Detection of LipL32-specific IgM by ELISA in sera of patients with a clinical diagnosis of leptospirosis

Kumaresan Vedhagiri¹, Sridhar Velineni², John F. Timoney²,
Santhanam Shanmughapriya¹, Paluru Vijayachari³, Ramasamy Narayanan¹,
Kalimuthusamy Natarajaseenivasan^{1,2}

¹Medical Microbiology Laboratory, Department of Microbiology, School of Life Sciences, Bharathidasan University, Tiruchirappalli, India, ²Gluck Equine Research Center, University of Kentucky, Lexington, KY, USA, ³Regional Medical Research Centre (ICMR), WHO Collaborating Center for Diagnosis, Research Training and Reference in Leptospirosis, Port Blair, India

Successful treatment of leptospirosis is heavily dependent on early diagnosis and prompt initiation of antibiotic therapy. An ELISA test to detect specific IgM antibodies against LipL32 for early diagnosis of leptospirosis is described and evaluated here. One thousand one hundred and eighty sera from clinically suspected leptospirosis cases were enrolled together with 109 healthy volunteers selected from an endemic area between October 2007 and January 2010. Patients were categorized based on their clinical signs and symptoms. Sera were screened for leptospiral antibodies by the microscopic agglutination test (MAT) using a panel of locally circulating serovars followed by enzyme-linked immunosorbent assay (ELISA) based on recombinant LipL32 from *Leptospira interrogans* serovar Autumnalis strain N2. The sensitivity and specificity of the ELISA test were determined to establish its diagnostic efficiency. The cut-off value was determined to be 0.205. Overall sensitivity and specificity compared to the MAT were found to be 96.4 and 90.4%, respectively. The LipL32-specific IgM ELISA had good sensitivity and acceptable specificity and may be a candidate for the early serodiagnosis of human leptospirosis.

Keywords: Leptospirosis, Outer membrane protein, LipL32, ELISA, IgM

Introduction

Leptospirosis is a life-threatening clinical manifestation caused by infection with pathogenic *Leptospira* species and early diagnosis in the acute phase of infection is needed for prompt initiation of antibiotic therapy.¹ Several enzyme-linked immunosorbent assays (ELISAs) have been developed for the rapid diagnosis in both humans and animals.^{2,3} These are often empirical and have low values of sensitivity and specificity during the acute stages of infection. Outer membrane proteins (OMPs), which include LipL21, Loa22, LipL32, LipL36, LipL41, LipL46, LipL48, LigA, LigB, and HbpA, predominate on the outer leptospiral membrane⁴⁻⁹ and are highly conserved among different *Leptospira* species. Thus, these OMPs may have potential in the development of new serodiagnostic tests. LipL32 is the most abundant outer membrane lipoprotein expressed *in vitro* by pathogenic,

but not by saprophytic leptospires.^{6,10,11} Based on whole-cell leptospiral antigen preparation, LipL32 was found to be expressed during both acute and convalescent phases of infection, suggesting a potential value for the diagnosis of human leptospirosis.¹²

An effective tool for the early detection of *Leptospira* spp. is the polymerase chain reaction (PCR).¹³ However, it requires trained personnel and sophisticated laboratory equipment, making difficult the establishment of this kind of tests in peripheral health centers. Thus, the development of sensitive and specific assays to diagnose the infection during the early acute phase of the disease would be attractive.

ELISA is the simplest tool for the detection of antibody response and can be used to test large numbers of samples to investigate outbreaks and epidemics, but its performance is limited by the source of the antigen to be used in the assay. In this regard, recombinant DNA technology has provided an alternative approach for the production of recombinant proteins in large amounts to be used

Correspondence to: K. Natarajaseenivasan, Medical Microbiology Laboratory, Department of Microbiology, School of Life Sciences, Bharathidasan University, Tiruchirappalli 620 024, India. Email: natarajaseenivasan@gmail.com

in different immunoserological assays, such as ELISA.

