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A STUDY OF LEPTOSPIRA DIAGNOSIS USING DIFFERENT LABORATORY TECHNIQUES AND THE IDENTIFICATION OF THE MOST COMMON SEROVARS IN DADRA AND NAGAR HAVELI

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ABSTRACT:
Leptospirosis is a neglected and re-emerging zoonoses caused by a Gram-negative bacterium belonging to the Spirochaetes phylum, Leptospiraceae family, Leptospira genus[1]. These microorganisms appear spiral-shaped, with a diameter of 0.1 µm and a length of 6–20 µm and a pointed end that is typically folded into a characteristic hook shape[1]. Leptospira is highly mobile and performs rotational movements around the central axis, translation, undulation, and flexion thanks to two periplasmic axial flagella located under the cell membrane[2]. Although Leptospira is a microaerophile, it develops well even in conditions of complete aerobiosis. The optimum temperature for its growth is between 28 °C and 30 °C, although it also grows at 37 °C. The ideal pH range is between 7.2 and 7.4[3]. Traditional classification divided the genus Leptospira in two species: L. interrogans, pathogenic strains, and L. biflexa, saprophytic strains. On the basis of antigenic agglutination reactions and cross absorption, both species are divided into serovars. There are over 60 serovars belonging to the L. biflexa species, while there are more than 200 Leptospira interrogans[3]. The invasion of Leptospira into the body occurs through skin lesions (even of minimal entity), via the mucous membrane (conjunctiva and oral mucosa), and by contact with wet skin or by inhalation[3]. Infiltrated microorganisms invade the bloodstream, causing bacteremia that persists for about 5–7 days[4]. Once a critical number of bacteria has been reached in the blood, the first symptoms related to their trans-endothelial migration appear. The pathogenetic mechanism of Leptospira is not yet fully understood, and it is hypothesized that virulence factors, such as toxins, adhesins, and other surface proteins, are expressed[5].

KEYWORDS: Leptospira, PCR, ELISA, and MAT

INTRODUCTION

Leptospirosis is worldwide distribution and occurs in man, cattle, buffaloes, pig, sheep, goat, dog, horse, etc. Due to its zoonotic nature, it is communicable from man to animals and vice versa. The earliest recorded outbreak of leptospirosis in India was reported among construction workers in the village of South Andaman in 1929 and was named as Andaman hemorrhagic fever[6]. It is still of common occurrences in South and North Andaman. In Dadra and Nagar Haveli 154 cases of Leptospirosis were diagnosed during five years study (2011-2015)[7]

Despite being common, the diagnosis of leptospirosis is often not made unless a patient presents with textbook manifestations of the so-called Weil's disease, such as fever plus jaundice, renal failure and pulmonary hemorrhage. Leptospiral infection often has minimal or no clinical manifestations; In both children and adults, leptospirosis commonly presents with fever, myalgia, and headache. Lethargy, emesis, abdominal pain, photophobia, arthralgia, cough, diarrhea, or constipation also may occur. Onset of symptoms often occurs abruptly after 2 to 20 days incubation period. Direct tissue injuries from leptospirae invasion and toxins, which have been theorized yet never clearly elucidated, characterize the acute phase. Symptoms then abate with cessation of the systemic proliferation of leptospires. The second or immune phase is characterized by increasing antibody titers and inflammatory infiltration of affected organ systems. Aseptic meningitis and renal dysfunction are hallmarks of the immune phase. Symptoms may persist for 6 days to more than 4 weeks, with a mean duration of 14 days. Approximately 10% of patients diagnosed with leptospirosis develop signs of Weil′s disease. The classic definition of Weil′s disease is severe leptospirosis presenting with jaundice, renal failure, and pulmonary hemorrhage. Mortality rates among these patients are 10%, despite care in an intensive care unit (ICU), and even higher in regions with less sophisticated care. Severe, fatal cases of leptospirosis may occur without associated jaundice. Of the cases in which fever develops, as many as 90% are undifferentiated febrile illnesses. Because of the variety of clinical symptoms seen in the symptomatic cases, leptospirosis at its onset is often misdiagnosed as aseptic meningitis, influenza, hepatic disease or fever (pyrexia) of unknown origin (Turner, 1967). In developing countries, laboratory facilities may be inadequate for diagnosis despite a high prevalence of the disease. Of substantial clinical

