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# Evaluation of serological diagnostic tests for Leptospirosis-A retrospective study

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**ABSTRACT**

**Introduction**

Leptospirosis is an emerging zoonotic bacterial infection that remains a major public health problem in tropical and subtropical regions majorly in coastal regions ofIndia. Laboratory diagnosis of leptospirosis is mandatory because of varied clinical picture and existence of similar infections in endemic regions which cause confusion in the diagnosis. We evaluated and analyzed two immunodiagnostic test, Leptocheck WB-Rapid immunochromatography test and Leptospira IgM ELISA (Panbio) test detecting Ig M antibodies (Ab) for Leptospira in serum and compared with MAT (Microscopic agglutination test) test.

**Materials and Methods**

Blood samples from suspected patients of leptospirosis were tested by ELISA and Rapid test for IgM Ab detection and MAT test from January 2022 to December 2023. The data were entered in Microsoft Excel and analyzed using Epi Info software version 3.5.1. Descriptive analysis based on bivariant and multi-variant analysis was done. Chi square test was applied and p-value found to demonstrate association between the tests.

**Results**

Out of 75 blood samples tested, 29 were positive by three different serological tests. ELISA and rapid test on comparison with MAT showed 96.29%, 85.18% of sensitivity respectively and 93.75% of specificity. Predominant serovar identified from MAT were L.Automonalis (77%) L. Australis (77%). **Conclusion:** This study suggests that diagnostic accuracy of IgM ELISA for leptospirosis is sensitive in acute stage of the thedisease. Immunodiagnostic methods are simple and easy to perform in detecting IgM antibody in acute phase compared to MAT in resource limited settings.

**Keywords**: Leptospirosis; ELISA; Rapid Immunochromatography test; MAT; Sensitivity; Specificity

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**Ethical Consideration:** The study protocol was approved by the Institutional Ethical Committee [GMCS/STU/RRC-1/Approval/9175/24]. Informed written consent was waived because the study was a retrospective data analysis. Confidentiality was maintained throughout the study by avoidance of use of personal identifiers during the formulation of the report.

## INTRODUCTION

Leptospirosis is an infectious zoonotic disease caused by pathogenic bacteria called Leptospira, which are transmitted directly or indirectly from animals to humans. [1] Globally, more than one million cases of leptospirosis occur, with more than 10% mortality. That may contribute up to 20% cases of fever of unknown origin in India.[2] High burden of disease has been reported from coastal districts of Andaman and Nicobar, Gujarat ,Kerala ,Maharashtra, Karnataka and Tamil Nadu where heavy monsoon, animal rearing practices, unplanned urbanization and agrarian way of life predispose to this infection. [3,4] Occupational exposure is a major cause of infection and the risk groups include agricultural and livestock farmers, workers in underground sewers, meat and animal handlers and veterinarians.[5] Leptospires are divided into more than 24 serogroups and 250 serovars of pathogenic Leptospira. [6] Human is an accidental host. Infection results from direct or indirect exposure to infected reservoir host animals that carry the pathogen in their renal tubules and shed pathogenic Leptospira in their urine. [7] Incubation period of Leptospira ranges from 3 to 30 days; usually 10- 12 days. [8] Leptospirosis presents a diverse array of clinical manifestations and shares common clinical symptomatology with many acute febrile diseases, such as influenza, dengue fever or malaria causing various degrees of infections. [9] These mimicking clinical features may delay the diagnosis of Leptospirosis and patient may succumb to complications.

