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RAPID SERODIAGNOSIS OF LEPTOSPIROSIS BY LATEX AGGLUTINATION TEST AND FLOW-THROUGH ASSAY

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Abstract

**Purpose:** Diagnosis of leptospirosis facilitates patient management and initiation of therapy. The microscopic agglutination test (MAT) is the serological test used in reference laboratories because of its high degree of sensitivity and specificity. But the results are not available quickly for patient management. In the present study, in order to develop a simple, rapid immunodiagnostic assay, one of the outer membrane proteins (OMPs), recombinant LipL41 (rLipL41) has been utilised in latex agglutination test (LAT) and flow-through assay. **Methods:** Part of LipL41 gene was expressed in *Escherichia coli* system and purified. The rLipL41 antigen of pathogenic *Leptospira interrogans* serovar Icterohaemorrhagiae, which is conserved in all pathogenic *Leptospira* spp. was used as capture antigen in the LAT and flow-through test. Both tests are very rapid and could be completed within 5 minutes. The sensitivity and specificity of rLipL41 was assessed and evaluated in LAT and flow-through assay in comparison with standard MAT. **Results:** The sensitivity and specificity of the LAT were 89.70 and 90.45% and flow-through assay were 89.09 and 77.70%, respectively. **Conclusions:** The developed LAT and flow-through assays were simple, rapid and economical for the detection of leptospira infection and suitable for large-scale screening of samples in endemic areas without any sophisticated equipment.

**Key words:** Diagnosis, flow-through assay, latex agglutination test, leptospirosis, rLipL41

Leptospirosis is a zoonosis of worldwide distribution, caused by infection with pathogenic spirochaetes of the genus *Leptospira*. The organisms are maintained in nature by chronic renal infection of carrier mammals, which excrete the organisms in their urine. Humans become infected through direct exposure to infected animals or their urine or through indirect contact via contaminated water or soil. Leptospirosis has been recognized as an emerging infectious disease, in part because of large-scale outbreaks associated with recreational activities. Investigation of outbreaks would be greatly enhanced by the availability of rapid and sensitive diagnostic assays, which can confirm the diagnosis early in the clinical illness.

Diagnosis is usually accomplished retrospectively by serology, because culture requires both special media and incubation for several weeks. Serological diagnosis by microscopic agglutination test (MAT) invariably requires testing of acute and convalescent sera, since agglutinating antibodies often are not detectable during the acute stage. IgM antibodies become detectable 5–7 days after the onset of symptoms and the use of IgM-ELISA assays for presumptive diagnosis has been evaluated in numerous populations.

From the clinical and epidemiological standpoint, a rapid method which is highly specific for the pathogenic strain of leptospires is urgently needed. In the present study, latex agglutination test (LAT) and flow-through immunosassay have been developed for direct screening of leptospiral antibodies in humans by simple visual identification. The assays developed in the study use a broadly reactive leptospiral antigen to capture *Leptospira*-specific antibodies present in the suspected sera. These developed assays for the detection of leptospiral infection in endemic areas were found to be simple, rapid and effective.

**Materials and Methods**

**Bacterial strains and media**

A panel of eight *Leptospira interrogans* serovars - viz., Australis, Autumnalis, Canicola, Javanica, Pomona, Icterohaemorrhagiae, Grippotyphosa and Pyrogenes - was used for MAT. They were grown in EMJH liquid and semi-solid media (Difco, USA) at 29-30 °C and the growth was assessed by darkfield microscopy regularly. These reference leptospiral strains were obtained from Koninklijk Institute Voor De Tropen (KIT), Amsterdam, The Netherlands and National Leptospirosis Reference Center, Port Blair, Andaman and Nicobar Islands, India. *Escherichia coli* DH5α strain was used as host cells for the expression of recombinant antigen and was grown and maintained in the LB broth and agar.

**Serum samples**

Three hundred and seventeen human serum samples clinically suspected for leptospirosis were collected from Leptospirosis Research Laboratory, Madhavaram Milk Colony,
Chennai and hospitals in and around Chennai city. Five blood samples collected from apparently healthy individuals were included to serve as negative controls. All the serum samples were stored in aliquots at −20°C until used.

