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**Original Article**

# SEROPREVALENCE OF LEPTOSPIROSIS IN SOUTH GUJARAT REGION BY EVALUATING THE TWO RAPID COMMERCIAL DIAGNOSTIC KITS AGAINST THE MAT TEST FOR DETECTION OF ANTIBODIES TO LEPTOSPIRA INTERROGANS

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## ABSTRACT

The study was conducted to evaluate the two rapid tests for the serologic diagnosis of leptospirosis namely Microplate Immunoglobulin M(IgM)-Enzyme Linked Immunosorbent Assay(ELISA) and IgM Rapid Leptocheck WB and the performance of each assay compared with that of the current standard, the microscopic agglutination test (MAT). The panels of 188 sera from 130 cases of leptospirosis from three different geographical locations were tested as well as 310 sera from healthy individual or individual with other infectious disease other than leptospirosis. Acute phase sera from cases (n=130) were collected <14 days after the onset of symptoms and convalescent phase sera (n=58) were collected ≥14 days after the onset of symptoms. By traditional method (two-by-two) contingency table, the sensitivity, specificity, PPV(Positive predictive value), NPV(Negative predictive value), Efficiency of test and □(Kappa) value for agreement (with MAT) for the Rapid Leptocheck WB were 98.36%, 86.95%, 86.95%, 98.36%, 92.37% and 0.81 in acute phase of disease. Corresponding values for IgM ELISA were 96.82%, 88.05%, 88.40%, 96.72%, 91.53% and 0.88 respectively. The sensitivity, specificity, PPV(Positive predictive value), NPV(Negative predictive value), Efficiency of test and □(Kappa) value for agreement (with MAT) for the Rapid Leptocheck WB were 87.87%, 88%, 90.82%, 84.61%,86.20% and 0.85 in convalescent phase of the disease. Corresponding values for IgM ELISA were 91.42%, 95.65%, 96.96%, 88%, 93.10% and 0.81 respectively. These values for the 2 tests were comparable, indicating that there was no difference in their efficacies. The second-generation assay included in study (Leptocheck and ELISA) showed significantly higher sensitivity with early acute phase sera than the reference or first generation method (MAT) while retaining high specificity and should greatly improve the rapid detection of leptospirosis in the field.

**KEY WORDS:** Leptospirosis, MAT test, IgM ELISA test, IgM Rapid Leptocheck test.

## INTRODUCTION

Leptospirosis is a zoonosis caused by spirochetes of the genus *Leptospira*, which has a worldwide distribution[1]. Humans become infected through contact with contaminated animal urine, tissues, or water[2] The clinical presentation is difficult to distinguish from dengue, malaria, influenza, and many other diseases characterized by fever, headache, and myalgia[3]. Although the patient's exposure history may assist in narrowing the differential diagnosis, a rapid and simple test with high sensitivity and specificity would be useful for early diagnosis and treatment and for public health surveillance[4]. Definitive laboratory diagnosis of leptospirosis requires detection of the organism in a clinical specimen or a fourfold or greater rise in microscopic agglutination test (MAT) titer in the setting of an appropriate clinical syndrome.

The most frequently used diagnostic approach for leptospirosis has been that of serology. The

MAT is the serological test used in reference laboratories, because of its high degree of sensitivity and specificity[5]. However, the MAT is a complex test that requires a large panel of live-cell suspensions to provide adequate coverage of the antigenic diversity represented in a given testing area. Moreover, antibody levels detectable by MAT usually do not appear before day 6 or 7 after development of symptoms; they usually peak by the fourth week, but detectable titers may persist for years[6, 7, 8]. Hence, interpretation of the results is difficult without paired specimens collected at the appropriate times; therefore, results are usually not available quickly enough to be useful for patient management.

