Evidence of cross reaction potential of recombinant leptospira LipL41 protein

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The present study was undertaken to compare the cross reactive potentiality of expressed recombinant protein (LipL41) with lipopolysaccharide (LPS) antigen of leptospira against different serovars of leptospira. Antigens from both recombinant LipL41 and the isolated LPS from Leptospira icterohaemorrhagiae were coated in latex beads and used to test the human sera samples of different serovars of leptospira using field based latex agglutination test. Recombinant antigen showed strong cross reaction with different serovars of leptospira, whereas LPS antigen showed very mild cross reaction with few serovars. This clearly indicates that outer membrane protein cross reacted with heterologous serovars and can be used as effective diagnostic agent as well as candidate antigen for development of vaccine for leptospira.

Keywords: Latex agglutination test, leptospira, lipopolysaccharide, recombinant protein, serovars

Introduction

Leptospirosis is a zoonotic disease of worldwide distribution caused by spirochetes of the genus Leptospira. In the traditional classification based on the serovar, the genus contains two species: the pathogenic species L. interrogans with 250 serovars, grouped into 23 serogroups, and the saprophytic non-pathogenic species L. biflexa.

Leptospires have been reported to possess several antigens expressed on their cell surface and prominently among these are lipopolysaccharides (LPS) and outer membrane proteins (OMPs). The immune serovar diversity among pathogenic leptospires has been attributed to differences in the structure and composition of LPS. These LPS are mostly serovar specific and provide only short term immunity. On the other hand, immunity developed by whole cell vaccine is demonstrated. However, cross protection against many of the 250 different serovars of pathogenic leptospira is lacking because the whole cell vaccine involves lipopolysaccharides, which confer protective immunity against homologous but not the heterologous leptospira. Unlike leptospiral LPS, the outer membrane proteins, viz., LipL41 and OmpL1, are surface exposed and antigenically conserved among pathogenic leptospira species.

Since highly conserved protein is needed for effective vaccination and diagnosis of leptospira against different serovars, the present study was undertaken to clone and express the LipL41 and its cross reaction potential was compared with LPS antigen of leptospira against different serovars of leptospira using latex agglutination test.

Materials and Methods

Cultivation of Leptospira and Collection of Sera Samples

L. icterohaemorrhagiae reference strain available in the Department of Biotechnology, Madras Veterinary College, Chennai, was inoculated aseptically in Ellinghausen-McCullough-Johnson-Harris (EMJH) medium (DIFCO Laboratories, USA) and the growth was checked in dark field microscope for the presence of the organism. Further, hyper-immune serum raised against L. icterohaemorrhagiae in rabbits and used in the present study was received from Leptospira Reference Laboratory, Tamil Nadu Veterinary and Animal Sciences University (TNVASU), Chennai. Sera samples obtained from suspected human beings were screened for leptospirosis using micro-agglutination test as per the method described earlier.

Isolation of Lipopolysaccharide

Lyophilized leptospiral cells were suspended in 200 µL of Trizol reagent. Phase separation was done with chloroform and LPS was separated from the
upper aqueous phase using speed vac and checked in 12% PAGE.

Polymerase Chain Reaction (PCR) and Sequencing of LipL41 Gene
DNA was extracted from Leptospira culture using high salt method. The primers and temperature profile for the amplification of LipL41 gene were used as described by Haake et al.

Forward Primer
5’ TG TTA CCCATG GGG AGA AAA TTA TCT TCT CT 3’

Reverse Primer
5’ AAA GGA CTC GAG TTA CTT TGC GTT GCT TTC 3’

The amplified product (5 µL) was checked by 1.5% agarose gel electrophoresis with 1 Kb DNA mol wt marker (Bangalore GeNei, India). The LipL41 gene PCR product was purified using Qiagen PCR product purification kit and sequenced. The nucleotide sequence of LipL41 gene was compared with other Leptospira using blast analysis.

Cloning and Expression of LipL41 Gene in Prokaryotic Vector
The PCR amplicons and the expression vector (pET15b) were digested with NcoI and XhoI restriction enzymes. The digested product was ligated with pET15b expression vector in the ratio of 3:1 using T4 DNA ligase and incubated at 16°C overnight. The 3 µL volume of the ligation mixture was transformed into Escherichia coli JM109 (DE3) cells. The transformants were spread onto LB ampicillin plates for selection of transformed colonies.

The colonies were screened for colony PCR and plasmid was isolated from the colonies positive by PCR. The plasmid was then digested with NcoI and XhoI enzymes (Promega, USA) and the insert release was checked in 1.5% agarose gel.

The positive cultures were induced with 1 mM IPTG and similarly the positive culture without IPTG was used as uninduced controls. The induced and uninduced cells were lysed by heat denaturation and resolved in 12% SDS-PAGE along with protein mol wt marker (Bangalore GeNei, India).

The recombinant LipL41 fusion proteins were purified by affinity chromatography using Ni²⁺-NTA affinity column as per the invitrogen protocol.

