Comparison of Immunoprotection of Leptospira Recombinant Proteins with conventional vaccine in experimental animals

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Leptospirosis is a bacterial disease caused by bacteria of the genus Leptospira affecting humans and animals. Untreated leptospirosis may result in severe kidney damage, meningitis, liver failure, respiratory distress, and even death. Virulent leptospirosis can rapidly enter kidney fibroblasts and induce a programmed cell death. Thus, it is a challenge for immunologists to develop an effective and safe leptospirosis vaccine. Here, we compared the commercial canine leptospira vaccine and recombinant proteins (OmpL1 and LipL41) with and without adjuvant in terms of immune response and challenge studies in hamsters and immune response studies alone in experimental dogs. The outer membrane proteins viz., lipL41 and OmpL1 of leptospira interrogans serovars icterohaemorrhagiae were amplified. The primers were designed in such a way that amplified products of OmpL1 and lipL41 were ligated and cloned simultaneously into a single vector. The cloned products were expressed in E.coli BL21 cells. The immunoprotection studies were conducted for both recombinant proteins and commercial vaccine. The challenge experiment studies revealed that combination of both rLip41 and rOmpL1 and commercial vaccine gave 83% and 87% protection, respectively. Histopathological investigation revealed mild sub lethal changes were noticed in liver and kidney in commercially vaccinated group alone. The immune responses against recombinant leptospiral proteins were also demonstrated in dogs.

Keywords: Leptospirosis, LipL41, Lipopolysaccharides, Membrane proteins, OmpL1, Spirochetes

Leptospirosis is a zoonotic disease of worldwide distribution caused by spirochetes of the genus Leptospira1 that contains two species; the pathogenic, Leptospira interrogans with 230 serovars, and the saprophytic non-pathogenic species, Leptospira biflexa2. Leptospira possess several antigens expressed on their cell surface, and the lipopolysaccharides (LPS) and outer membrane proteins (OMPs) are the prominent ones. The serovar diversity among pathogenic leptospira is attributed to differences in the structure and composition of LPS3. Commercially available vaccines, which rely up on bacterins and inactivated whole cells, are mostly serovar-specific and provide only short term immunity.

Certain leptospiral proteins expressed in cultivated organisms may or may not be expressed during infection4. Proteins expressed during infection may serve as determinants in leptospiral pathogenesis and as targets for the host immune response. LipL41, a major leptospiral outer membrane protein (OMPs), has been identified as a serodiagnostic marker for screening of leptospiral infection5. Similarly, OmpL1 is another outer membrane protein expressed in all pathogenic leptospira species6.

In the present study, we compared the commercial canine leptospira vaccine and recombinant proteins (OmpL1 and LipL41) with and without adjuvant in terms of immune response and challenge studies in hamsters and immune response studies alone in experimental dogs.

Materials and Methods

Cultivation of leptospira— Leptospira interrogans serovar icterohaemorrhagiae reference strain available in our laboratory was inoculated aseptically in EMJH medium (Difco Laboratories, USA) and maintained at 30°C. The growth was monitored in dark field microscopy.

Cloning and expression of OmpL1 and LipL41 genes— DNA was extracted from Leptospira culture using STET method (8% Sucrose, 50 mM Tris-HCl pH 8.0, 50 mM EDTA pH 8.0, 5% Triton-X-100) method as described by Bartlett & Stirling7. Leptospira outer membrane genes viz., OmpL1 and
LipL41 genes were amplified by PCR using specific primers with restriction enzyme sites. The primer sequences were as follows:

LipL41 forward: 5'-GGGCATATGCTTCAGAAATTCCTC GGAACCATT-3'; LipL41 reverse: 5'-GGGGGATCCTTACTTGGATTTGCTTTTCGTAAC-3'; OmpL1 forward: 5'-GGGAAGGGATCCATGATCCGTATCATTATG-3'; and OmpL1 reverse: 5'-GGGTGGCTCGAAGTTACTTGTGAATTCG-3'. The start codons and stop codons for the above primers are underlined.

The PCR amplicons were checked in 1.5% agarose gel electrophoresis. The PCR products of OmpL1 and LipL41 genes were purified using Qiagen PCR product purification kit. Then purified products were sequenced and analyzed using blast analysis.