Several alternatives to the MAT have been developed; those available commercially include an Immunoglobulin M (IgM) Enzyme-Linked Immunosorbent Assay (ELISA)[9], an IgM dipstick assay (LDS)[10], an IgM dot-ELISA dipstick test (DST)[11], and the indirect heamagglutination assay (IHA)[12]. Reported evaluations suggest that some of these assays are highly sensitive and specific[12, 13, 14, 15, 16, 17, 18], but they have not been systematically compared to each other and to the MAT. This study was designed to determine the performance of these serologic assays in detecting *Leptospira*-specific antibodies and to compare results obtained with each system to those obtained with the MAT. This information should assist diagnostic laboratories, especially those without the capacity to maintain the MAT, to select a suitable assay for screening serum samples from suspected cases of leptospirosis.

## MATERIAL AND METHODS

**Case sera:** The study was conducted at new civil hospital, Surat, India, a tertiary health centre in South Gujarat during the period May 2007 to July 2008. All suspected cases of Leptospirosis attending the outpatient department of these hospitals during the study period were included. A total of 188 sera from 130 cases were included in the study, the panel of case sera (188 specimens) consisted of 130 acute phase sera (obtained <14 days after the onset of illness) and 58 convalescent phase sera (obtained 14 to 28 days after the onset of illness). Paired sera were available for 58 cases. Samples were from different geographic location namely, 76 cases were from Surat district, 18 cases were from Valsad district and 36 cases were from Navasari district. **Control sera:** A total of 310 control specimen were collected which includes 50 healthy donors, 100 were from individuals known to have disease other than leptospirosis and 160 healthy control from different geographic locations. Information helpful in the interpretation of results such as agent or disease specific finding and place of residence was obtained.

**Criteria for clinical suspicion of leptospirosis:** Acute febrile illness with headache, myalgia and prostration associated with any of the following:

- Conjuctival suffusion
- Meningeal irritation
- Anuria or oliguria and/or proteinuria
- Jaundice
- Hemorrhages (from the intestines; lung bleeding is notorious in some areas)
- Cardiac arrhythmia or failure
- Skin rash and a history of exposure to infected animals or an environment contaminated with animal urine.
- Other common symptoms include nausea, vomiting, abdominal pain, diarrhea & arthralgia.

**MAT test:** The MAT test was performed using standard procedure[19]. Live leptospira (representing 11 serovars belonging to 11 serogroup) cultured in EMJH (Ellinghausen-McCullough- Johnson-Harris) media to detect agglutination antibodies from patient sera. Live leptospira cell suspension were added to serially diluted serum specimens in 96 well flat bottomed microtiter plates and incubated at $37^0$C for 2 hours. Agglutination was examined by dark field microscopy at a magnification of 100X. The reported titer was calculated as the reciprocal of the highest dilution that agglutinated at least 50% of the cells for each serovar.A MAT test is considered borderline at titre of $\geq$80 and positive at titre of $\geq$200 for single samples. Serogroup included in the antigen panel are as follows:
Australis (Australis), Autumnalis (Bangkinang), Ballum (Ballum), Sejroe (Hardjo), Grippotyphosa (Grippotyphosa), Canicola (Canicola), Hebdomadis (Hebdomadis), Pomona (Pomona), Semeranga (Patoc1), Pyrogen (Pyrogen), Icterohaemorrhagiae (Icterohaemorrhagiae).

**IgM ELISA test:** The ELISA was carried out as per the manufacturer's instruction. ELISA kit was obtained from Serion verion ELISA (classic leptospira IgM). Serum antibodies of the IgM

class, when present, combine with leptospira antigen attached to the polystyrene surface of the microwell test strips. Residual serum is removed by washing and peroxidase conjugated antihuman IgG, IgA, IgM is added. The microwells are washed and substrate system, para-nitrophenyl-phosphate is added. The substrate is hydrolysed by enzyme, and chromogen changes to yellow coloured. Case and control sera (10µL) were diluted 1:100 and tested according to the manufacturer's instruction. The result is read with a dual wavelength spectrophotometer at 405nm and a background of 620nm. The colour intensity is directly related to the concentration of Leptospira IgM antibodies in the test sample. Each set of tests is run with a positive control, negative control and cut-off calibrator in duplicate. The test is valid when the absorbance reading of the above meets the specification of the Serion ELISA instruction. The results were interpreted according to the manufacturer's recommendation. Specimens having an absorbent ratio greater than that of cutoff calibrator were defined as positive.

