

## Leptospiral proteins expressed during acute & convalescent phases of human leptospirosis

K. Natarajaseenivasan, P. Vijayachari, A.P. Sugunan, S. Sharma & S.C. Sehgal

*National Leptospirosis Reference Centre, Regional Medical Research Centre (ICMR)  
Port Blair, India*

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**Background & objectives:** The available serological techniques for the diagnosis of leptospirosis have less sensitivity during the early stage of the disease. Understanding of leptospiral proteins expressed during acute and convalescent phases of leptospirosis, would help the development of new serodiagnostic strategies. Therefore, the present study was carried out to identify (i) an antigen that is conserved among the various pathogenic leptospira; (ii) best protein antigen to which immune response can be identified in the acute phase; and (iii) best protein antigen which is present in convalescent sera which can be used for seroepidemiological studies.

**Methods:** Quantitative immunoblot analysis was performed using acute and convalescent phase human sera along with sera from normal healthy individuals and from patients with typhoid, malaria and hepatitis as the controls. All the samples were analyzed for the leptospiral protein recognition by using IgM and IgG immunoblots. Leptospiral cell fractionation was performed using triton X-114 and lysozyme and further the conservation of leptospiral proteins was also performed.

**Results:** In confirmed cases of leptospirosis, the IgG recognition in acute phase sera was 30.2, 39.5, 27.9, 55.8 and 27.9 per cent for the leptospiral proteins p32, p41/42, p58, p62 and p82 respectively. The IgG has considerably increased to 65.1, 55.8, 46.5, 67.4 and 48.8 per cent against the same proteins during convalescent phase. The IgM recognition was 32.6, 32.6, 30.2 and 37.2 per cent for acute phase sera and 32.6, 37.2, 44.2 and 41.9 per cent for convalescent phase sera for the leptospiral proteins p14, p25, p32 and p41/42, respectively. Leptospiral proteins like p62 and p82 were recognized among all the control groups with 3.3-15.3 per cent for IgG recognition.

**Interpretation & conclusion:** Leptospiral protein p32 was found to be highly sensitive and specific and could be useful for the development of newer techniques for diagnosis and seroepidemiological studies. Combination of p32 and p41/42 for IgG and p14, p25, p32, p41/42 for IgM would increase the sensitivity of these techniques further.

**Key words** Immunoblotting - leptospiral proteins - leptospirosis

Leptospirosis has worldwide distribution but is more prevalent in tropical and subtropical regions. The clinical spectrum of the disease ranges from mild

flu like illness to severe fatal form due to multi organ involvement<sup>1</sup>. The clinical presentation varies from patient to patient; hepato-renal failure, myocarditis,

severe pulmonary haemorrhage with respiratory distress and meningitis are some of the syndromes reported commonly<sup>2,3</sup>. Demonstration of leptospire, isolation of the organism, serological techniques for detection of specific antibodies and polymerase chain reaction (PCR) are the available laboratory methods for diagnosis<sup>4</sup>. Low success rate of isolation, unreliability of direct demonstration of leptospire in clinical samples using dark ground microscopy<sup>5</sup> and inaccessibility of molecular techniques to most of the peripheral hospitals and clinics make serological tests play an important role in the laboratory diagnosis of leptospirosis. Recently, various serological tests like microcapsule agglutination test (MCAT), Lepto Dipstick, Lepto Lateral Flow, Lepto dridot have been evaluated as rapid screening tests for leptospirosis<sup>6-9</sup>. Since all these techniques were found to be having low sensitivity during the acute stage of the disease, development of techniques that would be more sensitive during the early stage of the disease is the need for the hour.

Development of serological techniques for early diagnosis of leptospirosis needs comprehensive understanding of leptospiral proteins expressed during acute and convalescent phases. Identification of an antigen that is conserved among a wide spectrum of pathogenic leptospire will help the development of serological test for early diagnosis as well as for seroepidemiological purposes. Proteins expressed during infection against which antibodies appear during acute stage will be useful in the development of rapid diagnostic test, whereas those against which antibodies persist during convalescent stage of the disease would be of useful for developing a test system that can be used in seroepidemiological studies. With this background we attempted to identify (i) leptospiral antigens conserved among the various pathogenic leptospira; (ii) to identify the best protein antigen to which immune response can be identified in the acute phase; and (iii) to identify the best protein antigen which is present in convalescent sera, which can be used for seroepidemiological studies.

