Identification and evaluation of LPS antigen for serodiagnosis of uveitis associated with leptospirosis

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Leptospirosis is a widespread zoonotic disease that affects all mammals in different parts of the world. Though there are many commercial kits available for the diagnosis of systemic leptospirosis, the nature of the antigen has not been described. Therefore, identification of a specific antigen is important. Since ocular involvement in leptospirosis has been reported, there is a need to identify and characterize the leptospiral antigen for diagnosis of uveitis associated with past leptospiral infection (leptospiral uveitis) and for confirming the clinical diagnosis. Seven-day-old culture of Leptospira biflexa serovar Patoc was used for preparing the antigen. The present study included serum samples from 81 patients with clinical criteria for leptospiral uveitis, 15 cataract controls and 15 non-leptospiral uveitis controls. Serum samples were assayed by ELISA using our antigenic preparation and by a microscopic agglutination test (MAT) using 19 serovars. The antigen prepared had 280 µg LPS ml⁻¹ and no detectable amount of protein. Silver-staining of SDS-PAGE for protein and LPS, dot blot and Western blot analysis and proteinase K and periodate treatment showed that LPS (13–21 kDa and 28 kDa) in our preparation was the relevant antigen for serodiagnosis. IgG antibodies showed reactivity in both leptospiral uveitis patients and controls. However, on the basis of IgM response to LPS, 48 % of the leptospiral uveitis patients were significantly positive compared with controls; 58 % of leptospiral uveitis patients and none of the controls were positive for MAT. When MAT and IgM ELISA results were considered together, 77 % were significantly positive. LPS is identified as a candidate antigen for serodiagnosis of leptospiral uveitis and has sensitivity and specificity of 48 and 90 %, respectively, in ELISA for IgM antibodies. Confirmation of clinical diagnosis with a specific laboratory test would help to initiate the most appropriate treatment for leptospiral uveitis.

INTRODUCTION

Leptospirosis is a worldwide zoonosis caused by spirochaetes belonging to the genus Leptospira. Ocular involvement in leptospirosis following systemic infection was first reported by Adolf Weil in 1886. Uveitis can develop early or late in disease and has been reported up to a year after the initial illness (Farr, 1995; Martins et al., 1998). A large cluster of cases of uveitis was reported from Madurai, in the southern part of India, in 1994 following an outbreak of leptospirosis that occurred after heavy flooding in November 1993. This form of uveitis typically manifests as acute non-granulomatous, diffuse uveitis involving one or both eyes (Rathinam et al., 1997). An elevated antibody titre to leptospires by microscopic agglutination test (MAT) and the detection of leptospiral DNA in the anterior chamber fluid of uveitis patients suggested a possible leptospiral aetiology (Chu et al., 1998).

Detection of specific anti-leptospiral antibodies by MAT is the standard reference test for diagnosis of systemic infection, despite the limitations imposed by the need to maintain cultures of several pathogenic leptospiral serovars and the subjectivity involved in reading the results under dark-field microscopy. As an alternative method, more widely accessible ELISA and dipstick assays using crude extracts have been developed for the diagnosis of acute leptospiral infection (Terpstra et al., 1985). Serological assays like macroscopic agglutination (Wanyangu et al., 1987), indirect haemagglutination (Levett & Whittington, 1998) and microcapsule agglutination (Arimitsu et al., 1982) tests are less sensitive than MAT and identify fewer than 50 % of patients with early-phase leptospirosis. Assays that focus primarily on detecting IgM binding to crude antigen (Adler et al., 1980;
Gussenhoven et al., 1997; Winslow et al., 1997; Smits et al., 1999; Levett et al., 2001), though more sensitive for serodiagnosis, are subject to variations in specificity. However, the nature of IgM-binding antigen has not been identified in these studies. Recently, the immunodominant moiety of the cross-reactive antigen has been characterized as a disaccharide epitope (Matsuo et al., 2000a, b). Validation of such a specific antigen from leptospires in the diagnosis of leptospiral uveitis in humans has not been done so far. Therefore, the objective of the present study was to identify a candidate antigen purified from leptospires and to evaluate its potential to confirm clinical diagnosis of leptospiral uveitis.

