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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;

Abbreviations: HRP, horseradish peroxidase; MAT, microscopic agglutination test; NC, nitrocellulose.

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. The inclusion criteria were: acute, anterior or pan, non-granulomatous uveitis. When these patients had hypopyon, disc oedema, vasculitis and vitreous membrane or vitreous exudates, they suggested stronger association with past leptospiral infection (Rathinam *et al.*, 1997; Dana, 2002). Idiopathic uveitis and other entities associated with tuberculosis, leprosy, sarcoidosis and HLA B27 were excluded. A total of 81 patients with a specific combination of clinical criteria of leptospiral uveitis were selected for the study and, when presenting at Uvea Clinic, they did not have fever or other symptoms of acute leptospiral infection.

Fifteen patients undergoing surgery for age-related cataract were selected as controls. They showed no symptoms of systemic or ocular infection. Another set of controls was 15 non-leptospiral uveitis patients (tuberculous uveitis, leprosy, Vogt Koyanagi Harada syndrome and sarcoidosis). This study was approved by the ethical committee of Aravind Eye Hospital and informed consent was obtained from all patients before recruitment. Blood (5 ml) was obtained from both patients and controls for serological tests. All sera were stored at −70 °C.

Serum samples from three patients with confirmed systemic leptospirosis, though without symptoms of uveitis, were pooled and used as positive control for leptospiral infection.

Leptospiral serovars. Nineteen 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 culture (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 concentrator (Sartorius) and the protein concentration of the retentate thus obtained was measured according to Lowry *et al.* (1951) using BSA as standard. Since the antigenic epitopes of *L. biflexa* LPS have been identified as disaccharide units (Matsuo *et al.*, 2000a), we considered that the amount of LPS in our antigenic preparation is equal to total sugar estimated by the phenol/sulfuric acid method (Dubois *et al.*, 1956) using sucrose as standard. After estimation, 20 μg LPS per lane was separated on SDS-PAGE and silver-stained for protein (Bloom *et al.*, 1987) and LPS (Tsai & Frasch, 1982).

Table 1. Serovars used for MAT

Serogroup	Serovar	Strain
Australis	lora	Lora
Autumnalis	autumnalis	Akiyami A
Icterohaemorrhagiae	copenhageni	Wynberg
Louisiana	lanka	R740
Semaranga	patoc	Patoc 1
Icterohaemorrhagiae	lai	Lai
Djasiman	djasiman	Djasiman
Australis	bratislava	Jez Bratislava
Sejroe	wolffi	3705
Sejroe	hardjo	Hardjoprajitno
Pomona	pomona	Pomona
Bharathy	–	–
Javanica	menoni	Kerala
Celledoni	celledoni	Celledoni
Louisiana	louisiana	LSU1945
Pyrogenes	alexis	Hs616
Autumnalis	bulgarica	Nikolaevo
Cynopteri	cynopteri	3522C
Grippotyphosa	ratnapura	Wumalaseena
Andamana	andaman	Ch11
Australis	ballico	Ballico

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.

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 8·3] 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).

Proteinase K and periodate treatment. Antigen (70 μg LPS ml^{-1}) was treated with 10 mg 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 metabisulfite. After washing, treatment 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 0·03 % hydrogen peroxide) and incubation in the dark for 30 min. The reaction was stopped with 2 M sulfuric acid and the A_{490} was measured in a BioRad ELISA reader. Sera from three systemic leptospirosis patients were pooled and used as positive control in each assay in order to monitor variation in the assays. Similarly, pooled sera from five healthy controls were used as negative control. The cut-off value was defined as the mean+2SD absorbance value of cataract controls.

Statistical analysis. The data obtained from MAT and ELISA were analysed by Mann–Whitney U test and correlation coefficient analysis using SPSS 9.0 software. Indices of sensitivity and specificity of the ELISA were calculated as follows: sensitivity $= [a/(a + c)] \times 100$; specificity $= [d/(b + d)] \times 100$; 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 (Gerhardt & Keller, 1986).