### Material & Methods

**Leptospiral strains:** The leptospiral local isolates belonging to serogroups *icterohaemorrhagiae* (AF61), *autumnalis* (N2), *grippityphosa* (CH31), along with

other reference strains from the serovars like *australis* (Strain Jez-Bratislava), *autumnalis* (Akiyami A), *canicola* (Hond Utrecht IV), *grippityphosa* (Moskva V), *pyrogenes* (Salinem), *pomona* (Pomona), *hebdomadis* (Hebdomadis), *hardjo* (Hardjoprajitno) and *semaranga* (Patoc I) were used. All the strains were maintained in Johnson and Harris bovine serum albumin tween 80 medium<sup>10</sup> at National Leptospirosis Reference Centre, Regional Medical Research Centre, Port Blair, were received earlier from Royal Tropical Institute, Amsterdam, the Netherlands.

**Serum samples:** Forty three paired serum samples from patients with leptospirosis and 22 paired serum samples from patients clinically suspected but found to be negative for leptospiral serology, and 60 from other diseases (26 typhoid, 14 malaria and 20 hepatitis) were collected during October to December 2002 from G.B. Pant Government General Hospital, Port Blair. Apart from these 30 serum samples from apparently healthy controls were also collected. Paired sera were collected in a mean interval of 23 days. Patients fulfilling any of the following criteria were considered as cases of leptospirosis: (i) positive isolation of leptospire from blood or urine, (ii) seroconversion or four-fold rise in titre in microscopic agglutination test (MAT); and (iii) a MAT titre of 1:80 or more with a positive IgM ELISA (titre of 1:80).

**Whole cell solubilization:** Leptospiral cultures were centrifuged at 12,000 g for 10 min, pellets were washed twice in 5mM MgCl<sub>2</sub>-phosphate buffered saline (PBS) and then solubilized in sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer composed of 0.125M Tris.Cl (pH 6.8), 5mM EDTA, 2 per cent SDS, 0.1 per cent bromophenol blue, 1 per cent 2-mercaptoethanol<sup>11</sup>. This was utilized as the antigen in immunoblotting for probing patient serum for IgM and IgG recognition.

**Insoluble membrane (cytoplasmic and outer membrane) soluble cytoplasmic and periplasmic fractions:** Leptospiral cultures were harvested by centrifuging at 12,000 g for 10 min and the pellets were washed twice in 5mM MgCl<sub>2</sub>-PBS and resuspended in lysis buffer (50 mM Tris.Cl (pH8), 150 mM NaCl, 10 mM EDTA, 1 mg lysozyme/ml and 15% sucrose). The bacterial suspension was subjected to three cycles of freezing, thawing, and tip sonication

followed by centrifugation at 1,00,000 *g* for 30 min to separate the soluble supernatant fraction (periplasmic and cytoplasm) from the membrane pellet (cytoplasmic and outer membrane) fraction. The supernatant was precipitated with acetone.

*Detergent (Triton X-114) fractions:* Cultured leptospira cells were washed in 5mM MgCl<sub>2</sub>- PBS and then extracted in the presence of 1 per cent Triton X-114, 10 mM Tris (pH 8), and 1mM EDTA at 4°C. The insoluble material was removed by centrifuging at 17,000 *g* for 10 min. The Triton X-114 concentration in the supernatant was increased to 2 per cent. Phase separation was performed by warming the supernatant to 37°C and subjecting it to centrifugation for 10 min at 2,000 *g*. The detergent (outer membrane proteins (Omps)) and aqueous (periplasmic) phases were separated and precipitated with acetone<sup>12</sup>. Protein concentrations were measured by bicinchonic acid kits (Sigma, USA).

