Indian J Med Res 121, March 2005, pp 151-158

Comparison of immunoreactive proteins of commonly circulating serogroups of *Leptospira* in Andaman Islands, India

D. Biswas, S. Roy, P. Vijayachari, A.P. Sugunan, K. Natarajaseenivasan & S.C. Sehgal

WHO Collaborating Centre for Diagnosis, Research, Training & Reference in Leptospirosis, Regional Medical Research Centre (ICMR), Port Blair, Andaman & Nicobar Islands, India

Received May 26, 2004

***Background & objectives*: Early diagnosis is the key to the treatment of leptospirosis. For development of rapid diagnostic kits, a thorough knowledge about the nature of the proteins expressed by the pathogen during infection is necessary. The present study was undertaken to understand the nature of immunoreactive proteins from commonly circulating serogroups of *Leptospira* in the endemic Andaman and Nicobar Islands, India.**

***Methods*: Proteins were extracted from six strains of *Leptospira* representing five different serogroups following four different preparation methods, *viz*., whole cell lysis by sonication, detergent solubilization, outer and inner membrane isolations, and were subsequently characterized on sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Immunoblots were made from the sonicated proteins using hyperimmune rabbit antisera, homologous and heterologous patient sera separately.**

***Results*: The 67, 65, 45, 43, 35, 32 and 18 kDa major proteins in the whole cell lysate were common among all the five serogroups of *Leptospira*. The 67, 41, 35, 32, 28 and 22 kDa were the major outer membrane proteins, while 94, 32, 25 and 18 kDa protein were in inner membrane. Immunoblots with hyperimmune rabbit antisera detected 67, 65, 60, 45, 43, 41 and 32 kDa common proteins from the whole cell lysates of all strains while homologous and heterologous patient sera detected 32 kDa as the major immunoreactive protein in all pathogenic serogroups. This protein reacted against specific LipL32 antisera indicating that this protein was LipL32.**

***Interpretation & conclusion*: The circulating serogroups of *Leptospira* have common nature of expression of proteins during human infection. Among several immunoreactive proteins, three (67, 45 and 32 kDa) were recognized as major antigens by both rabbit hyperimmune sera and patients sera while the 32 kDa protein was recognized as the major immunoreactive protein by homologous and heterologous patient sera. These conserved immunoreactive proteins could be utilized in developing indigenous diagnostic tests for leptospirosis.**

Key words Detergent soluble antigen - immunoreactive protein - IMP - leptospirosis - OMP - sonicated antigen

Leptospirosis is a widespread and re-emerging zoonosis caused by pathogenic spirochetes belonging to the genus *Leptospira*. Serological classification of *Leptospira* at the subspecies level is based on antigenic similarities, serovar being the basic taxon. Based on variations in the carbohydrate side chains of lipopolysaccharide (LPS) of the cell membrane, more than 230 different antigenic types or serovars of pathogenic *Leptospira* have been recognized[1,2]. Genotypic classification based on DNA hybridization defined 21 genospecies of *Leptospira* that include 29 serogroups and 269 serovars[3]. Although the serological and genotypic classifications do not correlate, the latter provides a strong foundation for future classification[4]. The disease caused by all serovars exhibit more or less similar symptoms.

Although leptospirosis was known to be endemic from the early part of the twentieth century in Andaman Islands, the first authentic isolation of leptospires in 1931[5]. Very little was known on the disease status till 1988, when post-monsoon outbreaks of a febrile illness with haemorrhagic tendencies started appearing. The cause of these outbreaks was later proved as leptospirosis[6]. Since then, several isolates of leptospires have been obtained and attempts have been made to characterize these strains, both serologically and using molecular techniques. The commonest serovars isolated belong to serogroup Grippotyphosa[7,8]. Isolation of strains belonging to other serogroups such as Icterohaemorrhagiae were reported from these Islands but these are relatively rare[9]. Serogroup Autumnalis is common in southern States of India[10].

Several approaches have been made to identify and characterize the leptospiral antigens and elucidate their role in pathogenesis. Most of these are concerned with single serogroups of leptospira focusing on leptospiral LPS and outer membrane proteins (OMP)[11-16]. It is known that leptospiral pathogenesis is related to the expression of proteins during infection and these proteins become the targets for the host immune response[17]. Little is known about immunoreactive proteins present in different serogroups of leptospires. Hence a study was carried out to identify and characterize the common immunoreactive leptospiral proteins from different serogroups of *Leptospira* in Andaman islands that are expressed during the host immune response.

