

## Immunoprotection studies using Leptospiral recombinant proteins from *L. interrogans Icterohaemorrhagiae* in hamster models : A synergistic approach

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### ABSTRACT

Major immune-dominant proteins of *Leptospira interrogans Icterohaemorrhagiae* were cloned, expressed in prokaryotic system was purified and confirmed by western blot. The protective efficacy of purified recombinant proteins was assayed synergistically with adjuvant in hamster models. Three combination were assessed for homologous protection (i.e, rLipL41, rOmpL1, rHapl and rSphH; rLipL41, rOmpL1, rLigA and rLigB; rLigA, rLigB, rHapl and rSphH) each protein at 25 µg and 37.5 µg concentration. Among the combinations tested combination of immunoglobulin's (rLigA and rLigB) with hemolysins (rHapl and rSphH) conferred 100% protection in both the concentration tested that is evident through the elevated elicited antibody levels. The combination of outer membrane proteins and the immunoglobulin's stands next which is comparable with the former combination. The blend of recombinant outer membrane proteins and hemolysins did effect protection which is lesser than the coalescence of immunoglobulin's and hemolysins. Membrane fractions of *E. coli* harboring pET15b used as internal control showed neither homologous protection nor enhanced antibody levels used to compare the experimental proteins over hamster models. Placebo controls receiving PBS showed similar results as that of internal controls.

Key words : *Leptospiral recombinant protein, recombinant proteins, outer membrane proteins, hemolysins, immunoglobulin like proteins, immune response, challenge studies, , homology modeling*

### INTRODUCTION

Spirochate bacteria that belongs to the genus *Leptospira* (Faucher *et al.*, 2004) that causes leptospirosis which is a re-emerging epidemic zoonosis that has worldwide distribution. This invasive vector borne spirochaete spreads through diverse group of hosts (Merino *et al.*, 1995) and infests the accidental human host leading to potential lethal weils disease characterized by multiple organ dysfunction (Levett,

2001). The infective spirochete enters the host through the wound or through skin aberrations and get localized in the vital organs causing meningitis, blindness, pulmonary hemorrhage, jaundice and nephritis (Faine, 1994). Bacterins based vaccine mediated control of leptospirosis stands in vain due to its lesser protective immunity and inferior heterologous protection (Koizumi and Watanabe, 2004) that leads to subclinical lesions in tissues and secondary infections (Bolin *et al.*, 1991). *Leptospiral* surface and sub-surface localized proteins and the proteins that are expressed during its infective stage are of great importance for the design and development of vaccines (Pinnie and Haake 2009).

*Leptospiral* lipoproteins LipL41, LipL32, LipL21 (Isogai *et al.*, 1986) and outer membrane glycoproteins OmpL1 (Alves *et al.*, 1992), are the abundant proteins that are expressed as house keeping genes that beholds maximum homogeneity among the *Leptospira* genera. Certain proteins like Hsp's (Stamm *et al.*, 1991), flagellins *fla1*, *fla2* (Goldstein and Charon, 1990), Immunoglobulins, *LigA*, *LigB* and *LigC* (Koizumi and Watanabe, 2004) and hemolysins like phospholipases and sphingomyelinases are expressed during *in-vivo*

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infection in host (Lee *et al.*, 2002). The expression of these proteins are dropped down in medium adapted culture reveals that these are host inducible and possess vital role during pathogenesis invoking virulence to the organism (Matsunaga *et al.*, 2003). The proteins of these origins are of great interest as they serve as markers for virulence of the spirochete bacteria (Palaniappan *et al.*, 2004)