*Gel electrophoresis and immunoblotting:* For one dimensional SDS-PAGE, samples were solubilized in SDS-PAGE sample buffer. The samples were heated at 100°C for 10 min the day before electrophoresis was done and were stored at 4°C overnight<sup>11</sup>. The next day, samples were run in 10 per cent polyacrylamide gels<sup>13</sup>. Low molecular weight protein standards (Bangalore Genei) were also included on each gel. After electrophoresis, the proteins were electrophoretically transferred to nitrocellulose (0.45µm) membrane (Whatman, UK) with 20 per cent methanol in the tris-glycine buffer<sup>14</sup>. After transferring the membranes were blocked with 3 per cent bovine serum albumin (BSA) in tris buffered saline (TBS) and 0.1 per cent tween 20 and incubated with patients' sera followed by IgM/IgG (Sigma, USA). Colour was developed with a substrate solution of 25 mg of diaminobenzidine (DAB) and 30 µl of 3 per cent H<sub>2</sub>O<sub>2</sub> in 50 ml of 1X TBS. Blots were then rinsed in several changes of deionized water to stop the reaction. For conservation of proteins, samples prepared from different leptospiral reference strains were solubilized in SDS-PAGE sample buffer and run on one dimensional SDS-PAGE followed by immunoblotting and probed with pooled convalescent human sera followed by IgG recognition.

## Results

Sera of confirmed cases of leptospirosis analyzed by Western blot analysis recognized different leptospiral antigen bands with relative mobility (*Mr*) values in the range of 14 to 82 kDa for *L. interrogans* serovar Icterohaemorrhagiae strain AF61 (Fig.1). Against these antigens the predominant response observed was against IgG antibody in comparison to that of IgM antibodies. IgM antibody mainly detected the leptospiral protein in lower *Mr* from 14 to 45kDa (Fig.2). However we observed frequently the IgG response against leptospiral antigens in the range of 31kDa-82kDa. In subsequent immunoblot analysis the frequencies of IgG and IgM antibody reactivity to the leptospiral proteins were identified for acute and convalescent phase sera of leptospirosis patients and compared with that from sera of other control groups (Table I). Among the confirmed cases the IgG response was frequently observed against leptospiral proteins *viz.*, p32, p41/42, p45, p58, p62, p76 and p82 during acute phase and during the convalescent phase the recognition was considerably increased. The recognition was higher for p32 followed by p82, p41/42 and p62 respectively. Similarly, IgM recognition during acute phase for the proteins like p14, p25, p32 and p41/42 with a increase in recognition during the convalescent phase and the recognition was higher for p32 followed by p41/42, p25 and no further increase for p14. Apart from the predominant proteins second line proteins were also recognized, which included p20, p25, p31, p37, p44, p45, p48, p70 and p76 for IgG and p20, p31, p37, p44 and p45 for IgM recognition.

Overall, the leptospiral protein p32 reactivity was found to be the best with high specificity as it only showed 9 per cent reactivity for IgG and no recognition for IgM for serologically negative cases for leptospirosis in both the phases. Among the healthy controls, a positivity of 3.3-10.0 per cent for IgG and no recognition for IgM was observed. For IgG the p82 and p62 recognition was found among all the control groups. But other proteins like p32 IgG reactivity was seen in patients with hepatitis and healthy controls, and p76 reactivity was found in typhoid and hepatitis patients.

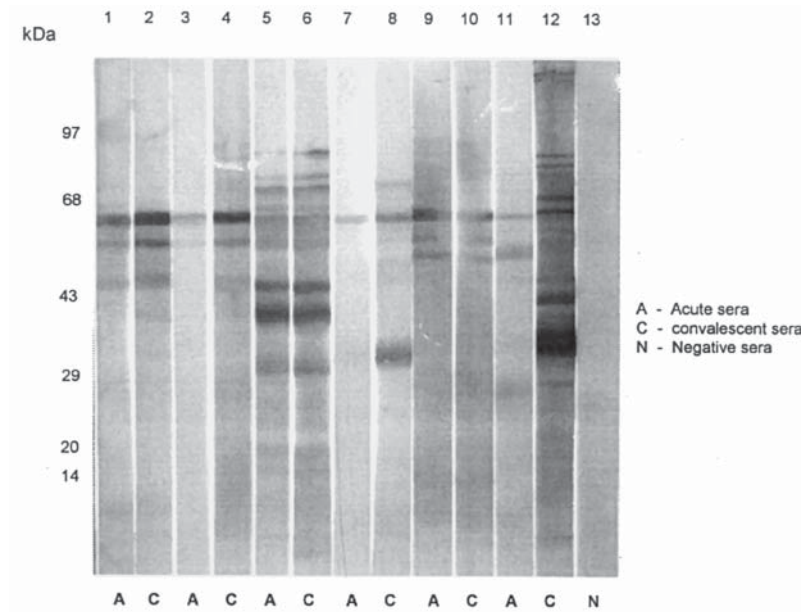
**Table I.** Leptospiral protein recognition by immunoblotting for confirmed and suspected cases of leptospirosis along with control group

