immunochromatographic Test, Microscopic Agglutination Test, Igm Elisa, Weil's Disease, Bacterial Zoonosis

Journal of Communicable Diseases (P-ISSN: 0019-5138 & E-ISSN: 2581-351X)
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Introduction

Leptospirosis is the most common zoonosis caused by the pathogenic strains belonging to the genus Leptospira. It is estimated that 1.03 million cases and 58,900 deaths occur every year, representing a major threat worldwide.[1] This disease remains endemic in several parts of India, including Tamil Nadu.[2] Transmission of the disease occurs to humans when they come in contact with infected animals directly or indirectly through the environment contaminated with the urine of the animals. While rodents are the most important primary reservoirs for leptospires, domestic animals like cattle, pigs, and dogs are considered potential sources of pathogenic leptospires.[3] The illness is often underdiagnosed or misdiagnosed because of the protean manifestations ranging from mild febrile anicteric illness similar to dengue, typhoid, malaria, etc., to severe icteric illness with haemorrhage and renal involvement, which is referred to as Weil's disease.[4,5] Hence, the diagnosis must be confirmed based on the microbiological laboratory tests. In developing countries, laboratory available may be insignificant for diagnosis despite a high prevalence of the disease.[6] Leptospirosis is under reported leading to increase in mortality.[7] The mortality rates range between 3% and 54% according to the affected organ system.[8] Diagnosis of leptospirosis in the microbiology laboratory is commonly carried out by various methods, which involve the detection of anti-leptospiral antibodies by Rapid Leptospira IgM/IgG (Immunochromatography), IgM ELISA, Microscopic Agglutination Test or by molecular methods like Real-time PCR. However, maintaining the isolates of Leptospira is more important for epidemiological investigations, studying strain diversity, serovar identification, and understanding transmission patterns of this zoonotic infection.[9] Leptospira isolation by culture is of limited use in diagnosing leptospirosis because of its low sensitivity; often, the isolation requires more than 2 weeks. However, the maintenance of a collection of Leptospira isolates remains crucial for epidemiological understanding of this complex zoonosis and to provide researchers with different isolates that can be novel and also the existing serovars.[10] The MAT is the serologically gold standard test for detecting human leptospirosis. However, the MAT is restricted to reference laboratories, requires maintenance of live Leptospira cultures, which are used as antigens for the test, and the performance of the test is complex and cumbersome.[11] There is also a need for the second sample (convalescent sera) to demonstrate seroconversion or rise in titre, making the test more challenging. MAT, when performed at the acute phase (earlier), may produce false-negative results as detection of IgM antibodies by this method appears after 7 days of fever.[12] However, the prevalent serovars in the study area can be identified.[2] In this context, IgM antibody detection by rapid Immunochromatography or by ELISA is commonly used in hospital-based laboratories. An Immunochromatography method gives results rapidly and is technically easy to perform. IgM ELISA is easier to perform, many laboratories have the facility to perform it, and it is more sensitive than the MAT test during the acute phase of the illness; however, it is not serogroup or serovar-specific.[13] This study was carried out to assess the diagnostic accuracy and utility of serological methods like rapid IgM and IgM Leptospira ELISA, and real-time PCR

