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# Comparative Analysis of ELISA, MAT, and RT-PCR for Early Diagnosis of Human Leptospirosis

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**ABSTRACT:** A neglected zoonotic illness that affects people all around the world is human leptospirosis. Contact with soil or water tainted by sick animals' urine is the primary way it spreads. Early identification is challenging since the symptoms frequently mimic those of other feverish diseases including dengue, malaria, or influenza. This study evaluated the effectiveness of the Enzyme-Linked Immunosorbent Assay (ELISA), Microscopic Agglutination Test (MAT), and Real-Time Polymerase Chain Reaction (RT-PCR) in a structured diagnostic process for suspected human leptospirosis. Blood samples were taken under sterile conditions and analyzed for both serological and molecular details. ELISA was used to detect IgM and IgG antibodies. MAT served as the serological standard, while RT-PCR targeted the LipL32 gene for direct detection of the pathogen. Clinical, demographic, and laboratory data were compiled into a standard database. After that, the data undergo preprocessing to ensure consistency. The study assessed diagnostic performance using Receiver Operating Characteristic (ROC) analysis. The Area under Curve (AUC) values were 0.942 for MAT, 0.904 for ELISA, and 0.981 for RT-PCR. Among these, RT-PCR showed the best diagnostic accuracy, especially within the first 7 days of illness, proving its advantage for early detection. These results highlight the supportive role of RT-PCR alongside serological tests, which aids in the quick, sensitive, and reliable diagnosis of leptospirosis.

## 1. INTRODUCTION

Leptospirosis is a bacterial disease caused by harmful species of the genus Leptospira. It is found all over the world, especially in tropical and subtropical areas, affecting both humans and animals [1]. The disease lives in the kidneys of reservoir hosts, mainly rodents, and spreads through contact with contaminated water, soil, and food. Human infection can happen, with an estimated annual incidence of over one million cases [2]. Outbreaks are often linked to flooding and poor sanitation, making leptospirosis a significant public health issue. Figure 1 show the leptospirosis transmission cycle [3].

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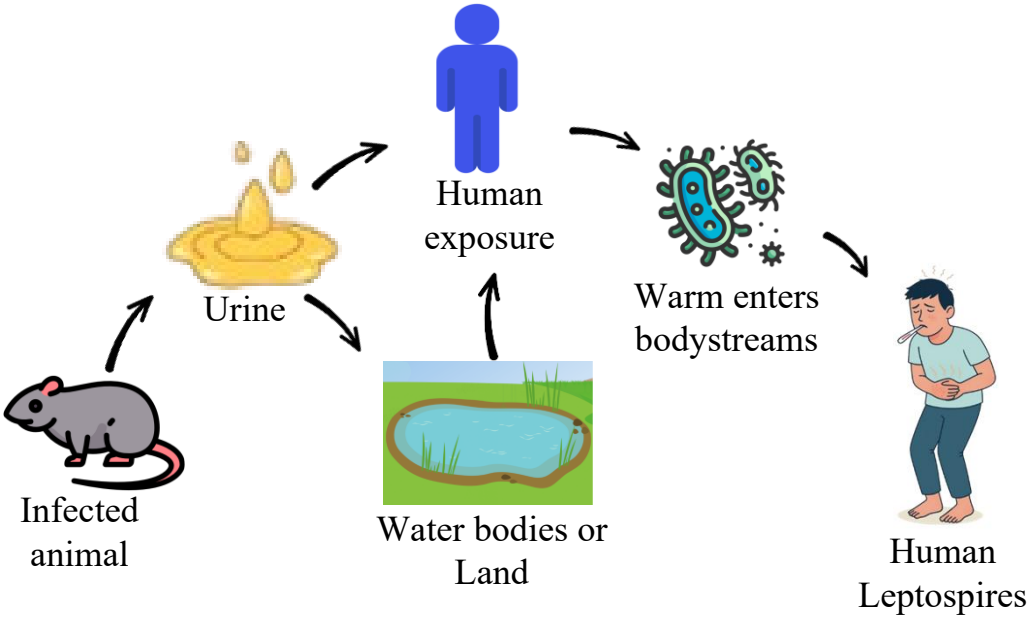


**Figure 1:** Leptospirosis Transmission cycle

It is caused by infection with spirochetes of the genus Leptospira. Spirochetes are bacteria carried by several mammals, ranging from rodents and cattle to some wildlife species that colonize the renal tubules in mammals and are ultimately excreted in urine, causing contamination of the surrounding environment [4]. In humans, symptoms can vary and range from mild and nonspecific issues like fever, headache, and muscle pain to severe disease that involves multiple organ failure [5]. Severe cases may include liver and kidney failure, bleeding in the lungs, and even death. Because early symptoms are nonspecific, it is hard to tell leptospirosis apart from other illnesses with fever, such as dengue, malaria, influenza, Hantavirus, Zika, and COVID-19 [6]. Both maintenance hosts and accidental hosts play crucial roles in the epidemiology of the disease. Figure 2 shows the leptospirosis symptoms.


**Figure 2:** Leptospirosis symptoms

Early diagnosis of leptospirosis is crucial to begin the treatment on time and lower illness and death rates. The symptoms often overlap with other infectious diseases, which leaves many patients undiagnosed and untreated [7]. This situation leads to high death rates. Detecting leptospirosis in the early stage is tough, since standard blood tests only find antibodies 5 to 7 days after symptoms begin. Blood culture and microscopy are difficult to perform, slow, and need skilled staff [8]. Fast and accurate testing methods are especially necessary for limited resources, where the disease burden is highest. Early medical help can greatly improve patient outcomes [9]. The samples collected in real time for leptospirosis testing are shown in Figure 3.

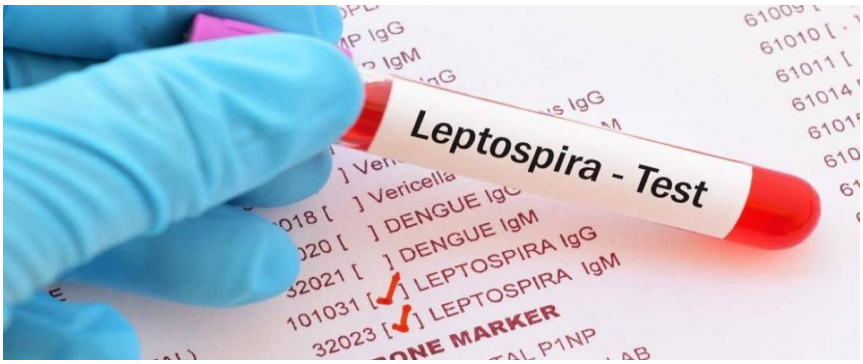


**Figure 3:** Leptospirosis test samples

It can be diagnosed using direct and indirect methods. Direct detection includes culture and microscopy [10]. Indirect methods rely on finding antibodies through serological tests. PCR offers high sensitivity and specificity during the acute phase, but its use is limited because it requires specialized equipment and skilled personnel [11]. The models remain the reference standard, especially for epidemiological studies, although they need paired samples and struggle with early detection. New diagnostic approaches are improving rapid and accurate detection [12]. These include Loop-Mediated Isothermal Amplification (LAMP) for field-friendly detection, Lateral Flow Assays (LFA) using recombinant leptospira proteins, and ultrasensitive Electrochemical DNA Sensors (EDS) that work with smartphones for Point-Of-Care (POC) testing. Label-free enzyme-based colorimetric sensors (LECS) and miRNA-based biomarkers are also being studied as new tools for early detection [13]. Furthermore, molecular typing techniques such as Variable-Number Tandem Repeat (VNTR), Matrix-Assisted Laser Desorption Ionization Time of Flight (MALDI-TOF), Multiple-Locus VNTR Analysis (MLVA), Multi-Locus Sequence Typing (MLST) and PCR-based serovar identification provide useful information for epidemiological surveillance and understanding disease severity [14]. Together, these advances aim to address the shortcomings of traditional diagnostics by enabling early, sensitive, and accessible detection of leptospirosis in both humans and animals [15].

Leptospirosis diagnosis is tough due to its unclear early symptoms. These symptoms look a lot like those of other febrile illnesses, such as dengue, malaria, or influenza. Traditional serological tests can detect antibodies only 5 to 7 days after symptoms start, which delays early diagnosis. Direct methods, like culture and microscopy, are slow and labor-intensive; they also need skilled staff. While molecular techniques like PCR have higher sensitivity, they require specialized equipment, which is not always available in places with fewer resources. New rapid tests are being developed for field use, but their sensitivity and specificity can differ. These challenges make it difficult to get timely treatment, resulting in more cases of illness and death. Finding effective early detection methods is still a crucial need for controlling the disease.