Protein	Sera with IgG reactivity									
	Confirmed cases for leptospirosis (n=43)			Clinically suspected but serologically negative (n=22)			Control groups (n=90)			
	I Phase	II Phase	Sero-conversion	I Phase	II Phase	Sero-conversion	Healthy (n=30)	Typhoid (n=26)	Malaria (n=14)	Hepatitis (n=20)
P82	12 (27.9)	21 (48.8)	9 (20.9)	2 (9.1)	2 (9.1)	0 (0.0)	1 (3.3)	1 (3.8)	2 (14.2)	3 (15.0)
P76	13 (30.2)	14 (32.5)	1 (2.3)					2 (7.6)		2 (10.0)
P70	3 (6.9)	4 (9.3)	1 (2.3)							
P62	24 (55.8)	29 (67.4)	5 (11.6)	4 (18.2)	4 (18.2)	0 (0.0)	1 (3.3)	4 (15.3)	1 (7.1)	1 (5.0)
P58	12 (27.9)	20 (46.5)	8 (18.6)	1 (4.5)	1 (4.5)	0 (0.0)	4 (13.3)			
P48	6 (13.9)	7 (16.3)	1 (2.3)							
P45	16 (37.2)	18 (41.9)	2 (4.6)	1 (4.5)	5 (22.7)	4 (18.2)	3 (10.0)			
P44	3 (6.9)	7 (16.3)	4 (9.3)							
P41/42	17 (39.5)	24 (55.8)	7 (16.3)	4 (18.2)	4 (18.2)	0 (0.0)				2 (10.0)
P37	6 (13.9)	10 (23.3)	4 (9.3)	1 (4.5)	1 (4.5)	0 (0.0)				1 (5.0)
P32	13 (30.2)	28 (65.1)	15 (34.9)	2 (9.0)	2 (9.0)	0 (0.0)	1 (3.3)			2 (10.0)
P31	2 (4.6)	5 (11.6)	3 (6.9)							
P25	10 (23.2)	10 (23.3)	0 (0.0)							2 (10.0)
P20	0 (0.0)	1 (2.3)	1 (2.3)				1 (3.3)			
P14										