importance, the syndrome of leptospiral pulmonary haemorrhage has emerged in recent years, in diverse places around the world[8].
The differential diagnosis of leptospirosis is difficult due to the varied and often "flu like" symptoms which may result in a missed or delayed diagnosis[9]. For example, patients presenting with severe fever and haemorrhage have symptoms which are difficult to distinguish from viral haemorrhagic fever[10].
The timely laboratory diagnosis of leptospirosis is a formidable challenge. Various methods are available for the diagnosis of leptospirosis which include point-of-care tests, serological tests (IgM ELISA) and microscopic agglutination test (MAT). Molecular diagnostics is a sensitive method for leptospira detection in acute care settings. Introduction of polymerase chain reaction (PCR) has greatly helped in the diagnosis of leptospirosis during the early days of infection. During early illness, it is the test of choice in terms of sensitivity and specificity which can aid in rapid diagnosis.
After the first week, detection of specific IgM antibodies by serology is a reliable test for diagnosis. The negative predictive values of IgM and PCR are very low in the first and second week respectively. Therefore, if PCR is used in conjunction with IgM, it helps in rapid and accurate diagnosis which is crucial for initiating proper and timely management[11]. The objective of this study was to determine the difference in diagnostic rates in suspected cases of leptospirosis—presenting during the first week of symptom onset—when using IgM alone versus using IgM in conjunction with PCR. Furthermore, all these samples were confirmed using MAT.
The MAT relies on the use of live cultures as the source of antigen, often performed using a panel of antigens representative of local serovars. A specific anti body response detectable by the MAT generally occurs at around 8–10 days after onset of the illness[8].
The MAT is generally performed by reference laboratories due to the inherent safety risks of handling cultures of live leptospiral organisms, the high cost of commercial media, and the need for ongoing maintenance of representative serovars or serogroups.

MATERIAL AND METHODS

Material

In this study, 727 Serum samples were collected from patients presenting with acute febrile illness from both the Out-Patient and In-Patient departments from NAMO Medical Education and Research Institute and NAMO Hospital conducted cross-sectional research. The research ran for a full year, from April 1, 2025, to March 31, 2026. Sample were obtained from all probable leptospira cases, regardless of age or gender. Leptospira IgM ELISA and PCR (Truenat) tests were performed on these samples. Subsequently, all samples that yielded positive results via either ELISA or PCR were sent to ICAR-NIVEDI, Bengaluru, for the Microscopic Agglutination Test (MAT); throughout this process, the 'cold chain' was strictly maintained, and all other relevant protocols were also fully adhered to.

Methods

All suspected cases were tested for IgM Enzyme-Linked Immunosorbent Assay (ELISA) and Microscopic Agglutination Test (MAT) along with Polymerase chain reaction (PCR) for Leptospira. All IgM ELISA and PCR tests were carried out at Namo Hospital, Silvassa, Nagar Haveli.

1. **Detection of antibody by Pan Bio Leptospira IgM ELISA:** - PanBio Leptospira IgM ELISA reagents were purchased from the manufacturer (Pan-Bio, Queensland, Australia). Sera to be examined were diluted 1:100 in serum diluent provided by the manufacturer, and 100 µL of the resultant dilutions were put to antigen-coated microwells. Every test run includes a calibrator serum ran in triplicate along with positive and negative controls. Plates were incubated at 37°C for 30 min before washing with an automatic plate washer. An anti-human IgM conjugate is then added followed by the substrate. The overall incubation time for this experiment was 1 hour, 10 minutes. Absorbance was read at 450 nm/600 nm. A Pan Bio unit was determined for each sample by dividing the absorbance of the sample by the mean absorbance of the calibrator sample duplicates and multiplying by 10. A score of < 9 units showed a negative result, 9–11 units an inconclusive result, and > 11 units a positive result, showing the existence of leptospira-specific IgM antibodies[12].