The diagnosis of Leptospirosis is usually based on the demonstration of serum antibodies by MAT (Microscopic agglutination test) and ELISA (enzyme linked immunosorbent assay) or isolation of Leptospira from blood and urine.[7, 10] As microscopy and isolation ischallenging and have poor sensitivity; serology and molecular methods remain mainstay for diagnosis. [7, 10, 11] Definitive diagnosis requires fourfold or greater rise in the MAT (Microscopic agglutination test) titre between acute and convalescent phase sera or seroconversion. [3, 10] The MAT is the serological test used in reference laboratories due to its high degree of sensitivity and specificity. However, the MAT is a complex test that requires expertise and a large panel of live-cell suspensions to provide adequate agglutination of different antigens. As antibody levels detectable by MAT usually appear after 1-2 week of illness and interpretation of the results is difficult without paired sera, results are usually not available quickly enough to be useful for patient management. Several other immunodiagnostics alternatives to MAT, such as Ig M detectable enzyme linked immune sorbent assay (IgM-ELISA), dot ELISA, indirect hemagglutination assay (IHA), Leptospira dipstick test and Leptospira immunochromatography test are relatively easier to perform when compared with MAT. [12] The simplicity and relatively low cost of these tests make them potentially well suited for use in resource-poor settings with limited laboratory and human capacity. [13] In this study, we evaluated and analyzed two immunodiagnostic test, Leptocheck WB-Rapid test and Leptospira IgM ELISA (Panbio) test detecting Ig M antibodies to Leptospira in serum with MAT test taking it as a gold standa

### Materials and Methods

This retrospective observational study was done at the Leptospirosis laboratory which is the State referral center for Leptospirosis testing. The centredelivers diagnostic services like Fontana staining, dark ground microscopy, microbial culture, PCR, MAT, IgM antibody detection by ELISA and rapid test for Leptospirosis infection. Leptospira infection is endemic in South Gujarat mainly Surat, Valsad, Navsari, Tapi districts. The centre caters diagnostic services to these districts and other parts of Gujarat State as well. A total of 75 blood samples from suspected patients of Leptospirosis were enrolled in the study from January 2022 to December 2023.MAT,Leptocheck WB-Rapid test and Leptospira IgM ELISA (Panbio) were performed for all samples to detect antibodies against Leptospira.

**Inclusion Criteria:** Blood samples (5ml) were collected in plain vacuette from the patients of different age groups who were suspected clinically of Leptospirosisand presented with acute febrile illness with headache, myalgia and prostration

associated with any of the following features i.e. conjuctival suffusion ,meningeal irritation ,anuria or oliguria and/or proteinuria ,jaundice , hepatosplenomegaly, hemorrhagic manifestations ,skin rash , Acute Respiratory Distress Syndrome and a history of exposure to infected animals or an environment contaminated with animal urine, occupational history.[3] Single serum sample of acute illness of Leptospirosis requisites for ELISA Ig M antibody testing ,Ig M rapid Test and MAT tests were included in the study.

**Exclusion criteria:**

1.Patients with other acute or chronic illnesses such as malaria, typhoid, dengue, hepatitis or with other comorbid febrile diseases were excluded from the study.