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 reduction 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

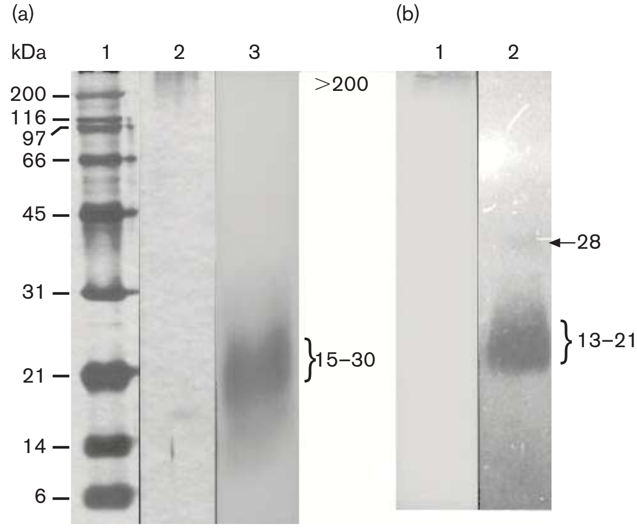

Fig. 1. Antigen prepared from *L. biflexa* serovar Patoc was analysed by SDS-PAGE (a) and Western blot (b). (a) Antigen (20 μg LPS per lane) was separated in 13 % SDS-PAGE and silver-stained for protein (lane 2) and LPS (lane 3). Lane 1: molecular mass marker, silver-stained for protein. (b) Separated antigens were transferred onto NC membrane and immunoblotted with pooled sera of patients with leptospirosis (1 : 100) for reactivity of IgG (lane 1) and IgM antibodies (lane 2) (1 : 1000). Numbers to the right indicate molecular mass (kDa), determined from R_f values. The gels in (a) and (b) are different.

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

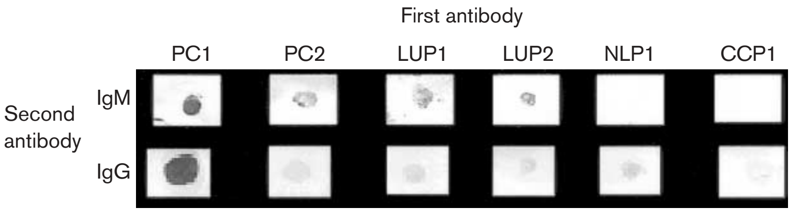

Fig. 2. Dot-blot analysis of antigen. Antigen (1 μg LPS per dot) was coated onto NC membrane. Dot-blot analysis was performed using pooled serum samples (1 : 50) from systemic leptospirosis patients (PC1 and PC2), leptospiral uveitis patients (LUP1, LUP2), non-leptospiral uveitis patients (NLP1) and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

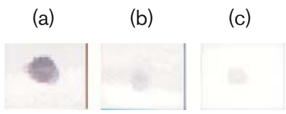

Fig. 3. Dot-blot analysis using untreated (a), proteinase K-treated (b) and periodate-treated (c) antigen. Serum samples (1 : 100) from positive leptospirosis patients were used for the analysis, followed by HRP-conjugated anti-human IgM (1 : 1000).

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.

Table 2. Absorption of agglutinating activity in sera of leptospiral uveitis patients

Agglutinating activity was measured by MAT. LU, Leptospiral uveitis; SL, systemic leptospirosis.

Absorption using	MAT titre	
	Before absorption	**After absorption**
PBS		
SL serum	1 : 200	1 : 200
LU serum 1	1 : 400	1 : 400
LU serum 2	1 : 200	1 : 200
LU serum 3	1 : 400	1 : 400
LPS antigen		
SL serum	1 : 200	Negative
LU serum 3	1 : 400	Negative
Anti-human IgM		
SL serum	1 : 200	Negative
LU serum 1	1 : 400	Negative
LU serum 2	1 : 200	Negative
LU serum 3	1 : 400	Negative
Anti-human IgG		
SL serum	1 : 200	1 : 200
LU serum 1	1 : 400	1 : 400
LU serum 2	1 : 200	1 : 200
LU serum 3	1 : 400	1 : 200