Currently, ELISA, MAT, and Real-time PCR tests have similar performance levels for diagnosing leptospirosis, but their effectiveness during the first week of infection is not clear. This study aims to compare these three diagnostic methods to see which one is best for early detection within seven days of symptom onset. By evaluating their sensitivity, specificity, and practicality, the research seeks to pinpoint the most reliable tool for timely diagnosis. The findings could enhance patient outcomes, inform public health strategies, and improve diagnostic procedures in both clinical and field settings. The major contributions of the research include:

- The primary objective of this research is to conduct a comparative analysis of three diagnostic assays ELISA, MAT, and RT-PCR for leptospirosis detection. This helps identify which method is most reliable for diagnosis within the first seven days of symptom onset.

- Patients were recruited from regions with known leptospirosis outbreaks using clear inclusion and exclusion criteria. Blood samples were collected following standardized aseptic protocols, tailored to each diagnostic test's requirements.
- A comprehensive database was created to link clinical, demographic and laboratory data for each patient. OD ratios, MAT titers, and RT-PCR Ct values were systematically recorded.

The research is organized as follows. Section 1 introduces leptospirosis, its challenges, and the motivation for the study. Section 2 presents the proposed model and the comparative analysis. Section 3 covers the experimental results and discussion. Section 4 concludes the study.

### 1.2 Problem Statement

The diagnosis of leptospirosis presents major challenges, especially in the early stage. Clinical symptoms are nonspecific and often overlap with other febrile illnesses. Current diagnostic methods have several drawbacks that make prompt and accurate detection difficult. Antigen-based apt sensors show promise but need validation in larger groups and require special equipment, which limits their use. Serologic tests, like those using recombinant proteins, can have cross-reactivity issues and can only detect antibodies, not active infections. PCR techniques have high sensitivity but require laboratory facilities, making them expensive for field use. Modified ELISAs, designed for local serovars, improve detection in specific areas but do not apply widely. Ultra-low-volume PCR methods seem promising, but their effectiveness with low-bacterial load samples is still uncertain. Strain isolation methods are limited to specific serogroups, which reduces their relevance for general diagnostics. Epidemiological studies provide valuable data on prevalence but do not help with clinical diagnosis, creating a gap in early and accessible detection tools.

## 2 PROPOSED METHODOLOGY

For the early diagnosis of human leptospirosis, a cohort of 50 patients with characteristic symptoms was recruited, and detailed demographic, clinical, and exposure information was recorded. Blood samples were collected and processed according to standardized protocols to ensure sample integrity and suitability for testing. These specimens underwent comparative analysis using three diagnostic methods: ELISA for IgM/IgG antibodies, MAT as the serological gold standard, and RT-PCR targeting the LipL32 gene for direct pathogen detection. Each test followed specific stepwise procedures to ensure accuracy and reproducibility. The resulting clinical and laboratory data were systematically organized into a comprehensive database linking patient information with test outcomes. To prepare the data for analysis, preprocessing steps including data cleaning, missing value imputation, and Min–Max normalization were applied to ensure consistency, completeness, and comparability across variables. This structured approach provides a robust foundation for evaluating the diagnostic performance of serological and molecular assays. The integrated workflow supports reliable early detection and aids in guiding clinical decision-making for leptospirosis. The overall study frame work has been presented in the Figure 4.

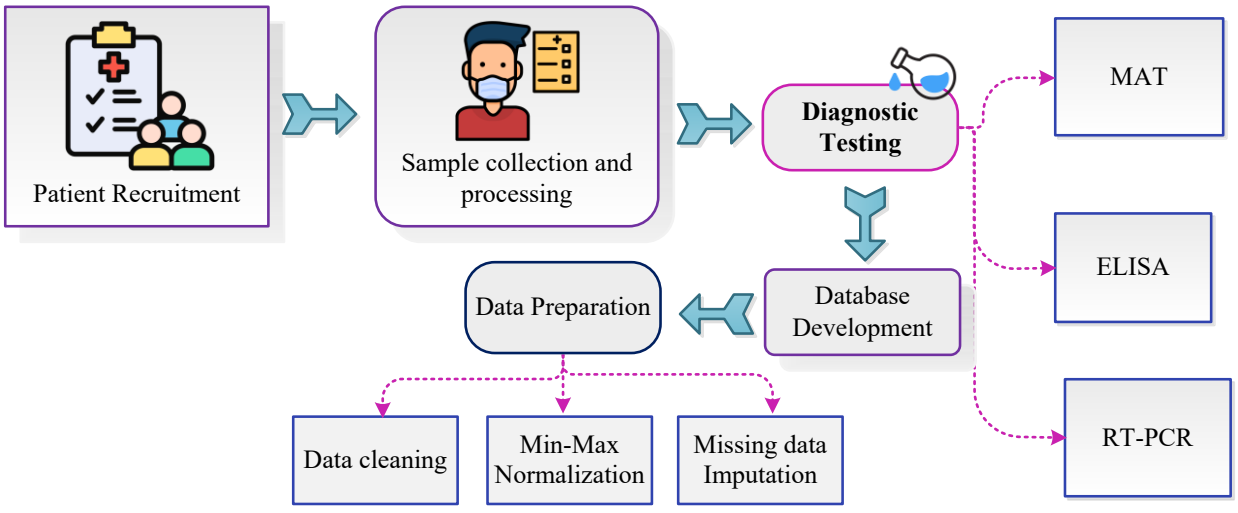


**Figure 4:** Overall Framework

## 2.1 Patient Recruitment

The sample for this study consisted of 50 patients between 15 and 50 years of age, that presented clinical features consistent with leptospirosis (fever, myalgia, headache, jaundice, conjunctiva suffusion). Patients were selected from regions with relevant leptospirosis history. Each patient had at least one of the characteristic symptoms of leptospirosis. Patients with known infectious diseases, chronic diseases, or other immune-compromised conditions were excluded from the study. Patient demographic information (age, sex, occupation, medical history) was recorded, as well as when symptoms began, duration of illness, exposure history (rain, stagnant water, contact with animal contaminated environments). This makes up a structured sampling of patients to source a population for the purpose of evaluating serological and molecular diagnostic tests.

## 2.2 Sample gathering and Processing

For Diagnosis the blood samples were gathered from the individuals enrolled under standardized aseptic protocols to ensure patient safety and sample integrity. An ideal blood sample size for a general laboratory test is between 2–5 mL; for leptospirosis testing, typically 5 mL is collected for ELISA and MAT, although this may vary, while RT-PCR requires much smaller volumes, sometimes as little as 1 μL of serum. For MAT, serum is separated from the collected blood, whereas ELISA can utilize either serum or serum. The amount of blood and type of container were chosen according to the specific diagnostic test requested, with PCR samples collected in EDTA tubes and MAT samples in clotted, gold-top tubes. Paired samples for MAT were taken 7–14 days apart when necessary to confirm infection. Samples are labeled with unique identifiers and stored at appropriate temperatures, and transported promptly to the laboratory. Standard protocols were strictly followed, which includes patient identification, site antisepsis, correct collection technique, and use of personal protective equipment. Factors such as stage of illness and prior antibiotic treatment were considered to ensure diagnostic accuracy. This approach ensured that the collected specimens were suitable for high-quality serological and molecular evaluation of leptospirosis.

## 2.3 Diagnostic Testing

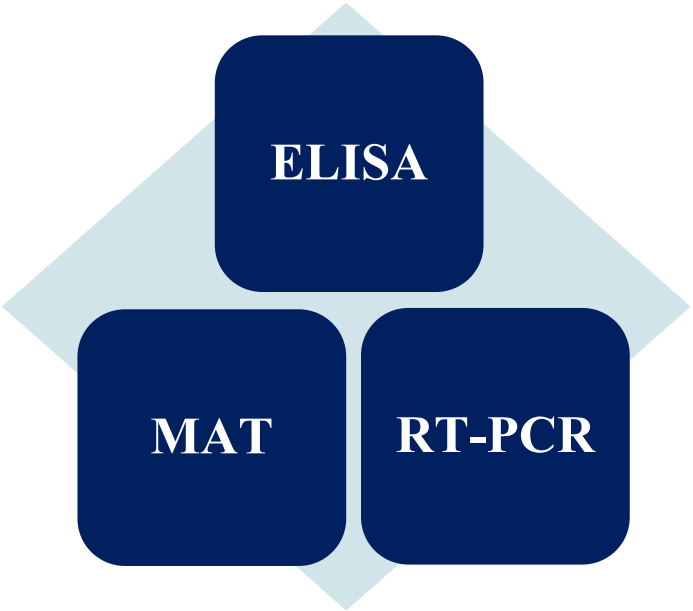


**Figure 5:** Diagnostic Testing Models

Early diagnosis of Leptospira infection was conducted using three complementary diagnostic tests like ELISA, MAT, and RT_PSCR as shows in Figure 5. ELISA for detecting IgM and IgG antibodies, MAT recognized internationally as the serological gold standard, and RT-PCR targeting the LipL32 gene for molecular detection. While ELISA and MAT assess the body humeral immune response, RT-PCR allows direct detection of the pathogen, making it particularly useful during the early acute phase of infection. Summary of Diagnostic Tests for Leptospirosis has been presented in table 2.