Materials and methods

- **Study design and population**: This prospective cross-sectional study was conducted at a teaching medical college hospital in Chennai. All patients presenting with fever, with or without headache, myalgia, conjunctival suffusion, and calf muscle tenderness who gave consent during the study period from December 2022 to June 2024 were included in this study. Ethical clearance was obtained from the institutional ethical committee. (IEC Ref No: KIMS/F/2022/27) Demographic details, epidemiological history, occupational history, and detailed information on fever duration and clinical symptoms were collected.
- **Sample processing:** After obtaining the consent, 5ml blood sample was collected in a plain vacutainer tube and allowed to clot. The serum was separated after centrifugation at 3000rpm for 5minutes and used for the tests
- **Leptospira IgG/IgM Combo Rapid Test - Rapid Immunochromatography**: Rapid Immunochromatography was performed using the CTK onsite Leptospira IgM, IgG combo rapid kit, intended to detect Leptospira-specific IgM and IgG antibodies. It is a lateral flow immunochromatographic assay. The presence of a band in both the Control and Test regions was interpreted as positive. The presence of a band only in the Control region (C) and absence in the Test region (T) was interpreted as negative.
- **Leptospira ELISA IgM**: The RecombiLISA kit is a qualitative relies on a solid-phase enzyme-linked indirect immunosorbent assay. It helps detect IgM antibodies against Leptospira interrogans in human serum or plasma. The kit's micro wells come pre-coated with Leptospira interrogans antigen. The conjugate solution includes monoclonal anti-human IgM antibodies. These link to horseradish peroxidase. The test samples were processed, as per manufacturer's instructions including positive and negative controls in duplicates, in a semi-automated ELISA analyzer. The instructions in the kit insert, RecombiLISA Leptospira IgM ELISA, were followed.
- **Microscopic Agglutination Test**: A panel of live cultures of *L. autumnalis, L. canicola, L. grippotyphosa L. icterohaemorrhagiae, L. australis,* and non-pathogenic

ISSN: 0019-5138
DOI: https://doi.org/10.24321/0019.5138.202624

L. biflexa (patoc 1 strain) were used as the antigens. A 5-to-7-day-old culture grown in Ellinghausen McCullough Johnson Harris (EMJH) medium was used as an antigen. The antigens were checked for purity and density before performing the test. It was also ensured that the cultures were free from auto agglutination. The density of the cultures was checked with a Biosan densitometer. The antigens with approximately 1 × 10^8 to 2 × 10^8 organisms/mL were used for the test. (200-250 leptospires/40X). MAT was done at doubling dilutions starting from 1 in 10.100µl of the test sample. was diluted with 900µl of Phosphate buffer saline in 1in 10 dilution. From this 50µl was taken and serially diluted in 50µl saline, making 1in 20, 1in40,1in80, and so on. 50µl of the live antigen of each serovar was added into the microtiter wells and incubated in a dark environment for 2-3hours at 27°C. after incubation, one drop of serum-antigen mixture was transferred with an inoculation loop and observed for agglutination under a darkfield Microscope. A MAT reaction was measured positive when at least 50% of leptospires were agglutinated, with ≤50% free leptospires remaining compared with the antigen control wells. Samples showing positive agglutination were further serially titrated to determine the end-point titre. A titre of 1:80 or more against any of the tested serovars was interpreted as positive.

- **Real-time PCR**: (True NAT LTS Molbio diagnostics).[14]: True Nat chip-based real-time PCR, which targets the *lipl32* gene of *Leptospira interrogans,* was used for all the samples. The bacterial DNA was reported as Ct (Cycle threshold). The Ct value is inversely proportional to the load of bacterial DNA present in the sample. At the end of the PCR, positive samples were displayed as "detected". The run was considered valid if Internal control was detected (IC)

Statistical Analysis

The SPSS software version 24.0 was used for statistical analyses of this study. We regarded MAT as the gold standard. A titer of 1 in 80 was considered positive. Samples positive by MAT were classified as true positives. The Specificity, Sensitivity, Positive Predictive Value (PPV), and Negative Predictive Value (NPV) for Rapid IgM, ELISA IgM, and real-time PCR methods were calculated using MAT positives as the gold standard.

Results

Out of the 208 febrile cases that were evaluated, 49 (23.5%) tested positive for leptospirosis by at least one of the diagnostic methods used. Among the various tests, the IgM ELISA method identified 46(22.1%) cases followed by the MAT, which confirmed 30 (14.4%), real-time PCR, which identified 19 (9.1%) and rapid IgM immunochromatographic assay which identified 5 (2.4%) cases.