The present study involved cloning and expression of LipL32 of *L. interrogans* serovar Autumnalis strain N2 and its evaluation in supporting the diagnosis of various clinical manifestations of leptospirosis by means of ELISA.

Materials and Methods

Bacterial strains, culture conditions, and plasmids

L. interrogans Autumnalis strain N2 a virulent human isolate,¹⁴ and 12 leptospiral reference strains Australis (serovar Australis, strain Jez-Bratislava), Autumnalis (serovar Autumnalis, strain Akiyami A), Ballum (serovar Ballum, strain Mus 127), Bataviae (serovar Bataviae, strain Swart), Canicola (serovar Canicola, strain Hond Utrecht IV), Icterohaemorrhagiae (serovar Icterohaemorrhagiae, strain RGA), Grippotyphosa (serovar Grippotyphosa, strain Moskva V), Hebdomadis (serovar Hebdomadis, strain Hebdomadis), Javanica (serovar Poi, strain Poi), Pomona (serovar Pomona, strain Pomona), Sejroe (serovar Hardjo, strain Hardjoprajitno), and Pyrogenes (serovar Pyrogenes, strain Salinem) were maintained in Ellinghausen McCullough Johnson Harris media (Difco Laboratories, Detroit, MI, USA) at the Medical Microbiology Laboratory, Department of Microbiology, Bharathidasan University, India. *Escherichia coli* DH5alpha was used for transformation of recombinant DNA. *E. coli* BL21 (DE3) containing PlyS (Stratagene, Santa Clara, CA, USA) was used for the expression of recombinant protein. *E. coli* strains were maintained in Luria Bertani medium (Sigma, St Louis, MO, USA). The pRSETA plasmid vector (Invitrogen, Carlsbad, CA, USA) was used for cloning.

Patients and diagnostic criteria

Blood samples (2–3 ml) were collected during the acute phase and a second sample 10–14 days later from the suspected cases. One thousand one hundred

and eighty sera samples were obtained from suspected patients of Tiruchirappalli district during an outbreak of leptospirosis in 2007 and were stored in the serum bank of the Medical Microbiology Laboratory, Bharathidasan University. A positive IgM ELISA and microscopic agglutination test (MAT) was considered as an indication of possible current leptospiral infection. Isolation of leptospires from the blood was considered as a confirmatory evidence of leptospiral infection. Seroconversion (negative to a titer of 1:160 or more) or four-fold rise in titer was considered as strong evidence in favour of current leptospiral infection in cases. A titer of 1:160 was taken as the cut-off for seroconversion as well for the diagnosis of possible current leptospiral infection in the controls as it was the closest titer to 1:200, which is the ideal cut-off for endemic areas.¹⁵

Out of the 1180 patients who were hospitalized with a clinical suspicion of leptospirosis, 444 had laboratory confirmed diagnosis for leptospirosis and 736 were subsequently diagnosed as having other illness based on laboratory and radiological evidence. Of the 736 cases, 97 had typhoid, 96 had malaria, and 101 had hepatitis (Table 1). Control sera were obtained from 109 seronegative healthy individuals. All 1180 sera samples and 109 seronegative negative sera were included in the study to analyse the sensitivity and specificity of the rLipL32-based ELISA. Informed written consent was obtained from both cases and controls before blood sampling, and the study protocol was approved by the Institutional Ethics Committee of Bharathidasan University (DM/2007/101/373/Project No. 2) as well as permitted by the Directorate of Health Services (Ref. No. 5796/TV 1/07), Tamilnadu. The obtained sera samples were aliquoted after collection and stored at –20°C.

Cloning and expression of rLipL32

LipL32 was amplified by PCR from *L. interrogans* serovar Autumnalis N2,¹⁶ ligated into pRSET A

Table 1 Case definition and grouping of the patients included in the present study

S. no.	Group no.	Cases	Number of cases
1	A	Severe form of pulmonary Leptospirosis*	93
2	A1	Severe form of pulmonary Leptospirosis†	101
3	B	Renal failure*	121
4	B1	Renal failure†	132
5	C	Pediatric*	137
6	C1	Pediatric†	109
7	D	Pregnant*	93
8	D1	Pregnant†	100
9	Controls	Seronegative healthy controls	109
10	E	Typhoid	97
11	F	Malaria	96
12	G	Hepatitis	101

Note: *Confirmed cases for leptospirosis.