2. **Truenat™ micro RT-PCR:** This is a chip-based micro RT-PCR system. Proprietary matrix enclosed cartridge designed to extract and purify nucleic acid, amplification and result prediction. The principle of Truenat is the same as that of conventional RT-PCR. The equipment is miniaturized and procedure, reading the results and interpretation are automated. The cartridge also contains an internal positive control for the whole process of the test namely nucleic acid extraction, amplification and result prediction. Positive control and negative control are coded in the Microchip, CT value of positive control, and sample (CT value of positive control ranges between 25 and 35).
Processing of clinical samples: For DNA extraction 500 µl of serum/plasma was added to sample pre-treatment tube which contained lysis buffer and incubated at room temperature for two minutes and the content was transferred to the sample chamber of the cartridge. The run for 20 min was performed and the elute from the cartridge was collected and stored. Six microlitre of eluted DNA sample was added to the master mix tube and the elute was allowed to dissolve for 30 sec and then was placed in the microchip. The elute was added to the well and the reaction was run for 40 min[13].

3. **For MAT detection** of anti-Leptospira antibody, all IgM ELISA and PCR positive findings were forwarded to ICAR-NIVEDI, Bengaluru, using twenty known live reference strains (serovars) of leptospira encompassing 18 serogroups while following all required safety procedures[14].

RESULTS:

Leptospirosis cannot be diagnosed based on clinical grounds alone due to the variability in clinical manifestations, similarity of signs and symptoms with those of other bacterial, viral and parasitic infections and frequent occurrence of the disease in atypical forms. Confirmation of the diagnosis requires laboratory support. A small number of organisms can cause infection. The incubation period usually ranges from 7-10 days. Leptospirosis may follow a biphasic course. During the first 10 days, there is a phase of leptospiraemia when the leptospires multiply in blood and spread to different organs. The chances of recovery of leptospires from blood or other tissues or body fluids is usually high during this stage. This phase is followed by immune phase or leptospirurea phase when the organisms are excreted in the urine.

The antibodies usually develop within 2-12 days after the onset of illness. IgM antibody starts appearing early in the course of the disease and reaches detectable levels within one week or as early as on third or fourth day of illness. They reach peak levels during third or fourth week and then decline slowly over months and become undetectable within six months. Rarely IgM may persist at low level for several years.

In about 10% of patients microscopic agglutinating antibodies appear at detectable levels only after about a month of illness. Hence the sera collected from these patients during the first month of illness may give negative result in Microscopic Agglutination Test (MAT). Serovar-specific antibodies are long-lasting than serogroup specific antibodies.

Laboratory diagnosis is broadly divided into two categories- those that give direct evidences and those that give indirect evidences. The direct evidences include either demonstration of leptospires or its DNA and isolation of organism from clinical specimens. Detection of specific antibodies to leptospires (serological diagnosis) is indirect evidence[15].