**Table 2:** Summary of Diagnostic Tests for Leptospirosis

| Diagnostic Test | Sample Type | Input | Output |
|---|---|---|---|
| ELISA | Serum | 5 mL blood | OD ratio = Sample OD/Cutoff OD;<br><0.9 = Negative<br>0.9–1.1 = Equivocal<br>>1.1 = Positive |
| MAT | Serum or plasma | 0.5–5 mL | MAT titer;<br>≥1:100 =positive |
| RT-PCR | Serum | 1 mL typical;<br>100 µL possible | Ct value;<br>Ct <35 = positive<br>lower Ct = higher bacterial load |

**i.ELISA:** It was performed to detect IgG and IgM antibodies against Leptospira antigens in patient serum. Approximately 5 mL of blood was collected, serum separated, and incubated with micro plates coated with Leptospira-specific antigens. A colorimetric substrate reaction produced a signal proportional to antibody levels, allowing semi-quantitative measurement. Commercially available kits were used, and procedures strictly followed the manufacturer's instructions. The input for the test was patient serum, and the output included Optical Density (OD) values interpreted as positive, negative, or equivocal. ELISA is quick and accessible but may have reduced sensitivity during early infection stages. Repeated testing can confirm seroconversion. Positive results indicate humeral response, while negative results in early illness may require follow-up testing. Controls were included in every assay to validate performance. ELISA test process for Leptospira antibody detection has been presented in Figure 6.

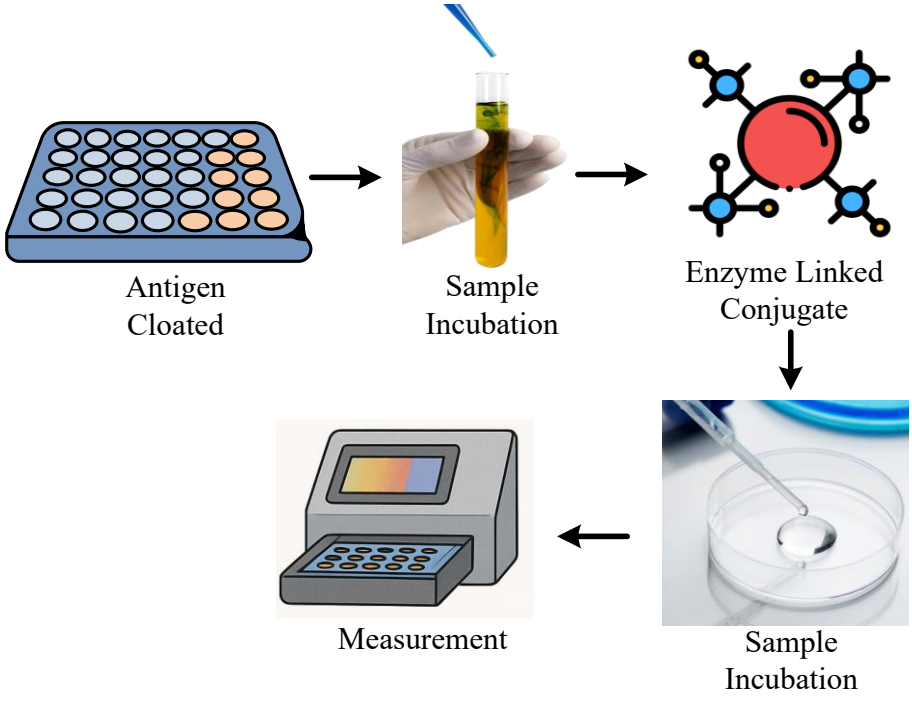


**Figure 6:** ELISA test process for Leptospira antibody detection

**Extraction Procedure:**

1) **Sample Collection:** Collect 5 mL blood; centrifuge to obtain serum.
2) **Plate Coating:** Add 100 µL Leptospira antigen (1–10 µg/mL) per well; incubate 30 min at 37°C or overnight at 4°C.
3) **First Wash:** Wash wells 3 times with PBS + 0.05% Tween 20.
4) **Blocking:** Add 200 µL blocking buffer (5% BSA/milk), incubate 1–2 h at room temperature.
5) **Serum Addition:** Add 100 µL diluted patient serum; incubate 2 h at room temperature.
6) **Second Wash:** Wash wells 3times with wash buffer.
7) **Secondary Antibody:** Add 100 µL enzyme-conjugated; incubate 1 h at 37°C.
8) **Third Wash:** Wash wells 3 times with wash buffer.
9) **Substrate Addition:** Add 100 µL chromogenic substrate; incubate 10to 30 min in dark.
10) **Stop and Read:** Add 50 µL and 2 molar sulfuric acid and read OD at 450 nm and interpret results versus controls.

**Stepwise Process of ELISA for Leptospira Diagnosis**

***Antigen Coating:*** Micro plate wells are pre-coated with Leptospira-specific antigens. These immobilized antigens act as capture molecules, ensuring that any antibodies specific to Leptospira in the patient's serum will bind selectively. This step establishes the foundation for specific antigen–antibody interactions.

***Sample Incubation:*** Patient serum is added to the coated wells and allowed to incubate. If antibodies (IgM/IgG) against Leptospira are present, they will specifically attach to the antigens on the plate. Unbound antibodies or other serum proteins are washed away, leaving only the target antibody–antigen complexes.

***Enzyme-Linked Conjugate:*** An enzyme-labeled secondary antibody (conjugate) is introduced. This conjugate binds to the patient's antibodies already attached to the antigens, forming a stable antigen–antibody–enzyme complex. The enzyme acts as a detectable marker that enables visualization in the next step.

***Substrate Addition:*** A chromogenic substrate is added to the wells. The enzyme linked to the conjugate reacts with the substrate, producing a measurable color change. The degree of color development is proportional to the amount of anti-Leptospira antibodies present in the sample.

***Measurement:*** The intensity of the developed color is measured using an ELISA reader at a specific wavelength. The OD values obtained are compared to control wells and interpreted as positive, negative, or equivocal, reflecting the antibody concentration in the patient's serum.

**ii. MAT:** It detects antibodies against multiple Leptospira serovar and was used as the reference gold-standard test. Approximately 0.5–5 mL of serum or plasma was required for the assay. Patient serum was serially diluted and incubated with live Leptospira organisms representing different serovar. Agglutination was observed under a microscope, with visible clumping indicating antibody presence. The test input was patient serum, and the output was the highest dilution showing ≥50% agglutination, reported as the MAT titer. MAT requires specialized laboratories, expertise in maintaining live cultures, and longer processing time. Paired samples were collected 7–14 days apart for confirmation when necessary. The test can identify infecting serovar, aiding epidemiological studies. Results were interpreted according to standardized cutoffs, with controls included in every run. Figure 7 shows the MAT test process.

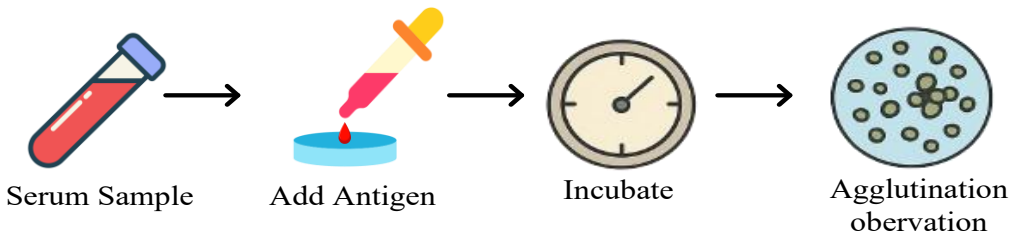


**Figure 7:** MAT test process

**Extraction Procedure:**

1) **Sample Collection:** Collect 0.5–5 mL patient serum or plasma.
2) **Serum Dilution:** Perform serial dilutions.
3) **Antigen Prep:** Prepare live Leptospira cultures of multiple serovars.
4) **Mixing:** Combine equal volumes of diluted serum and live Leptospira.
5) **Incubation:** Incubate mixtures at 30°C for 1–2 hours.
6) **Observation:** Examine under a dark-field microscope.
7) **Agglutination Check:** Look for visible clumping of Leptospira.
8) **Titer Determination:** Identify the highest dilution showing ≥50% agglutination.
9) **Controls:** Include positive and negative serum controls for each serovar.
10) **Reporting:** Report MAT titer; paired samples may be collected after 7–14 days to confirm seroconversion.

iii. **RT-PCR:** It was performed to detect Leptospira DNA directly in serum samples. Serum was collected in EDTA tubes. Nucleic acids were extracted, and PCR amplification targeted the conserved LipL32 gene, a reliable molecular marker. Fluorescence monitoring during each cycle allowed real-time detection of bacterial DNA. The input was extracted DNA, and the output was the Cycle threshold (Ct) value, with lower Ct indicating higher bacterial load. Samples were ideally collected early in infection, within the first 7 days, for maximum sensitivity. Controls included positive Leptospira DNA and negative no-template controls. RT-PCR provides rapid, sensitive detection, complementing serological assays. Thermal cycling was performed under specific conditions, including enzyme activation, denaturation, and combined annealing and extension steps has been presented in the Table 3. The reaction components and their volumes used for each 25 µL PCR reaction are detailed in Table 4.Results support early diagnosis, especially when antibodies are not yet detectable. Proper storage and handling were critical to maintain nucleic acid integrity. A step-by-step procedure for nucleic acid extraction and subsequent amplification under RT-PCR is described below, detailing the specific steps and reagents used to ensure accurate and reproducible results.