†Clinically suspected but serologically negative.

(Novagen, Madison, WI, USA) in frame with his-6 tag sequence, and introduced into *E. coli* DH5alpha by CaCl₂ transformation using standard methods. Expression of rLipL32 was induced by IPTG (isopropylthio beta-D-galactoside) from *E. coli* BL21 (DE3) pLysS following transformation by *pRSET A + LipL32*. His-tagged rLipL32 was purified by Ni²⁺ metal affinity chromatography (Bio-Rad, Hercules, CA, USA) and its molecular weight and reactivity with specific antiserum (David Haake, UCLA School of Medicine, Los Angeles, CA, USA) were verified by SDS-PAGE and immunoblot, respectively.

Immunoblot analysis

SDS-PAGE was performed in a 12% polyacrylamide gel using a discontinuous buffer system as described elsewhere.¹⁷ The affinity-purified rLipL32 protein (1 mg/ml) was mixed with 2× loading buffer (125 mM Tris-HCl, 4% SDS, 2% glycerol, 1% beta-mercaptoethanol, and 0.5% bromophenol blue) and boiled for 5 minutes. Electrophoresis was carried out in a vertical electrophoretic unit (Bio-Rad) at a constant voltage of 120V for 1 hour and 45 minutes.

After electrophoresis, the rLipL32 was transferred to PVDF membrane (0.2 µm pore size; Schleicher and Schuell, Keene, NH, USA) and blocked with 4% non-fat dry milk in TBST (20 mM Tris, 150 mM NaCl, 0.05% Tween 20, pH 7.5). The membranes were incubated with specific LipL32 rabbit hyper-immune serum/pooled MAT-positive human sera for 1 hour, followed by incubation with secondary antibody (anti-rabbit/anti-human IgG conjugated to horseradish peroxidase; Sigma) for 1 hour and bands visualized by using 4-chloro-alpha-naphthol (Sigma).

Detection of IgM antibodies to rLipL32

rLipL32 (0.2 µg/well) was coated on flat-bottom polystyrene microtiter plates using carbonate coating buffer (pH 9.6) overnight at 4°C. Wells were washed three times with PBS-Tween 20 (PBST) and blocked with (200 µl/well) of blocking buffer (4% non-fat milk powder in PBST) for 1 hour at room temperature. Patient and control sera were diluted two-fold in PBST and added to the plates (100 µl/well) and incubated for 1 hour at 37°C. After washing three times in PBST, horseradish peroxidase-conjugated rabbit anti-human IgM (Sigma) was diluted in 1:8000 and added to the plates (100 µl/well) and incubated for 1 hour at 37°C. After washing, the chromogenic substrate (o-Phenylenediamine) was added to the plates (100 µl/well) and incubated for 2 minutes at room temperature. The enzymatic reaction was stopped by adding 1 N H₂SO₄ (50 µl/well) and the optical density were measured at 490 nm. All serum samples were tested in duplicate and the mean values were recorded.

MAT

The MAT was performed using different *Leptospira* serovars cultured in Ellinghausen McCullough Johnson Harris medium (7 days at 30°C). Serum samples were analyzed at two-fold dilutions (starting at 1:20 and ending at 1:10240). Positive cut-off titer was set at 1:160. The serovar that gives the highest titer was recorded as the probable cause of the infection.

Statistical analysis

The sensitivity and specificity of the ELISA test on comparison with MAT were analyzed. GraphPad Prism software (version 5.0; GraphPad Prism Software, Inc., San Diego, CA, USA) was used for preparation of graphs. A cut-off value for recombinant ELISA was determined as mean±2SD.

