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 leptospirosis 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°C236 g LPS ml°C1 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 (Levitt & 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;
that the amount of LPS in our antigenic preparation is equal to total
using sucrose as standard. After estimation, 20


table 1. Serovars used for MAT

<table>
<thead>
<tr>
<th>Serogroup</th>
<th>Serovar</th>
<th>Strain</th>
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<tbody>
<tr>
<td>Australis</td>
<td>lora</td>
<td>Lora</td>
</tr>
<tr>
<td>Autumnalis</td>
<td>autumnalis</td>
<td>Akiyama A</td>
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<td>Wynerberg</td>
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<td>lanka</td>
<td>R740</td>
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<tr>
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<td>patoc</td>
<td>Patoc 1</td>
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<td>Lai</td>
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<td>djasiman</td>
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<tr>
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<td>bratislava</td>
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<td>Hardiyanto</td>
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<tr>
<td>Bharathy</td>
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<td>Javanica</td>
<td>menoni</td>
<td>Kerala</td>
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<tr>
<td>Cynopteri</td>
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<tr>
<td>Grippotyphosa</td>
<td>ratnapura</td>
<td>Wumalaseena</td>
</tr>
<tr>
<td>Andamanana</td>
<td>andamanan</td>
<td>Ch11</td>
</tr>
<tr>
<td>Australis</td>
<td>ballico</td>
<td>Ballico</td>
</tr>
</tbody>
</table>

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 leptospirosis 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.

For 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 1 h at room temperature. Blots were washed with PBS-T after each incubation and developed with 4-chloro-1-naphthol (Sigma).

Proteasease K and periodate treatment. Antigen (70 µg LPS ml⁻1) was treated with 10 mg proteasease K ml⁻1 (water for control) by incubating at 37 °C for 1 h and then stored at −20 °C. Proteasease 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 9.6). After incubation at 37 °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 IgM 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 0.01% hydrogen peroxide) and incubation in the dark for 30 min. The reaction was stopped with 2 M sulfuric acid and the absorbance was read at 490 nm.

**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), and (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.

**Statistical analysis.** The data obtained from MAT and ELISA were subjected to statistical analysis using SPSS 9.0 software. Indices of sensitivity and specificity of the ELISA were calculated as follows: sensitivity = a/(a+c) × 100; specificity = d/(b+d) × 100; where a is the number of true positive samples, b is the number of false positive samples, c is the number of false negative samples and d is the number of true negative samples.

**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 with our antigenic preparation resulted in the elimination of bacterial agglutinating activity (Table 2). Taken together, these results indicate the importance of LPS in our antigenic preparation in serodiagnosis.

**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
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.

Fig. 2. Dot-blot analysis of antigen. Antigen (1 μg LPS per dot) was coated onto NC membrane. Dot-blot analysis was performed using pooled serum samples (1 : 50) from systemic leptospirosis patients (PC1 and PC2), leptospiral uveitis patients (LUP1, LUP2), non-leptospiral uveitis patients (NLP1) and cataract controls (CCP1) for both IgG and IgM antibodies (1 : 1000).

Fig. 3. Dot-blot analysis using untreated (a), proteinase K-treated (b) and periodate-treated (c) antigen. Serum samples (1 : 100) from positive leptospirosis patients were used for the analysis, followed by HRP-conjugated anti-human IgM (1 : 1000).

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.
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

Table 3. MAT with 19 leptospiral serovars

A titre of 1 : 100 dilution of serum was taken as positive. The serovar with the highest titre was taken as the causative serovar.

<table>
<thead>
<tr>
<th>Serovar</th>
<th>No. positive for titre</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>patoc</td>
<td>8</td>
<td>13</td>
</tr>
<tr>
<td>louisiana</td>
<td>7</td>
<td>16</td>
</tr>
<tr>
<td>icterohaemorrhagiae</td>
<td>6</td>
<td>11</td>
</tr>
<tr>
<td>australis</td>
<td>2</td>
<td>6</td>
</tr>
<tr>
<td>djasiman</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Negative</td>
<td>34</td>
<td></td>
</tr>
</tbody>
</table>

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).

<table>
<thead>
<tr>
<th>Results</th>
<th>n</th>
<th>%</th>
</tr>
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<tr>
<td>MAT⁺ ELISA⁺</td>
<td>24</td>
<td>30</td>
</tr>
<tr>
<td>MAT⁺ ELISA⁻</td>
<td>23</td>
<td>28</td>
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<tr>
<td>MAT⁻ ELISA⁺</td>
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<td>18</td>
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<tr>
<td>MAT⁻ ELISA⁻</td>
<td>19</td>
<td>24</td>
</tr>
<tr>
<td>Total</td>
<td>81</td>
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</tr>
</tbody>
</table>

leptospirosis patients towards 15, 23 and 28 kDa LPS bands (Chapman et al., 1988) and a proteinase K-resistant, diffuse band of 148–22 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.

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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 leptospirosis uveitis patients compared with controls and a good correlation with MAT results, it was not useful for serodagnosis due to its low sensitivity.

A good correlation was observed between the levels of IgG and IgM antibodies in sera of patients with leptospiral uveitis. It has been reported that early host immune response to leptospiral infection is characterized by IgM antibodies specific for whole leptospiral antigen preparation and IgG antibodies for recombinant leptospiral 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 & Wilske, 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 serodagnosis of systemic leptospirosis (Flannery et al., 2001; Guerreiro et al., 2001). Utilization of such recombinant proteins in serodiagnosis of leptospirosis uveitis patients needs further analysis.

Confirmation of aetiology

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

Development of leptospiral uveitis can be due to a number of pathogenic mechanisms. In spite of the PCR-positivity for leptospirosis in these patients, as suggested by Silva et al. (1995), the pathogenic mechanisms in the pathogenesis of leptospiral uveitis are not clear. Two probable reasons may be recent reinfection or persistence of IgM in the serum after initial infection (Blackmore et al., 1984). Further analysis is required to understand the specific role of LPS in the pathogenesis of leptospiral uveitis.

ACKNOWLEDGEMENTS

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REFERENCES