**Table 3:** Thermal Cycling Conditions

| Step | Temperature (°C) | Time (minutes) | Number of Cycles |
|---|---|---|---|
| Enzyme Activation | 95 | 15 | 1 |
| Denaturation | 95 | 20 | 50 |
| Annealing & Extension | 56 | 60 | 50 |

**Extraction Procedure:**

1) **Preparation:** Take a 1.5 mL micro centrifuge tube. Add 180 µL of buffer ATL (lysis buffer) and 20 µL of proteinase K to a mix of tissue for serum.
2) **Lysis and Incubation:** Vortex and incubate the mixture at 56°C. After this, add 200 µL of sample.
3) **Mixing:** Mix the contents thoroughly by vortexing for 15 seconds.
4) **Ethanol Addition:** Add 200 µL of ethanol. Vortex for 15 seconds.
5) **Centrifugation:** Briefly centrifuge the tube to remove drops from the lid.
6) **Spin Column Transfer:** Transfer the entire volume to a spin column. Centrifuge at 8000 rpm for 1 minute. Discard the flow-through.
7) **Washing:** Add 500 µL of wash buffer (AW1) and centrifuge at 8000 rpm for 1 minute.
8) **Washing (AW2):** Add 500 µL of buffer AW2. Centrifuge at 14000 rpm for 3 minutes. Discard the flow-through.
9) **Drying:** Empty the spin column and spin for 2 minutes at 8000 rpm.
10) **Elution:** Add 200 µL of elution buffer to a 1.5 mL centrifuge tube. Centrifuge at 8000 rpm for 1 minute to elute the DNA.

**Table 4:** Reaction Components and Volumes

| Component | Volume per Reaction (25 µL total) |
|---|---|
| qPCR Master Mix | 12.5 |
| Forward Primer (LipL32) | 1.0 |
| Reverse Primer (LipL32) | 1.0 |
| MGB Probe | 0.5 |
| Nuclease-free Water | 5.0 |
| Extracted DNA Template | 5.0 |
| Total Reaction Volume | 25.0 µL |
| Component | Volume per Reaction |

***Primer:*** In this study, specific primers targeting the LipL32 gene of Leptospira spp. were used for molecular detection. The LipL32 gene encodes a conserved outer membrane lipoprotein found exclusively in pathogenic Leptospira, making it a reliable molecular marker for diagnosis.

The forward primer (F) and reverse primer (R) were designed to amplify a gene fragment specific to Leptospira DNA.

- ***Forward primer (F):*** 5'–AAG CAT TAC CGC TTG TGG TG–3'
- ***Reverse primer (R):*** 5'–GAA CTC CCA TTT CAG CGA TT–3'

The primers were synthesized and validated to ensure high specificity and amplification efficiency. The optimal annealing temperature was maintained at 58°C to maximize binding precision. These primers target a 242 bp region of the LipL32 gene, ensuring accurate detection of pathogenic Leptospira in clinical samples.

## 2.4 Database Development

A database was created to organize and manage the clinical and laboratory findings from the enrolled patients. The dataset included demographic information, symptom details, and exposure histories along with laboratory results from the three diagnostic methods. Each patient entry received a unique identifier to maintain confidentiality and to help connect multiple test results. Clinical details such as fever, muscle pain, jaundice, and eye swelling were recorded, along with laboratory values like blood counts, liver function tests, and serological or molecular outcomes. For ELISA, OD ratios were entered with classifications as positive, negative, or equivocal; for MAT, titers were recorded with thresholds indicating positivity; and for RT-PCR, threshold values were saved to measure bacterial load. The database structure allowed for comparisons of results among the three methods, making it possible to evaluate diagnostic sensitivity at different stages of infection. Data were standardized to reduce entry errors and keep consistency across variables. Quality control checks, including double entry verification and random audits, were carried out to ensure correctness. This organized dataset serves as a basis for comparative analysis, providing solid evidence to evaluate the diagnostic performance of serological and molecular tests for leptospirosis.

## 2.5 Data Preparation

Following the initial database development process, the dataset underwent a form of data preprocessing so that it could be prepared for valid analysis purposes. Preprocessing can be viewed as part of the broader process of data preparation which provides a systematic, organized, and analyzable structure to processed raw data. Preprocessing was viewed to be necessary given issues of inconsistency, scale deviations, and entries with missing data would have clearly introduced bias into the results.

**Data cleaning:** It is a process of identifying removing inconsistencies in the data to improve data quality and reliability. It was chosen over existing, because of its ability to retain maximum data while ensuring accuracy. It also enhances the dataset integrity by removing duplicate records, correcting typos, and standardizing formats. Its process involves validating entries against predefined rules, handling outliers, and ensuring uniform coding of categorical variables. This step ensures the dataset is consistent, accurate, and ready for advanced analysis.

**Min-Max Normalization:** It is the transformation of continuous parameters to a common scale for uniformity. It was chosen over raw data because variables measured in different units would otherwise dominate analysis unfairly. Its advantage is that it makes models more stable and comparable by ensuring no single feature skews results. This method scales continuous data to a common range, typically between 0 and 1, ensuring that variables with larger units do not dominate the analysis. It improves model stability and comparability by preventing any single feature from skewing results. The choice of Min–Max scaling or other standardization depends on whether a bounded range or zero-centered data with unit variance is needed.

**Missing data Imputation:** It is a process of replaces missing entries with estimated values rather than discarding incomplete records. It was chosen over deletion because removal could shrink the dataset and introduce bias, especially in a cohort of only 50 patients. Its advantage lies in retaining valuable patient information while preserving dataset size and analytical validity. The process involves using statistical or model-based approaches, such as mean substitution, regression estimates, or nearest-neighbor methods, depending on the variable type and distribution. This ensures that the dataset remains as complete as possible without compromising reliability.

## 3 RESULTS AND DISCUSSION

The human leptospirosis testing analysis was carried out through a comparative study of laboratory methods, computational resources, and diagnostic performance. Laboratory hardware such as centrifuges, biosafety cabinets, freezers, PCR systems, ELISA readers, microscopes, and pipettes enabled safe and efficient sample handling. Computational resources included high-performance CPUs, sufficient RAM, SSD storage, optional GPUs, and backup drives to support smooth data processing. Software tools such as Microsoft Excel, and Python were used for preprocessing, statistical analysis, and visualization. Raw data, including ELISA results, MAT titers, RT-PCR outcomes, and clinical information, were cleaned and validated against MAT as the initial reference, followed by a composite reference for stage-specific evaluation. The analysis highlights the comparative performance of diagnostic tests and confirms the reliability of the processed dataset. Table 5 shows the Configuration Summary.

**Table 5: Configuration Summary**

| Category | Tools | Purpose |
|---|---|---|
| Laboratory | Centrifuge, Biosafety Cabinet, Freezers, PCR system, ELISA reader, Microscope, Pipettes | Sample handling, storage, and molecular testing |
| Computational | CPU ≥3.0 GHz, RAM ≥16 GB, SSD ≥1 TB, Backup HDD | Data processing, redundancy |
| Software | Excel, Python (pandas, numpy, sklearn, matplotlib), R (ggplot2) | Data entry, preprocessing, analysis, visualization |