**Microscopic agglutination test**

The gold standard serodiagnostic test for leptospirosis is MAT, which was performed as per the method of Cole et al.\(^1\) Briefly, the sera from the patients were serially diluted from 1:50 to 1:3200 in phosphate buffered saline (PBS), pH 7.2 and allowed to react with live antigen suspensions of the panel of eight reference leptospiral serovars. After two hours incubation at 37°C, the serum-antigen mixtures were examined by darkfield microscopy for the presence of agglutination/clearance and the titres were determined. Reciprocal agglutination titres of greater than or equal to 100 were considered as positive reactions.

**Recombinant LipL41 antigen preparation**

The outer membrane protein (OMP) gene LipL41 was partially amplified from the reference *Leptospira interrogans* serovar Icterohaemorrhagiae (strain RGA) by polymerase chain reaction (PCR). The PCR primers used to amplify part of the leptospiral OMP gene LipL41 were forward oligonucleotide primer with an *NcoI* restriction endonuclease site (underlined): 5′- TG TTA CCC ATG GGG AGA AAA TTA TCT TCT CT –3′ and reverse oligonucleotide primer with an *XhoI* restriction endonuclease site (underlined): 5′ AAA GGA CTC GAC TTA CTT TGC GTT GCT TTC –3′, which were selected based on the LipL41 primer sequences of *L. kirschneri* species by Haake et al.\(^2\) The PCR amplicon size of 1077 bp (Genbank accession no. DQ 132992) was cloned into an expression vector pProEXHTb′ (Invitrogen). The expression of recombinant fusion protein (rLipL41) of approximately 39 kDa was achieved by adding isopropyl β-D thiogalactoside (IPTG) at 1 mM final concentration. Purification of the expressed protein was carried out by sequential addition of buffer A (50 mM Tris-HCl, pH 8.0, 7.5% glycerol, 0.1 mM EDTA and 50 mM NaCl), buffer B (50 mM EDTA, 10% Triton X-100, 2.5 mg/mL lysozyme and 2 mM PMSF or β-Mercaptoethanol) and 5 mM MgCl₂ to the cell pellet and incubation in ice for one hour. Then the cells were sonicated at constant pulse twice for 30 seconds. The lysate was clarified at 17,950 × g for 30 minutes at 4°C. The supernatant was subjected to affinity purification. The polyhistidine tag (6X-His) of the fusion protein was utilized for purification of fusion protein by Ni²⁺-NTA affinity chromatography (Qiagen). The purified protein was analysed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting. The concentration of the protein was determined by Lowry’s method and stored in aliquots at −80°C until used.

**Latex agglutination test**

LAT was standardized with rLipL41 fusion protein as per the method of Dey et al.\(^3\) with minor modifications. Briefly, a 10% suspension of the latex beads (0.8 µm dia, Sigma, USA) was coated with rLipL41 antigen (25 µg/dL) using 0.06 M sodium carbonate-bicarbonate buffer (Na₂CO₃ 1.59 g and NaHCO₃ 2.93 g in 1 L of distilled water, pH 9.6) and kept at 37°C for 6 hours with constant shaking. The sensitized beads were centrifuged at 6800×g for 3 min and the pellet was resuspended as a 1% suspension in PBS, pH 7.2 containing 5 mg/dL of bovine serum albumin (BSA). The latex beads were left at 37°C overnight with constant shaking. Latex beads were centrifuged as before and resuspended in PBS, pH 7.2 containing 0.5 mg/dL of BSA and 0.1% sodium azide as 0.25% suspension. The sensitized latex beads were stored at 4°C until used. The LAT was performed on glass slides (VDRL slides) by mixing equal volumes of serum samples and sensitized beads (20 µL each). The slide was rocked briefly for 2 minutes to mix the coated beads and serum samples. The results were read well within 2 minutes by the naked eye. The test was positive if agglutination had occurred, which was indicated by the formation of fine granular particles, which tend to settle at the edge of the beads-serum mixtures. If the suspension remained homogenous, the test was declared negative.

**Flow-through enzyme immunoassay**

The test was performed in an immunofiltration device, in which the nitrocellulose membrane (NCM) was pressed tightly over a water-absorbing layer. The antigen (rLipL41) dotted onto the NCM would capture the specific antibodies in the serum samples. The protein A gold colloidal conjugate would bind to the captured antibodies and impart a reddish colour over the antigen-dotted area. If the samples are negative, the protein A gold colloidal conjugate would not be retained after the washing step.