The vector (pET15b) plasmid was extracted using Qiagen kit and checked in agarose gel electrophoresis. The primers were designed to ligate PCR products of both LipL41 & OmpL1 gene as shown in Fig. 1 and the ligated product was cloned into pET15b vector and the cloned product (containing both LipL41 & OmpL1) was transformed into BL21 DE3 cells.

The recombinant colonies were screened using colony PCR and digestion of recombinant plasmid with restriction enzyme digestion (Nde I and Xho I) to check the insert release. The insert size of

![Fig. 1 — Strategy for ligating PCR products of LipL41 and OmpL1 gene for cloning.](image)
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2036 bp was eluted from the gel and sequenced. The recombinant plasmid was induced with 1 mm IPTG. The induced and uninduced cells were lysed by heat denaturation and resolved in 12% SDS-PAGE.

**Purification of LipL41 and OmpL1 recombinant protein**— The recombinant LipL41 and OmpL1 fusion proteins were purified by affinity chromatography using Ni²⁺-NTA affinity column as per the manufacturer’s instructions (Invitrogen, USA) and analyzed in 12% SDS-PAGE. The purified recombinant antigens were estimated using nanodrop. The purified recombinant antigen yielded the concentration of 2.1 mg/L of rLipL41 and 1.9 mg/L of rOmpL1 protein.

**Western Blotting**— The recombinant cells after induction were lysed and transferred to Nitrocellulose membrane (NCM, Millipore, USA) from PAGE. The NCM was treated with anti-hamster serum raised against LipL41 and OmpL1 proteins to check the specificity of the expressed protein.

**Determination of lethal dose (LD₅₀)**— The 8-9 wk old hamsters were obtained from National Institute of Nutrition, Hyderabad and maintained at TANUVAS animal house facility as per the guidelines. After obtaining the ethical clearance from 20th Institutional Animal Ethical Committee held at Madras Veterinary College, they were infected intraperitoneally with 10 fold serial dilution (10⁻⁶-10⁻⁵) with low passage *Leptospira interrogans serovar icterohaemorrhagiae*. The LD₅₀ was calculated by the method of Reed & Muench. The LD₅₀ value was found to be 10⁶.

**Immunization of hamsters with commercial vaccine and recombinant proteins**— Hamsters of 4 wk old were grouped into 8 groups each comprising 8 animals. Group I, Control (membrane fractions of *E. coli* without leptospira outer membrane protein); Group II, 50 µg of recombinant LipL41 protein; Group III, 50 µg recombinant LipL41 protein + Aluminum hydroxide adjuvant; Group IV, 50 µg of recombinant OmpL1 protein; Group V, 50 µg recombinant OmpL1 protein + Aluminum hydroxide adjuvant; Group VI, 50 µg combination of LipL41 and OmpL1 proteins; Group VII, as in group VI + Aluminum hydroxide adjuvant; Group VIII, commercial vaccine containing 4 serovars viz., *Icterohaemorrhagiae*, *Canicola*, *Pomona* and *Grippotyphosa*. The groups were immunized subcutaneously with recombinant proteins as per the given schedule. On day 0 and subsequently boosted on day 21st for recombinant proteins whereas single injection on day 0 was given for commercial vaccine. Blood for serum separation was collected on day 0 (pre vaccination), 21st and 42nd directly from saphenous vein. Immunized hamsters were challenged on 42nd day with 10LD₅₀ of *L. interrogans serovar icterohaemorrhagiae*. Three such trials were conducted. The animals were monitored and sacrificed on day 71 and blood was collected by cardiac puncture. The tissue from infected animals was collected aseptically for culture and histopathological analysis.

**Immunization of experimental dogs with commercial vaccine and recombinant proteins**— Two groups comprising 4 experimental dogs each were immunized with commercial leptospira and combination of both rLipL41 and OmpL1 proteins, respectively as per the schedule given above for hamsters to study the humoral response.

**ELISA with recombinant antigens**— Sera samples collected on days 0, 21, 42 and 71 from hamsters and dogs receiving recombinant antigens OmpL1, LipL41, combination of OmpL1 and LipL41 and commercial vaccine groups were evaluated for presence of specific IgG using ELISA assay with rOmpL1, rLipL41, combination of rOmpL1 and rLipL41and whole cell antigens, respectively.