Gender wise distribution showed that out of 208 patients, 120 were males. Of those, 25(20.8%) were positive. Of the 88 female patients tested, 24 (27.3%) were positive. In our study, females showed a slightly higher positivity rate than males.

Among the different methods of detection, IgM ELISA detected 46(22%) cases. followed by serovar-specific MAT, which detected 30(14.4%) cases, Real-time PCR detected Leptospira antigen in 19 (9%) and rapid Immunochromatographic test detected 5 (2.4%) cases, (Table 1).

The diagnostic accuracy of different serological and molecular testing methods is described in Table 2. The sensitivity and specificity of IgM ELISA were found to be 100% and 91%. Real-time PCR showed a moderate sensitivity of 53.3%, but specificity was 98.4%. The rapid IgM Immunochromatographic test showed a very low sensitivity (16.6%) and greater specificity (100%). The Highest accuracy was detected in IgM ELISA, which was estimated as 92.3%, followed by Real-time PCR and Rapid IgM, which was 92.5% and 87.6%. Positive predictive values were significant for the rapid test (100%) and PCR (84.2%). Only ELISA showed 100% in negative predictive value. So, it emerges as the most reliable screening option among these.

Table 1.Results of Serology and Molecular Methods

Method	No of positives	Percentage (%)	No of Negatives	Percentage (%)
Leptospira IgM/IgG Rapid	5	2.4%	203	97%
Leptospira IgM ELISA	46	22%	162	78%
Serovar-specific MAT	30	14.4%	178	86%
Realtime PCR	19	9%	189	91%

Table 2.Diagnostic Accuracy of Serology and Molecular Tests

Indices	Rapid IgM (%)	ELISA IgM (%)	Real-time PCR (%)
Sensitivity	16.6	100	53.3
Specificity	100	91	98.4

ISSN: 0019-5138
DOI: https://doi.org/10.24321/0019.5138.202624

Positive predictive value	100	65.2	84.2
Negative predictive value	87.6	100	92.5
Accuracy	87.9	92.3	91.8

Serovar distribution highlights *Leptospira grippotyphosa* as the prevalent serotype. It appeared in 15 (50%) cases. Of which, 9 samples reacted at 1:80 dilutions, 4 at 1:160 and 2 at 1:320. This was followed by *L. icterohaemorrhagiae* in 5 (16.7%), 2 samples at 1:80 dilution and 3 at %1:160. L. australis was positive in 2 (6.7%) and both samples were positive at 1:160. L. autumnalis tested positive in 4 (13.3%), three at 1:160 and one at 1:640. L. patoc was positive in 4 (13.3%). of which, 2 samples each showed a dilution of 1:80 and 1:160. L. canicola was detected in none. The data suggest L. grippotyphosa was the most common, followed by L. icterohaemorrhagiae, L. autumnalis, L. patoc and L. australis serotype. (Table 3).

Table no: 4 shows that among 208 patients with varying durations of fever: less than 3 days (20.2%), 4–7 days (63.0%), 8–14 days (14.9%), and >15 days (1.9%) the diagnostic sensitivity varied significantly ($P < 0.05$). in the fever category of less than 3 days duration Rapid IgM/IgG , MAT, and PCR demonstrated 0% positivity while IgM ELISA provided the earliest reactivity in only one patient (2.4%). However, in fever category of 4-7 days duration, the diagnostic efficacy increased for all methods. IgM ELISA confirmed 32 cases (24.4%); MAT (15.3%); PCR (11.5%), and rapid assays (3.1%) Beyond the first week of fever, IgM ELISA sensitivity peaked significantly at 41.9% while the molecular (12.9%) and rapid IgM/IgG (3.2%) had shown a downward trend of positivity. However, the MAT titre remained consistent, showing positivity in 32.3% of cases. In four patients with a fever lasting 15 days or longer, all diagnostic modalities (Rapid tests, ELISA, MAT, and PCR) yielded negative results. The lower PCR positivity in patients with a history of prolonged fever may be explained by the natural decline in leptospiremia during the later stages of infection, which reduces detectable bacterial DNA in blood samples. In contrast, serological methods such as IgM ELISA and MAT rely on the host antibody response rather than on direct bacterial detection. IgM ELISA showed consistently higher positivity across different fever durations, likely due to sustained IgM antibody detection even after the acute bacteremic phase. The Rapid IgM/IgG test showed lower reactivity, particularly during the early stage of infection, suggesting lower sensitivity compared with ELISA.

