Original Article

Evaluation of High Performance Liquid Chromatography Purified Leptospiral Antigen for the Diagnosis of Leptospirosis

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SUMMARY: Leptospirosis is a re-emerging infection, and often occurs in outbreaks. Early diagnosis is important for appropriate control measures and treatment. The sensitivity and specificity of the diagnostic test depends on the antigen, which is used for the detection of antibodies. In the present study, an effort was made to purify and characterize leptospiral antigens, and their performance in the laboratory was assessed. Leptospira biflexa semaranga, Patoc was subjected to processing by the heat extraction method, and the crude antigenic preparation was subjected to high-performance liquid chromatography for purification. The purified antigen fractions were subjected to enzyme-linked immunosorbent assay (ELISA) to determine their reactivity. Only the reactive fractions were tested for specificity by ELISA by allowing the fractions to react with positive sera of patients with non-leptospiral illness. H1, the purified antigen fraction of the heat-extracted preparation, was found to be the most reactive and specific of all fractions tested. The protein was found to have a molecular weight of 50 kDa. The performance of H1 in the laboratory was assessed by formulating an in-house ELISA using the H1 antigen. The results were compared with those of microscopic agglutination testing and commercial ELISA. The specificity and sensitivity of in-house ELISA using the H1 antigen were found to be 93.3 and 85.0%, respectively.

INTRODUCTION

Leptospirosis is an infectious disease with a worldwide distribution that is caused by pathogenic spirochetes belonging to the genus Leptospira. The disease poses a diagnostic dilemma to clinicians, as the clinical presentation ranges broadly from a mild influenza-like illness to multiorgan involvement. Laboratory confirmation is essential to reach an accurate diagnosis.

The rapid and precise diagnosis of leptospirosis is important to provide early and specific treatment and to control infection. Microscopy and culture methods are unreliable, and neither has adequate sensitivity (1). The gold-standard microscopic agglutination test (MAT) requires the maintenance and handling of live cultures of leptospires, which is laborious and associated with risk to laboratory technicians. The specificity of currently available commercial enzyme-linked immunosorbent assay (ELISA) kits is questionable. Thus, a sensitive and specific diagnostic test for the early diagnosis of leprospirosis remains to be developed.

The present study was aimed at the identification of an antigen that could be successfully employed for the development of a serological test for the diagnosis of leptospirosis.

MATERIALS AND METHODS

Selection of antigen for in-house ELISA: A bulk culture of Leptospira biflexa serovar Patoc was washed three times by centrifugation at 12,320 × g with phosphate buffered saline (PBS; pH 7.2). The bacterial pellet was suspended in bicarbonate buffer (pH 9.6), and was then treated with formalin (final concentration, 0.5% v/v), heated in a boiling water bath for 30 min, and centrifuged for 30 min at 12,400 × g (2). The supernatant was subjected to high-performance liquid chromatography (HPLC; 2410 Refractive Index Detector; Waters Corp., Milford, Mass., USA; column: sugar pak I, column temperature, 70°C; flow rate, 0.4 ml/ml). The peaks obtained by HPLC were collected with the sample in the mobile phase (warm, deionized, bacteria-free water containing approximately, 0.0001 M calcium EDTA), and the sample was lyophilized and stored in purified fractions at −20°C.

The eluted purified fractions were then subjected to ELISA according to the method described by Matsuo et al. (3) to evaluate reactivity and specificity. Sera from three systemic leptospirosis patients (clinically diagnosed and MAT- and IgM ELISA-positive) were pooled and used as a positive control, and the pooled sera from five healthy controls were used as the negative control.

For reactivity testing, each purified fraction was allowed to react with positive control serum and negative control serum at 1:20 dilution. The conjugate used was anti-human IgM (Mu chain specific)-peroxidase conjugate (Sigma-Aldrich, St. Louis, Mo., USA) and the substrate tetramethylbenzidine/hydrogen peroxide (Bangalore Genei, Bangalore, India). If the absorbance of the positive serum was twice that of the negative serum using either of the antigen fractions, the antigen was considered as a reactive antigen (3). Only reactive fractions were tested for specificity by allowing them to react with sera from patients with non-leptospiral illness (i.e., typhoid, malaria, dengue fever, syphilis, and hepatitis). The serum dilutions used were ranged from 1:20 to 1:320, as described by Matsuo et al. (3).