Different stages of leptospirosis: -

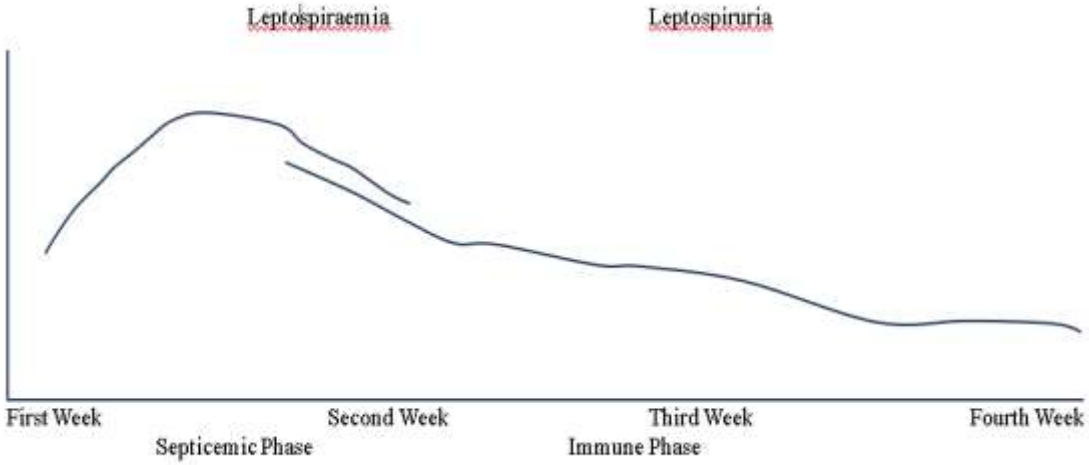
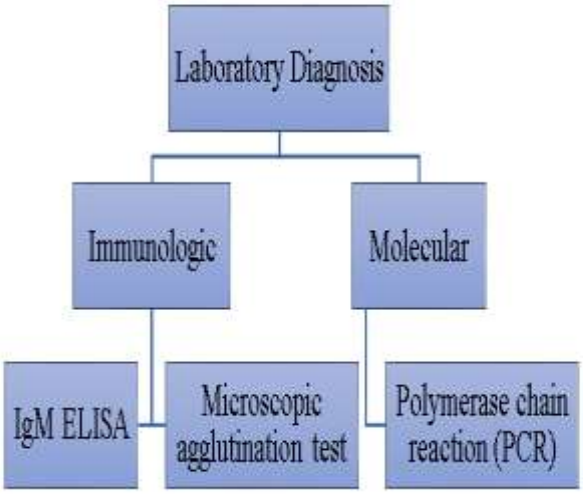
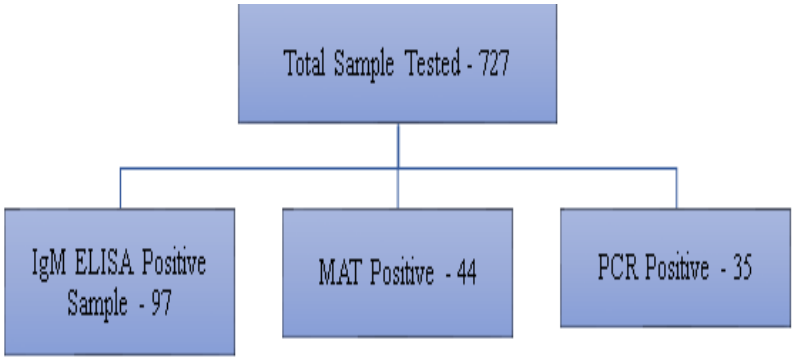