Table 4 illustrates the link between fever duration and the positivity of each diagnostic test.

In the overall positivity prevalence, only one sample tested positive by all four methods. Out of 4 methods, a significant number of samples that showed positivity by 3 different methods. 15 samples tested positive by 3 methods ELISA, MAT and PCR, and 4 samples by Rapid, ELISA and MAT. 5 samples tested positive by both ELISA and MAT. 16 samples were positive only by ELISA alone, followed by PCR (3). The overall distribution of positive samples by one or more diagnostic methods is summarized in Table 5.

Table 3.Serovar distribution of Microscopic Agglutination test

Serovar (n=30)	Number of positives in each dilution				
	1 in 80	1 in 160	1 in 320	1 in 640	Total
L.grippotyphosa	9	4	2	0	15
L.icterohaemorrhagiae	2	3	0	0	5
L.australis	0	2	0	0	2
L.autumnalis	0	3	0	1	4
L.cannicola	0	0	0	0	0
L.patoc	2	2	0	0	4

Table 4.Fever duration and positivity of tests

Fever Duration days	Total n=208(%)	Rapid IgM/IgG		ELISA IgM		MAT		PCR	
		Pos (n=5)	Neg (n=203)	Pos (n=46)	Neg (n=162)	Pos (n=30)	Neg (n=178)	Pos (n=19)	Neg (n=189)
≤ 3	42 (20%)	0	42 (100%)	1 (2%)	41 (97.6%)	0	42 (100%)	0	42 (100%)
4-7	131 (63%)	4 (3%)	127 (96%)	32 (24%)	99 (75%)	20 (15%)	111 (85%)	15 (11%)	116 (88%)

ISSN: 0019-5138
DOI: https://doi.org/10.24321/0019.5138.202624

8-14	31 (15%)	1 (3%)	30 (97%)	13 (42%)	18 (58%)	10 (32%)	21 (68%)	4 (13%)	27 (87%)
>15	4 (1%)	0	4 (100%)	0	4 (100%)	0	4 (100%)	0	4 (100%)

Table 5.Prevalence of positivity by one test or more

Number of samples that were positive by all 4 methods	
No of samples positive by all methods Rapid, ELISA, MAT, and PCR	1
Number of samples that were positive by any 3 methods	
No of samples positive by ELISA, MAT and PCR	15
No of samples positive by Rapid, ELISA and MAT	4
Samples positive by 2 methods	
No of samples positive by ELISA and MAT	5
Samples positive by only 1 method	
No of samples positive by ELISA alone	16
No of samples positive by PCR alone	3