Results

Expression and purification of rLipL32

A 761-bp gene fragment was obtained by PCR amplification of genomic DNA from *L. interrogans* serovar Autumnalis N2. Sequencing and BLAST analysis revealed a high degree of homology (99%) with LipL32 of *L. interrogans*. This sequence encodes a mature protein of 254 amino acids with a predicted molecular mass of 32.8 kDa. Subsequent analysis by SDS-PAGE of the rLipL32 revealed an apparent molecular mass of 32 kDa, confirming the prediction of its previous amino-acid sequence. The recombinant protein reacted strongly with LipL32-specific antiserum (Fig. 1).

MAT

From all patients with the suspect of leptospirosis, 69.4% had positive reactions with the MAT. Sera from pediatric patients had the highest percentage of positive titers (80.3%), followed by pregnant women, pulmonary, and renal cases. The serovars most frequently reactive were Australis (Jez Bratislava), Autumnalis (N2), and Icterohaemorrhagiae (RGA). Serum samples from healthy volunteers or patients with typhoid, malaria, or hepatitis were negative.

rLipL32 ELISA

The mean±2SD (0.205) of the absorbance values from control individuals was defined as the cut-off values to achieve diagnostic specificity. The mean optical densities were 0.57, 0.64, 0.56, and 0.67 for sera from cases categorized as pulmonary, renal, pediatric, and pregnancy, respectively (Fig. 2). When compared to the MAT, the ELISA test demonstrated an overall sensitivity and specificity of 96.4 and 90.4%, respectively, in sera from clinically diagnosed cases of leptospirosis (Table 2). Within this group, the sensitivities of ELISA test for sera from pulmonary, renal failure, pediatric, and pregnancy cases were 96.4, 96.2, 91.1, and 94.9%, respectively. The overall agreement between MAT and rLipL32 ELISA was 94.8%,

Figure 1 Immunoblot reactivity of rLipL32 of *L. interrogans* serovar Autumnalis N2. Lane M: SDS low range molecular marker; lane 1: rLipL32 probed with antiserum against LipL32; lane 2: rLipL 32 probed with pool of MAT positive patients' sera.

kappa co-efficient is 0.894, and Z value is 27.3. The P value was <0.001 and found to be statistically significant. The cross-reactivity of these ELISAs was in the range of 1.7–3.9% among the sera from diseases confirmed other than leptospirosis.

Discussion

Leptospirosis is a serious zoonotic human disease which is endemic in India and elsewhere in the world. Humans are infected directly or indirectly from wildlife rodents, including bandicoots and from cattle via urine and milk or contaminated water. Traditional diagnosis of leptospiral infection can be made directly (by dark-field microscopic observation of leptospires, isolation by culture, or detecting leptospiral DNA by molecular assays) or indirectly by detection of serum antibodies using immunoserological tests. Nevertheless, both microscopic observation and culture present low rates of success, and the availability for molecular assays in peripheral hospitals and health care centers in endemic areas makes the immunoserological tests more reliable to carry out. Unfortunately, some types of immunoserological tests based on agglutination of living leptospires or serovar lipopolysaccharide antigens have the limitations of periodic subcultures in special media or the detection of specific antibodies against a particular serovar, respectively.² Therefore, an immunogenic protein shared by pathogenic *Leptospira* species, such as LipL32, clearly provides broader specificity in the detection of antibodies to unknown virulent *Leptospira* spp. using enzyme immunoassays such as ELISA with a reasonable sensitivity to assess

Figure 2 Evaluation of recombinant LipL32-ELISA with sera from patients' with different clinical manifestations. x-axis depicts groups with different clinical manifestations as per Table 1 and the y-axis represents the OD_{490 nm} obtained by recombinant LipL32-ELISA.