The flow-through assay was developed as per the method of Paepens et al.\(^4\) with minor modifications. Briefly, NCMs (0.45 µm, Advanced Microdevices Pvt. Ltd., Ambala Cantt., India) were cut into small squares (2 cm × 2 cm). The immunofiltration device designed for the study was a small rectangular plastic module with a test window at the centre. The module was filled with absorbent pad and the NCM was pressed tightly over the absorbent pad in the test window. One microlitre capture antigen, rLipL41 was diluted in PBS, pH 7.2 (2 mg/dL) and was added to the NCM in the “T” side of the test window and 1:100 diluted negative human serum was added (1 µL) at the “C” side of the test window to serve as reagent control. The modules were kept at 37°C for 1 hour, sealed in plastic bags and stored at room temperature.

The assay was performed by adding 100 µL of washing buffer (1 mM Na₂HPO₄, 1 mM NaH₂PO₄, pH 6.0, 0.9% saline, 0.5 dL Triton-X 100 and 1% BSA) to the test window twice to wet and block the NCM. The serum samples to be tested were diluted to 1:10 in the wash buffer (100 µL), then added to the test window and allowed to absorb completely. After washing with washing buffer, 100 µL of protein A colloidal
gold conjugate (Advanced Microdevices Pvt. Ltd., India) was added to the test window. Finally, 100 µL of washing buffer was added to remove all unbound conjugate. The protocol can be completed within 2 minutes and the results can easily be determined by the naked eye. The appearance of two red colour dots in the ‘T’ and ‘C’ sides of the test window indicated a positive reaction and the absence of a red colour dot in the ‘T’ side of the test window indicated a negative reaction. The test was considered invalid if there were no red dots in the test window.

Relative sensitivity, specificity and concordance

The relative sensitivity, specificity and concordance (in %) of the LAT and flow-through assay for the detection of leptospiral antibodies were determined in comparison with the MAT as described below.

\[
\text{Sensitivity} = \frac{a}{a + c} \times 100
\]

where “a” is the number of serum samples positive by the test and MAT, “c” the number of serum samples positive by MAT but negative by the test.

\[
\text{Specificity} = \frac{d}{b + d} \times 100
\]

where “d” is the number of serum samples negative by the test and MAT, “b” the number of serum samples negative by MAT but positive by the test.

\[
\text{Concordance} = \frac{a + d}{a + b + c + d} \times 100
\]

Results

Latex agglutination test

The test was performed with recombinant antigen-sensitized beads and suspected serum samples. The sensitivity and specificity were assessed in comparison with MAT. A total of 322 human serum samples were tested by LAT; among them, 163 samples were positive and 159 samples were negative (Table 1). The five negative samples were found to be negative by both the tests.

Optimization of flow-through assay

A series of experiments were performed to determine the optimal conditions and to adjust the test sensitivity to an appropriate level. Checkerboard titrations were carried out with various concentrations of antigen (10 mg/dL, 5 mg/dL, 2 mg/dL, 100 µg/dL and 50 µg/dL) and the intensities of colour development to determine the optimal concentration of the capture antigen (Table 2). Various dilutions of serum samples and the intensities of colour development were also checked (Data not shown). The assay was optimized using 2 mg/dL concentrations of capture antigen (rLipL41) and 1:10 dilution of the serum samples to be tested (Fig. 1). The sensitivity and specificity of the assay were compared with MAT (Table 3).

Table 1: Comparison of latex agglutination test with microscopic agglutination test

<table>
<thead>
<tr>
<th>Microscopic agglutination test</th>
<th>LAT</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>148 (a)</td>
<td>163</td>
</tr>
<tr>
<td>-</td>
<td>17 (c)</td>
<td>159</td>
</tr>
<tr>
<td>Total</td>
<td>165</td>
<td>322</td>
</tr>
</tbody>
</table>

\[\chi^2 = 206.72^{**}; \ k = 0.80, \text{ Sensitivity: 89.7%; specificity: 90.45%; concordance: 90.06%}\]

Table 2: Determination of antigen concentration for Flow-through assay

<table>
<thead>
<tr>
<th>Concentration of rLipL41</th>
<th>Human serum samples with reciprocal microscopic agglutination test titres in brackets</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 mg/dL</td>
<td>+++ + + +</td>
</tr>
<tr>
<td>5 mg/dL</td>
<td>+ + + + +</td>
</tr>
<tr>
<td>2 mg/dL</td>
<td>– + + + +</td>
</tr>
<tr>
<td>1 mg/dL</td>
<td>– – + + +</td>
</tr>
<tr>
<td>100 µg/dL</td>
<td>– – – + +</td>
</tr>
<tr>
<td>50 µg/dL</td>
<td>– – – – –</td>
</tr>
</tbody>
</table>