Calculation for Serion ELISA classic leptospira IgM:

- Serion units of <15 gives a negative result interpreted as no evidence of recent infection.
- A Serion unit of 15-20 is a low positive or borderline result and may suggest a recent infection.
- Serion units of >20 is a positive result suggestive of a recent or current infection.

Samples giving borderline results should be tested in parallel with a further sample taken from the patient 1-2 weeks later.

**Rapid Leptocheck Test:** Case and control sera (10µL) were used and tested according to the manufacturer's instruction. It utilizes the principle of immunochromatography, a unique two-site immunoassay on a membrane. As the test sample flow through the membrane assembly of the test device, the anti-human IgM colloidal gold conjugate forms a complex with IgM antibodies in the sample. This complex moves further on the membrane to the test window 'T' where it is immobilized by the broadly reactive leptospira genus specific antigen coated on the membrane, leading to the formation of a red to deep purple coloured band at the test region. 'T' which confirms a positive test result. Absence of this coloured band in test region 'T' indicates a negative test result. The unreacted conjugate and the unbound complex if any move further on the membrane and are subsequently immobilized by the anti-rabbit antibodies, coated on the control window "C" of the membrane assembly, forming a red to deep purple coloured band. The control band shows to validate the test result.

**Criteria for laboratory confirmation:** The suspected patients fulfilling any of the following criteria were considered as a case of leptospirosis:(1) isolation of leptospira from clinical specimen (2) Seroconversion in IgM ELISA and MAT test from seronegative to a titre of at least 100, (3) Fourfold or greater increase in MAT or ELISA titre between acute and convalescent phase serum specimens obtained 2 weeks apart and studied at the same laboratory (4) a titre of >100 in IgM ELISA or >200 in MAT if only a single sample was available.

## DATA ANALYSIS

Sensitivity, specificity, positive predictive values(PPV), negative predictive values(NPV), Kappa value were calculated based on MAT cutoff of $\geq$80 dilution , using standard equations:

- % sensitivity =true positive / (true positive + false negative) × 100.
- % specificity = true negative/ (false positive + true negative) × 100.
- PPV (Positive predictive value) = true positive/all positive test.
- NPV (Negative predictive value) = true negative/ all negative test.
- Efficiency of test= (true positive +true negative)/total samples

## RESULTS

The sensitivity, specificity, PPV(Positive predictive value), NPV(Negative predictive value), Efficiency of test and □(Kappa) value for agreement (with MAT) for the Rapid Leptocheck WB were 98.36%, 86.95%, 86.95%, 98.36%,92.37% and 0.88 in acute phase of disease. Corresponding values for IgM ELISA were 96.82%, 88.05%, 88.40%, 96.72%, 91.53% and 0.88 respectively. These values for the 2 tests were comparable, indicating that there was no difference in their efficacies.

The sensitivity, specificity, PPV(Positive predictive value), NPV(Negative predictive value), Efficiency of test and □(Kappa) value for agreement (with MAT) for the Rapid

Leptocheck WB were 87.87%, 88%, 90.82%, 84.61%,86.20% and 0.85 in convalescent phase of the disease. Corresponding values for IgM ELISA were 91.42%, 95.65%, 96.96%, 88%, 93.10% and 0.81 respectively. So, the changes in the values of these tests, depending on the stage of the disease are shown in table-1 and chart- 1& 2 below.

**Table 1:** Comparison of two rapid tests in acute and convalescent phase

| Tests | Phases | Sensitivity | Specificity | PPV | NPV | Efficiency |
|---|---|---|---|---|---|---|
| **Leptocheck WB** | **Acute Phase (< 14 days)** | 98.36% | 86.95% | 86.95% | 98.36% | 92.37% |
| | **Convalescent phase (14-28 days)** | 87.87% | 88.00% | 90.62% | 84.61% | 6.20% |
| **IgM ELISA** | **Acute Phase (< 14 days)** | 96.82% | 88.05% | 88.40% | 96.72% | 91.53% |
| | **Convalescent phase (14-28 days)** | 91.42% | 95.62% | 96.96% | 88.00% | 93.10% |

The sensitivity of the MAT for diagnosis of leptospirosis was also tested which showed sensitivity of 44.61% during 1st week and 60.38% during second to fourth week. These values were lower than the corresponding values for the Leptocheck WB and IgM ELISA.