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

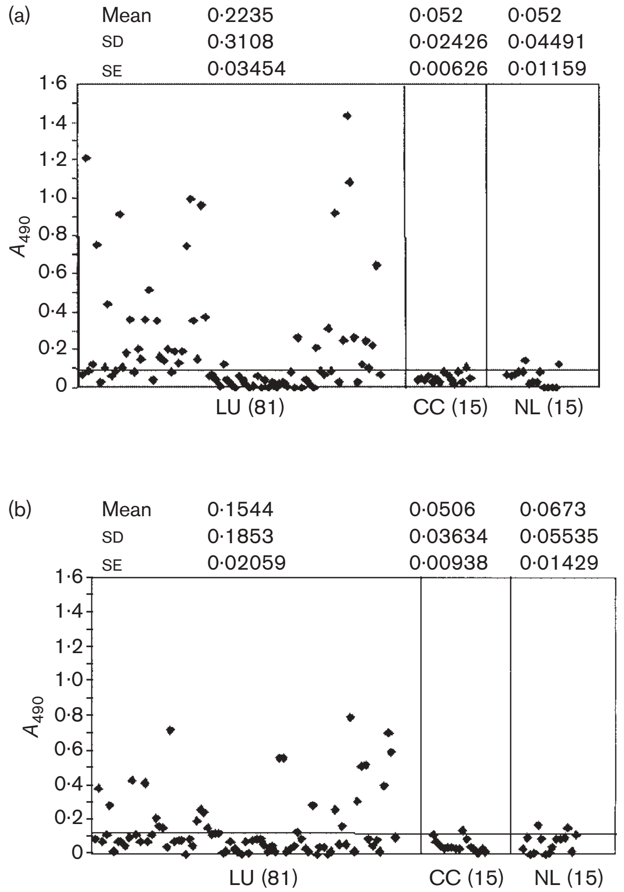

Fig. 4. IgM (a) and IgG (b) antibody response to LPS antigen in leptospiral uveitis patients (LU) and controls (CC, cataract controls; NL, non-leptospiral uveitis). ELISA plates were coated with 50 μl antigen (5 μg LPS ml^{-1}). Plates were incubated with 1 : 800 dilution of patient and control serum 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).

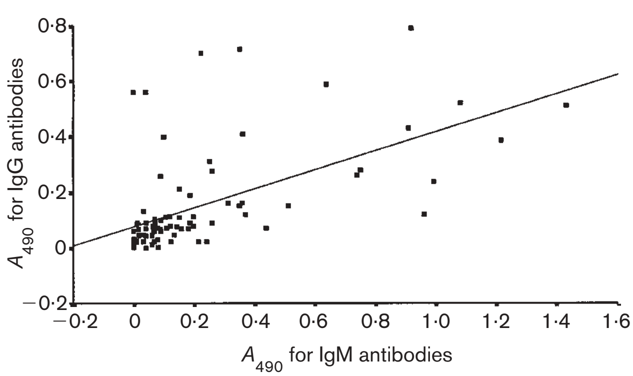

Fig. 5. Correlation of absorbance for anti-leptospiral uveitis IgM and IgG antibodies at 1 : 800 dilution of serum samples. There was a statistically significant correlation ($P < 0·01$) between the IgM and IgG antibody responses to LPS antigen.

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.

Serovar	No. positive for titre			Total
	1 : 100	1 : 200	1 : 400	
patoc	8	3	2	13
louisiana	7	9	0	16
icterohaemorrhagiae	6	5	0	11
australis	2	2	2	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	*n*	%
MAT^{+} $ELISA^{+}$	24	30
MAT^{+} $ELISA^{-}$	23	28
MAT^{-} $ELISA^{+}$	15	18
MAT^{-} $ELISA^{-}$	19	24
Total	81	–

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 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 leptospiral 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 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; Magnarelli *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; Magnarelli *et al*., 2000; Schmidt *et al*., 2000). More recently, a recombinant protein antigen 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 leptospiral 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. Hartskeerl, 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 leptospires in the aqueous fluid, it has not been possible to isolate the bacteria (C. Gowri Priya and K. Bhavani, unpublished results). Since leptospiral uveitis is a late complication of systemic infection, the reason for the presence of IgM antibodies in the serum of leptospiral uveitis patients is 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.

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REFERENCES

Adler, B., Murphy, A. M., Locarnini, S. A. & Faine, S. (1980). Detection of specific anti-leptospiral immunoglobulins M and G in human serum by solid-phase enzyme-linked immunosorbent assay. *J Clin Microbiol* **11**, 452–457.

Arimitsu, Y., Kobayashi, S., Akama, K. & Matuhasi, T. (1982). Development of a simple serological method for diagnosing leptospirosis: a microcapsule agglutination test. *J Clin Microbiol* **15**, 835–841.

Blackmore, D. K., Schollum, L. M. & Moriarty, K. M. (1984). The magnitude and duration of titres of leptospiral agglutinins in human sera. *N Z Med J* **97**, 83–86.

Bloom, H., Beier, H. & Gross, H. S. (1987). Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels. *Electrophoresis* **8**, 93–99.

Chapman, A. J., Adler, B. & Faine, S. (1988). Antigens recognised by the human immune response to infection with *Leptospira interrogans* serovar *hardjo*. *J Med Microbiol* **25**, 269–278.

Chu, K. M., Rathinam, R., Namperumalsamy, P. & Dean, D. (1998). Identification of *Leptospira* species in the pathogenesis of uveitis and determination of clinical ocular characteristics in south India. *J Infect Dis* **177**, 1314–1321.