**METHODS**

**Recruitment of cases.** Leptospiral uveitis patients attending the Uvea Clinic, Aravind Eye Hospital, Madurai, were recruited for the study based on the following inclusion and exclusion criteria. Clinical diagnosis of leptospiral uveitis was based on a detailed clinical history, an extensive review of systems, a complete ophthalmic examination by slit lamp and indirect ophthalmoscopy and laboratory and ancillary tests.

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**Antigen preparation.** Bulk culture was washed three times by centrifugation at 6600 × g with PBS to remove medium particles. The bacterial pellet suspended in bicarbonate buffer (pH 9.6) 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 10 400 g (Terpstra et al., 1985). Seventeen serovars of Leptospira were obtained from the Royal Tropical Institute (KIT), Amsterdam, The Netherlands (Table 1) and grown in Ellinghausen and McCullough medium as modified by Johnson and Harris in continuous cultures. Samples of bacteria after 4 days of culture were used for MAT. Seven-day-old broth (250 ml) of Leptospira biflexa serovar Patoc was used for preparation of antigen.

**Leptospiral sera.** Nineteen sera of Leptospira were obtained from the Royal Tropical Institute (KIT), Amsterdam, The Netherlands (Table 1) and grown in Ellinghausen and McCullough medium as modified by Johnson and Harris in continuous cultures. Samples of bacteria after 4 days of culture were used for MAT. Seven-day-old broth (250 ml) of Leptospira biflexa serovar Patoc was used for preparation of antigen.

**Antigen preparation.** Bulk culture was washed three times by centrifugation at 6600 × g with PBS to remove medium particles. The bacterial pellet suspended in bicarbonate buffer (pH 9.6) 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 10 400 g (Terpstra et al., 1985). The supernatant was filtered through a 10 kDa membrane. For Western blot analysis, 20 µg LPS per lane was separated on SDS-PAGE and silver-stained for protein (Bloom et al., 1987) and LPS (Tsai & Frasch, 1982).

**Dot blot and Western blot.** In order to have sufficient quantity of the same sera for several assays, serum samples from clinically diagnosed cases of leptospiral uveitis were pooled. Each serum pool was made by mixing equal amounts of MAT-positive sera from three leptospiral uveitis patients and two pools of sera were used. Similarly two pools for non-leptospiral uveitis and two for cataract controls were included in all the assays.

**Dot blot analysis.** 1 µg LPS per dot was bound onto the nitrocellulose (NC) membrane. For Western blot analysis, 20 µg LPS per lane was subjected to SDS-PAGE (Laemmli, 1970; Gallagher & Smith, 1994) and blotted to NC membrane in a semi-dry trans-blot apparatus (BioRad) at 15 V for 15 min using the electrode buffer [25 mM Tris/HCl, 182 mM glycine, 20 % (v/v) methanol, pH 8.5] described by Towbin et al. (1979). After blocking with 5 % skimmed milk powder in PBS for 2 h at room temperature, blots were incubated consecutively with pooled sera from uveitis patients and controls (1:100) and horseradish peroxidase (HRP)-conjugated anti-human IgG or IgM (Sigma) diluted 1:1000 in 1 % BSA in PBS/Tween 20 (PBS-T) for 2 h at room temperature. Blots were washed with PBS-T after each incubation and developed with 4-chloro-1-naphthol (Sigma).

**Proteinase K and periodate treatment.** Antigen (70 µg LPS ml⁻¹) was treated with 10 µg proteinase K ml⁻¹ (water for control) by incubating at 37 °C for 1 h and then stored at −20 °C. Proteinase K-treated antigen and untreated control antigen were subjected to dot-blot analysis using pooled positive serum from leptospirosis patients as described above. Periodate treatment of the antigen was based on the method of Xu et al. (1998) with minor modifications. Briefly, NC membranes with dotted antigen after blocking were treated with 100 µl 200 mM sodium acetate buffer (pH 5.5) and 100 µl 30 mM sodium metaperiodate for 1 h at 23 °C in the dark. The reaction was stopped by addition of 100 µl 20 mM sodium metabsulphite. After washing, treat-
ment with pooled positive serum from leptospirosis patients and enzyme-conjugated second antibody was performed as described above.

Absorption studies. MAT-positive serum samples from both systemic leptospirosis patients and leptospiral uveitis patients were absorbed with (i) anti-human IgG (Sigma), (ii) anti-human IgM (Sigma), (iii) antigenic preparation or (iv) PBS for 1 h at room temperature (30°C). After absorption, they were centrifuged at 10,400 g for 30 min and the supernatant thus obtained was tested for MAT titre.