## DISCUSSION

Leptospirosis is an acute febrile disease, widely recognized as being emergent or re-emergent in tropical and subtropical regions, the disease is endemic and exposure to infection is widespread. In temperate climates, the disease is primarily one of occupational, recreational expose. Leptospirosis is frequently under-diagnosed, because of the non-specific symptoms early in the disease and the difficulty of performing the culture.

In leptospirosis, antibodies begin to appear within a few days of onset of symptoms and in a significant proportion of patients the antibodies persist in detectable quantities for several months (Silva et al, 1995). As has been described, genus specific antibodies appear earlier than the serovar specific microscopic agglutinating antibodies. At this earlier stage of the disease, genus-specific tests, especially IgM immunoassays, are expected to be positive though more serovar specific tests such as MAT may not be able to detect the presence of antibodies owing to nil or low immune response (Christie, 1980). From the clinical point of view, the ability to detect the infection early in the course of the disease is of extreme importance for initiating appropriate treatment to avoid serious complications. In this context, the genus specific IgM immunoassays would be of great use for detecting leptospirosis at an early stage of the disease.

One of the drawbacks of IgM immunoassays and Rapid Leptocheck WB is their inability to give any information about the infecting serovars. But such information is mainly of epidemiological importance, as differentiation between the infecting serovars does not affect the clinical course of management. The usefulness of these rapid genus-specific immunoassays is at the peripheral level, where the only information required is whether or not a patient has a leptospiral infection.

The sensitivities of both rapid Leptocheck WB and IgM ELISA are at acceptable levels even during the first week of illness when the IgM antibodies start to appear. This indicates that the assays are highly responsive to even low levels of IgM antibodies. As the tests have high PPV during all stages of the disease, these tests are useful for screening. Since these tests detect IgM antibodies, which persist for a shorter period than IgG antibodies, their NPV begin to decline after 1 month of infection. Because of this, these tests will have only limited usefulness in epidemiological studies on prevalence of infection among a population.

As MAT detects both IgM and IgG antibodies, it is difficult to differentiate between current clinical infection and past exposure to leptospira using a single MAT. In this regard there is a need to define criteria for a positive MAT when MAT is used alone for serodiagnosis of leptospirosis. Based on our criteria, MAT on a single sample had shown only 44.61% sensitivity during the acute phase (0 to 14 days) of illness. This comparatively ≥1:80 cut-off value was used

because the study was conducted in an endemic zone with high seroprevalence among the healthy population. The sensitivity of MAT rose to 60.38% during the convalescent phase (14 to 28 days) of disease. Some of the patients who had negative MAT results during the first weeks of disease and they became positive by seroconversion and showed rising titres when another sample obtained 14 days after the onset of illness was examined.

Therefore, this test is a useful tool for epidemiological purpose.

- We observed that more patients were male in our study. Almost are working class male farm workers.
- We observed that there were 71 (seventy one) i.e. more number of cases in the age group of 20-39 years. This reflects as they are active earning adult age groups and from history majority of these had occupational history as farmer.

Among the 100 serum samples from patients with disease other than leptospirosis (malaria, dengue, hepatitis, typhoid, HIV). There were no false positive reactions observed with Leptocheck WB or IgM ELISA. It may be due to we used limited numbers diseased groups. We did not observe any significant difference in the cross-reactivity rate in different disease by ELISA & Leptocheck WB. None of the sera from the above groups of patients had given significant titres by MAT. However, low titres by MAT (1:20-1:40) were obtained for some of the patients, which reflects that it may be IgG antibody.