Material & Methods

Bacterial strains and media: Six *Leptospira* strains D22, Moskva V, AF61, CH11, Aut N, and Patoc1 belonging to five different serogroups (Grippotyphosa, Icterohaemorrhagiae, Andamana, Autumnalis and Semaranga) were included in this study. Among these strains, D22 (serogroup Grippotyphosa), AF61 (serogroup Icterohaemorrhagiae), CH11 (serogroup Andamana) and Aut N (serogroup Autumnalis) were isolated from South Andaman Island and other parts of India. Moskva V (*L. interrogans* Sensu Lato) was the reference strain of serogroup Grippotyphosa and Patoc1 (*L. biflexa*) was the reference strain of serogroup Semaranga. However, CH11 (serogroup Andamana) was an isolate from a patient that was later found to be a member of saprophytic *L. biflexa*. All strains were serologically characterized using microscopic agglutination test (MAT)[18] with group sera and also with monoclonal antibodies[19]. All cultures were maintained in Ellinghausen, McCollaugh, Johnson, Harris (EMJH) culture medium (Difco, USA) at 30°C.

Serum samples: Paired serum samples from nine confirmed cases of leptospirosis and 11 patients showing symptoms similar to leptospirosis in whom leptospirosis was later ruled out (based on MAT), were used in the study. The diagnosis in the confirmed cases was based on seroconversion or four-fold rise in titre in MAT and these patients' sera had given high MAT titres against different serogroups. These patients were selected randomly from among 70 consecutive cases of suspected leptospirosis attending a rural primary health centre in South Andaman during the period 2001-2002 and sera were collected during 1st - 3rd wk of illness. MAT was performed on all the serum samples following standard procedure[18] using 12 live leptospiral strains (1-2 x 10^8 organism/ml) as antigens (Table). Sera from patients and controls were used in immunoblots against antigenic preparations.

Table. Details of the leptospiral strains used in MAT

S.No.	Serogroup	Serovar	Strain
1.	Autumnalis	rachmati	Rachmati
2.	Australis	australis	Ballico
3.	Bataviae	bataviae	Swart
4.	Canicola	canicola	H.Uterecht IV
5.	Grippotyphosa	grippotyphosa	CH 31
6.	Hebdomadis	hebdomadis	Hebdomadis
7.	Icterohaemorrhagiae	lai	Lai
8.	Javanica	poi	Poi
9.	Pomona	pomona	Pomona
10.	Sejroe	hardjo	Hardjoprajitno
11.	Tarasslovi	tarasslovi	Perepelicin
12.	Pyrogenes	pyrogenes	Salinem

Rabbit hyperimmune sera: Hyperimmune sera were raised in rabbits against the strains D22 (Grippotyphosa), AF61 (Icterohaemorrhagiae), CH11 (Andamana) and Aut N (Autumnalis) as recommended by the International Committee on Systematic Bacteriology, Sub-Committee on the Taxonomy of Leptospira (TSC)[20]. Serum samples collected from non-immunized healthy rabbits that showed negative results in MAT were used as negative controls. Antisera raised in rabbits against the leptospiral proteins LipL32 and Flagellin A (gifted by Dr David A. Haake of VA Greater Los Angeles Healthcare System, Los Angeles, USA) were also used as the outer membrane marker for confirmation of the sarcosyl treated outer membrane fractions of leptospiral strains used in the study.

Whole cell lysis by sonication: Six well-grown formalin-inactivated cultures (1-2X10^8 organisms /ml) of *Leptospira* were centrifuged separately at 4000 *g* for 30 min. The pellets were washed with 0.15 *M* phosphate buffered saline (PBS), *p*H 7.4 (Sigma, USA) thrice and suspended in PBS containing phenylmethyl sulfonyl fluoride (PMSF,100 µg/ml, Sigma) and lysed using a sonicator (Labsonic, B.Braun, Germany). The sonicated suspension was centrifuged at 4000 g for 30 min for removing the cell debris. The supernatant was collected and centrifuged again at 100,000 g for 1 h in an ultra-centrifuge (Discovery 100 SE, Sorvall, USA). The pellets were dissolved in MilliQ water and used as whole cell lysates.