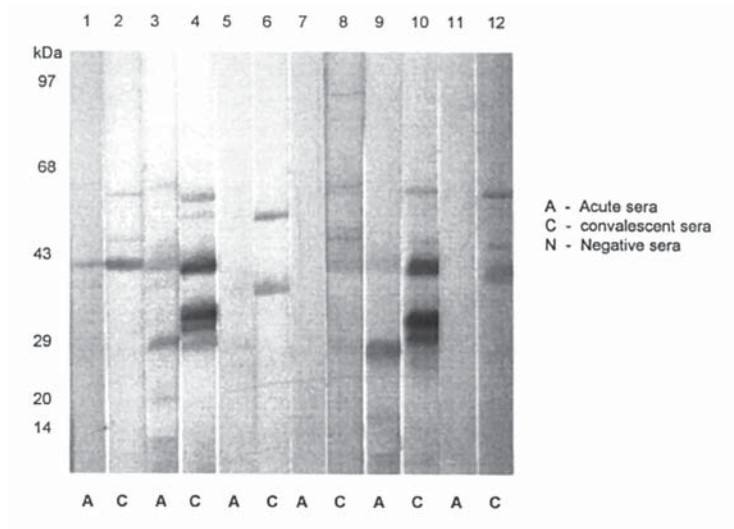
  

Protein	Sera with IgM reactivity									
	Confirmed cases for leptospirosis (n=43)			Clinically suspected but serologically negative (n=22)			Control groups (n=90)			
	I Phase	II Phase	Sero-conversion	I Phase	II Phase	Sero-conversion	Healthy (n=30)	Typhoid (n=26)	Malaria (n=14)	Hepatitis (n=20)
P82										
P76										
P70										
P62										
P58										
P48										
P45	3 (6.9)	4 (9.3)	1 (2.3)							
P44	2 (4.6)	3 (6.9)	1 (2.3)							
P41/42	16 (37.2)	18 (41.9)	2 (4.7)	2 (9.0)	2 (9.0)	0 (0.0)				
P37	8 (18.6)	8 (18.6)	0 (0.0)							
P32	13 (30.2)	19 (44.2)	6 (14.0)							
P31	3 (6.9)	4 (9.3)	1 (2.3)							
P25	14 (32.6)	16 (37.2)	2 (4.7)							
P20	4 (9.3)	4 (9.3)	0 (0.0)							
P14	14 (32.6)	14 (32.6)	0 (0.0)	1 (4.5)	1 (4.5)	0 (0.0)				

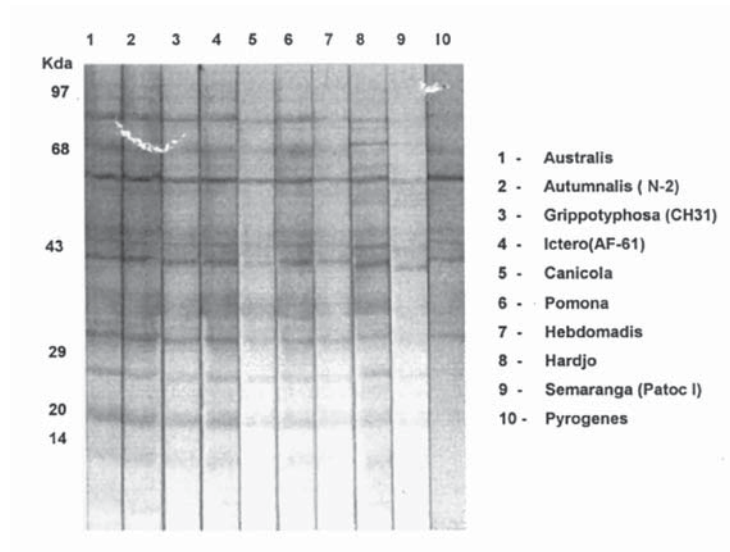
Values are shown as number (%)



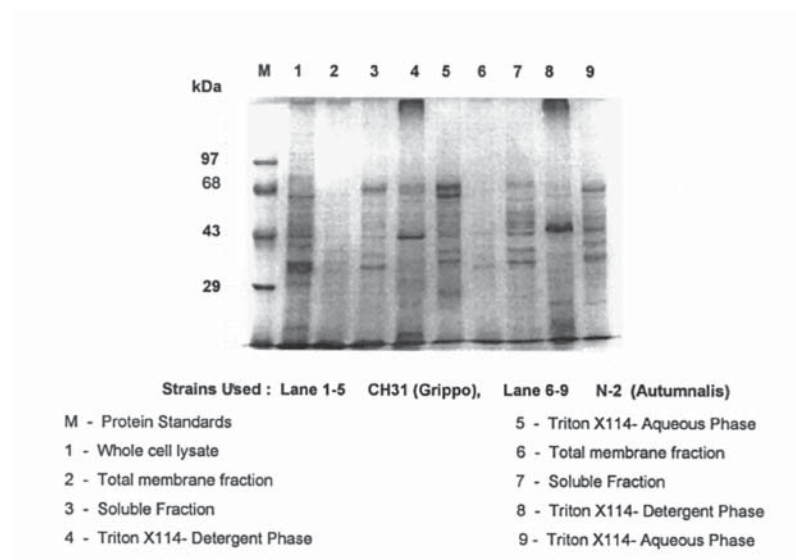
**Fig.1.** Immunoblots for IgG recognition using paired sera of leptospirosis patients.