Cumberland, P., Everard, C. O. R. & Levett, P. N. (1999). Assessment of the efficacy of an IgM-ELISA and microscopic agglutination test (MAT) in the diagnosis of acute leptospirosis. *Am J Trop Med Hyg* **61**, 731–734.

Dana, M. R. (2002). Leptospirosis. In *Diagnosis and Treatment of Uveitis*, pp. 273–277. Edited by C. S. Foster & A. T. Vitale. Philadelphia: W. B. Saunders.

Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A. & Smith, F. (1956). Colorimetric method for the determination of sugar and related substances. *Anal Chem* **28**, 350–356.

Eapen, C. K., Sugathan, S., Kuriakose, M., Abdoel, T. & Smits, H. L. (2002). Evaluation of the clinical utility of a rapid blood test for human leptospirosis. *Diagn Microbiol Infect Dis* **42**, 221–225.

Engstrom, S. M., Shoop, E. & Johnson, R. C. (1995). Immunoblot interpretation criteria for serodiagnosis of early Lyme disease. *J Clin Microbiol* **33**, 419–427.

Faine, S., Adler, B., Bolin, C. & Perolat, P. (1999). *Leptospira and Leptospirosis*, 2nd edn. Melbourne: MediSci.

Farr, R. W. (1995). Leptospirosis. *Clin Infect Dis* **21**, 1–8.

Flannery, B., Costa, D., Carvalho, F. P. & 8 other authors (2001). Evaluation of recombinant *Leptospira* antigen-based enzyme-linked immunosorbent assays for the serodiagnosis of leptospirosis. *J Clin Microbiol* **39**, 3303–3310.

Gallagher, S. R. & Smith, J. A. (1994). One-dimensional gel electrophoresis of proteins. In *Current Protocols in Immunology*, pp. 8.4.1–8.4.19. Edited by J. E. Coligan, A. M. Kruisbeek, D. H. Margulies, E. M. Shevach & W. Strober. Chichester: Wiley.

Gerhardt, W. & Keller, H. (1986). Evaluation of test data from clinical studies. I. Terminology, graphic interpretation, diagnostic strategies, and selection of sample groups. *Scand J Clin Lab Investig Suppl* **181**, 5–45.

Goossens, H. A. T., van der Bogaard, A. E. & Nohlmans, M. K. E. (1999). Evaluation of fifteen commercially available serological tests for diagnosis of Lyme borreliosis. *Eur J Clin Microbiol Infect Dis* **18**, 551–560.

Guerreiro, H., Croda, J., Flannery, B., Mazel, M., Matsunaga, J., Galvao Reis, M., Levett, P. N., Ko, A. I. & Haake, D. A. (2001). Leptospiral proteins recognized during the humoral immune response to leptospirosis in humans. *Infect Immun* **69**, 4958–4968.

Gussenhoven, G. C., van der Hoorn, M. A., Goris, M. G., Terpstra, W. J., Hartskeerl, R. A., Mol, B. W., van Ingen, C. W. & Smits, H. L. (1997).

LEPTO dipstick, a dipstick assay for detection of *Leptospira*-specific immunoglobulin M antibodies in human sera. *J Clin Microbiol* **35**, 92–97.

Hauser, U. & Wilske, B. (1997). Enzyme-linked immunosorbent assays with recombinant internal flagellin fragments derived from different species of *Borrelia burgdorferi* sensu lato for the serodiagnosis of Lyme neuroborreliosis. *Med Microbiol Immunol* **186**, 145–151.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.

Levett, P. N. & Whittington, C. U. (1998). Evaluation of the indirect hemagglutination assay for diagnosis of acute leptospirosis. *J Clin Microbiol* **36**, 11–14.

Levett, P. N., Branch, S. L., Whittington, C. U., Edwards, C. N. & Paxton, H. (2001). Two methods for rapid serological diagnosis of acute leptospirosis. *Clin Diagn Lab Immunol* **8**, 349–351.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). Protein measurement with Folin phenol reagent. *J Biol Chem* **193**, 265–275.

Magnarelli, L. A., Ijdo, J. W., Padula, S. J., Flavell, R. A. & Fikrig, E. (2000). Serologic diagnosis of Lyme borreliosis by using enzyme-linked immunosorbent assays with recombinant antigens. *J Clin Microbiol* **38**, 1735–1739.

Martins, M. G., Matos, K. T. F., da Silva, M. V. & de Abreu, M. T. (1998). Ocular manifestations in the acute phase of leptospirosis. *Ocul Immunol Inflamm* **6**, 75–79.