Fig. – 1 Laboratory Diagnosis flow-chart

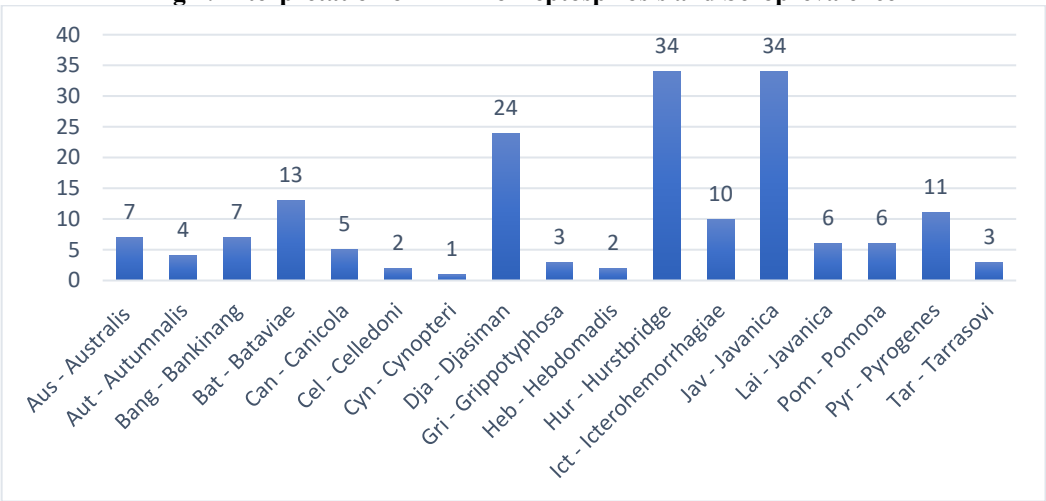


A flowchart of the protocol developed in our laboratory is depicted in Figure 1; this same flowchart has also been utilized in the *Leptospirosis Laboratory Manual* published by the Regional Medical Research Centre (Indian Council of Medical Research) located in Port Blair[15].



In this study, out of 727 samples, 97 were found positive for ELISA. PCR tests were also performed on all ELISA-positive samples, of which 35 samples were found to be positive. 65 IgM ELISA positive samples sent to ICAR-NIVEDI, Bengaluru, Karnataka for screening of MAT detection of anti – leptospira antibody using twenty known live reference strains (serovars) of Leptospira, 44 were found to be MAT-Positive.

Fig 1: Interpretation of MAT for leptospirosis and Seroprevalence



For MAT testing, we submitted 65 samples, each exceeding a volume of 1 ml, all IgM ELISA positive with a Pan Bio unit more than 11, to ICAR-NIVEDI in Bengaluru. The results indicated that several samples tested positive for MAT, revealing the following serovars: Aus, Aut, Bang, and Bat, among others, which were identified in 44 samples. The least prevalent serovar was Cyn (Cynopteri), detected in only one sample, while the most prevalent were Hur and Jav, each found in 34 samples. This data highlights the diversity of serovars present in the tested samples and points out the need for ongoing surveillance in the region.

Table 1: Reactivity titer of 44 Positive serum samples with different reference leptospiral serovars by MAT

Reactivity Titer	Serogroup	No. of Serum Samples
1:50	Bat, Hur	02
1:100	Aus, Bang, Bat, Cel, Cyn, Dja, Gri, Heb, Hur, Jav, Lai, Pyr, Tar	54
1:200	Aus, Aut, Bang, Bat, Can, Dja, Gri, Heb, Hum, Hur, Ict, Jav, Lai, Pom, Pyr, Tar	91
1:400	Dja, Hum, Hur, Ict, Jav	25
Total		172

Among the 172 serum samples screened, 2 samples had a titer of 1:50, while 54 samples demonstrated a titer of 1:100. Out of the total, the highest number tested positive, amounting to 91 samples; the titer was 1:200, and 25 samples showed a titer of 1:400. The identified serovars included Daj, Hum, Hur, Ict, and Jav. Further analysis indicated that the prevalence of these serovars varied significantly, suggesting possible geographical or environmental factors that may influence infection rates. This information is essential for developing targeted intervention strategies to effectively manage and mitigate outbreaks.

Fig 2: Distributions of cases based on illness duration.