Discussion

Early diagnosis of leptospirosis is essential to initiate therapy, as severe leptospirosis or Weil's disease, is often fatal if untreated. Because of its clinical presentation often resembling other infections, there is a need to confirm leptospirosis by laboratory methods. Serological methods are often employed for diagnosing leptospirosis. This study shows differences in sensitivity among various diagnostic tests. ELISA IgM demonstrated a high sensitivity of 100%, followed by Real-Time PCR with 53%, while rapid IgM Immunochromatography showed a lower sensitivity of 16.6%. Regarding Leptospira IgM ELISA, the results are similar to those of Kucerova et al., who reported a sensitivity of 100% and a specificity of 88.6% in their study.[15] Other researchers have documented sensitivity ranges between 48% and 100%.[16,17] In this group of 208 patients, fever durations varied. Forty-two had fever for 3 days or less (20%). One hundred thirty-one patients had fever for 4 to 7 days (63%). Thirty-one (15%) had 8 to 14 days. Only 4 (1%) had fever lasting beyond 15 days. For those with short duration of fever, averaging 3 days, rapid IgM over IgG were negative in all the samples. IgM ELISA was positive in 1 case (2%). MAT and PCR tests were negative for all samples. In cases where fever range was of 4 to 7 days, the rapid test was positive in 4 cases (3%), ELISA 24%, MAT 15% and PCR 15 (11%). Detection improved here for ELISA, MAT, PCR. For the day between 8 to 14, the rapid test was positive in 1 case (3%), ELISA 13 (42%), MAT 10 (32%), PCR 4 (13%) percent. ELISA remained strongly sensitive, where PCR and rapid tests were less sensitive. Of the 4 patients with fever over 15 days, results were mixed. Rapid tests yielded no positives while other tests ranged from zero to 100 percent. But no extra cases from ELISA, MAT, and PCR. Declining bacterial load might explain it, Antibodies levels declined, too. Overall, IgM ELISA showed highest positivity rates among different fever duration, and showed highest detection rate among different tests. Whereas the lowest positivity rate was detected in Rapid IgM/IgG test, especially in earlier and fever with more than 15 days. Regarding PCR, the highest positivity rates were detected in earlier stage which declined in later stage.

Early detection of leptospirosis is crucial to prevent progression to severe forms like Weil's disease.[18] Concerning the duration of illness and leptospirosis detection, ELISA IgM identified most cases (32) During the early stage of illness (patients with fever for <7 days), MAT detected fewer positive cases, likely because detectable agglutinating antibodies against Leptospira serovars may not have reached sufficient titres during the acute phase. Seroconversion in leptospirosis generally occurs within 6–10 days after infection; therefore, MAT may show reduced sensitivity or false-negative results during the early phase of illness when antibody titres remain low. In contrast, IgM ELISA identified a higher number of early cases, suggesting better utility as a screening test during acute infection.[6,9,11]

Rapid IgM Immunochromatography in other studies shows a sensitivity ranging from 15-100%. In our research, the Rapid IgM had a sensitivity of 16.6% with a specificity of 100%. In a study by Alia et al., a specificity of 90.32% and sensitivity of 15.79% of were reported, almost similar to our findings.[20] The poor performance in sensitivity (16.6%) of the Immunochromatography method may be due to the specific kit, the timing of sample collection, or circulating serovars among the study population. Most samples were collected during the acute phase (4-7 days).

Real-time PCR showed a specificity of 98% and sensitivity of 53%; Mulla et al. (52%) observed a similar sensitivity.[21]

ISSN: 0019-5138
DOI: https://doi.org/10.24321/0019.5138.202624

Real-time PCR was positive in patients with fever during the acute bacteraemia phase (4-10 days). As diagnosing and treating leptospirosis early with antibiotics is important, this test may help clinicians identify the cause of the illness before the antibodies appear.

Our study showed a slightly higher prevalence in females (21% in males vs. 26% in females), while most studies showed the male preponderance.[15,16,17] In a study by Das et al in 2025, a similar finding was reported.[22] This difference in gender distribution in the study may be due to a change in epidemiological exposure patterns and occupational exposure that requires further investigation.

Leptospira grippotyphosa was the predominant serovar in our study (50%), followed by *Leptospira icterohaemorrhagiae*. Padmakumari et.al reported a similar finding in their research grippotyphosa, as this is one of the commonly reported serovars from South India, where agricultural activities and rodent reservoirs are common.[23] This information suggests the importance of epidemiological surveillance and vaccine development.