The most reactive and specific fraction was selected as the candidate antigen, and was chemically characterized by the measurement of the total amount of proteins (Lowry’s...
method), total carbohydrate (phenol-H$_2$SO$_4$ method), and fatty acid content (gas chromatography). The candidate antigen was subjected to polyacrylamide gel electrophoresis (PAGE) according to the protocol of Laemmli with minor modifications (4). The gel was stained by coomassie brilliant blue (CBB) (5).

The bands obtained were blotted onto a polyvinylidene difluoride membrane (electrophoresis-grade; Sigma, St. Louis, Mo., USA) and were treated separately with pooled IgM-positive serum (1:20), pooled IgM-negative sera, and sterile distilled water at room temperature for 1 h. This procedure was followed by treatment with conjugate solution (anti-human IgM, peroxidase conjugate; 1:100 dilution using PBST + BSA) for 1 h at room temperature. Substrate solution (diaminobenzidine; Sigma Aldrich—3 mg + 10 μl H$_2$O$_2$ [Qualigen-GlxoSmithKline, Mumbai, India]) added to 5 ml PBS was then allowed to react until the color developed.

In-house ELISA using the candidate antigen: (i) Standardization of the antigen concentration: Initial assays were performed to determine the antigen concentration using serum samples from confirmed leptospirosis cases. Checkerboard titration was performed with 25-, 50-, 100-, and 200-fold serum dilutions at antigen concentrations of 5, 10, and 15 μg per well. In subsequent assays, the optimal dilution rate of the conjugate was determined.

(ii) Determination of cut-off for positivity: To determine the cut-off value for positivity, 42 serum samples from patients with non-leptospiral illness and 40 samples from healthy volunteers were tested.

(iii) In-house ELISA: The wells of polyvinylchloride microtiter plates (Tarsons, Kolkata, India) were coated overnight at 4°C with 50 μl (5 μg) of candidate antigen in carbonate buffer. After incubation at 37°C for 2 h, the plates were kept at 4°C overnight. After washing the wells with PBS (pH 7.2) containing 0.05% (vol/vol) Tween 20 (PBS-T), blocking was carried out with 1% bovine serum albumin (HiMedia, Mumbai, India) for 3 h at room temperature. After the samples were washed three times with PBS-T, 100 μl of an appropriate dilution of test serum (see below) was added to the plates, which were then incubated at 37°C for 1 h. After six washes in PBS-T, 100 μl of anti-human IgM conjugated to peroxidase was added, and the samples were incubated for 1 h at 37°C. This procedure was followed by the addition of 100 μl of substrate (tetramethylbenzidine/hydrogen peroxide), and the samples were incubated in the dark for 30 min. The reaction was stopped by 1N sulfuric acid, and absorbance was measured at 450 nm using a microplate reader (Bio-Rad, Hercules, Calif., USA).

Testing of the performance of in-house ELISA: Fifty serum samples from patients with suspected leptospirosis (based on clinical history) were included in the study. Patients with a fever lasting for more than 8 days with concomitant body aches, myalgia, and conjunctival suffusion were enrolled in the study.

Thirty serum samples were obtained from healthy blood donors as a negative control. Another set of controls was containing sera from 30 patients showing non-leptospiral illness (i.e., typhoid, malaria, dengue, syphilis, and hepatitis). All serum samples were stored at −70°C until use. The Ethical Committee of B. J. Medical College and Sassoon General Hospital (Pune, India) approved the study.

All serum used here was subjected to in-house ELISA according to the protocol described above, as well as to commercial IgM ELISA (Pan-Bio, Brisbane, Australia) according to the manufacturer’s instructions.

MAT and commercial IgM ELISA: All serum samples from patients with suspected leptospirosis were subjected to MAT using 12 serovars according to the protocol of Faine (6). The following serovars were used: *L. interrogans* serovars Rachnati, Pomona, Icterohaemorrhagiae, Canicola, Tarassovi, Butembo, Australis, Bataviae, Pyrogenes, Hebdomadis, Ballum, and *L. biflexa* serovar Patoc. The criterion for a positive MAT was a fourfold rise in antibody titer in paired sera, seroconversion to a titer of at least 200, or a single serum sample showing a titer of 200 or more (7). In situations in which multiple serovars reacted with serum samples, the one that reacted most strongly was considered as the infecting serovar (7).

Statistical analysis: Chi-square, Kappa, and t test analysis were performed where appropriate. Results were considered significant when the *P* value was less than 0.001.