**Leptospira culture**— The leptospira growth was checked in the culture after ‘challenge studies. The hamster tissues including liver and kidney collected aseptically from different vaccinated groups after 28 days of post challenge were homogenized in 0.5 ml EMJH medium and transferred to 20 ml EMJH medium and maintained at 30°C for 4 wk. The growth of organism was checked using dark field microscopy.

**Histopathology**— In case of leptospira infection, the sub-lethal infection was commonly noticed in liver and kidney. Hence, histological examinations of survived animals’ kidney and liver in the vaccinated groups after challenge studies were performed to find out the sub lethal infection. Similarly, unvaccinated animal tissues were also examined to study the leptospira induced histopathological changes.

**Statistical analysis**— Graph Pad Prism version 5 was used for analysis of the results. The survival of hamsters was monitored after post challenge to assess the efficacy of the recombinant protein administered. The data collected based on the survival of hamsters were subjected to student’s t-test to compare the significance among different treatment groups.
Results

PCR amplification and sequence analysis of OmpL1 and LipL41 genes—The expected amplicon size of 1077 bp and 961 bp was observed in 1.5% agarose gel electrophoresis for LipL41 and OmpL1, respectively (Fig. 2 A and B). The LipL41 & OmpL1 nucleotide sequences showed 99 and 98 percent homology with other Leptospira sequences, respectively using NCBI nucleotide blast analysis.

Cloning and expression of LipL41 and OmpL1 genes—Figure 3 shows the presence of insert in the recombinant colonies by restriction enzyme digestion in 1.5% agarose gel electrophoresis. The insert was eluted from the gel and sequenced. The expression of Lip41 and OmpL1 gene was noticed in 1st, 2nd, 3rd, 4th, and 5th h of post induction with a molecular weight 39 and 49 kDa, respectively compared to un-induced controls. (Fig. 4A). The nitrocellulose membrane was treated with anti-hamster serum raised against LipL41 and OmpL1 proteins to check specificity of the expressed protein. The antiserum reacted with protein band of molecular weight of approximately 39 and 49 kDa (Fig. 4B).

Antibody response to recombinant antigens and commercial vaccine—ELISA results clearly indicate that enhanced levels of antibodies detected against recombinant antigens and commercial vaccine. On the other hand, no significant levels of antibodies were detected in controls immunized with membrane fractions of E.coli without leptospiral genes (Fig. 5A). Similarly, the sera collected from dogs in vivo showed elevated levels of antibodies (Fig. 5B).
**Challenge studies**—The protective efficacy of recombinant proteins of LipL41 and OmpL1 with commercial vaccine in hamster model is presented in Table-1.

**Leptospira culture from tissues**—Tissues from the animals, control as well as challenged, showed growth in EMJH medium, and the presence of organism was observed on dark microscopic examination. In contrast, tissues from survived animals! of recombinant and commercial vaccine group had no growth.

**Histopathological analysis**—All the control animals after challenge experiment showed lesion in both liver and kidney (Fig. 6 A and B). In the hamsters vaccinated with commercial vaccine 70%

<table>
<thead>
<tr>
<th>Groups</th>
<th>Experiment</th>
<th>Total</th>
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<tbody>
<tr>
<td></td>
<td>I</td>
<td>II</td>
</tr>
<tr>
<td>LipL41 M</td>
<td>4/8 (50%)</td>
<td>5/8 (62.5%)</td>
</tr>
<tr>
<td>LipL41 Adjuvant</td>
<td>5/8 (62.5%)</td>
<td>4/8 (50%)</td>
</tr>
<tr>
<td>Control</td>
<td>2/8 (25%)</td>
<td>1/8 (12.5%)</td>
</tr>
<tr>
<td>Significance</td>
<td>P&lt;0.03*</td>
<td>Ns</td>
</tr>
<tr>
<td>OmpL1 M</td>
<td>4/8 (50%)</td>
<td>4/8 (50%)</td>
</tr>
<tr>
<td>OmpL1 Adjuvant</td>
<td>4/8 (50%)</td>
<td>4/8 (50%)</td>
</tr>
<tr>
<td>Control</td>
<td>1/8 (12.5%)</td>
<td>1/8 (12.5%)</td>
</tr>
<tr>
<td>Significance</td>
<td>P&lt;0.03*</td>
<td>Ns</td>
</tr>
<tr>
<td>LipL41+OmpL1</td>
<td>6/8 (75%)</td>
<td>7/8 (87.5%)</td>
</tr>
<tr>
<td>LipL41+OmpL1 Adjuvant</td>
<td>6/8 (75%)</td>
<td>6/8 (75%)</td>
</tr>
<tr>
<td>Control</td>
<td>1/8 (12.5%)</td>
<td>1/8 (12.5%)</td>
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<tr>
<td>Significance</td>
<td>Ns</td>
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<tr>
<td>Commercial Vaccine</td>
<td>7/8 (87.5%)</td>
<td>7/8 (87.5%)</td>
</tr>
<tr>
<td>Control</td>
<td>1/8 (12.5%)</td>
<td>1/8 (12.5%)</td>
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<tr>
<td>Significance</td>
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Numbers of Surviving animals at 28 days after challenge. Statistical analysis were performed by using Student’s test for two independent means (Significant, P<0.05) NS, not significant.