The fever duration and test positivity analysis showed that IgM ELISA and PCR had identified the maximum cases during the acute phase, 4-7 days of fever duration. Since diagnosis and treatment with antibiotics is critical during the acute phase, both ELISA IgM and PCR can serve as screening tests. In a facility where MAT testing is unavailable, the samples that tested positive by ELISA IgM can be treated for leptospirosis. In a facility where MAT testing is available, ELISA IgM reactive samples can be confirmed using MAT. However, due to the good negative predictive value of IgM ELISA (100%), the samples that tested negative can be considered negative for leptospirosis, and other tropical infectious diseases need to be considered. Regarding the real-time PCR True Nat, it can be used as a confirmatory test where MAT facilities are not available. Our study suggests the need for combined testing for the diagnosis of leptospirosis. Although the Immunochromatography-based Rapid IgM method is easy to perform and rapid, because of its poor sensitivity, we suggest that this method can be replaced with the ELISA IgM.

More patients tested positive with ELISA. Fewer with ICT than MAT or PCR. ICT offered higher specificity than ELISA.ELISA captured more leptospirosis cases but risks overdiagnosis. False positives happen often, as studies note.[24,25] In a facility where MAT or PCR is available, IgM ELISA-positive samples should be subjected to these tests for confirmation. In a facility where these are not available, ELISA IgM positives should be considered as a probable case of leptospirosis, and treatment with antibiotics needs to be initiated to prevent complications and mortality.

Positivity prevalence among different diagnostic methods showed that ELISA has the highest detection rate compared to MAT and PCR. The high clinical sensitivity of the IgM ELISA is probably due to the fact that universally cross-reacting, genus-specific antibodies against recombinant lip l32 antigen of pathogenic Leptospira interrogans appear earlier than the serovar-specific antibodies detected by MAT.[9] Besides, as the analytical sensitivity of the ELISA technology is higher than the agglutination principle used in the MAT, antibodies are detected earlier in IgM ELISA.[9] PCR positivity was higher in the very early phase of the illness (leptospiraemic phase) than the late immune phase. The decrease PCR positivity is probably due to migration of leptospira from the blood stream to the kidneys [20] However, PCR can improve the diagnostic accuracy when it is used along with serological tests. Compared to MAT and PCR, ELISA reported higher positivity in our study, possibly due to its ability to detect genus-specific IgM antibodies during the early and intermediate stages of infection. MAT may show lower positivity during the acute phase because detectable agglutinating antibody titres may not yet be sufficiently developed. In contrast, PCR detects Leptospira DNA and is most useful during the early leptospiremic phase; its sensitivity may decline in later stages as bacterial load in blood decreases. In addition, PCR sensitivity may be influenced by low DNA concentration or the presence of PCR inhibitors in blood samples, which can reduce amplification efficiency. Additionally, the higher ELISA positivity may partly reflect broader antibody detection and possible cross-reactivity, which can contribute to false-positive results.

Conclusion

In this study, IgM ELISA was shown to be the most sensitive and detected leptospirosis consistently in various stages of the febrile illness, proving it a trustworthy option for the early diagnosis. In other cases, real-time PCR is a highly valuable test for confirmation during the acute phase. However, the rapid IgM Immunochromatographic test showed less sensitivity and not suggested as a standard diagnostic method. The circulating serovar *Leptospira grippotyphosa* was the most common, suggesting the need for ongoing regional epidemiological screening and monitoring. This showed a main screening tool of IgM ELISA and MAT or PCR as confirmatory whenever possible. Early and precise detection is essential, as it can help prevent the disease from progressing to severe forms like Weil's disease and reduce the associated morbidity and mortality of leptospirosis.

Implication

The IgM ELISA can be used as a screening test to replace Rapid IgM Immunochromatography due to its significant sensitivity.

ISSN: 0019-5138
DOI: https://doi.org/10.24321/0019.5138.202624

Limitation

Our study has some limitations. It is a single-centre study with a small sample size. The absence of convalescent serum samples may have impacted the MAT sensitivity. The PCR results could be affected by when the samples were collected and any previous antimicrobial treatment.

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ISSN: 0019-5138
DOI: https://doi.org/10.24321/0019.5138.202624