RESULTS

Selection of antigen for in-house ELISA: Heat-extracted antigenic preparation of *L. biflexa* serovar Patoc after purification by HPLC gave 4 peaks (Fig. 1). The peaks were designated as H1, H2, H3, and H4, respectively. Out of these four antigen fractions, only two (H1 and H3) were found to be reactive. The H1 fraction was more reactive (absorbance of positive control sera and negative control sera with H1 was 1.73 and 0.19, respectively) than H3 (absorbance of positive control sera and negative control sera with H3 was 0.23 and 0.08, respectively). Both H1 and H3 reacted with serum from patients with syphilis at a 1:20 serum dilution. The H1 fraction was selected as the candidate antigen, as it was more reactive than H3.

Chemical characterization revealed H1 to be protein with no detectable carbohydrate or fatty acids. CBB staining of SDS-PAGE revealed a broad band between 45 and 66 kDa, approximately at 50 kDa (Fig. 2). In the immunoblot analysis, H1 reacted with the positive sera. As the candidate antigen was a protein with a molecular weight of 50 kDa, it was designated as p50.

In-house ELISA using the candidate antigen: (i) Antigen concentration: Checkerboard titration revealed 5 μg as the appropriate antigen concentration to be used in the assay. The conjugate dilution was determined to be 1:1,000. Ten positive and five negative serum samples were used as the panel sera for in-house ELISA. To avoid the non-specific reaction observed when a lower dilution of the panel sera

![Fig. 1. Heat-extracted antigen showed four peaks on high-performance liquid chromatography (HPLC).](image-url)
was used, we selected a serum dilution of 1:100, which was used for all subsequent test sera.

(ii) Determination of cut-off: The means (M) of the OD of all negative sera for the anti-IgM conjugate was calculated as 0.17. The standard deviation was calculated to be 0.11. The means plus two standard deviations (M + 2SD) were used for all subsequent test sera.

Testing of the performance of in-house ELISA: Since MAT is considered the gold-standard test for leptospirosis, all serum samples were subjected to MAT. Out of 50 samples collected, 20 were positive according to the MAT results (40.0%). When the results of in-house ELISA were compared to those obtained with MAT, 90.0% overall serological agreement was observed (P < 0.001; Kappa, 78.0%). Comparison of commercial ELISA with MAT also yielded significant serological agreement of 84.0% (P < 0.0001; Kappa, 66%). Table 1 shows the MAT results obtained with the serum used to evaluate the in-house ELISA. A diversity of serovars was obtained, yet when the in-house ELISA was evaluated with respect to MAT, the in-house ELISA had a specificity of 93.3% and a sensitivity of 85.0%. Table 2 shows the comparison of results obtained with the p50-in-house ELISA and the commercially available ELISA. Across all parameters, the p50-in-house ELISA was found to perform better than the commercially available kit.

Statistical analysis: When the results of the in-house ELISA was compared with those obtained by MAT, 90.0% overall serological agreement was observed (P < 0.0001; Kappa, 78.0%). Comparison of the commercial ELISA kit with MAT showed 84.0% serological agreement (P < 0.0001; Kappa, 66.0%).

**DISCUSSION**

Improved diagnostic tests for leptospirosis are urgently needed not only to facilitate clinical diagnosis during the initial phase of the disease in individuals, but also for rapid case confirmation during outbreaks. Antibiotic therapy initiated soon after the onset of symptoms can prevent severe complications and death (8).

Serology is the gold-standard approach to diagnosing leptospirosis. The sensitivity and specificity of a serological test depends on the antigen used. The complexity of the leptospiral antigenic structure has posed particular challenges to researchers. Several antigenic preparations have been used for diagnosis so far (9-11). Efforts to develop new, highly sensitive diagnostic tests for use in the acute phase of disease have focused primarily on detecting IgM binding to whole-cell antigen preparations. The immunodominant moiety in the whole cell preparation appears to be a broadly reactive antigen that is a disaccharide epitope present in non-pathogenic leptospires as well as in a diverse group of non-leptospiral species (12).

In the present study, we used a heat-extraction method to obtain the antigen preparation according to the published protocol of Terpstra et al. (2). In 1985, they used a crude heat-extracted preparation to formulate an ELISA for the detection of specific IgM and IgG in human leptospirosis, however, the antigen was not characterized or purified (2). In 2003, Gowri Priya et al. (13) used a heat extract prepared according to the protocol of Terpstra et al. (2), and purified the lipopolysaccharide (LPS) antigen by filtration, and the purified LPS was used to develop both an IgG and an IgM ELISA, which performed better than tests with the unpurified antigen.