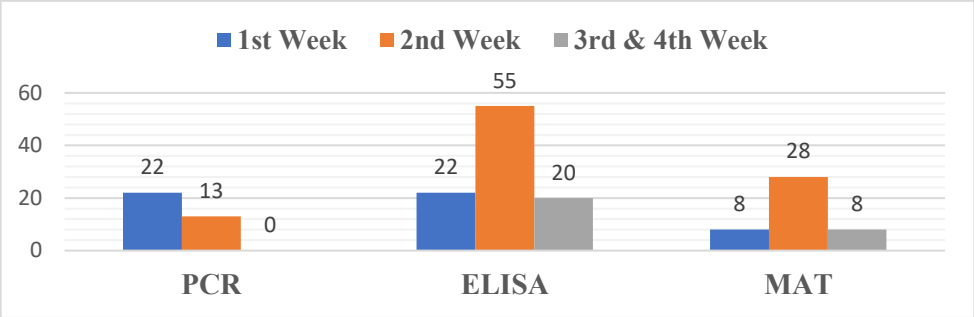


Fig 2 showed, based on the tests we conducted, we found that during the first week of the illness, our molecular tests showed a higher positivity rate compared to other testing methods. Specifically, of the 35 molecular tests performed, 22 yielded positive results in the first week. In contrast, only 13 tests were positive in the second week. After the second week, no further positive results were obtained from our molecular tests.
The positivity rate of the IgM ELISA is higher in the second week compared to the first, third, and fourth weeks. The maximum number of positive samples were detected during the second week of illness.
The MAT test also reveals that the highest positivity is observed in the second week, which helps to confirm our results.

Fig 3: "Distribution of deaths based on duration: Number of death cases."

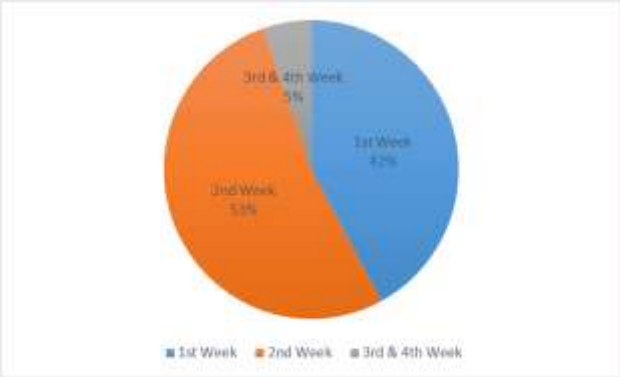


Fig 3 showed, The IgM ELISA test and either molecular testing or MAT were positive for the 19 deceased persons. Additionally, the second week accounted for 53% of the fatalities, the first week for 42%, and the third and fourth weeks for 5%.

DISCUSSION

Leptospirosis is widely recognized as acute febrile disease and being emergent or re-emergent in tropical and sub-tropical regions. Leptospirosis is frequently under-diagnosed, also challenging as culture of Leptospira and seroconversion require weeks. Isolation of Leptospira from the clinical specimen is difficult because leptospires are fastidious, slow growing that requires special growth media and it is time consuming and laborious. Therefore, PCR assay is highly useful as a contemporary method for diagnosis in acute phase of leptospirosis. Moreover, PCR positivity peaked from 4th day to 8th day after onset of symptoms; even cases were detected upto 15 days without any significant difference in sensitivity of assay[16].In leptospirosis, IgM antibodies begin to appear only by the end of the first week and therefore the sensitivity of these tests in early stages of illness is very low. Therefore, in the first week of illness, a significant number of cases will be missed with IgM ELISA whereas a majority can be picked up with PCR. Hence, testing protocols using both PCR and IgM ELISA will improve diagnostic yield significantly[17].
Although MAT is considered to be the serological reference test for the diagnosis of leptospirosis, it has many inherent issues in performance. The inability to standardize the test conditions may lead to interpersonal variations in the interpretation of results. The necessity to maintain live cultures for the MAT panel is a laborious procedure due to the fastidious nature of the species, and imposes a risk of laboratory acquired infections. Apart from these technical problems, the sensitivity of MAT in the acute stage has been questioned. Patients might not elicit detect able immune responses in the acute stage and sensitivity can be significantly low in such cases. Therefore, for a proper interpretation, paired sera should be used. How ever, relatively higher specificity of MAT has been reported in both acute and paired sera for the diagnosis[18].
The findings of this study have several important implications. In the present study, 727 serum samples were collected from individuals in endemic coastal regions who were either admitted, or suspected of having leptospirosis. Initially, IgM ELISA for leptospira was performed on each serum sample. Out of the total samples, 97 (13.34%) tested positive for IgM using ELISA. P.B. Praveen Kumar et al. conducted a similar study in 2024, which showed a positivity rate of 17%[19]. All IgM ELISA-positive samples were subsequently tested using PCR, resulting in 35 samples being positive out of the 97 IgM ELISA-positive serum samples. This indicates a significant correlation between the two testing methods. The findings