Detergent lysis: 0.5 per cent formalin-inactivated leptospiral cultures (1-2 x 10^8 organisms/ml) were centrifuged for 30 min at 4000 g. Pellets were washed twice with 0.15*M* PBS and re-suspended in Triton X 100 (4% in 0.01 *M* PBS) at a proportion of 5 ml Triton per g net weight of the pellet. The whole suspensions were then kept at 45^0C for 4 h in a shaking water bath. After incubation, the cell suspensions were centrifuged at 4000 g for 30 min and the supernatants were collected, concentrated in a lyophilizer (Christ, B. Braun Biotech International, Germany) and used as detergent soluble proteins.

Preparation of outer and inner membrane proteins: The outer and inner membrane proteins were prepared following the method of McCarter & Silverman[21] with minor modifications. In brief, six well grown cultures (1-2X10^8 organisms/ml) of leptospires were centrifuged. The pellets were washed twice with normal saline and once with 10 m*M* HEPES buffer *p*H 7.0 (Sigma) and suspended in reagent A (50 m*M* Tris HCl, 50 m*M* EDTA, 15% sucrose and 0.3 mg/ml lysozyme) for 1 h on ice. After incubation, the suspensions were centrifuged at 4000 g for 10 min to eliminate unlysed cells and the supernatants were centrifuged at 12000 g for 30 min to obtain the total cell membranes. The membrane pellets were then treated with 1 per cent N-lauryl sarcosine. The sarcosine insoluble fractions were removed by centrifugation at 100,000 g for 1 h in an ultra-centrifuge (Discovery 100 SE, Sorvall), washed once with PBS, *p*H 7.4 and used as outer membrane protein fractions. The supernatants (sarcosine-soluble fractions) were dialyzed against cold water for 48 h for removal of sarcosine. The dialyzed samples were ultracentrifuged at 200,000 g for 2 h and the pellets were dissolved in MilliQ water and used as inner membrane protein fractions. The protein content of different types of preparations was estimated using bicinchoninic acid assay (Sigma).

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE): Four different types of protein preparations (sonicated whole-cell lysates, detergent soluble, outer membrane fractions, and inner membrane fractions) of the six strains of *Leptospira* were subjected to SDS-PAGE individually using a Mini Protein II cell (Bio Rad) following the method of Laemmli[22]. Equal amounts (20 µg) of each type of protein preparations were heated at 100^0C for 5 min in 1:2 diluted sample buffer [62.6m*M* Tris.HCl, *p*H 6.8, 25% (v/v) glycerol, 2% (v/v) SDS and 5% (v/v) b-mercaptoethanol] before separation on 10 per cent polyacrylamide gels. Proteins were stained with Coomassie blue (Brilliant Blue R; Sigma). Relative molecular mass of proteins was determined using standard protein marker (Amersham Pharmacia, USA) in the same gel and analyzing the migration with BioGene software in Gel documentation system (Vilber Lourmat, France).

Immunoblotting: Sonicated and outer membrane preparations of the six leptospiral strains were subjected to SDS-PAGE separately. The separated

proteins were transferred from unstained 10 per cent SDS-PAGE onto membrane filters (0.45 mm immobilon NC HAHY nitrocellulose membrane, Millipore, USA) according to method described by Towbin *et al*[23]. After transfer, membranes were blocked with blocking buffer [PBS containing 2% (w/v) bovine serum albumin (BSA), 0.05% (v/v) Tween 20] for 2 h at room temperature (RT) and rinsed three times with blocking buffer without BSA. Human convalscent phase sera from confirmed patients of leptospirosis and hyperimmune rabbit antisera (at a dilution of 1:100); rabbit polyclonal antisera raised against Flagellin A and LipL32 outer membrane proteins (at a dilution of 1:3000, for immunoblotting of the sarcosyl treated fraction of outer membrane proteins) were used separately as primary antibody and incubated overnight at 4^0C. The conjugates used were alkaline phosphatase-conjugated anti-rabbit IgG whole molecules (Sigma) for rabbit antisera, and anti-human IgM Fc specific conjugate for human sera (Sigma) at 1:2000 dilution. Colour was developed with *p*-nitroblue tetrazolium chloride (NBT; 0.33 mg/ml Sigma), 5-bromo-4-chloro-3-indolyl phosphate (BCIP; 0.165 mg/ml, Sigma) as substrate in alkaline phosphatase buffer (0.1*M* Tris-0.1 *M* NaCl-0.005*M* $MgCl_2$: *p*H9.5) and documented in Gel documentation system.