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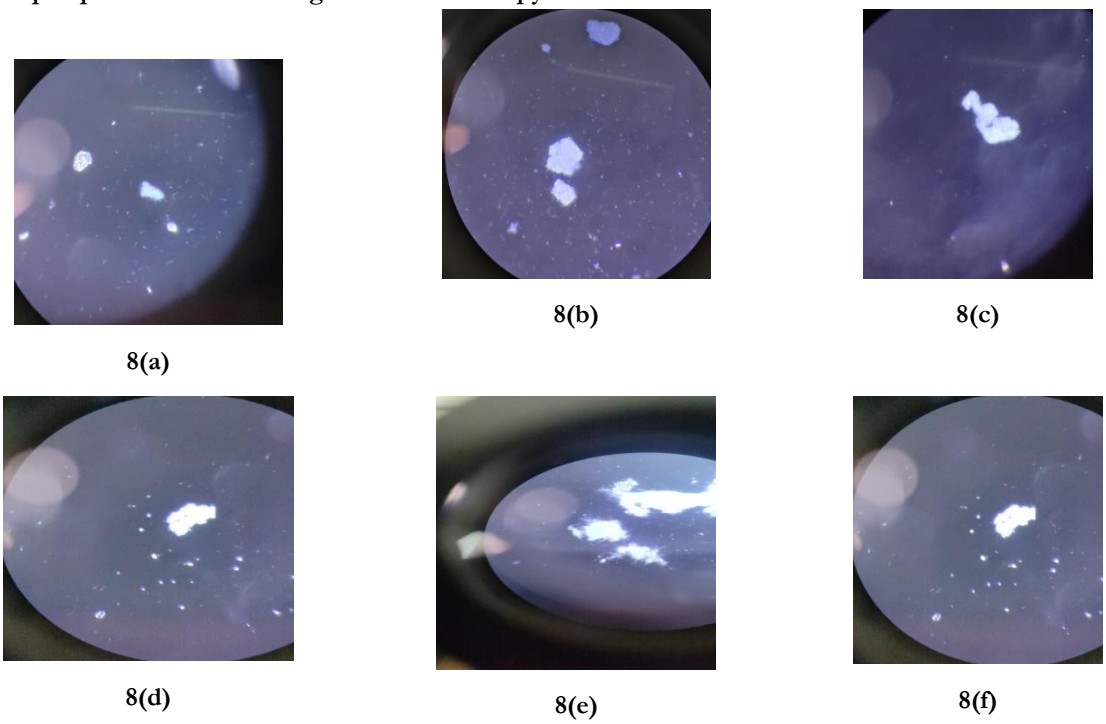
### 3.1 Leptospira Detection through MAT Microscopy



8(a) 8(b) 8(c) 8(d) 8(e) 8(f)

**Figure 8:** Microscopic visualization of Leptospira agglutination

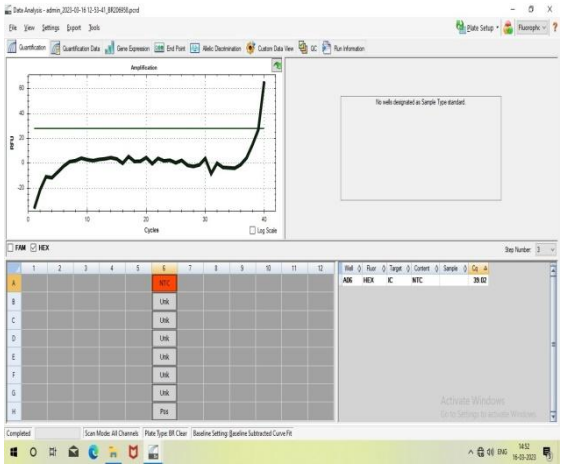
The figures 8(a)-8(f) shows microscopic visualization of Leptospira agglutination observed during the Microscopic Agglutination Test (MAT), where clumping of spirochetes indicates positive antigen and antibody reactions under dark-field microscopy

### 3.2 ELISA Test Kit Used for Leptospira IgM Detection

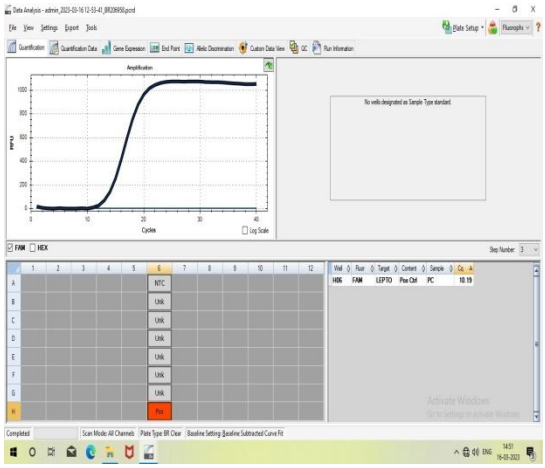

**Figure 9:** ELISA kit

Figure 9 shows the Lepto IgM Microlisa microwell ELISA test kit (TMB, India) used for the detection of Leptospira IgM antibodies in human serum and plasma samples, enabling serological screening and confirmation of leptospirosis cases.

### 3.3 PCR Amplification Results for Leptospira Detection



(a)

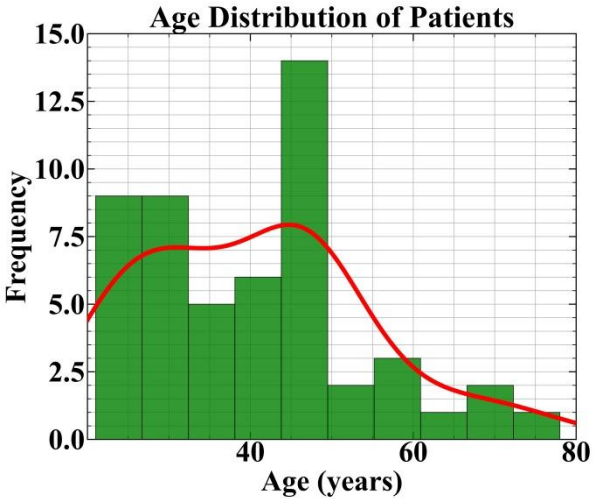


(b)

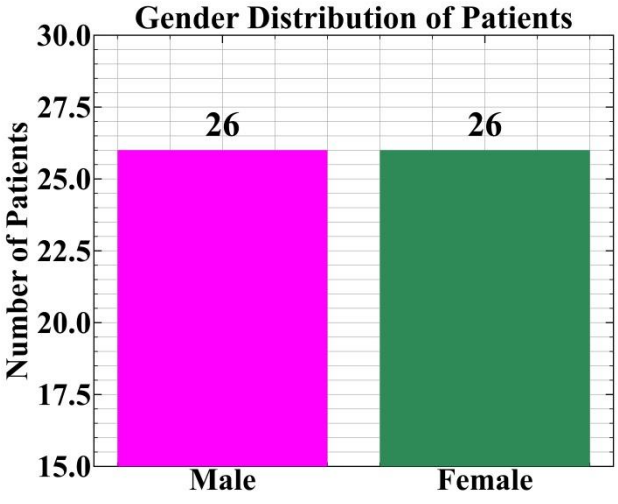
**Figure 10:** Leptospira NTC and PC

Figure 10 illustrates the polymerase chain reaction (PCR) results for Leptospira detection. The Negative Template Control (NTC) shows no visible amplification band, confirming the absence of contamination and ensuring the specificity of the reaction. In contrast, the Positive Control (PC) exhibits a distinct amplification band corresponding to the LipL32 gene of Leptospira spp., validating the proper functioning and sensitivity of the PCR assay. Together, these controls confirm the reliability and accuracy of the molecular detection protocol.

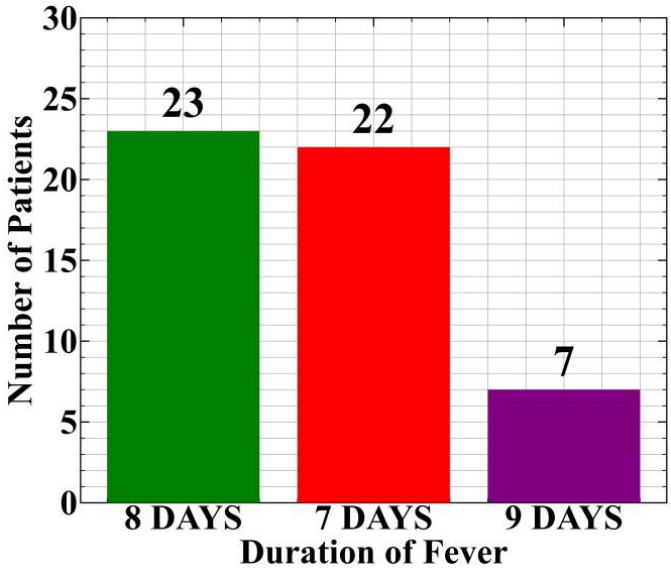
### 3.4 Descriptive Analysis of Patients




(a)




(b)




(c)

**Figure 11:** Distribution of Patients by (a) Age (b) Gender and (c) Duration of Fever

Figure 11 shows the dataset analysis of the 52 patients using three different perspectives. In (a), it shows the age distribution of patients ranging from 20 to 80 years, with the highest frequency around 45 years. The frequency gradually declines after 50 years, with very few patients above 70, as indicated by the red trend curve peaking near the mid-40s. In (b), it shows the gender distribution, with 26 males and 26 females. In (c), it shows the distribution of patients based on the number of days they experienced fever like 23 patients had 8 days of fever, 7 patients had 9 days, and 22 patients had 22 days.