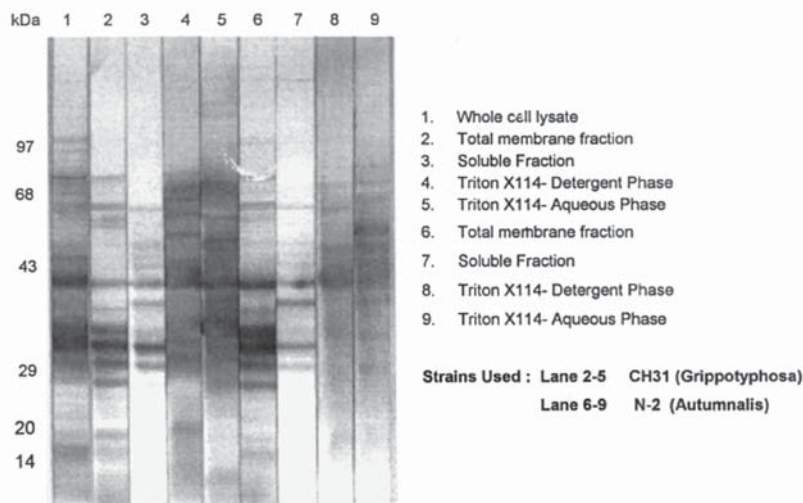
**Fig.2.** Immunoblots for IgM recognition using paired sera of leptospirosis patients.



**Fig.3.** Immunoblots for conservation of protein antigens among leptospiral patients.



**Fig.4.** SDS PAGE profile of total membrane and soluble fractions and Triton X114 fractions of leptospiral strains N2 and CH31.



**Fig.5.** Immunoblots of total membrane and soluble fractions and Triton X114 fractions of leptospiral strains N2 and CH31.

It was found that the p32 and other proteins like p41/42, p58, p62 and p82 were frequently recognized for IgG and p41/42 for IgM among leptospirosis cases. The combination of these proteins with p32 has increased the sensitivity further (Table II). The combination of p32 with p41/42 had increase the sensitivity to 74.4 per cent without compromising the specificity (81.8%) with high PPV (88.9%). The combination of other proteins along with these two proteins increased the sensitivity but the specificity was decreased (77.3%). p41/42 was recognized by the serum of 10 per cent hepatitis patients only. This suggested that the combination of p32 with p41/42 could be useful for the diagnosis of leptospirosis. Though p62 reactivity was higher and it was detected even in phase I sera by IgG, it gave about 3.3-15 per cent false positivity among other controls. This particular protein may be useful for seroepidemiological studies since in the present study all the control groups were selected from the endemic areas. For IgM recognition the combination of p32 with p14, p25 and p41/42 increased the sensitivity to 76.7 per cent without compromising the specificity (90.9%) with a positive predictive value (PPV) of 94.3 per cent. The proteins like p14 and p25 were predominantly recognized by IgM and p62 by IgG. For these three proteins, the seroconversion during infection was 0-11.6 per cent only (Table I). This showed that these proteins were expressed at the early stage of infection and the other proteins like p32, p41/

42 showing increased seroconversion expressed during active infection.

Conservation of protein antigens among leptospiral strains, belonging to various locally predominant serovars was also analyzed using pooled convalescent phase sera of leptospirosis patient for IgG recognition (Fig.3). Based on this analysis 14 leptospiral bands were recognized for the leptospiral proteins, which included p14, p20, p25, p31, p32, p35, p41/42, p45, p48, p58, p62, p76 and p82. There was no polymorphism observed among the leptospiral proteins of different pathogenic strains used. The non pathogenic leptospiral strain Patoc I did not show any bands except p62. The p62 recognition may be common among pathogenic and non pathogenic leptospire. Other predominant proteins like p32, p41/42, p58 and p82 were recognized only by using the pathogenic leptospire as antigen.

Leptospiral cell fractionation studies were performed using two local isolates *viz.*, CH31 and N2 belonging to the serogroups Grippotyphosa and Autumnalis (Figs 4,5). The leptospiral protein p31, p32, p41, p76 were prominently seen in the total membrane fraction which included the cytoplasmic and outer membrane. P42, p62 appeared in the soluble fraction, which mainly comprised cytoplasmic and periplasmic materials. In Triton X-114 fraction detergent phase p41, p32 and p31 were observed,

**Table II.** Sensitivity, specificity and predictive values of leptospiral proteins in single and combination for IgG and IgM recognition

