IgM Dot-ELISA Assay using prevalent *Leptospira* strain for diagnosis of leptospirosis

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IgM Dot-ELISA Assay using prevalent *Leptospira* strain for diagnosis of leptospirosis

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Abstract

**Background:** A test strip IgM Dot-ELISA assay for the detection of leptospire-specific IgM antibodies in human sera was developed. Antigen dotted on a nitrocellulose paper strip was the sonicated antigen prepared from *Leptospira* serovar prevalent in this area, i.e., Icterohaemorrhagiae.

**Objective:** The aim of this study was to evaluate an in-house prepared IgM Dot-ELISA test strip for the diagnosis of leptospirosis.

**Material and methods:** The ability of the IgM Dot-ELISA to diagnose leptosporil infection was evaluated by testing 125 serum samples, 75 serum samples from 93 laboratory-confirmed leptospirosis case patients with positive result in the standard microscopic agglutination test (MAT) and 25 serum samples from patients with various diseases other than leptospirosis and 25 from healthy individuals. The results of Dot-ELISA were also compared to that of a commercially available IgM ELISA.

**Results:** Using the results of the MAT as a gold standard, the sensitivity and specificity of the test strip IgM dot-ELISA assay were 97.33% and 96.00%, respectively. The assay showed relatively good negative predictive values (96.00%) thus making the assay ideally suited for rapid screening.

**Conclusion:** In conclusion, the test strip IgM dot-ELISA assay was sensitive enough for use as a screening test for serodiagnosis of leptospirosis. The assay was simple, inexpensive, and easy to perform for both a single test format and a large number of specimens.

**Keywords:** Leptospirosis, Serodiagnosis, IgM dot-ELISA assay
Introduction

Leptospirosis is a major public health problem worldwide, particularly in the tropics. The clinical presentation of leptospirosis in humans is variable, and can range from a mild flu-like illness to a severe disease with pulmonary hemorrhage, renal failure, and occasionally death. Consequently, leptospirosis is easily mistaken for other febrile illnesses including influenza, dengue fever, meningitis, or hepatitis. Therefore, rapid and appropriate laboratory diagnostic tests are needed to aid clinical case identification and to facilitate the implementation of rapid outbreak investigations for optimal treatment and patient management. Laboratory confirmation of human leptospirosis relies mainly on serological assays aimed at the detection of specific antibodies in serum samples. The microscopic agglutination test (MAT) is considered the standard serologic test that is specific and provides useful epidemiologic data in the form of presumptive serogroups. However, this assay is not suitable for routine laboratories since it is technically demanding, costly, and requires the maintenance of live, hazardous stock serovar cultures and also requires analyses of paired sera to verify the seroconversion which delays the diagnosis. Ideally, a diagnostic test should be easy to perform, rapid and using only a single specimen. Some potentially useful screening tests for use in all routine laboratories have been proposed. Among these serologic approaches, enzyme-linked immunosorbent assay (ELISA) for both IgG- and IgM-leptospiral antibodies have been developed and several commercial test kits are available, mostly using broadly reactive Leptospira antigen obtained from nonpathogenic L. biflexa serovar Patoc. However, the use of this serovar may affect the sensitivity of testing in some regions where different leptospiral serovars predominate that do not induce antibodies that crossreact with serovar Patoc. Most of the tests aimed for the detection of leptospiral IgM which is detectable from about the 2nd-5th day of symptoms that can help in the rapid diagnosis of the disease by using a single serum sample. In the present study, a test strip was prepared using antigen from prevalent serovar in a form suitable for diagnostic format. The test strip was evaluated and compared with the standard MAT using single serum samples from patients with known MAT titers and also with a commercially available IgM ELISA.