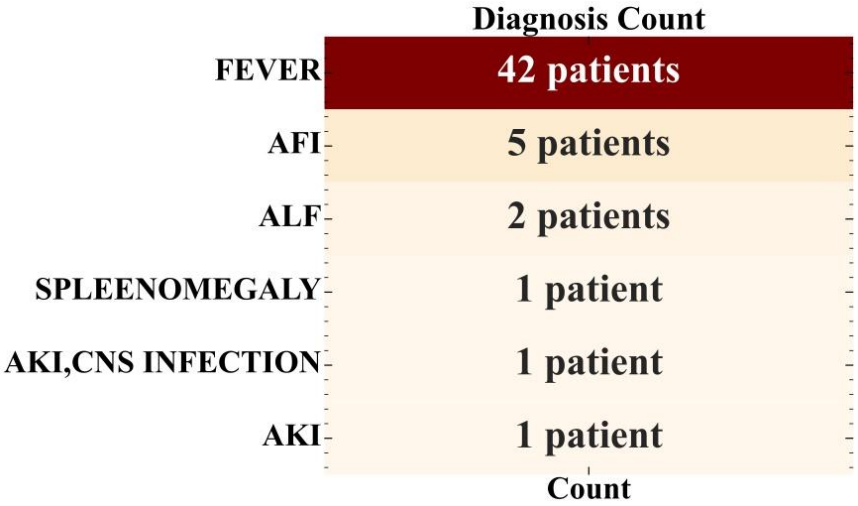



**Figure 12:** Distribution of Diagnoses

Figure 12 illustrates the distribution of 6 different diagnoses among 52 patients with leptospirosis [16]. The majority of 42 patients presented with fever, 5 patients with Acute Febrile Illness (AFI), 2 with Acute Liver Failure (ALF), and 1 with splenomegaly. One patient had three diagnoses like AKI, CNS involvement, and infection, while another had only Acute Kidney Injury (AKI).

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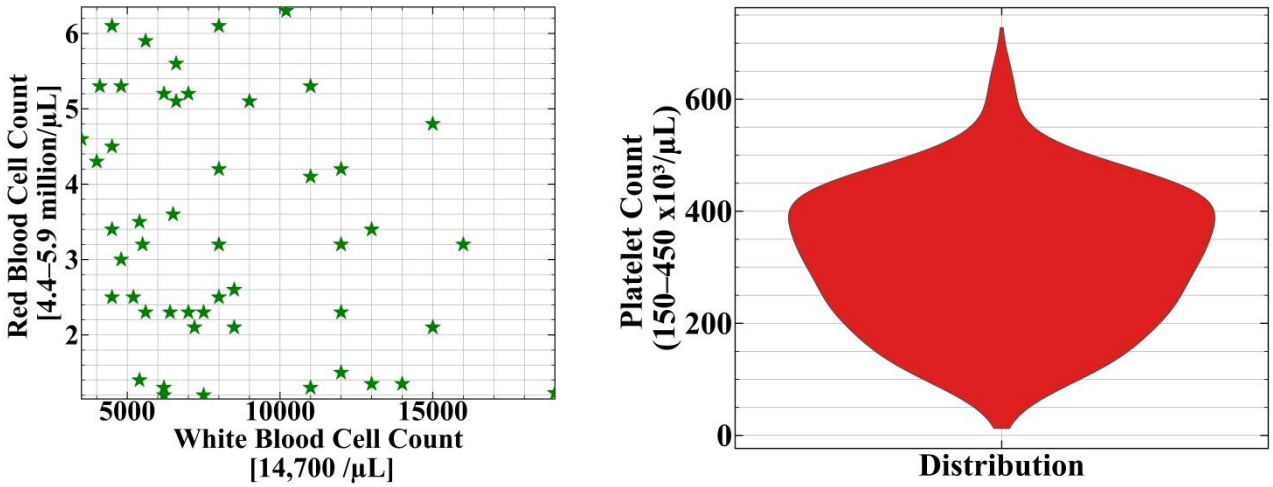
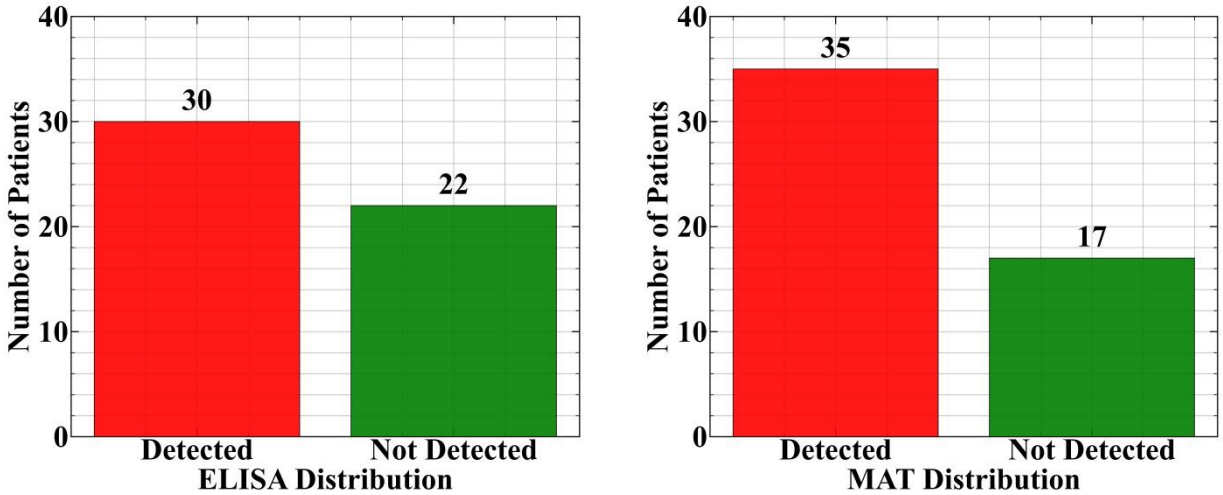



(a) (b)

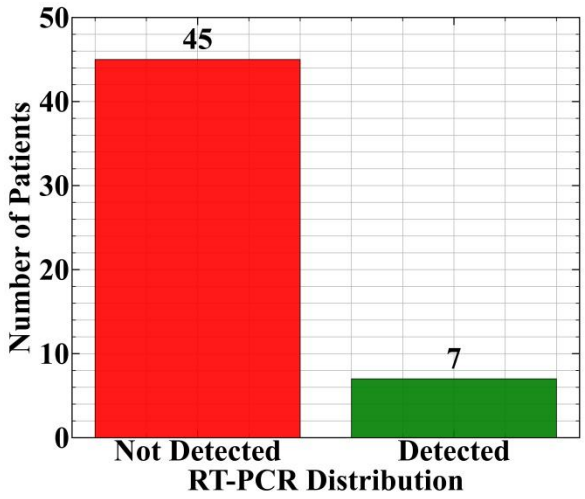
**Figure 13:** Analysis of Hematological Parameters in Patients (a) Relationship between RBC and WBC counts of patients (b) Distribution of Platelet count among patients

Figure 13 illustrates the analysis of hematological parameters, focusing on WBC, RBC, and platelet counts among patients. In subfigure (a), the scatter plot compares RBC and WBC values, where WBC counts are centered on $14{,}700/\mu L$ and RBC counts range between $4.4-5.9$ million/µL. The distribution shows scattered points without a clear linear trend, indicating no strong correlation between WBC and RBC levels. This suggests that variations in one parameter do not directly predict changes in the other. In subfigure (b), platelet counts are analyzed, with values falling within the normal range of $150-450\times10^3/\mu L$. Most platelet measurements cluster around $400\times10^3/\mu L$, highlighting their concentration near the upper-normal range. Together, these findings provide an overview of the hematological variability in the studied patients, reflecting typical distributions without abnormal deviations [17].

### 3.5 Diagnostic Test Analysis




(a) (b)




(c)

**Figure 14:** Diagnostic test results (a) ELISA (b) MAT (c) RT-PCR

Figure 14 presents the analysis of diagnostic tests conducted on 52 patients to detect Leptospirosis. In(a), it shows the results of the ELISA test, with 30 patients testing positive for Leptospirosis and 22 testing negative. In (b), it presents the MAT test results, where 35 patients tested positive and 17 tested negative. In(c), it shows the RT-PCR results, with 7 patients testing positive and 45 testing negative for the infection. These results indicate that the majority of patients in the study were not infected with Leptospirosis, highlighting the relatively low prevalence of the disease within this population [18]. Overall, the analysis provides a clear comparison of infection status across different diagnostic methods.