The 5th h induced lysed-cells were transferred to nitrocellulose membrane (NCM) (Amersham International PLC, UK) from PAGE. The NCM was treated with anti-rabbit polyclonal serum raised against Leptospira to check the specificity of the expressed protein along with pre-stained protein marker (GeNei, Bangalore, India).

Latex Agglutination Test
The carboxylate modified polystyrene latex beads (size 0.8 µm, Sigma Aldrich, USA) were used. The recombinant antigen (LipL41) and LPS antigen were used for coating the latex beads and used to test MAT (macroscopic agglutination test) positive human sera samples (4 sera samples for each serovar) against L. icterohaemorrhagiae, Australis, Hardjo, Hebdomadis, Autumnalis, Javanica, Pomona and Canicola as per the method described by Jiafeng.

Results
PCR and Sequencing of LipL41 Gene
The amplified product (5 µL aliquot) was electrophoresed and the expected amplicon size of 1077 bp was observed in 1.5 % agarose gel along with 1 Kb DNA ladder. BLAST analysis revealed that the LipL41 nucleotide sequence showed 98% homology with other L. interrogans serovar icterohaemorrhagiae available in GenBank.

Cloning and Expression of LipL41 Gene
The 5 recombinant colonies were positive by colony PCR (Fig. 1) and these colonies were further confirmed by digesting plasmid with NcoI and XhoI enzymes (Promega, USA). The plasmid with insert release was observed in agarose gel (Fig. 2).

The IPTG induced and uninduced cells were lysed by heat denaturation and resolved in 12% SDS-PAGE. The expression of LipL41 protein was

Fig. 1—Colony PCR for screening of the recombinant LipL41 gene. [Lane 1, 1 kb DNA ladder; Lanes 2, 4 & 6, Negative colonies; & Lane 3, 5 & 7, Positive colonies]
noticed in 2nd, 3rd, 4th, 5th and 6th h of post induction as a band of 39 kDa. No band was observed in uninduced controls of equivalent mol wt (Fig. 3a).

**Purification of Recombinant LipL41 Protein**
Nickel (Ni²⁺-NTA) affinity column purified protein was checked in 12% SDS-PAGE and a band with mol wt of approx 39 kDa was observed (Fig. 3b).

**Identification of Expressed Recombinant LipL41 Protein**

**Western Blot**
The 5th h induced lysed cells were transferred to nitrocellulose membrane (NCM) from polyacrylamide gel. The NCM was treated with anti-rabbit polyclonal serum raised against Leptospira to check the specificity of the expressed protein. The antiserum reacted with the protein and gave a single band with mol wt of approx 39 kDa (Fig. 4).

**Latex Agglutination Test**
Recombinant LipL41 and LPS antigens were used as coating antigen onto latex beads and the results of agglutination reaction with different serovars of leptospiros were presented in Figs 5 and 6. Recombinant antigen cross reacted with different serovars, viz., *L. icterohaemorrhagiae*, *Australis*, *Hardjo*, *Hebdomadis*, *Autumnalis*, *Javanica*, *Pomona* and *Canicola*. On the other hand, LPS antigen reacted with few serovars mildly.

**Discussion**
The leptospiral LPS are extremely heterogeneous among various strains and they are generally serovar specific. So they are unsuitable for diagnosis and vaccine production. On the other hand, the LipL41...
was identified as an immunoreactive protein. Unlike leptospiral LPS, LipL41 were antigenically conserved among the *Leptospira* sp. and expressed during the infection of mammalian host. Hence, recombinant LipL41 proteins were produced and used in latex agglutination reaction to check the cross reactivity against different serovars.

The amplified LipL41 gene yielded the product size of 1077 bp as also reported earlier. The blast analysis of nucleotide sequence of LipL41 gene showed 98% homology with the sequence of *L. interrogans* (GenBank Acc. No. AB240677.1), *Copenhageni* (Acc. No. AE016823.1), *Wolffi* (Acc. No. AY622686.1) and *Pomona* (Acc. No. AY776295.1) as reported by Cheema et al. In the present study, *E. coli* JM109 was taken as a host strain for the *in vitro* expression of LipL41 protein. *E. coli* has been considered as the most favoured organism for the expression of many proteins.

In our study, recombinant LipL41 antigen showed strong cross reaction with different serovars of *Leptospira*, whereas LPS antigen showed very mild cross reaction with few serovars. This clearly shows the cross reacting potential of rLipL41. MAT is a serovars specific complex tests to control, perform and interpret. However, in the present study, latex agglutination test using recombinant antigen detected all the serovars. The advantage of latex agglutination test is its usefulness in detecting IgM antibodies in the early stage of infection. Further, it can also be used for screening large number of samples without sophisticated equipments and trained personnel. The multi-epitope recombinant leptospiral antigen has shown high potential for detecting both IgM and IgG antibodies. Earlier, rLipL41 was used as diagnostic protein to assess the seropositivity of *Leptospira* among bovine population and serovars, viz., *Sejroe* and *Pomona*, were predominantly reported based on the ELISA titers. In the present study, rLipL41 reacted with different serovars of human beings and, therefore, could be used as diagnostic protein to assess the seroprevalence of *Leptospira* in human beings.

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