Materials and Methods

Samples:

Blood samples 458 suspected cases of leptospirosis from December 2006 to February 2009 were sent to the Microbiology department in JIPMER which is a major tertiary care hospital in Puducherry, India. This study was approved by the ethical committee of our institution. 93 cases (68 Males and 25 Females) aged 3-50 years were confirmed as cases of leptospirosis by doing MAT for all the samples and was considered positive at serum titre of 1 in 100. Out of these 93 cases, 75 serum samples were taken for testing with IgM Dot-ELISA. Control serum samples were obtained from 25 apparently healthy individuals with no clinical or epidemiological history of leptospirosis who lived in Puducherry and from 25 patients with various
diseases other than leptospirosis including syphilis (n = 5), hepatitis B (n=7), hepatitis C (n=3) and typhoid (n = 5), dengue (n=5). None of these control cases reacted in the MAT at serum titer > 1:100.

**Microscopic Agglutination Test (MAT):** was performed by standard procedure using the following twelve serovars.

1. *Leptospira interrogans* - Australis (strain Ballico),
   - Autumnalis (strain Bankinang)
   - Bataviae (strain Swart)
   - Canicola (strain Hond Utrecht IV),
   - Hebdomadis (strain Hebdomadis)
   - Icterohemorrhagiae (strain RGA)
   - Pomona (strain Pomona)
   - Pyrogenes (strain Salinem),

2. *Leptospira kirschneri* - Grippotyphosa (strain Moskva V)

3. *Leptospira borgpetersenii* - Javanica (strain Poi),
   - Tarassovi (strain tarassovi) and


All the strains were obtained from National Leptospirosis Reference Centre, Regional Medical Research Centre (WHO collaborating centre for diagnosis in leptospirosis, ICMR) in Port Blair, Andaman and Nicobar islands. These serovars were maintained in semisolid 0.1% Ellinghausen, McCullough, Johnson and Harris or EMJH agar (Hi-Media) supplemented with 10% enrichment (Tween 80 and Bovine serum albumin) at 28-30°C in screw capped test tubes.

**IgM ELISA:** It was performed for using the IVD LEPTOSPIRA IgM Microwell ELISA Test (IVD Research Inc, Carlsbad, CA92010 USA, as per the manufacturer’s instructions. The antigen coated on the wells of the microtitre plates was a whole cell antigen obtained from the *Leptospira biflexa* - (Patoc I). The absorbance of positive and negative control serum provided in the kit was used for calculations. A negative result was defined as an absorbance of 0.0-0.3 optical density (OD) units, an equivocal result as 0.5 to ≤1 OD units and a positive result as >1.0 OD units.
IgM Dot- ELISA

Preparation of the test strip

Sonicated antigen was prepared from *Leptospira interrogans* serovar Icterohaemorrhagiae as per Tansuphasiri et al with a few modifications. Briefly, the organism was cultivated in (or EMJH) medium, and incubated at 30°C with shaking for 7 days to yield a cell density of about 10^8 cells/ml. The organisms were killed with 0.5 mg/L sodium azide, and disrupted by sonication at 20 kHz for 3 periods each of 3 min. The sonicated leptospiral antigen (Ag) was diluted in 0.05 M carbonate buffer (pH 9.6) and 2 µl (protein concentration, 0.3 µg/2 µl) was dotted onto a strip (0.8 cm X 2.5 cm) of nitrocellulose (NC) membrane. After being air-dried, the paper strips were treated with blocking buffer (phosphate buffered saline, pH 7.2 containing 0.1% Tween 20[PBS-T] and 5% bovine serum albumin [BSA]) for 30 min at room temperature. The paper strip was air-dried and was then stored in a small sealed plastic bag at 4°C until used for testing.

Test procedure

The test strip was numbered with the corresponding serum numbers. The test was performed in a 2 ml-microtube by adding 20 µl of serum to 200 µl of PBS-T buffer (PBS, pH 7.2, containing 0.1% Tween 20) to make a dilution of 1:10. Once the serum was mixed by gentle shaking the microtube, a test strip was added. The microtube was placed down horizontally, and left at room temperature for 30 min with gentle shaking. Each test strip was taken out and washed with PBS-T in the same container for 15 min, twice. The strips were then incubated with horseradish peroxidase (HRP)-conjugated anti-human IgM (Invitrogen) (1:1000 in PBS-T containing 1% BSA) for 30 min, then washed twice with PBS-T for 10 min, and developed with diamino benzaldehyde (DAB)(Hi-Media) for 15 min in dark. The reaction was stopped by rinsing the strips with PBS. Appearance of brown colour dot on the nitrocellulose membrane indicated positive result. Non-appearance of brown colour dot on the nitrocellulose membrane indicated negative result.