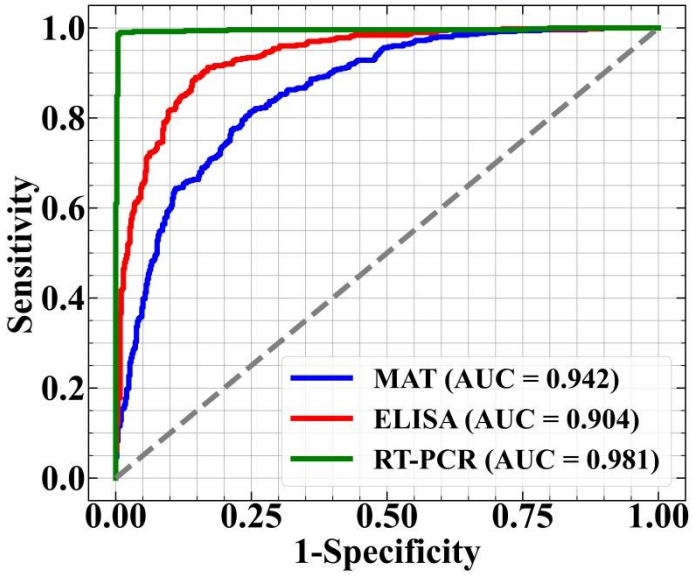



**Figure 15:** ROC Curve Analysis

Figure 15 presents the ROC analysis comparing the sensitivity and specificity of MAT, ELISA, and RT-PCR tests. The AUC values were 0.942 for MAT, 0.904 for ELISA, and 0.981 for RT-PCR, showing that among the three methods, RT-PCR achieved the highest outcome. This test detects Leptospira DNA directly from serum collected in EDTA tubes, targeting the conserved LipL32 gene as a reliable molecular marker. Nucleic acids were extracted, and real-time fluorescence monitoring during amplification enabled sensitive detection, with lower Ct values indicating higher bacterial load. RT-PCR was most effective when samples were collected within the first 7 days of infection, before antibodies appear. Positive and negative

controls ensured accuracy, and careful handling preserved nucleic acid integrity. Thus, RT-PCR provides the most rapid and sensitive method, complementing serological assays for early and reliable leptospirosis diagnosis [19].

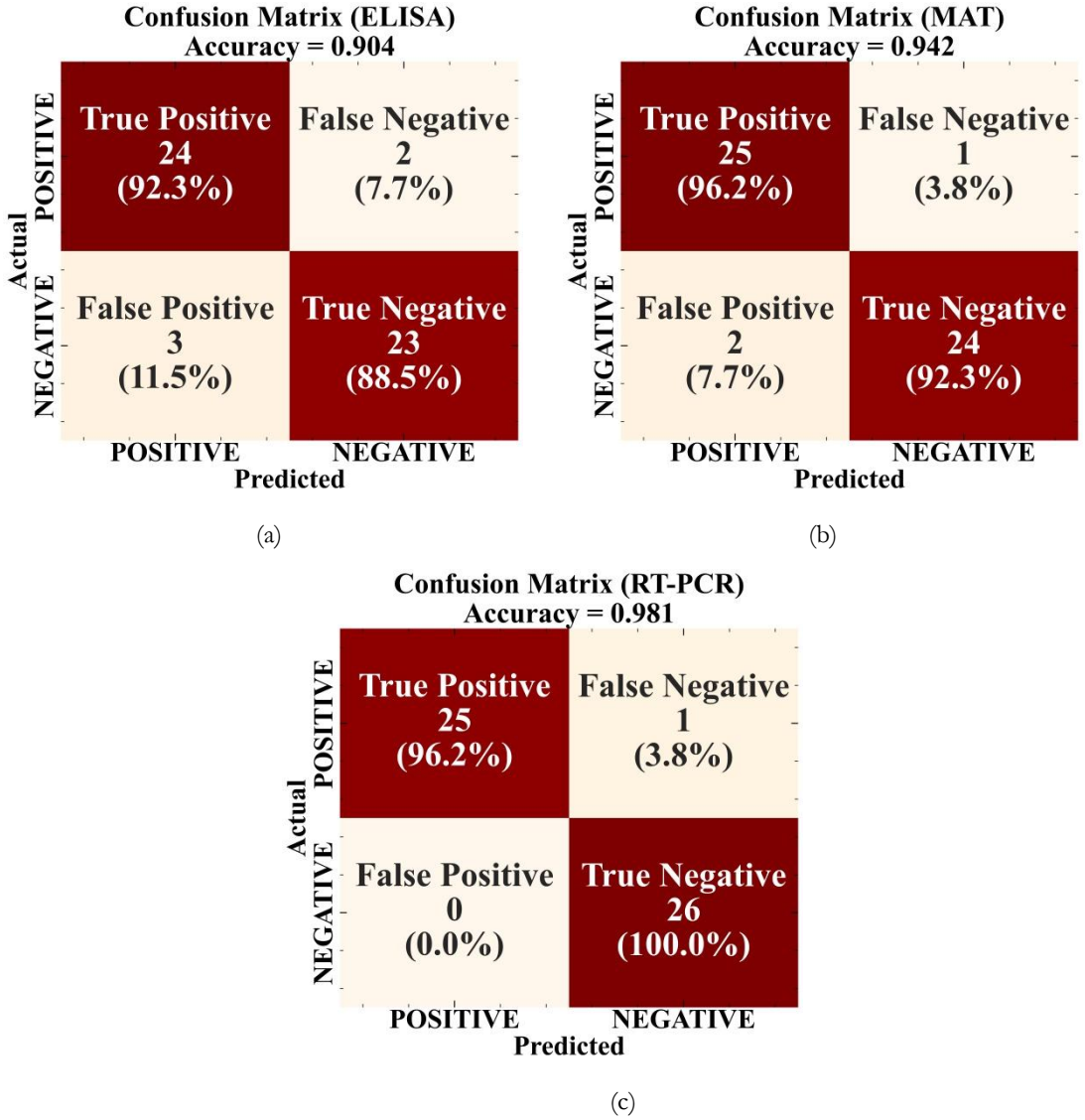



**Figure 16:** Confusion Matrix Analysis over test (a) ELISA (b) MAT (c) RT-PCR

Figure 16 presents the confusion matrix analysis for the three tests. In (a), the EISA test results are shown: out of 26 actual positive cases, 24 were correctly predicted as positive while 2 were misclassified. For the 26 actual negative cases, 23 were correctly identified as negative and 3 were misclassified. The test achieved an AUC of 0.904. In (b), the MAT test results indicate that out of 26 actual positive cases, 25 were correctly predicted, and for 24 actual negative cases, all were correctly identified as negative, yielding an AUC of 0.942. In (c), the RT-PCR test analysis shows 25 positive cases correctly predicted and all 26 negative cases correctly identified, with only one misclassification, resulting in an AUC of 0.981. These results demonstrate the high effectiveness and accuracy of the RT-PCR test compared to the other methods [20].

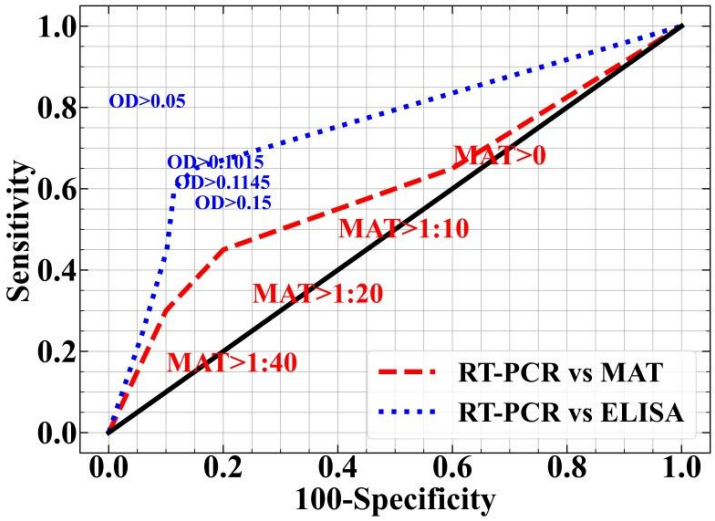
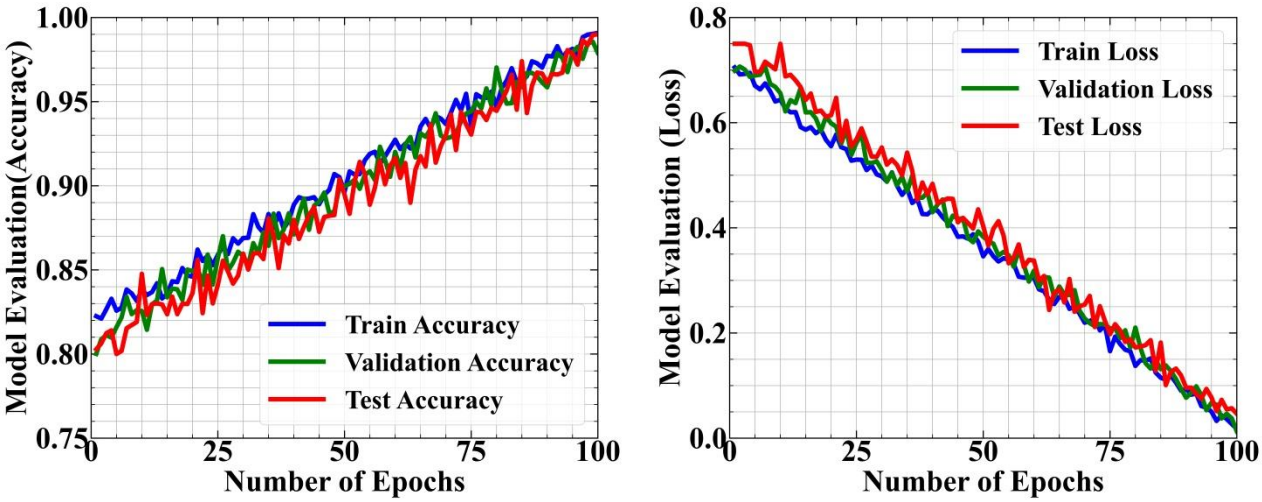



**Figure 17:** Sensitivity and Specificity analysis

Figure 17 presents the sensitivity and specificity analysis of the test models. A comparison between RT-PCR and the MAT test was performed, considering MAT titers ranging from >1:40 to >0.RT-PCR was compared with the ELISA test, with OD values ranging from >0.05 to >0.15. The analysis highlights the performance differences between the diagnostic methods in detecting the target pathogen. Overall, these results provide insight into the relative accuracy and reliability of each test model [21].