Results

The amount of protein obtained per 100 ml culture varied from strain to strain and was in the range of 1.0-2.0 mg for whole cell lysates, 2.0-3.0 mg for detergent soluble proteins, 1.7-2.0 mg for outer membrane fractions and 3.0-4.0 mg for inner membrane fractions. The electrophoretic banding patterns of the different types of antigens showed similarities among all the six strains of *Leptospira* irrespective of their serogroup. In whole cell lysates, seven proteins having molecular weights of 67, 65, 45, 43, 35, 20 and 18 kDa were the major proteins that gave prominent bands in SDS-PAGE (Fig.1). The prominent components of outer membrane proteins were 67, 41, 35, 32, 28 and 22 kDa in size (Fig.2), while 94, 32, 25 and 18 kDa proteins were the prominent ones in the inner membrane fractions (Fig.3). Since the 67 and 32 kDa proteins were present in both outer membrane, as well as detergent soluble preparations, it appears that these proteins present in

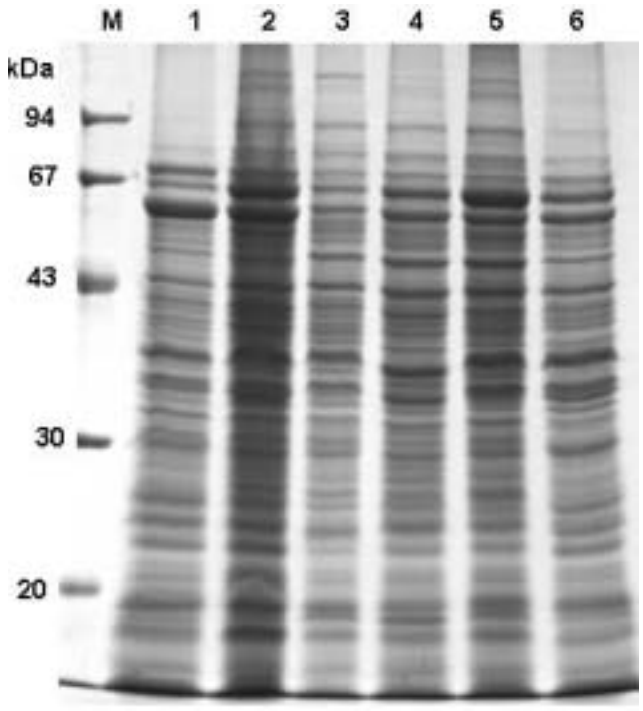

Fig.1. SDS-PAGE profiles of whole cell lysates by sonication; M, molecular weight marker. Lane 1: AF61, 2: D22, 3: Aut N, 4: Moskva V, 5: CH11, 6: Patoc1.

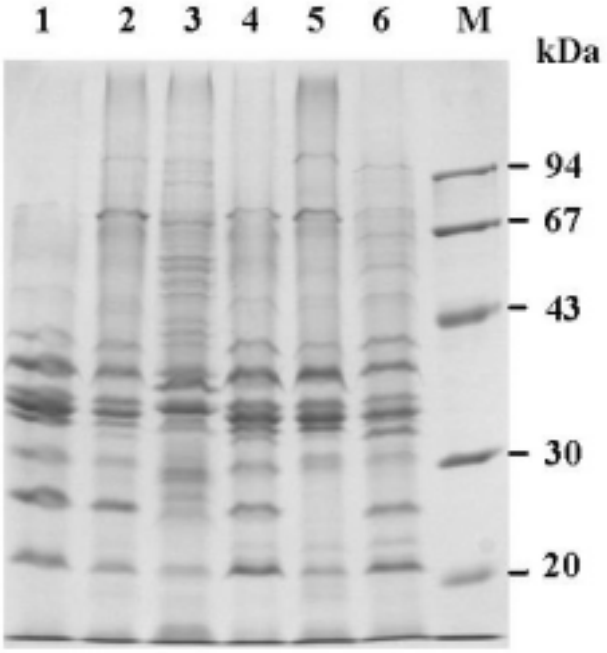

Fig.2. SDS-PAGE profiles of outer membrane proteins; M, molecular weight marker. Lane 1: AF61, 2: D22, 3: Aut N, 4: MoskvaV, 5: CH11, 6: Patoc1.