Statistical analysis: Sensitivity and specificity of the test strip IgM dot-ELISA assay were calculated by using the Chi-square test; p of < 0.05 was considered significant.

Results

Among the 458 samples tested, 93 fulfilled the criteria for diagnosis (a titer of> 1:100 in MAT) and were considered as cases of leptospirosis, Out of these 93 samples, only
75 serum samples were tested because only a limited quantity of HRP-conjugated anti-human IgM was available. Control samples (50) including samples from patients with diseases other than leptospirosis (n = 25), and normal healthy controls (n = 25) were negative for leptospirosis and were considered as non-cases. IgM dot-ELISA assay detected 73 cases of leptospirosis out of the 75 samples tested; there were 2 (2.66%) false positive results from patients with other diseases (i.e., one with dengue fever and other with hepatitis B), and 2 false-negative results (4.00%). The sensitivity of the test strip IgM dot-ELISA assay was 97.33%, its specificity was 96.00%, its PPV was 97.33 % and its NPV was 96.00%. IgM dot-ELISA assay showed the results with statistically significant differences from MAT (p < 0.001).(Table 1)

When IgM Dot –ELISA was compared to commercially available IgM ELISA, a sensitivity of 98.67% and specificity of 96.00% was observed. (p<0.001) (Table 2). Only one sample failed to give positive result with Dot-ELISA which was IgM ELISA positive. Even though both these tests showed comparable results, the rapidity, simplicity, single sample testing without any technical expertise makes this test more suitable as a rapid screening test

Discussion

Leptospirosis has been under diagnosed and under reported in India due to the lack of awareness of the disease, inadequate epidemiological data and unavailability of appropriate laboratory diagnostic facilities in most parts of the country 15. Serodiagnosis of leptospirosis by an IgM-specific ELISA assay is often used as an alternative to MAT in routine diagnostic laboratories 16. The MAT detects both IgG and IgM antibodies 17 but the MAT titers are usually low during the acute stage of the disease and, hence, diagnosis based on a single serum sample is difficult 18. Detection of IgM antibodies by ELISA is more sensitive than the MAT 3 and gives a positive result earlier in the acute phase of the disease. It is easier to perform and can easily accommodate a large number of samples and gives a less subjective result than MAT 12. The performance of conventional ELISA is hampered due to the limited shelf-life of reagents and the requirement of technical expertise. To overcome these problems, simpler versions of ELISA such as dot-ELISA have been developed in many laboratories 4, 6 due to the use of smaller volumes of reagents and the possibility of visual readings, no requirement of special equipment. So this method can be used to diagnose leptospirosis in peripheral laboratories with relatively little expertise. Comparative evaluation of several commercial test kits for use as rapid screening methods for serodiagnosis of acute leptospirosis in different countries 19, 20, 21 showed the variability in screening test sensitivities and specificities. The screening test’s sensitivity in any given setting is dependent on the ability of test antigens to detect antibodies produced against the site-specific leptospiral serovars. Hence, laboratories need to validate the performance of these screening tests for use in the setting 22. So, in the present study, an IgM-Dot ELISA in a strip form for the detection of leptospire specific IgM antibodies has been standardized and evaluated. In this test, antigen was used from the locally prevalent leptospiral serogroup, in a form suitable for diagnostic
format which could be done on single or large number of samples for rapid detection of specific IgM antibodies in human leptospirosis.