2. Sample received for PCR and culture for Leptospirosis and second sera samples were excluded from the study.

**Study Procedure:** Blood samples along with duly filled laboratory request form were received in microbiology department. Blood samples were allowed to clot at room temperature and centrifuged at 2000 RPM (Revolution Per Minute) for 10 minutes. Separated serum sample was used for Leptospirosis testing.Samples were tested for Ig M Ab detection by ELISA using commercial kits (Panbio Leptospira IgM Elisa, Abott Diagnostics, Korea) as per manufacturer's instructions and results were recorded. Result of < 9 panbio unit was considered negative, 9-11 panbio units were considered equivocal and >11 panbio unit was considered as positive for Leptospirosis. MAT (Microscopic agglutination test) Procedure: Doubling dilutions of serum from 1 in 50 to 1 in 1600 were prepared by adding PBS (Phosphate buffered solution) in 96 well flat bottomed microtitre plates. All 96 wells of microtitre plate column 1 to column 7 were filled with 25µl PBS . Another 23 µl PBS (Phosphate buffer saline) and 2 µl of patient's serum were added to the column 2 of microtitre plate (dilution became 1:25) Mixing was done and 25 µl PBS was transferred from column 2 well to the next wells till column 7, last 25 µl PBS was discarded. 25 µl Leptospira cultures were added to all wells from column 1 to 7. So, Column 1 well had only the antigen without addition of antibody and served as the antigen control. The final dilutions after adding the antigen were from 1 in 50 in column 2 to 1 in 1600 in column 7.. Mixing thoroughly was done on a micro shaker and incubated at 37°C for 2 hours.[14] Reading of the MAT test results: The serum antigen mixtures were examined under a dark field microscope for agglutination. For observation, one drop mixture was transferred with a pipette from a well to a microscopic slide and examined under a dark field microscope with 20x magnification objective lens without cover slip. The reported titre was calculated as the reciprocal of the highest dilution that agglutinated at least 50% of the cells for each serovar or a reduction in the number of leptospiral cells. This was compared to the antigen control and considered as end point titer. A titer of 1 in 100 or more was considered significant. Serovars tested were L.Autumonalis, L.Patoc, L. Australis, L.Pyrogenes, L.Icterohemorrhagiae, L. Pomona, L. Grippotyphosa, L. Hebdomadis. Strains were obtained from the Regional Medical Research Center (WHO collaborating center for diagnosis, reference, research and training in leptospirosis, ICMR) in Port Blair, Andaman and Nicobar Islands. These serovars were maintained in semisolid EMJH (Ellinghausen-McCullough-Johnson-Harris medium) media supplemented with 10% enrichment (Difco, USA) at 30°C in screw capped test tubes. [14] Rapid test for IgM Ab detection was performed by Immunochromatography (Leptocheck WB) as per manufacturer's instructions and results were recorded.

**Data collection and analysis:**Demographic and clinical details were obtained and recorded from laboratory request forms. A structured data sheet was created using Microsoft Excel software version 2013. Data were analysed retrospectively to know seropositivity of Leptospirosis infection in patients whose samples were tested by all 3 tests includingELISA, Rapid and MAT tests. Comparison of ELISA and rapid test was done with MAT test separately, considering MAT as gold standard to evaluate sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) by using Open epi software version 3.01. Statistical significance for the association between categorical variables was performed using the Chi-squared test and p value by open epi software. The observations

of the data were described in the form of tables, bar diagrams, and pie charts and measured in percent.