Table 2 Sensitivity, specificity, PPV, and NPV among different group of leptospirosis patients and their cumulative data for comparison

Test statistics (%)	Leptospirosis cases				Cumulative
	Pulmonary	Renal failure	Pediatric	Pregnant	
Sensitivity	96.4	96.2	91.1	94.9	94.78
Specificity	90.5	97.1	90.9	86.4	91.2
PPV	93.2	98.1	96.7	91.5	94.82
NPV	95.4	94.4	80.4	92.9	90.7

Note: PPV, positive predictive value; NPV, negative predictive value.

the immune response at early phases of infection by detecting IgM antibodies.

In this study, *LipL32* was cloned from *L. interrogans* serovar Autumnalis strain N2 and the expressed rLipL32 protein was used for the detection of IgM antibodies by means of ELISA. During infection, pathogenic leptospires express numerous proteins, which are targets for host humoral immune responses during natural infections. These proteins include p76, p62, p48, p45, p37, p32, and HbpA.^{9,10,12} Among these, LipL32 (formerly named as p32), the most abundant outer membrane protein,¹¹ showed high sensitivity and specificity during the acute and convalescent phases, leading to a substantial evidence that LipL32 has a role in pathogenesis.⁶

Using the MAT as 'gold standard', the sensitivity of 96.4% and specificity of 90.4% were achieved with rLipL32-based ELISA. The sensitivity and specificity obtained in this study were similar to the values found for recombinant LipL41, OmpL1, and GroEL. ELISA tests based on these recombinant proteins showed sensitivities of 89.3, 91.1, and 90.6%, respectively, and specificities of 89.2, 86.5, and 94.9%, respectively.¹⁸ Thus, these recombinant proteins have higher sensitivity and specificity than whole-cell preparations in serodiagnostic assays. In this context, an ideal serodiagnostic assay requires an antigen that may be both expressed and conserved only among the pathogenic leptospiral serovars. This kind of antigen would be helpful to rule out diseases with clinical presentations that overlap with leptospirosis and help to reduce the burden of leptospirosis in developing countries.

Our findings show that rLipL32 is an excellent antigen for the serodiagnosis of leptospirosis during the acute stage of the disease. IgM antibodies, directed primarily against carbohydrate epitopes,¹ are believed to be the predominant humoral response during the early acute phase of infection.¹⁹ Our study confirms that IgM antibodies to *Leptospira* infections include LipL32. A recent study that focused on the recognition of LipL32 during human infection showed that the IgM antibodies were directed against the C-terminal domain of LipL32 during both the acute and convalescent phases. In contrast, the IgG antibodies

change the epitope during the phases of infection, because most sera from patients recognized a central region of the protein during convalescence. Five of 12 patients responded to the C-terminus in both the acute and convalescent phases. These findings suggest the possibility that differential immune recognition of LipL32 domains during infection and convalescence may play a role in protective immunity.

LipL32 is a lipoprotein whose protein component is external and anchored to the outer membrane and fatty acids are covalently attached to the amino-terminal cysteine residue. The lack of sequence similarity between LipL32 and other functionally characterized proteins in the database has hindered progress in determining structural domains that relate to its function. Therefore, structural determination of LipL32 is important in understanding its role in infection, immunity, inflammatory response, and interaction with host extracellular matrix proteins. These aspects are currently under investigation.

The conserved nature, high-level expression, and immunogenicity of LipL32 among pathogenic *Leptospira*¹⁰ were the reason for the development of an ELISA test to detect LipL32-specific IgM. Application of ELISA to sera from patients residing in regions in which the most prevalent *Leptospira* serovars are Autumnalis, Javanica, and Icterohaemorrhagiae, the etiological agents for urban and rural epidemics indicated excellent potential as a substitute for MAT in detection of antibody in patients with different clinical manifestations of leptospirosis.

Competing interests

The authors have declared that no competing interest exists.

Author contributions

KNS and PV conceived and designed the experiments. KV, SS, and RN performed the experiments. KNS, SS, KV, JFT, and SV analyzed the data. KNS, JFT, and PV contributed reagents/materials/analysis tools. KNS, SS, SV, and JFT wrote the paper. KNS, SS, and JFT critically reviewed the paper.

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