### 3.6 Model and Statistical Evaluation




(a) (b)

**Figure 18:** Training, Testing and Validation Analysis (a) Accuracy (b) Loss

Figure 18 shows the training, testing, and validation performance of the model over 100 epochs after preprocessing. In (a), it shows the accuracy, starting between 0.80 and 0.83 at epoch 0 and progressively improving to 0.97–0.99 by epoch 100. This demonstrates the model's strong ability to learn and generalize effectively across training, testing, and validation sets. In (b), it shows the loss analysis, beginning with values between 0.70 and 0.73 at epoch 0 and gradually decreasing to 0.0–0.1 at epoch 100. The decreasing loss highlights the model improved optimization and minimized error during training. Together, these analyses confirm that the model achieves high accuracy and low loss, indicating excellent performance and stability throughout the learning process.

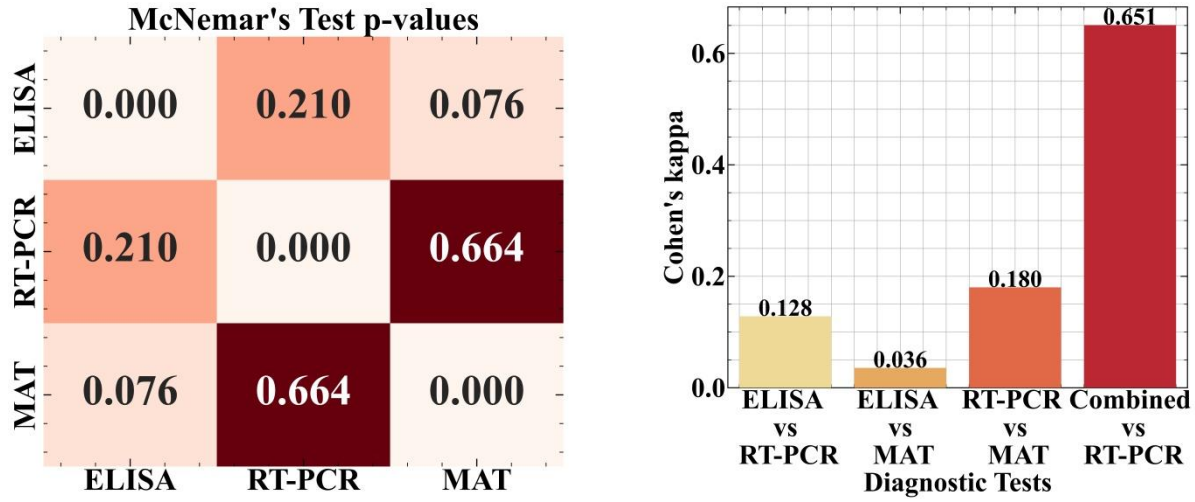



**Figure 19:** Statistical analysis Through (a) McNemar's test (b) Cohen's kappa coefficient

Figure 19 illustrates the statistical evaluation of the test models. In (a), it presents the McNemar's test results, showing values of 0.076 for MAT vs ELISA, 0.664 for MAT vs RT-PCR, and 0 for MAT vs mAT. In (b), it depicts the agreement analysis using Cohen's kappa coefficient, with values of 0.128 for ELISA vs RT-PCR, 0.036 for ELISA vs MAT, 0.180 for RT-PCR vs MAT, and 0.651 for the combined MAT and ELISA vs RT-PCR. These results highlight both the significance and agreement levels among the different diagnostic methods. Overall, the combined test comparison shows the highest agreement with RT-PCR [22].

### 3.7 Ablation Study

**Table 6:** Ablation study

| Test | Sample | Sensitivity (%) | Specificity (%) | AUC |
|---|---|---|---|---|
| Electrochemical apt sensor | Plasma | 100 | 80 | 0.90 |
| rErpY-like protein ELISA | Human sera | 60 | 76 | 0.68 |
| ELISA (IgM/IgG) | Serum | 92.3 | 88.5 | 0.904 |
| MAT | Serum or plasma | 96.2 | 100 | 0.942 |
| **RT-PCR** | **Serum** | **96.2** | **100** | **0.981** |

Table 6 presents a comparative analysis of various diagnostic tests for human leptospirosis. The Electrochemical aptamer sensor, using plasma from the acute phase, showed a sensitivity of 100%, specificity of 80%, and an AUC of 0.90. The rErpY-like protein ELISA, performed on human sera, has the lower performance with 60% sensitivity, 76% specificity, and an AUC of 0.68. Conventional ELISA for IgM/IgG in serum achieved 92.3% sensitivity, 88.5% specificity, and an AUC of 0.904. The MAT using serum or plasma had 96.2% sensitivity, 100% specificity, and an AUC of 0.942. RT-PCR on serum also showed 96.2% sensitivity, 100% specificity, and the highest AUC of 0.981. This highlights RT-PCR as the most accurate method for early diagnosis within the first 7 days of infection. Overall, it emphasizes the effectiveness of RT-PCR in early and precise leptospirosis detection.

### 3.8 Discussion

Diagnosing human leptospirosis is often complicated, as the first clinical signs are similar to other febrile illnesses. Laboratory confirmation of leptospirosis should never be avoided since there are still other clinical possibilities. Serological tests like ELISA and MAT are important in detecting antibodies; however, the various tests will not be useful during the acute stage if antibodies have not yet formed. RT-PCR proved to have superior diagnostic performance when compared to ELISA and MAT because it directly detects Leptospira DNA in the blood samples. It has the ability to identify the infection in the earlier stages when the immune response is not detectable. Early detection is essential for making timely treatment decisions on potential severe complications. In real-time clinical application, the RT-PCR provides faster and more reliable results, though it requires advanced infrastructure and technical expertise. ELISA and MAT remain more accessible options, particularly in resource-limited settings, but they are better suited for epidemiological studies. Computational approaches like data preprocessing have the ability to strengthen the reliability of test comparisons and support robust statistical validation. However, the cost and complexity of RT-PCR may limit its standalone use in routine settings. Thus RT-PCR is the most effective tool for early diagnosis, while ELISA and MAT continue to play supportive roles in comprehensive leptospirosis detection strategies.

## 4 CONCLUSION

A comparative evaluation of ELISA, MAT, and RT-PCR was conducted to identify the most effective diagnostic method for the early detection of human leptospirosis. ELISA and MAT, which assess the host immune response by detecting IgM/IgG antibodies, are well-established and widely used; however, their reliability is limited during the acute phase when antibody levels are insufficient for accurate detection. In contrast, RT-PCR enables direct detection of Leptospira DNA in serum, providing higher diagnostic accuracy during the first week of infection, before seroconversion occurs. Its rapid and sensitive detection makes RT-PCR particularly suitable for real-time clinical application, allowing timely therapeutic interventions and reducing the risk of severe complications. The implementation of RT-PCR in routine clinical workflows can facilitate prompt diagnosis and improve patient outcomes, though its use is currently constrained by requirements for specialized equipment, trained personnel, and higher operational costs. ELISA and MAT remain essential for broader epidemiological studies and for confirming infection at later stages, highlighting the complementary role of serological and molecular approaches. Despite its advantages, challenges remain in scaling RT-PCR for widespread use, particularly in resource-limited regions. Laboratory infrastructure, reagent availability, and cost constraints can limit accessibility, while serological tests may produce false negatives in early infection. Addressing these challenges will be crucial for optimizing leptospirosis detection on a global scale. Future work should focus on developing cost effective, portable RT-PCR platforms suitable for point-of-care use, particularly in endemic and resource-constrained regions. Integration of AI-based data analysis could further enhance predictive accuracy and streamline interpretation. Expanding cohort sizes and including diverse patient populations will strengthen the generalizability of findings. Moreover, combining molecular and serological methods into a unified diagnostic algorithm could offer a comprehensive, rapid, and reliable solution for global leptospirosis management and outbreak control.

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