## RESULT

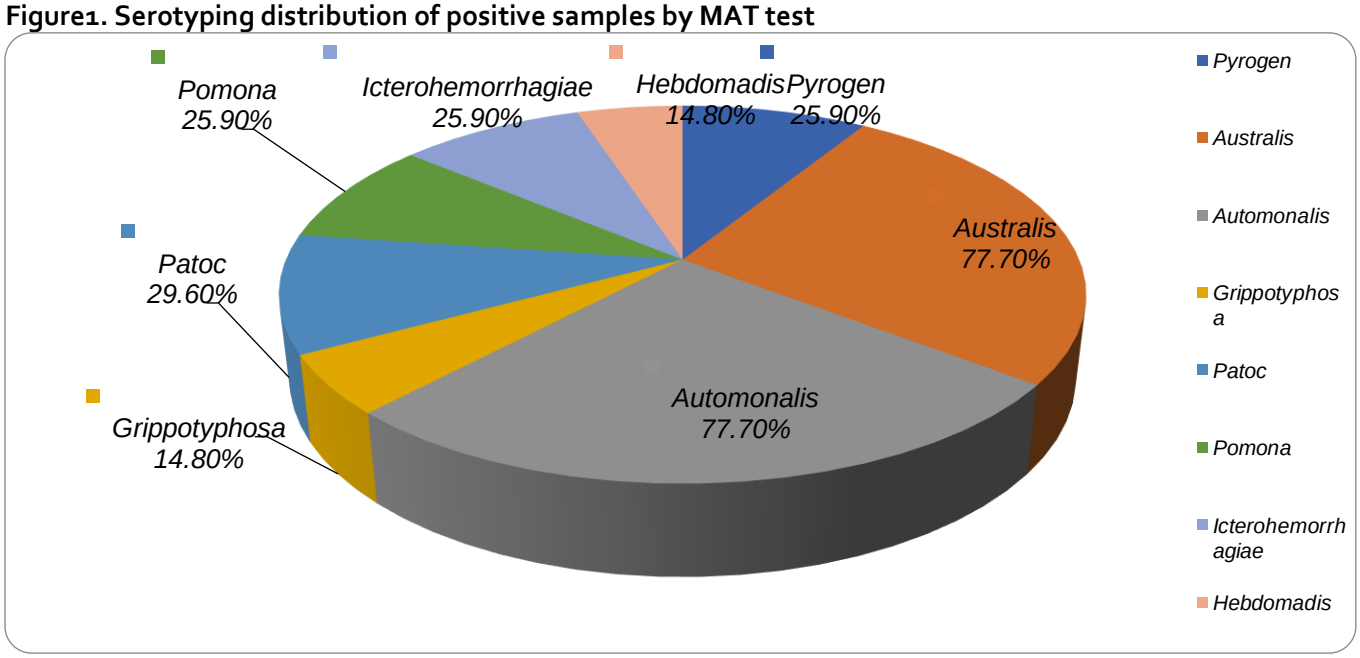
A total of 75 clinically suspected Leptospirosis patients' serum samples from January 2022 to December 2023 were tested by three serological tests for Leptospirosis. Genus specific tests were performed by detection of IgM Ab by ELISA (Panbio Leptospira IgM Elisa, Abott Diagnostics, Korea) and Leptocheck WB rapid immunochromatography test and MAT was done for serovar specific test for Leptospirosis. Results of these tests are demonstated in Table1.

**Table 1.Positive samples for ELISA, MAT, Rapid tests**

| Test | Positive, N=75 | Negative, N=75 |
|---|---|---|
| Ig M antibody ELISA | *29 (38.7%)* | *46 (61.3%)* |
| MAT | *27 (36%)* | *48 (64%)* |
| Rapid test for Ig M antibody Detection | *26 (34.7%)* | *49 (65.3%)* |

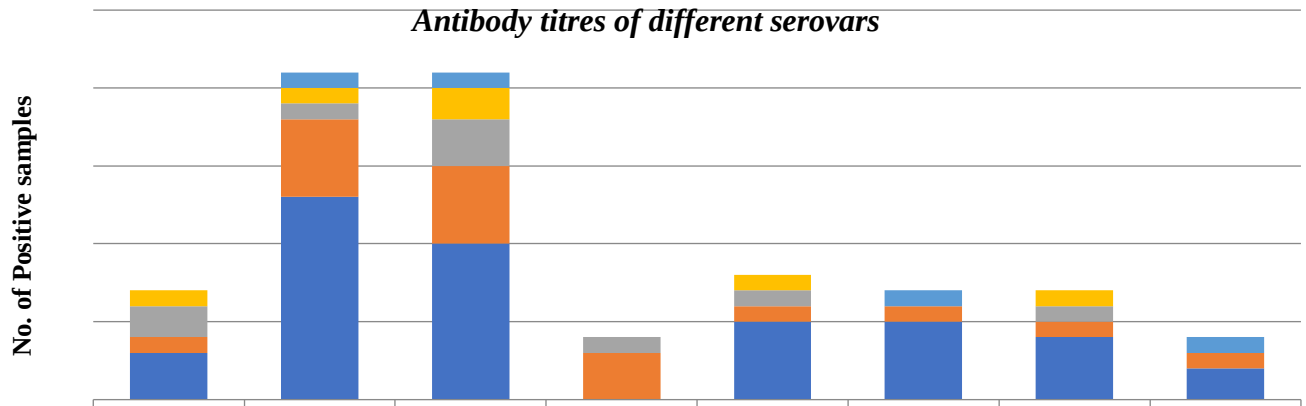
The number of samples labeled IgM antibody positive for Leptospirosis by ELISA and rapid tests were 29(38.7%), 26(34.7%) respectively. 27(36%) samples were showing ≥100 antibody titer in MAT result. Many MAT positive samples showed antibodies against multiple serovars in serum samples. Predominant serovar identified from MAT positive samples were L.Automonalis (77%) L. Australis (77%) followed by L.Patoc (29.6%), L.Pyrogenes (25.9%), L.Icterohemorrhagiae (25.9%), L. Pomona (25.9%), L. Grippotyphosa (14.8%), L. Hebdomadis (14.8%). (Figure-1).