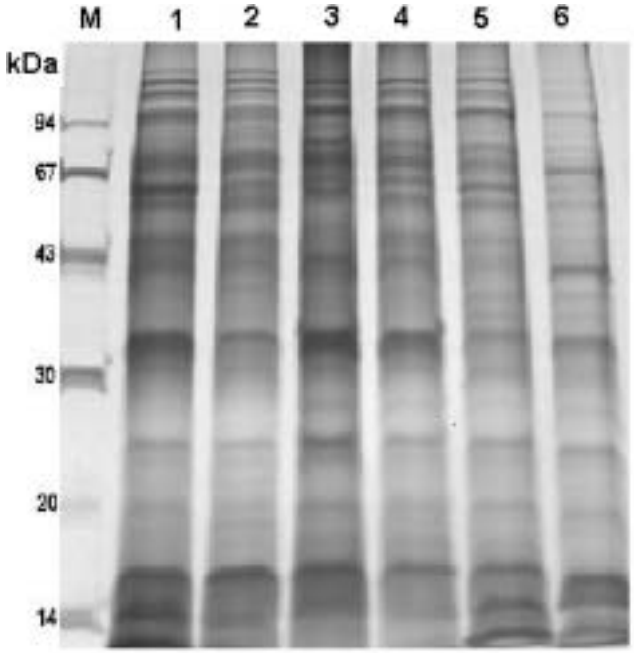

Fig.3. SDS-PAGE profiles of inner membrane proteins; M, molecular weight marker. Lane 1: AF61, 2: D22, 3: Aut N, 4: Moskva V, 5: CH11, 6: Patoc1.

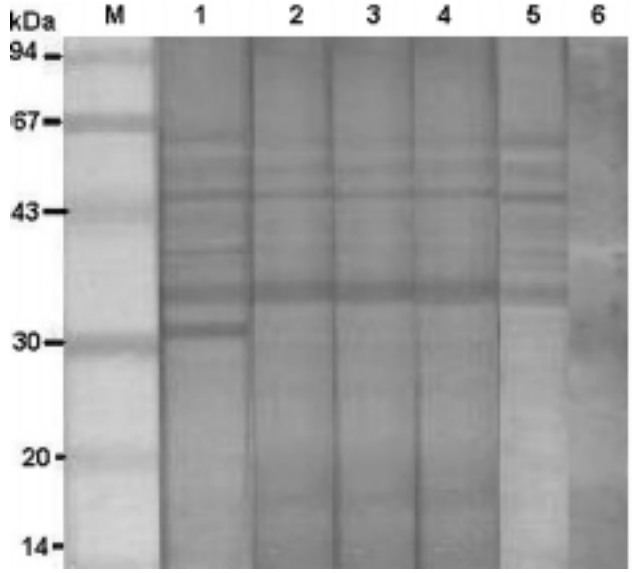

Fig.5. Immunoblots of whole cell lysates using rabbit hyperimmune sera; M, molecular weight marker. Lane 1: AF61, 2: D22, 3: Aut N, 4: Moskva V, 5: CH11, 6: Negative control.

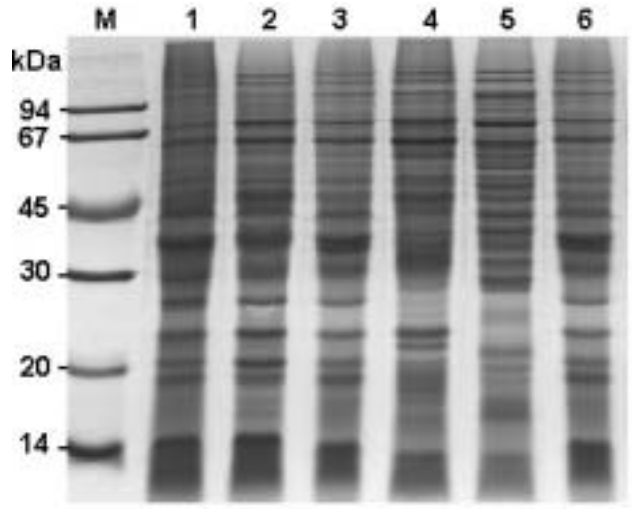

Fig.4. SDS-PAGE profiles of detergent soluble proteins; M, molecular weight marker. Lane 1: AF61, 2: D22, 3: Aut N, 4: Moskva V, 5: CH11, 6: Patoc1.