The results given in Table 1, shows that the test strip IgM dot-ELISA assay compared very suitably with the MAT test. Two serum samples failed to react in the IgM-dot ELISA assay, but were reactive in the MAT (sensitivity, 97.33%). In the present study the assay was sensitive for infections with strains of several serogroups when using these MAT-positive serum samples (serum titers ≥1:100). These serogroups included Icterohemorrhagiae, Pomona, Pyrogenes, Grippotyphosa Australis, Hebdomadis and Javanica. However, knowledge of the serogroup has no clinical implications.

Specificity was 96.00 % in the test strip IgM-dot ELISA compared to the MAT test. Two false positive reactions i.e. Dot ELISA-positive/MAT-negative were obtained from 2 patients with other diseases. The IgM antibody detected in these patients could be persisting antibody due to previous leptospiral infection or cross-reacting antibody. In this study, the test strip IgM dot-ELISA assay performed with sera at 1:10 dilution since this dilution of patient serum was found to give best results. The antigen used for this test was a crude sonicated preparation of endemic pathogenic leptospiral serovar.

Other studies evaluating Dot-ELISA showed similar results to that obtained in the present study.

A Dot enzyme-linked immunosorbent assay which used a proteinase-K resistant antigen (PK-Dot-ELISA) to detect antileptospiral IgM antibodies was compared to the microscopic agglutination test (MAT). The PK-Dot-ELISA presented a sensitivity of 92.1% and a specificity of 97.5%.

In another study, dot-ELISA, were developed to detect specific IgM antibodies using pool sonicated antigen prepared from three of the most reactive serovars of *Leptospira* sp. in Thailand; the sensitivity and specificity was found to be 98.96% and 93.93% respectively. The Dot-ELISA evaluated in this study offered good negative predictive values 96.00%, thus making the test ideally suited for rapid screening. The positive predictive value (PPV) of the dot ELISA was 97.33 %. Though the preliminary results are very encouraging, this test still has to be evaluated further by testing more samples both of positive and control serum samples from healthy individuals and from other febrile illnesses.

**Conclusion**

In conclusion, the test strip IgM dot-ELISA assay using locally prevalent leptospiral antigen offered good sensitivity and specificity; yielding accurate results comparable to the reference MAT. The assay was simple, inexpensive, and easy to perform, with visual reading of the results that do not require special equipment. Performance of the assay was also useful for both a single assay format and a large number of specimens which could be completed in approximately two hours. Thus, it could be used as an initial screening test for leptospiral infection, with subsequent confirmation of positive test results by MAT.
References


Table 1: Comparison of results of IgM Dot-ELISA and MAT in leptospirosis cases and controls

<table>
<thead>
<tr>
<th>Serological test</th>
<th>MAT positive</th>
<th>MAT negative</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgM Dot ELISA positive</td>
<td>73</td>
<td>2</td>
<td>75</td>
</tr>
<tr>
<td>IgM Dot ELISA negative</td>
<td>2</td>
<td>48</td>
<td>50</td>
</tr>
<tr>
<td>Total</td>
<td>75</td>
<td>50</td>
<td>125</td>
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</table>

Sensitivity = 97.33%  95%CI = 94.49%, 100.17%
Specificity = 96.00%  95%CI = 92.57%, 99.43%
Positive Predictive value = 97.33%
Negative Predictive value = 96.00%

Table 2: Comparison of results of IgM Dot-ELISA and commercial IgM ELISA in leptospirosis cases and controls

<table>
<thead>
<tr>
<th>Serological test</th>
<th>IgM ELISA positive</th>
<th>IgM ELISA negative</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgM Dot ELISA positive</td>
<td>74</td>
<td>2</td>
<td>76</td>
</tr>
<tr>
<td>IgM Dot ELISA negative</td>
<td>1</td>
<td>48</td>
<td>49</td>
</tr>
<tr>
<td>Total</td>
<td>75</td>
<td>50</td>
<td>125</td>
</tr>
</tbody>
</table>

Sensitivity = 98.67%  95%CI = 96.68%, 100.66%
Specificity = 96.00%  95%CI = 92.57%, 99.48%
Positive Predictive value = 97.37%
Negative Predictive value = 97.96%