Matsuo, K., Isogai, E. & Araki, Y. (2000a). Occurrence of [→3)-β-D-Man*p*-(1→4)-β-D-Man*p*-(1→]$_n$ units in the antigenic polysaccharides from *Leptospira biflexa* serovar patoc strain Patoc I. *Carbohydr Res* **328**, 517–524.

Matsuo, K., Isogai, E. & Araki, Y. (2000b). Utilization of exocellular mannan from *Rhodotorula glutinis* as an immunoreactive antigen in diagnosis of leptospirosis. *J Clin Microbiol* **38**, 3750–3754.

Rathinam, S. R., Rathnam, S., Selvaraj, S., Dean, D., Nozik, R. A. & Namperumalsamy, P. (1997). Uveitis associated with an epidemic outbreak of leptospirosis. *Am J Ophthalmol* **124**, 71–79.

Ribeiro, M. A., Sakata, E. E., Silva, M. V., Camargo, E. D., Vaz, A. J. & De Brito, T. (1992). Antigens involved in the human antibody response to natural infections with *Leptospira interrogans* serovar *copenhageni*. *J Trop Med Hyg* **95**, 239–245.

Schmidt, B. L., Edjlalipour, M. & Luger, A. (2000). Comparative evaluation of nine different enzyme-linked immunosorbent assays for determination of antibodies against *Treponema pallidum* in patients with primary syphilis. *J Clin Microbiol* **38**, 1279–1282.

Sehgal, S. C., Vijayachari, P., Sharma, S. & Sugunan, A. P. (1999). LEPTO dipstick: a rapid and simple method for serodiagnosis of acute leptospirosis. *Trans R Soc Trop Med Hyg* **93**, 161–164.

Silva, M. V., Camargo, E. D., Batista, L., Vaz, A. J., Brandao, A. P., Nakamura, P. M. & Negrao, J. M. (1995). Behaviour of specific IgM, IgG and IgA class antibodies in human leptospirosis during the acute phase of the disease and during convalescence. *J Trop Med Hyg* **98**, 268–272.

Smits, H. L., Ananyina, Y. V., Chereshsky, A. & 17 other authors (1999). International multicenter evaluation of the clinical utility of a dipstick assay for detection of *Leptospira*-specific immunoglobulin M antibodies in human serum specimens. *J Clin Microbiol* **37**, 2904–2909.

Smits, H. L., van der Hoorn, M. A. W. G., Goris, M. G. A., Gussenhoven, G. C., Yersin, C., Sasaki, D. M., Terpstra, W. J. & Hartskeerl, R. A. (2000). Simple latex agglutination assay for rapid serodiagnosis of human leptospirosis. *J Clin Microbiol* **38**, 1272–1275.

Smits, H. L., Eapen, C. K., Sugathan, S. & 8 other authors (2001). Lateral-flow assay for rapid serodiagnosis of human leptospirosis. *Clin Diagn Lab Immunol* **8**, 166–169.

Terpstra, W. J., Ligthart, G. S. & Schoone, G. J. (1985). ELISA for the detection of specific IgM and IgG in human leptospirosis. *J Gen Microbiol* **131**, 377–385.

Towbin, H., Staehelin, T. & Gordon, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci U S A* **76**, 4350–4354.

Tsai, C. M. & Frasch, C. E. (1982). A sensitive silver stain for detecting lipopolysaccharides in polyacrylamide gels. *Anal Biochem* **119**, 115–119.

Vinh, T., Shi, M.-H., Adler, B. & Faine, S. (1989). Characterization and taxonomic significance of lipopolysaccharides of *Leptospira interrogans* serovar *hardjo*. *J Gen Microbiol* **135**, 2663–2673.

Wanyangu, S. W., Palmer, M. F., Zochowski, W. J. & Waitkins, S. A. (1987). Comparison of the DIFCO and Patoc I slide antigens in the screening of leptospirosis. *Comp Immunol Microbiol Infect Dis* **10**, 155–161.

Winslow, W. E., Merry, D. J., Pirc, M. L. & Devine, P. L. (1997). Evaluation of a commercial enzyme-linked immunosorbent assay for detection of immunoglobulin M antibody in diagnosis of human leptospiral infection. *J Clin Microbiol* **35**, 1938–1942.

Xu, Y., Murray, B. E. & Weinstock, G. M. (1998). A cluster of genes involved in polysaccharide biosynthesis from *Enterococcus faecalis* OG1RF. *Infect Immun* **66**, 4313–4323.