ELISA. ELISA plates were coated with 50 µl antigen (5 µg LPS ml⁻¹) in carbonate buffer (pH 9.4). After incubation at 35°C for 1 h, the plates were kept at 4°C overnight. After washing (Immunowash; BioRad) in PBS-T, blocking was done with 1% BSA for 2 h at room temperature. Aliquots of 100 µl test serum at 1:800 and 1:1600 dilutions were added to each well and incubated for 1 h at room temperature. After washing, 100 µl anti-human IgG or anti-human IgG conjugated to HRP (Sigma) (1:6000) was used as second antibody. This was followed by addition of substrate (0.04% orthophenylene diamine in phosphate/citrate buffer, pH 5.0, with 0.03% hydrogen peroxide) and incubation in the dark for 30 min. The reaction was stopped with 2 M sulfuric acid and the pH 5.0, with 0.03% hydrogen peroxide) and incubation in the dark for 30 min. After absorption, they were centrifuged at 10,400 g for 30 min and the supernatant thus obtained was tested for MAT titre.

Statistical analysis. The data obtained from MAT and ELISA were analysed by Mann–Whitney U test and correlation coefficient analysis to monitor variation in the assays. Similarly, pooled sera from five healthy controls were used as negative control. The cut-off was determined from Rf values. The gels in (a) and (b) are different.

RESULTS

Purification and characterization of antigen

The antigen thus prepared contained 280 µg LPS ml⁻¹, but no detectable protein by Lowry’s method. Silver-staining for protein in SDS-PAGE revealed the presence of high-molecular-mass bands (> 200 kDa), while silver-staining for LPS detected a broad band at 15–30 kDa (Fig. 1a). Further analysis of our antigenic preparation was carried out by dot blot using pooled serum samples from systemic leptospirosis patients, leptospiral uveitis patients, non-leptospiral uveitis patients, cataract controls and healthy individuals. Fig. 2 shows that sera of systemic leptospirosis patients were positive for both IgG and IgM antibodies to our antigenic preparation. There was a stronger reactivity of IgM than that of IgG in leptospiral uveitis patients for the same amount of antigen. Furthermore, IgM reactivity was absent in both non-leptospiral uveitis and cataract controls, thereby indicating that the IgM response is relevant for diagnosis of leptospiral uveitis. For the same dilution of the leptospirosis serum, the reaction in the IgM response was more pronounced with periodate treatment than with proteinase K, thus indicating that the IgM antibody reacts with LPS moiety in the antigenic preparation (Fig. 3). Further analysis of the antigen by Western blot (Fig. 1b) revealed that the IgG antibodies were directed against high-molecular-mass proteins (> 200 kDa) and the IgM antibodies towards the low-molecular-mass LPS bands (13–21, 24 and 28 kDa). Absorption of MAT-positive serum samples from both systemic leptospirosis (1:100) for reactivity of IgG (lane 1) and IgM antibodies (lane 2) (1:10000). Numbers to the right indicate molecular mass (kDa), determined from Rf values. The gels in (a) and (b) are different.

Anti-leptospiral antibody levels in serum

ELISA results of the IgM and IgG antibody response are shown in Fig. 4. The cut-off values for IgM and IgG were respectively 0.1 and 0.12 for the first-antibody dilution of 1:800. On the basis of IgM response, 48 % leptospiral uveitis patients were significantly positive for anti-LPS leptospiral antibodies in comparison with cataract controls (P < 0.005). Furthermore, it is significant that only 10% of non-leptospiral uveitis patients were positive, however with low titre (Fig. 4a). Even though the IgG response was also significant (P < 0.02), the sensitivity was low (30%). Furthermore, this reaction was towards the high-molecular-mass proteins, as evident from the Western blot results (Fig. 1b). A significant correlation was seen between IgM and IgG antibody response to LPS in leptospiral uveitis patients (Fig. 5).

Agglutinating activity of leptospiral uveitis serum was eliminated after absorption with anti-human IgM but not with IgG, thus confirming that MAT positivity was due to IgM antibodies (Table 2). The results of MAT for serum samples

Statistical analysis. The data obtained from MAT and ELISA were analysed by Mann–Whitney U test and correlation coefficient analysis to monitor variation in the assays. Similarly, pooled sera from five healthy controls were used as negative control. The cut-off was as defined as the mean±2SD absorbance value of cataract controls.