**Table 2:** Results of our study in comparison with other studies

| Test | Results | WYsekhar EH Soo[4, 8] | SC Sehgal, PV Vijaychari[4, 2] | Present study |
|---|---|---|---|---|
| Rapid test Leptocheck or Dipstick | Sensitivity | 83.3% | 78.7% | 93.81% |
| | Specificity | 93.8% | 88.3% | 86.81% |
| | PPV | 95.29% | 91.0% | 88.34% |
| | NPV | 79% | 73.4% | 92.94% |
| IgM ELISA | Sensitivity | 54.2% | 78.5% | 93.81% |
| | Specificity | 96.9% | 87.6% | 90.10% |
| | PPV | 96.3% | 90.5% | 91.00% |
| | NPV | 58.5% | 73% | 93.81% |

Our study was compared with other studies (table- 2), our study sensitivity for rapid test is 94.68 % which is comparable to the other two studies (WY Sekhar, EH, Soo[20], P. Vijayachari et al[21]). It is slightly higher than the other two studies which may be due to the difference in test as they have used Dipstick as a rapid method which is based on immunochromatography principal, and in our study we have used Leptocheck WB (lateral flow method).

The specificity of P. Vijayachari et al[21] & W.Y. Sekhar EH Soo[20] ranges from 88% to 94%. In our study, it was 87.23% which correlates well with their studies.

In case of IgM ELISA, the sensitivity of WY Sekhar study was very low, which may be due to difference in kit mode. They have used PanBio for their study, where as we have used Serion Virion IgM ELISA which was evaluated according to Indian geographical areas.

The sensitivity of P. Vijayachari study was also slightly lower than our study but it is comparable. The specificity of two studies correlates well with our study.

The agreement between Rapid test with MAT and IgM ELISA with MAT test were 80% and 84% respectively which are comparable to SC Sehgal, P. Vijayachari et al study.

Additionally one of the major limitations for any evaluation of assays for serologic diagnosis of leptospirosis is the paucity of cases confirmed by culture. As a result, findings from new serologic assays are comparable with those from cases that are primarily defined by another serologic assay. Consequently, there are very few reports of sensitivity and specificity of the MAT, because it is the gold standard against which other assays are usually compared.

## CONCLUSION

This study was conducted at New Civil Hospital, Surat during the period May 2007 to July 2008. There were 130 clinically suspected cases from different regions of South Gujarat. Majority of patients were young adults. There was male preponderance, and majorities were farm workers.

The Rapid Leptocheck WB test is easy to perform and it requires only a single dilution and does not require any special equipment. The kit reagents have a long shelf-life even at room temperature. The test has good sensitivity (98.36%) and specificity (86.95%) in acute phase and sensitivity of 87.87% and specificity of 88% in convalescent phase considering MAT as Gold Standard. So, it is now the test of choice for the diagnosis of current leptospirosis, and for routine use at the peripheral level in developing countries. IgM ELISA is also very good test for early detection of leptospiral infection which has good sensitivity (96.82%) and specificity (88.05%) in acute phase and sensitivity of 91.42% and specificity of 95.62% in convalescent phase considering MAT as Gold standard. The limitation of this test includes its ability to give information about the infecting serovar because of these both are genus-specific nature. Therefore MAT test is a useful tool for epidemiological purpose.

The microscopic agglutination test (MAT) (WOLFF, 1954) is still the 'corner-stone' of leptospirosis diagnosis. However, the test has many disadvantages. Considerable laboratory infrastructure and skilled manpower are required for performing MAT. Many strains of leptospires have to be maintained in the laboratory for use as antigens in the test. Standardisation of the test can detect both IgM and IgG antibodies, but it may fail to demonstrate low levels of IgM antibodies during the early stage of the disease. The value of MAT lies in its ability to recognize the infecting serogroup, especially in repeat sample collected 10-14 days after the first specimen. Therefore, this test is a useful tool for epidemiological purposes.

So, the second-generation assay included in our study (Leptocheck and ELISA) showed significantly higher sensitivity with early acute phase sera than the reference or first generation method (MAT) while retaining high specificity and should greatly improve the rapid detection of leptospirosis in the field.

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