*Slightly haemolysed serum sample, +++ High intensity of the colour; ++ Moderate intensity of the colour; + Poor intensity of the colour; –Negative
## Table 3: Comparison of Flow-through assay with standard microscopic agglutination test

<table>
<thead>
<tr>
<th>Microscopic agglutination test</th>
<th>Flow-through</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>+</td>
<td>147 (a)</td>
<td>35 (b)</td>
</tr>
<tr>
<td>–</td>
<td>18 (c)</td>
<td>122 (d)</td>
</tr>
<tr>
<td>Total</td>
<td>165</td>
<td>157</td>
</tr>
</tbody>
</table>

\[ \chi^2 = 130.22 \]; k = 0.63, Sensitivity: 89.09%; specificity: 77.70%; concordance: 83.54%

## Discussion

Leptospirosis is a major public health problem throughout the world, particularly in the tropics.[2,3] Clinical recognition of leptospirosis is usually difficult because leptospires can affect different organs, resulting in a wide variety of clinical presentations. Timely diagnosis of leptospirosis is essential because prompt and specific treatment, as early as possible, is important in ensuring a favourable clinical outcome.[15] The most commonly used immunological methods for antibody detection are indirect haemagglutination test (IHA), IgG-ELISA, IgM-ELISA as well as the dot-ELISA. Most of these tests are quite sensitive and specific, but these tests are all time-consuming, sometimes requiring overnight incubation steps. Moreover, these tests need a laboratory equipped with proper instruments and trained personnel. These distinct drawbacks make them unsuitable for direct use in outbreak situations. Hence, there is a need to develop a simple, novel and a rapid test for screening of sera in endemic areas.

The focus of the present study is to develop a rapid diagnostic kit using recombinant antigen, which is conserved among all the pathogenic serovars of leptospires. In this study, LAT and Flow-through assay were developed using recombinant antigen (LipL41) and their results were analysed with standard MAT. The MAT result indicated that commonly occurring *L. interrogans* serovars in human beings were Australis and Icterohaemorrhagiae. The predominance of these serovars in humans has been reported earlier in the country. The results of the developed assays showed relatively higher sensitivity and specificity. Both the tests were very easy to perform and could be completed within five minutes. The flow-through assay developed in this study for leptospiral diagnosis showed the sensitivity as that of LAT. Eliades *et al.*[16] developed a dot immunobinding assay for the serodiagnosis of human hydatidosis. The assay was performed with samaron blue colloidal dye particles conjugated to hydatid antigen or protein A as visualizing agents for detecting antibodies. They concluded that the detection of anti-HA antibodies had agreed closely with the data from ELISA. Xiao Xiang *et al.*[17] developed a colloidal dye immunofiltration assay (CDIFA) for antibody detection in schistosomiasis using a red colloidal dye, R-3, as the antigen-antibody detecting reagents. In the present study, colloidal gold dye conjugated to protein A was used for the detection of leptospiral antibodies. Experiments to determine the sensitivity of the flow-through test revealed that a 1:10 serum dilution was optimum. Serum dilutions less than 1:10 generated false-positive results. Haemolysed serum samples also showed false-positive results. We therefore suggest that haemolysed serum samples should not be included. All the negative samples in the study were negative by MAT, LAT and flow-through assays. The sensitivity of the flow-through assay was found to be 89.09%, which was in agreement with the MAT for serodiagnosis of leptospirosis.

In leptospirosis, antibodies usually appear within 5-7 days after the onset of symptoms and antibodies persist in detectable quantities for many months.[18,19] From a clinical point of view, early detection of disease is very important. The fact that the specific immunological reactions are characterized by their increased production of antibodies, which permits the development of sensitive, early-detection assays, forms the basis for these assays. The sensitivity of the LAT was equal to or slightly higher than that of MAT. The overall sensitivity and specificity of this test were 89.7 and 90.45%, respectively, which was in agreement with the MAT for the diagnosis of acute human leptospirosis as described by Smits *et al.*[20] whose overall test sensitivity and specificity were 82.3 and 94.6%, respectively. From this study, we conclude that LAT and flow-through test are potential formats for rapid large-scale screening of samples in endemic areas without any sophisticated equipment.

## Acknowledgements

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## References


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