**Figure1. Serotyping distribution of positive samples by MAT test**



Titre of different serovars was in range from 1:100 to 1:1600. Analysis of different serovars and their antibody titer are demonstrated in Figure-2.

**Figure 2.Antibody titre of different serovars by MAT test**




| | Pyrogen | Australis | Automonalis | Grippotyphosa | Patoc | Pomona | Icterohemorrhagiae | Hebdomadis |
|---|---|---|---|---|---|---|---|---|
| 1600 | 0 | 1 | 1 | 0 | 0 | 1 | 0 | 1 |
| 800 | 1 | 1 | 2 | 0 | 1 | 0 | 1 | 0 |
| 400 | 2 | 1 | 3 | 1 | 1 | 0 | 1 | 0 |
| 200 | 1 | 5 | 5 | 3 | 1 | 1 | 1 | 1 |
| 100 | 3 | 13 | 10 | 0 | 5 | 5 | 4 | 2 |

The serological sensitivity and specificity of ELISA and rapid test were checked with MAT test which is a gold standard test for leptospirosis. ELISA test on comparison with MAT showed 96.29% of sensitivity, 93.75% of specificity, 89.65% of positive predictive value (PPV) and 97.80% of negative predictive value (NPV). Rapid test showed sensitivity of 85.18%, specificity of 93.75%, PPV of 88.46% and NPV of 91.83% in comparison with MAT.[Table 2] Highest level of association was found for ELISA results with MAT test results by using chi square test and calculating p value (<0.0001)