Serodiagnosis of leptospiral uveitis using LPS

![Fig. 1.](http://jnm.sgmjournals.org)
are presented in Table 3. A titre of 1 : 100 dilution of serum was considered as positive: 58 % of clinically leptospiral uveitis patients were positive for MAT, and some were positive for more than one serovar. None of the cataract controls and none of the non-leptospiral uveitis patients tested was positive for MAT.

Table 4 shows that only 24 cases were positive for both MAT and ELISA, there was no correlation in 38 cases and 19 were negative for both. When positivity in both tests was considered, 62 cases (77 %) were positive for leptospiral antibodies in their serum. Interestingly, there was a good correlation of IgG response in ELISA with MAT (75 %).

**DISCUSSION**

Several commercial kits are available for the diagnosis of systemic leptospiral infection using broadly reactive Leptospira antigen (Cumberland et al., 1999; Smits et al., 1999, 2000, 2001; Sehgal et al., 1999; Eapen et al., 2002). However, the nature of the antigen has not been described in the literature. This antigen, prepared following the published protocol of Terpstra et al. (1985), contained culture medium particles and formalin. Since the concentration of antigen is not known, the amount of antigen used for each assay may vary from batch to batch. Moreover, the drying method was used for antigen coating. To overcome these problems, the method of Terpstra et al. (1985) was modified in our study as follows. The bulk culture was washed initially to remove medium particles, the bacterial pellet was treated with formalin and supernatant was filtered (10 kDa) to eliminate formalin. After estimation of protein and total sugar, a specific amount of antigen was coated to the ELISA plate (incubation at 37 °C for 1 h) for consistent results.

**LPS as a candidate antigen for serodiagnosis**

Biochemical analysis of the antigenic preparation showed the presence of LPS, on the basis of total sugar estimation. Though the protein content varied in different bacterial preparations, the total sugar/protein ratio was about 4:1 (K. Bhavani and C. Gowri Priya, unpublished results). The LPS profile of the antigen in SDS-PAGE revealed a simple pattern, similar to LPS extracted from *Leptospira interrogans* serovar Hardjo, in contrast to the ladder-like pattern of other enterobacterial LPS (Vinh et al., 1989).

Immunoblotting with the antigen showed that IgM antibodies reacted with the diffuse band of LPS in the pooled sera of leptospiral uveitis patients tested and not in controls. Similar observations have been made with sera of systemic...
leptospirosis patients towards 15, 23 and 28 kDa LPS bands (Chapman et al., 1988) and a proteinase K-resistant, diffuse band of 14.8–22 kDa (Ribeiro et al., 1992). Removal of agglutinating antibodies in MAT-positive serum after absorption with the antigen specifies LPS to be the main component in our antigenic preparation, confirming the earlier finding that the bacterial agglutinating antibodies are directed against LPS (Faine et al., 1999). Furthermore, a significant reduction was observed in the IgM response by dot blot after periodate treatment. The above findings demonstrate that LPS in our antigenic preparation is the immunologically relevant antigen for diagnosis.

Serodiagnosis using LPS antigen

Whether the agglutinating activity was due to IgG or IgM antibodies from leptospirosis patients in MAT was not clear (Faine et al., 1999). Interestingly, in our study, absorption of agglutinating antibodies in sera of leptospiral uveitis patients by anti-human IgM but not by IgG revealed that bacterial agglutination was mediated by IgM antibodies that showed specificity towards LPS in our antigenic preparation. Therefore, detection of IgM antibody with specificity to LPS antigen in ELISA forms a good diagnostic tool for leptospiral uveitis patients, as these antibodies are significantly absent in non-leptospiral uveitis patients and controls. However, the sensitivity of 48% may be due to the fact that LPS of leptospirosis patients towards 15, 23 and 28 kDa LPS bands (Chapman et al., 1988) and a proteinase K-resistant, diffuse band of 14.8–22 kDa (Ribeiro et al., 1992). Removal of agglutinating antibodies in MAT-positive serum after absorption with the antigen specifies LPS to be the main component in our antigenic preparation, confirming the earlier finding that the bacterial agglutinating antibodies are directed against LPS (Faine et al., 1999). Furthermore, a significant reduction was observed in the IgM response by dot blot after periodate treatment. The above findings demonstrate that LPS in our antigenic preparation is the immunologically relevant antigen for diagnosis.