Proteins	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
<i>IgG reactivity</i>				
P82	48.8	90.9	91.3	47.6
P62	67.4	81.8	87.9	56.3
P58	46.5	95.5	95.2	47.7
P41/42	55.8	81.8	85.7	48.6
P32	65.1	90.9	93.3	57.1
<i>Combination of proteins</i>				
P32+P41/42	74.4	81.8	88.9	62.1
P32+P41/42+P58	74.4	81.8	88.9	62.1
P32+P41/42+P58+P62	91.0	77.3	88.6	81.0
P32+P41/42+P58+P62+P82	93.0	77.3	88.8	85.0
<i>IgM reactivity</i>				
P41/42	41.8	90.9	90.0	44.4
P32	44.2	100.0	100.0	47.8
P25	37.2	100.0	100.0	44.8
P14	32.5	95.4	93.3	42.0
<i>Combination of proteins</i>				
P32+P14	51.2	95.5	95.7	50.0
P32+P14+P25	62.8	95.5	96.4	56.8
P32+P14+P25+P41/42	76.7	90.9	94.3	66.7

PPV, positive predictive value; NPV, negative predictive value

which were released from outer membrane. In the aqueous phase p37 and p25 recognitions were observed, which were predominantly from the periplasmic material.

### Discussion

Guerreiro *et al*<sup>15</sup> analysed the leptospiral protein expression on the basis of IgG reactivity<sup>15</sup>, but in the present study the IgG reactivity was compared with IgM to analyse the proteins expressed during acute and convalescent stages. Pathogenic leptospira strains possess a number of proteins that are expressed during mammalian infection. The expression of leptospiral Omps components was analysed during renal infections in hamsters<sup>16</sup>. In the present study apart from the Omps, the expression of other membrane proteins was also recognized and the leptospiral protein p32 was found to be specific and a predominant protein both in acute and convalescent phase sera for IgG and IgM recognition. Reactivity of p32 was demonstrated as specific because it showed only 0-10 per cent false

positivity among the controls, and the recognition for p32 was only observed among the pathogenic serovars. These results are consistent with the recent characterization of LipL32 as an outer membrane lipoprotein, which is expressed at high levels by pathogenic leptospira species<sup>17</sup>. P41/42 is a complex with at least two antigen bands with *Mr* of 41 to 42kDa that were not consistently discriminated in immunoblot analysis, and p41 is outer membrane protein and p42 is inner membrane protein<sup>1</sup>. The leptospiral protein p62 is a GroEL<sup>15</sup> of cytoplasmic origin and its expression was found to be recognized even against non pathogenic leptospiral serovar *Patoc*. Compared to other predominant leptospiral proteins, p62 showed less specificity since it was also recognized in all control groups showing 3.3-15 per cent reactivity. Though the recognition of p62 was more frequent than that of p32, the seroconversion among the confirmed cases was less. The inner membrane protein p82 recognition was appreciable with an increased seroconversion among the confirmed cases next to p32. Based on the above it is clear that the p32 is

the major outer membrane protein, which is expressed during early phase of infection with an increased seroconversion. The other protein p41/42 complex also seems to be important because of its recognition in both acute and convalescent phase using IgG and IgM with increased specificity, showing only 4.4 per cent reactivity among controls.

In conclusion, the combination of the leptospiral proteins p14, p25, p32 and p41/42 may be a good candidate antigen for the diagnosis of leptospirosis during acute phase using IgM. This combinations the leptospiral proteins showed a PPV of 94.3 per cent for IgM recognition. This is higher in comparison to the commercially available IgM based tests kits like LEPTODipstick (91.4%)<sup>7</sup>, Lepto Lateral flow (90.7%)<sup>8</sup> and Lepto Dri Dot (82.5%)<sup>9</sup>. The high PPV indicates that it detects only the true cases. In case of IgG, the combination of p32 and p41/42 was found to be useful to diagnose the cases, with high sensitivity and PPV. For seroepidemiological studies the combination of p32, p41/42, p58, p62 and p82 may be useful using IgG because of high sensitivity. Further cloning and expression of these proteins and usage in these combinations would be helpful for the development of rapid diagnostic test systems.

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*Reprint requests:* Prof. S.C. Sehgal, WHO Collaborating Centre for Diagnosis Reference Research and Training in Leptospirosis Regional Medical Research Centre (ICMR), Port Blair 744101, India  
e-mail: pblicmr@sancharnet.in