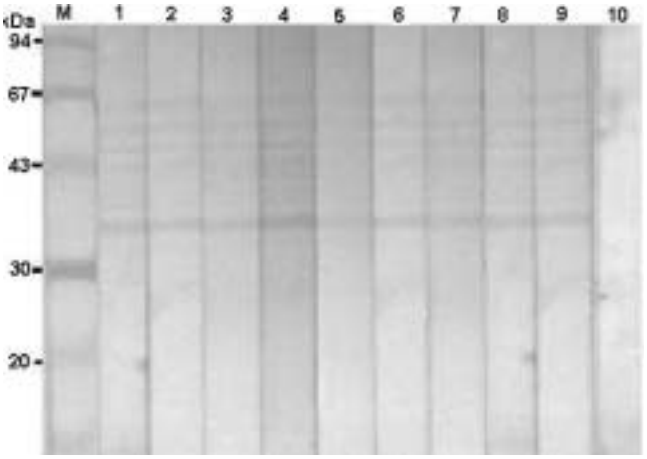

Fig.6. Immunoblots of whole cell lysates using patient sera; M, molecular weight marker, Lanes 1-9 Patients' sera, 10-Negative control.

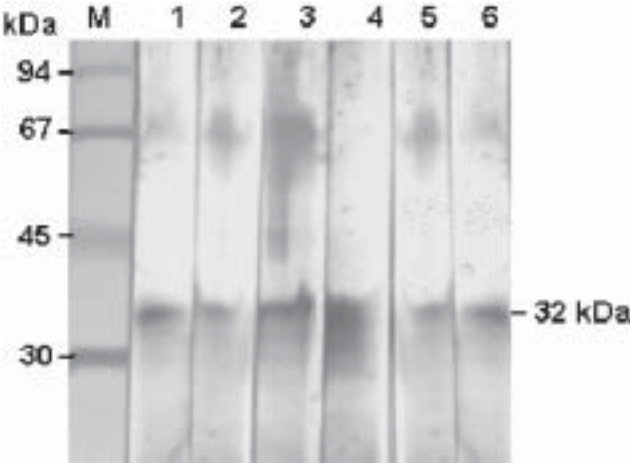

Fig.7. Immunoblots of outer membrane proteins using LipL32 specific antisera; M, molecular weight marker. Lane 1: AF61, 2: D22, 3: Aut N, 4: Moskva V, 5: CH11, 6: Patoc1.

outer membrane are detergent soluble in nature (Fig.4). Immunoblots of whole cell lysates using hyperimmune rabbit antisera detected seven distinct antigens of molecular weights 67, 65, 60, 45, 43, 41 and 32 kDa among all the four serogroups of *Leptospira* (Fig.5). However, among the seven immunoreactive proteins the 45 and 32 kDa proteins were the most reactive and gave the strongest bands on nitrocellulose filter. The antisera against AF61 (serogroup Icterohaemorrhagiae) detected an additional band (30 kDa), which was not very prominent in the SDS-PAGE of whole cell lysates. No band was observed on the immunoblots of control sera (Fig.5). Immunoblots on whole cell lysates of different strains of *Leptospira* using homologous and heterologous sera gave similar results.

The position, number, and the staining intensity of immunoreactive protein bands recognized by patient sera from confirmed cases were similar to those of immunoreactive proteins detected by hyperimmune rabbit antisera. Sera from all patients except one detected a 32 kDa protein as the major immunoreactive protein (Fig.6) in all the six strains used. Antibodies against the antigens 67, 45 and 32 kDa, were specific as none of the control sera reacted against these proteins. Immunoblots using LipL32 specific antisera were able to recognize the 32 kDa proteins from outer membrane of all the six strains suggesting that this protein was LipL32 (Fig.7).