![Fig. 6—(A) & (B), Kupffer cells hypertrophy in liver section and Tubular epithelial degeneration in kidney section seen in unvaccinated challenged control hamsters; (C) & (D), Mild degeneration of hepatocytes and mild degeneration of tubular epithelial cells seen in commercial vaccine immunized hamsters; and (E) & (F), No changes seen in recombinant proteins immunized hamster liver and kidney cells](image-url)
of animals showed mild lesions in kidney and liver (Fig. 6 C and D). On the other hand, in the recombinant antigen vaccinated group, all the survived animals did not show any histopathological evidence of disease (Fig. 6 E and F).

Discussion

Hamsters have been chosen for experimentation as they are the accepted model for leptospirosis. Mouse could not used as it is resistant to challenge with virulent leptospira. LipL41 and OmpL1 protein are surface exposed outer membrane proteins of virulent leptospira, and therefore, potential targets of a protective immune response. In this study, the single purified recombinant OmpL1 and LipL41 did not demonstrate the immunoprotection in hamsters, even though these proteins are surface exposed and containing immunoprotective epitopes. On the other hand, combination of both OmpL1 and LipL41 proteins produced significant level of immunoprotection and the results correlate well with previous research work. However, they used two vectors viz., pET15b and pMM66 for LipL41 and OmpL1, respectively for cloning and expression. In our work, we ligated both LipL41 and OmpL1 genes and cloned in single vector and the expressed protein did not affect the viability of host cell Escherichia coli. LipL41 and OmpL1 primers have separate start and stop codons. Hence, they expressed as individual proteins during expression in E.coli system as indicated in the Fig. 1. The intact OmpL1 and LipL41 including region of signal peptide site was chosen for cloning and expression to attain the structure resembling native proteins so as to act as better immunogens. E. coli is used for expression of leptospiral outer membrane protein because of its simple genetic manipulation, high rate of cell growth and low cultural conditions. The Leptospira interrogans LipL32-OmpL1 fusion gene was expressed in eukaryotic system with high efficiency in Pichia pastoris and the expressed fusion protein showed specific immunoreactivity which can be used as potential antigen for developing a novel vaccine for Leptospira interrogans.

The increased dosage of recombinant antigen does not influence the level of immune response significantly. Hence, a single dose of 50 µg of recombinant antigen was used for immunization in this study. Based on humoral immune response and protection studies, there was not much variation noticed between the hamsters vaccinated with and without adjuvant group. However, in addition to humoral immune response, cell mediated immune response was also reported in Leptospira by Naiman et al. On the other hand, the leptospiral recombinant Lig A antigen produced 100% immunoprotection in hamsters without adjuvant also. In our study, the commercial vaccine gave 87% protection against homologous challenge with serovar icterohaemorrhagiae. The experimental vaccine has been reported to give 100% protection against homologous challenge with serovar copenhageni.

Even though the protection efficiency of combination of both OmpL1 and LipL41 were slightly lower than the commercial vaccine, the sub lethal infection was observed histopathologically only in commercial vaccinated groups, and not in recombinant based vaccine. This is the first report on humoral antibody responses against leptospira recombinant antigens in vivo in dogs.

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References