The heat extract used in the present study was subjected to HPLC for purification. Among the four fractions obtained by HPLC, H1 was selected as the candidate antigen, a protein with a molecular weight of 50 kDa (designated as p50). In 1985, Nunes-Edwards et al. (14) identified antibody-accessible proteins on the cell surface of the strain hardjoprajitno. Anti-hardjoprajitno serum efficiently recognized nine distinct protein bands with molecular weights of 63, 55, 51.5, 38, 36, 35, 33, and 21 kDa. They also documented that the cell-surface-exposed proteins of the strain hardjoprajitno exhibit antigenic cross-reactivity with cell-surface proteins of other pathogenic strains. Brown et al. (15) resolved seven outer-
membrane proteins (OMPs) (60, 50, 42.7, 40, 38, 32, and 25 kDa) by SDS-PAGE. All of these seven proteins were observed in all of the *Leptospira interrogans* serovars (e.g., Hardjo, Pomona, Icterohaemorrhagiae, Grippotyphosa, and Canicola). The protein antigen obtained in the present study that had a molecular weight of 50 kDa resembled the 50-kDa OMP identified by Brown et al. (15).

The MAT used in the current study identified a diverse set of serovars (Table 1), which indicated p50 as a broadly reactive genus-specific antigen. The relative sensitivity of the in-house ELISA using the p50 antigen was 85.0%, which suggested that this test would be more useful for detecting true-positive cases than the commercially available ELISA (sensitivity, 75.0%). However the specificities of the in-house and commercial ELISAs were comparable (in-house ELISA, 93.3% versus commercial ELISA, 90.0%) (Tables 2 and 3). The observation that both in-house and commercial ELISA results were positive in MAT-negative cases could have been due to the fact that MAT was performed using a limited number of leptospiral serovars.

Tansuphasiri et al. (10) in 2005 developed a conventional IgM ELISA using pooled sonicated antigens prepared from three of the most reactive serovars of leptospira associated with disease in Thailand. The sensitivity and specificity obtained in that study were 98.9 and 93.3%, respectively, after comparison with MAT results. Ribeiro et al. (9) evaluated IgM ELISA, which used a proteinase K-treated antigen, as a rapid serodiagnostic test. When compared with MAT, IgM ELISA showed 89.9% sensitivity and 97.4% specificity. These results, taken together with those of the present analysis, indicate that purification of the antigen reduced the sensitivity of the test used in the present study. Flannery et al. (16) evaluated IgM ELISA, which had been standardized using recombinant pure proteins (i.e., LipL32, OmpL41, LipL36, and Hsp58). IgM ELISA using all of these pure antigens, with the exception of LipL32, showed low sensitivity in the acute phase of leptospirosis. Such results suggest that the sensitivity of the test will depend on the nature of the antigen used, and that sensitivity may decrease if the specific antigen is used. Another important point to be considered in this context is the location of the selected antigen in the organism. Usually, surface-exposed antigens (e.g., cell-wall and cell-membrane antigens) play an important role in triggering the antibody response. Sometimes antigens react strongly with specific antibodies in vivo. In the case of the p50 antigen, it may occupy a surface or a subsurface location, and thus could be a major antigen involved in antibody production and the immune response. The location of the p50 antigen still needs to be confirmed, and amino acid sequencing of this protein could enable detection of the gene responsible for its production.

The samples tested in the current study were acute-phase samples. The antigen will still need to be evaluated in terms of the role(s) it can play at various other stages of the disease as well.

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**REFERENCES**

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**Table 3. Comparison of in-house ELISA and commercial ELISA**

<table>
<thead>
<tr>
<th>Test</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>PPV (%)</th>
<th>NPV (%)</th>
<th>Serological agreement (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>In-house ELISA</td>
<td>85.0</td>
<td>93.3</td>
<td>89.4</td>
<td>90.3</td>
<td>90.0</td>
</tr>
<tr>
<td>Commercial IgM ELISA</td>
<td>75.0</td>
<td>90.0</td>
<td>75.0</td>
<td>84.3</td>
<td>84.0</td>
</tr>
</tbody>
</table>

PPV, positive predictive value; NPV, negative predictive value.