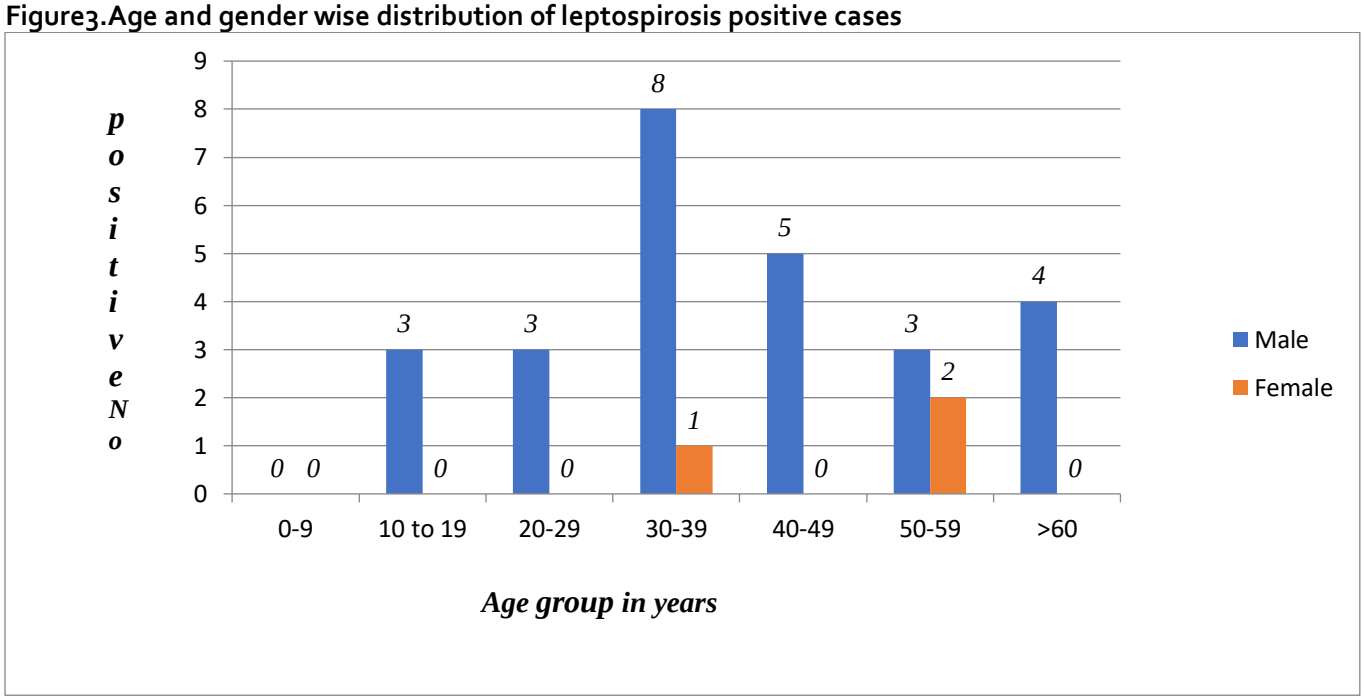
**Table 2. Comparison of ELISA and Rapid tests with MAT.**

| Sample Number | MAT | | | | MAT | | |
|---|---|---|---|---|---|---|---|
| **ELISA** | **Positive** | **Negative** | **Total** | **Rapid Test** | **Positive** | **Negative** | **Total** |
| **Positive** | *26* | *3* | *29* | **Positive** | *23* | *3* | *26* |
| **Negative** | *1* | *45* | *46* | **Negative** | *4* | *45* | *49* |
| **Total** | *27* | *48* | *75* | **Total** | *27* | *48* | *75* |

| Comparison with MAT | ELISA Test | Rapid Test |
|---|---|---|
| Combined Sensitivity | *96.29%* | *85.18%* |
| Combined Specificity | *93.75%* | *93.75%* |
| Positive predictive value (PPV) | *89.65%* | *88.46%* |
| Negative predictive value (NPV) | *97.80%* | *91.83%* |
| Yates corrected chi square value | *55.34* | *44.12* |
| P value in comparison with MAT Test | *<0.0001* | *<0.0001* |

Among 29 seropositive patients, 26 (89.7%) were male and 3 (10.3%) were female and majority adults of 30 to 49 years of age group were affected. [Figure 3].During the study period, highest cases were tested and positive (93.1%) in monsoon season between months of July to October. 55.2% of study positive cases were from Surat rural area, 17.3% from Navsari and 3.4% each from Valsad, Tapi and Bharuch and 17.3% fromotherdistricts (Chotaudepur,Dahod,Vadodara,Ahmedabad,Nandurbar)

**Figure3.Age and gender wise distribution of leptospirosis positive cases**




44.8% Leptospirosis positive cases were critical, requiring ICU support while 55.2% were in stable condition. Thrombocytopenia (82.8%) was the commonest hematological abnormality along with raised leucocytes count (79.3%), raised liver enzymes (SGOT-79.3%, SGPT-75.9%), high serum bilirubin (69%) and raised blood urea (79.3%) and creatinine (79.3%) .[Table 3].