Discussion

We studied commonly circulating serovars of *Leptospira* to find out the nature of common immunoreactive proteins that are expressed during the acute stage of the disease. Brown *et al*[24] compared the whole cell lysates and detergent (SDS) soluble outer envelope-enriched proteins of *Leptospira interrogans* serovars present in commercially available pentavalent vaccine by SDS-PAGE and Western blotting using antisera raised specifically against the cellular component of strains hardjobovis and hardjoprajitno. They found similarities among the protein profiles of both the whole cell and detergent soluble proteins indicating that common antigens are shared among the *Leptospira* strains studied. Our study was based on clinical isolates obtained from patients. The present study highlights the use of sarcosyl for the first time for fractionating the outer membrane and inner membrane proteins in *Leptospira*. Use of sarcosyl, though is an established method for fractionating outer and inner membrane fractions in the case of other Gram negative bacteria[21], has not been used in *Leptospira*. Immunoblots of outer membrane proteins using polyclonal antisera against Flagellin A and LipL32 raised in rabbit separately were able to identify the respective immunoreactive proteins in the outer membrane sarcosyl fraction in all the strains used in this study, suggesting that sarcosyl treatment could separate the leptospiral membrane proteins into outer and inner fractions.

To characterize the immunoreactive proteins of the commonly circulating serogroups of *Leptospira*, four protein preparations were made using different methods from representative members of the five serogroups. SDS-PAGE profiles of all five preparations showed similarities in their banding patterns. Seven distinct bands in the whole cell lysates were immunoreactive and these were common to all the six strains used in the study. Among these immunoreactive proteins the 67, 45 and 32 kDa proteins were the most prominent ones. These proteins were detected by homologous and heterologous rabbit antisera and human sera, which gave significant MAT titres against different serogroups. Homologous and heterologous patient sera however, detected the 32 kDa proteins as the major immunoreactive band. None of

the sera collected from non leptospirosis patients reacted against these proteins. Among the immunoreactive proteins, the 32 kDa (outer membrane) protein was LipL32 whose expression is related to mammalian infection[14], as it reacted with specific LipL32 antisera. In our study, a 67 kDa Triton-X-100 soluble and heat stable protein was also observed to be immunoreactive. Haake *et al*[12] characterized the LipL36 protein and demonstrated that its expression is down-regulated during mammalian infection and can be taken as a marker for studying the mechanisms by which pathogenic leptospires adapt to the host environment. Guerreriro *et al*[17] identified seven proteins p76, p62, p48, p45, p41, p37 and p32 as the targets of the humoral response during natural infection. In our study we did not find any reactive protein at the molecular weight region of 76 kDa. The position of all immunoreactive proteins identified in the present study was found similar among the six representative strains of five serogroups of *Leptospira*. These observations reveal that though there is a difference in the antigenic nature of the commonly circulating leptospires in the Andaman Islands, they have a common nature of expression of proteins during infection in humans. Use of these immunoreactive proteins as an antigen may help to develop an indigenous diagnostic kit for leptospirosis in future.

Acknowledgment

The work was supported by a grant from the Ministry of Science and Technology, Department of Biotechnology, Government of India. The authors thank Dr David A. Haake, VAGLAHS, Los Angeles, CA, USA for providing the antisera.

References

1. Faine S, Adler B, Palit A. Chemical, serological and biological properties of a serotype-specific polysaccharide antigen in *Leptospira*. *Aust J Exp Biol Med Sci* 1974; *52* : 311-9.

2. Kmety E, Dikken H. Classification of the species *Leptospira interrogans* and history of its serovars. University Press Groningen, Groningen, The Netherlands; 1993.

3. Brenner DJ, Kaufmann AF, Sulzer KR, Steigerwalt AG, Rogers FC, Weyant RS. Further determination of DNA relatedness between serogroups and serovars in the family Leptospiraceae with a proposal for *Leptospira alexanderi* sp. nov. and four new *Leptospira* genomospecies. *Int J Syst Bacteriol* 1999; *49* : 839-58.

4. Levett PN. Leptospirosis. *Clin Microbiol Rev* 2001; *14* : 296-326.

5. Taylor J, Goyle AN. Leptospirosis in the Andamans. Indian Medical Research Memories, supplementary series to the *Indian J Med Res* Memor No. 20, 1931; 55-6.

6. Sehgal SC, Murhekar MV, Sugugnan AP. Outbreak of leptospirosis with pulmonary involvement in North Andaman. *Indian J Med Res* 1995; *102* : 9-12.