suggest that while IgM ELISA can be a useful preliminary screening tool, PCR remains crucial for confirming active infections. Sreevalsan TV et al. conducted the same investigation in a central Keralan tertiary care facility, and the PCR method's positive was 76.6%[20].

Out of 97 serum samples that tested positive for IgM ELISA, 65 were sent to ICAR-NIVEDI for MAT testing. Among these, 44 samples were positive for 17 different serovars. According to Sujit Kumar Behera et al., MAT is the gold standard test for leptospira detection[21]. **Figure 1** illustrates that the majority of the identified serovars were Jav-Javanica and Hur-Hurstbridge, detected in 34 IgM ELISA-positive samples. Dja-Djasiman was present in 24 samples, whereas Cyn-Cynopteri was identified in only one. This distribution underscores the prevalence of specific serovars within the tested population. Further analysis of these findings may offer insights into the disease's epidemiology and aid in developing targeted control measures.

Table 1 shows, the majority of the serovar titers were 1:100 and 1:200. Only 2 samples had a titer of 1:50, while 25 samples had a titer of 1:400 among the 44 MAT samples. This indicates a significant prevalence of certain serovars within the tested population. The results suggest that further investigation may be necessary to understand the implications of these findings for disease management and prevention strategies. Future studies could focus on identifying specific factors that contribute to the high prevalence of these serovars. Additionally, exploring potential correlations between serovar distribution and environmental or demographic variables may be beneficial.

Fig 2 shows, 22 tested positive via PCR. All samples were collected during the first week of infection, while 13 were positive in the second week. No positivity was observed in the third and fourth weeks of infection in PCR testing. These findings suggest that PCR useful early indicator of infection, but its reliability decreases as the infection progresses. Further studies are needed to explore the correlation between ELISA and PCR results over time. Out of 97 IgM ELISA-positive samples, 22 were positive in the first week of infection, 55 samples were positive in the second week of infection, and 20 were positive in the third and fourth weeks of infection. The majority of IgM ELISA samples tested positive in the second week of infection, which is higher compared to the first and third weeks. Out of 44 MAT-positive samples, the highest number of positive samples was observed in the second week of infection, with an equal number found in the first and third to fourth weeks of infection. This pattern indicates a peak in the immune response during the second week, suggesting it may be a critical period for intervention.

Fig. 3 shows that 19 (19.58%) died out of 97 IgM ELISA-positive individuals, along with anyone with positive PCR and MAT results. In five-year research by Thottathan Athira et al., the death rate from leptospira varied from 9.2% to 15.3%. The research was conducted in a tertiary care facility in North Kerala between 2018 and 2022[22]. 42% of deaths happened in the first week of infection and 53% of deaths were in the second week of infection of leptospira. These findings highlight the critical importance of early diagnosis and intervention in managing leptospirosis. Timely treatment could potentially reduce mortality rates significantly, particularly during the initial stages of the disease.

Limitation: - As the present study was restricted to a single healthcare setup, the rate of leptospira could not be compared with other institute of same geographical areas. As the identification of leptospira was solely based on immunologic & Molecular methods, we would have missed few leptospira cases.

CONCLUSIONS: - Leptospirosis is the most prevalent, underestimated, and re-emerging zoonotic infection. PCR is very sensitive and specific test in the acute phase of illness. Manifestations of leptospirosis and early laboratory diagnosis will help to reduce morbidity and mortality associated with disease in humans. The identity of infection serovars has significant epidemiological and public health measures. This study involved PCR, ELISA and MAT which helped to save lives.

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