**Table 3.Distribution of symptomatic positive cases by combined ELISA, MAT, Rapid tests**

| Profile of Test positive cases | | Positive cases n (%) with each characteristic N 29 |
|---|---|---|
| **Condition** | Critical | *13 (44.8%)* |
| | Stable | *16 (55.2%)* |
| **Onset of fever** | *<5* days | *10 (34.6%)* |
| | *5-10* days | *15 (51.7%)* |
| | *10-15* days | *3 (10.3%)* |
| | *>15* days | *1 (3.4%)* |
| **Clinical profile** | Chills | *17 (58.6%)* |
| | Vomiting | *18 (62.1%)* |
| | Conjuctival suffusion | *12 (41.4%)* |
| | Epistaxis | *2 (6.9%)* |
| | Myalgia/arthralgia | *20 (69%)* |
| | Tendeness of calf muscle | *9 (31.03%)* |
| | Photophobia | *2 (6.9%)* |
| | Fatigue | *21 (72.4%)* |
| | Drowsiness | *8 (27.6%)* |
| | Retroorbital pain | *6 (20.7%)* |
| | Cough | *8 (27.6%)* |
| | Headache | *15 (51.7%)* |
| | Jaundice | *23 (79.3%)* |
| | hemoptysis | *5 (17.3%)* |
| | severe joint pain | *8 (27.6%)* |
| | rash/petechie | *5 (17.3%)* |
| | renal failue | *20 (69%)* |
| | Abdominal pain | *13 (44.8%)* |
| | altered sensorium | *8 (27.6%)* |
| **Blood Investigation** | Haemoglobin (*<10*g/dL) | *19 (65.5%)* |
| | Total count (*>10000*/µL) | *23 (79.3%)* |
| | Pletelet count (*<100000*/µL) | *24 (82.8%)* |
| | SGOT(*>60* IU/mL) | *23 (79.3%)* |
| | SGPT(*>60* IU/mL) | *22 (75.9%)* |
| | Serum Billirubin-Total (>2mg/dL) | *20 (69%)* |
| | Blood Urea (*>40* mg/dL) | *23 (79.3%)* |
| | Serum creatinine (*>1.5* mg/dL) | *23 (79.3%)* |

## DISCUSSION

Leptospirosis is endemic in south Gujarat since 1994. The endemic districts are Valsad, Navsari and Surat. Cases are seen during the monsoon months. Conditions such as heavy rainfall, high water table, and clay soil in South Gujarat favor endemicity for Leptospirosis. [15] The public health importance of Leptospirosis lies in its occupational, seasonal, sex, and age-related incidence. Males outnumbered the females and adults of 2nd, 3rdand 4th decade in the affect by Leptospirosis infection in this study which coincides with published studies by Deshmukh et al [2], Sethi S et al [4], Holla et al [5], Shukla et al [16] who observed that majority of the patients of Leptospirosis were males in the age group 26-40 years.