Serodiagnosis of leptospiral uveitis using LPS

Table 3. MAT with 19 leptospiral serovars

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<thead>
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<th>Serovar</th>
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<td>1:100</td>
<td>1:200</td>
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<tr>
<td>patoc</td>
<td>8</td>
<td>3</td>
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<tr>
<td>louisiana</td>
<td>7</td>
<td>9</td>
</tr>
<tr>
<td>icterohaemorrhagiae</td>
<td>6</td>
<td>5</td>
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australis            | 2                      | 2     | 0     | 6    |
djasiman             | 0                      | 1     | 0     | 1    |
|Negative             | 34                     |       |       |      |

Table 4. Correlation between MAT and IgM ELISA in serum samples of leptospiral uveitis patients

MAT was considered positive at 1:100 dilution of serum. ELISA was considered positive at 1:800 dilution of serum when the absorbance was above the cut-off value (0.1).

Results

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<th>Results</th>
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<td>MAT+ ELISA+</td>
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<td>Total</td>
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Fig. 4. IgM (a) and IgG (b) antibody response to LPS antigen in leptospiral uveitis patients (LU) and controls (CC, cataract controls; NL, non-leptospiral uveitis). ELISA plates were coated with 50 µl antigen (5 µg LPS ml⁻¹). Plates were incubated with 1:800 dilution of patient and control serum followed by HRP-conjugated anti-human IgM (a) or IgG (b) at 1:8000 dilution. Cut-off values were determined as the mean+2SD of the cataract control absorbance (a, 0.1; b, 0.12).

Fig. 5. Correlation of absorbance for anti-leptospiral uveitis IgM and IgG antibodies at 1:800 dilution of serum samples. There was a statistically significant correlation (P < 0.01) between the IgM and IgG antibody responses to LPS antigen.
leptospires are serovar-specific (Faine et al., 1999). Therefore, it would be necessary to make use of LPS preparations from several serovars, as suggested by Silva et al. (1995). Though there was a significant difference in the IgG antibodies in the serum of leptosporial uveitis patients compared with controls and a good correlation with MAT results, it was not useful for serodiagnosis due to its low sensitivity.

A good correlation was observed between the levels of IgG and IgM antibodies in sera of patients with leptosporial uveitis. It has been reported that early host immune response to leptosporial infection is characterized by IgM antibodies and IgG antibodies in sera of patients with leptosporial infection is specific for whole leptosporial antigen preparation and IgG antibodies for recombinant leptosporial protein (Flannery et al., 2001), as observed in the early response to infection by Borrelia (Engstrom et al., 1995; Magarelli et al., 2000) and Treponema (Schmidt et al., 2000).

Serological tests based on purified proteins like recombinant antigens are widely used in screening for systemic spirochaetal infections such as Lyme disease and syphilis (Hauser & Wilks, 1997; Goossens et al., 1999; Magarelli et al., 2000; Schmidt et al., 2000). More recently, a recombinant protein rLipL32 has been proposed as a useful antigen for the serodiagnosis of systemic leptospirosis (Flannery et al., 2001; Guerreiro et al., 2001). Utilization of such recombinant proteins in serodiagnosis of leptosporial uveitis patients needs further analysis.

Confirmation of aetiology

Results of MAT and ELISA indicate the presence of anti-leptosporial antibodies in sera of patients with leptosporial uveitis (77 %) and not in other uveitis patients or cataract controls. Furthermore, detection of leptosporial DNA by PCR in the aqueous fluid of 75 % of the leptosporial uveitis patients and not in the controls (G. Neethirajan, C. Gowri Priya and R. A. Haritskeerl, unpublished results) confirms the leptosporial aetiology in these patients.

Development of leptosporial uveitis can be due to a number of pathogenic mechanisms. In spite of the PCR-positivity for leptosporial antigens in the aqueous fluid of 75 % of the leptosporial uveitis patients and not in the controls (G. Neethirajan, C. Gowri Priya and R. A. Haritskeerl, unpublished results) confirms the leptosporial aetiology in these patients.

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ACKNOWLEDGEMENTS

This work was supported by the grants from Indian Council of Medical Research, New Delhi, India, and the Aravind Medical Research Foundation, Madurai, Tamil Nadu, India.

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