7. Roy S, Biswas D, Vijayachari P, Sugunan AP, Sehgal SC. Antigenic and genetic relatedness of *Leptospira* strains isolated from the Andaman Islands in 1929 and 2001. *J Med Microbiol* 2003; *52* : 1-3.

8. Vijayachari P, Sehgal SC, Goris MGA, Terpstra WJ, Hartskeerl RA. *Leptospira interrogans* serovar Valbuzzi: a cause of severe pulmonary hemorrhages in the Andaman Islands. *J Med Microbiol* 2003; *52* : 913-8.

9. Sehgal SC, Vijayachari P, Smythe LD, Norris M, Symonds M, Dohnt M, *et al*. Lai like leptospira from the Andaman Islands. *Indian J Med Res* 2000; *112* : 135-9.

10. Ratnam S, Subramanian S, Madanagopalan N, Sundararaj T, Jayanath V. Isolation of leptospires and demonstration of antibodies in human leptospirosis in Madras, India. *Trans R Soc Trop Med Hyg* 1983; *77* : 455-8.

11. Jost BH, Adler B, Vinh T, Faine SA. Monoclonal antibody reacting with a determinant on leptospiral lipopolysaccharide protects guinea pigs against leptospirosis. *J Med Microbiol* 1986; *22* : 269-75.

12. Haake DA, Martinich C, Summers TA, Shang ES, Pruetz JD, McCoy AM, *et al*. Characterization of leptospiral outer membrane lipoprotein LipL36: Downregulation associated with late log phase growth and mammalian infection. *Infect Immun* 1998; *66* : 1579-87.

13. Haake DA, Mazel MK, McCoy AM, Milward F, Chao G, Matsunaga J, *et al*. Leptospiral outermembrane proteins OmpL1 and LipL41 exhibit synergistic immunoprotection. *Infect Immun* 1999; *67* : 6572-82.

14. Haake DA, Chao G, Zuerner RL, Barnett JK, Barnett D, Mazel M, *et al*. The leptospiral major outer membrane protein LipL32 is a lipoprotein expressed during mammalian infection. *Infect Immun* 2000; *68* : 2276-85.

15. Haake DA, Matsunaga J. Characterization of the leptospiral outer membrane and description of three novel leptospiral membrane proteins. *Infect Immun* 2002; *70* : 4936-45.

16. Cullen PA, Cordwell SJ, Bulach DM, Haake DA, Adler B. Global analysis of outer membrane proteins from *Leptospira interrogans* serovar Lai. *Infect Immun* 2002; *70* : 2311-8.

17. Guerreriro H, Croda J, Flannery B, Mazel M, Matsunaga J, Reis MG, *et al. Leptospira* proteins recognized during the humoral immune response to leptospirosis in humans. *Infect Immun* 2001; *69* : 4958-68.

18. Kmety E, Dikken H. Serological typing methods of leptospires. In: Bergan T, Norris JR, editors. *Methods in microbiology*. London: Academic Press; 1978 p. 260-308.

19. Korver H, Kolk AHJ, Vingerhoed J, Van Leeuwen J, Terpstra WJ. Classification of serovars of the lcterohaemorrhagiae serogroup by monoclonal antibodies. *Isrl J Vet Med* 1988; *44* : 15-8.

20. International Committee on Systematic Bacteriology. Sub-Committee on the Taxonomy of Leptospira. Minutes of the meeting 6-10 August 1982, Boston, Massachusetts. *Int J Syst Bacteriol* 1984; *34* : 258-9.

21. Mc Carter LL, Silverman M. Phosphate regulation of gene expression in *Vibrio parahaemolyticus. J Bact* 1981; *169 :* 3441-9.

22. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970; *227* : 680-5.

23. Towbin H, Stachclin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. *Proc Natl Acad Sci USA* 1979; *76* : 4350-4.

24. Brown JA, LeFebvre RB, Pan MJ. Protein and antigen profiles of prevalent serovars of *Leptospira interrogans*. *Infect Immun* 1991; *59* : 1772-7.

Reprint requests: Dr S.C. Sehgal, Director, WHO Collaborating Centre for Diagnosis, Research, Training & Reference in Leptospirosis, Regional Medical Research Centre (ICMR) Post Bag No.13, Port Blair 744101, Andaman & Nicobar Islands, India
e-mail: pblicmr@sancharnet.in