Prevalence rate was more in farmers (55.3%)due to working in fields and more exposure to animals. The remaining samples were laborer (27.6%), vegetable vendor and local business man (3.4%), unemployed students and housewives (10.3%). The study observed a significant association of monsoon season and farm workers as the most vulnerable population suffered from Leptospirosis Farming is associated with higher exposure to contaminated water, especially the farming crops (paddy, sugarcane, banana) requiring substantial water irrigation. [17] This is also noted by study done by Shukla et al [16], Thalva et al [18], Prakash et al [19], Velineni et al. [20] Fever was the most consistent feature along with myalgia, headache, vomiting, fatigue, chills, conjuctival suffusion, and abdominal pain. Major complications noted in our study were jaundice (79%) and renal failure (69%) which are comparable with study done by Sethi et al [4] and Prakash et al [19] in which 73%, 96% had jaundice and 60.5% , 40% had renal failure respectively. This study evaluates the efficacy of Immunodiagnostic methods (ELISA and rapid test) in comparison to MAT test. Out of 75 serum samples, 27 showed anti Leptospiral antibodies by MAT analysis. Many MAT positive samples showed antibodies against multiple serovars in serum samples. The predominant infecting serovar observed were L.Autumanalis (77%) and L.Australis (77%). Velineni et al [20] study have reported L.Icterohemorrhagiae (68%) as prevalent serovar followed by Australis (22%), Autumnalis (8%) and Javanica (2%) in Hyderabad. while L.copenhageni was predominant strain in Mumbai in study by Bharadwaj et al. [21] Different serovars of Leptospira can be demonstrated by MAT in multiple regions. [7] Percentage positivity of MAT test was 36% (27/75) in comparison to ELISA and rapid test which was 38.7% and 34.7% respectively. Similar analysis was published by Bharadwaj RS [21] and Shekatkar et al [23] in which positivity rate of MAT and ELISA was 43.7%, 46.1% and 36%, 37% respectively. Although MAT is immunological gold standard test, it shows less detection rate in comparison to ELISA in acute cases of leptospirosis. Difference in percent positives of both tests may be due to the fact that genus specific antibodies appear earlier than serovar specific antibodies in serum. So, at the acute stage of infection, genus specific tests (Ig M Ab detection-based methods) were expected to give positive results early in comparison to serovar specific tests (MAT). [22] Single MAT positive result was considered the gold standard for comparison with ELISA and Rapid test. ELISA had combined sensitivity of 96.29% and rapid test had combined sensitivity of 85.18%. Combined specificity of the both tests was same (93.75%). This depicts the ability of the tests to identify the negative cases. High sensitivity and specificity of IgM-ELISA during the acute phase of illness in single sample was good enough for early as well as definitive diagnosis. This test had high PPV and NPV during the early phase of infection. Rapid test also had a good sensitivity and specificity to diagnose the cases early. Moreover, it is easy to perform, rapid method that takes only 15–20 minutes, not requiring any special equipment and can be conducted at resource poor settings at peripheral areas of the State.

**Table 4. Comparative analysis of the ELISA and Rapid tests with other studies**

| Test | References | Sample size | Sensitivity | Specificity |
|---|---|---|---|---|
| **IgM ELISA** | **The Study** | *75* | *96.29%* | *93.75%* |
| | Velineni et al [23] | *32* | *86.7%* | - |
| | Ooteman et al [10] | *125* | *96.6%* | *93.3%* |
| | Bajani et al [12] | *132* | *90.7%* | *97.4%* |
| | Khan et al [24] | *207* | *100%* | *87.6%* |
| | Ganesh et al [26] | *54* | *89.7%* | *100%* |
| **Rapid Test** | **The study** | *75* | *85.18%* | *93.75%* |
| | Niloofa et al [22] | *888* | *86.1%* | *84.5%* |
| | Goris et al [25] | *197* | *78%* | *98%* |
| | Ganesh et al [26] | *54* | *75%* | *96%* |

The analysis of sensitivity and specificity of ELISA and rapid tests of present study are comparable with previous studies mentioned in Table 4. IgM antibody detection by Panbio ELISA shows comparable sensitivity and specificity in studies done by Ooteman et al, [10] Bajani et al, [12] Khan et al. [24] Rapid test done by Leptocheck-WB showed comparable sensitivity and specificity with Niloofa et al. [22] Both tests shows significant agreement and highest level of association ( p value <0.0001) with MAT results in present study.

**Conclusion**

According to the study immunodiagnostic assays are equally sensitive when compared with MAT. Definitive diagnosis based on MAT paired sera is difficult because mostly convalescent sample is not available. Leptospira IgM detection based immunodiagnostic methods are gold standards in acute stage from single serum sample. Sensitivity is high for ELISA and Rapid test with MAT and also independently and can be used when MAT test results are awaited. Furthermore, these tests can be used by standard laboratories without extensive instrumentation, or in field settings where MAT testing is quite impossible.

**Limitations**

The study includes only acute samples for comparison. Paired samples are better for comparative analysis of different tests. The study includes only samples which conducted all 3 test (MAT, ELISA, Rapid test).

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