Several studies revealed these surfaces exposed and sub-surface exposed proteins are conserved among the leptospires serves as potential immunogens (Matsunaga *et al.*, 2006). The lipoprotein *LipL41* and *OmpL1* showed immunoefficacy was reported on a previous study. Although the purified *LipL41* and *OmpL1* showed immunoefficacy the protective efficiency increased on synergistic studies (Haake *et al.*, 1999). The fusion protein of *LipL41* and *OmpL1* showed enhanced homologous protection over individually tested *rLipL41* and *rOmpL1* proteins was reported. (Parthiban *et al.*, 2015). Further, the *LipL32* being an abundant surface and membrane bound protein in pathogenic *Leptospira*, the purified recombinant protein efficiency towards the homologous protection over challenge was limited (Lucas *et al.*, 2011). Further, hamsters receiving *LipL32* expressing recombinant BCG showed partial protection over lethal challenge (Seixas *et al.*, 2007), as well the DNA based viral vector containing *LipL32* (*Hapl*) showed protective immunity (Branger *et al.*, 2005) and served as cross-protective immunogens over *L. interrogans* serovar (Branger *et al.*, 2001). *Leptospiral* hemolysins are class of virulent factors that encompasses *Leptospiral* phospholipases and sphingomyelinases (Trowbridge *et al.*, 1981). The role of these hemolysins in direct cell damage by pore formation and cytolysis and enhanced expression during *in-vivo* pathogenesis was reported (Lee *et al.*, 2002). Recent reports indicated that *Leptospiral* serovars expressing the sphingomyelinases (*SphA*, *SphC*) possess cytotoxicity and hemolytic activity during *in-vivo* pathogenesis and its expression implies that it has a direct role in virulence (Bernheimer and Bay, 1986). Eventually, *in-vitro* investigations added insights on the hemolysis of RBC through pore formation and cytotoxic activities over mammalian cells revealed *SphH* protein as a putative pore-forming protein (Lee *et al.*, 2002). Sphingomyelinases activity in *L. interrogans* and its absence or non-sphingomyelinase activity in *L. biflexa* contrasts the possible contribution of sphingomyelinases in pathogenicity, virulence and survival of *Leptospira* inside the host (Picardeau *et al.*, 2008).

*Leptospiral* immunoglobulins are group of bacterial immunoglobulins were reported as effective immunogens towards humoral response as well for its homologous protection. These high molecular weight immunogens (*rLigA*) confer complete protectivity but with subclinical lesion in the challenged models from *L. interrogans* serovar Pomona (Palaniappan *et al.*, 2002). Studies on these virulence markers are not affected even with targeted gene disruption (*rLigB*) as reported previously. (Croda *et al.*, 2008). The expression of

these immunoglobulins is inducible on physiological osmolarity of the host which effects in binding with the extracellular matrix proteins of the host (Choy *et al.*, 2007). Several reports suggests that even purified recombinant conserved domains (Silva *et al.*, 2007) and the targeted domains of *LigA* also confers protective immunity rather than the complete ORF of high molecular weight of *rLigA* protein (Coutinho *et al.*, 2011). *E. coli* expressing lapidated form of *rLigA* from *L. interrogans* of copenhageni showed protective immunity over hamsters models when administered orally was reported (Lourdault *et al.*, 2014). These immunoglobulins also serve as effective serodiagnostic markers for acute leptospirosis (Croda *et al.*, 2007).

In the present study *Leptospiral* recombinant protein genes of lipoproteins, outer membrane proteins, hemolysins and immunoglobulins from *L. interrogans* serovar Icterohaemorrhagiae serogroup RGA were individually, cloned and expressed in prokaryotic (*E. coli*) host. The expressed recombinant proteins purified were assessed synergistically for its humoral response and its protective potential in hamster models by challenge studies.

## Materials and Methods

### Cultivation of *Leptospira*

*L. interrogans* serogroup Icterohaemorrhagiae serovar RGA type reference culture used in this study was maintained in our laboratory. The Strain was grown and maintained in liquid EMJH medium supplemented with *Leptospira* supplement (BD Biosciences, USA) at 30 °C under aerobic conditions and collected at a density of approximately  $5 \times 10^8$  bacteria per mL by direct counting of motile bacteria under dark field microscopy. The virulence of the *L. interrogans* was maintained by repeated infection and re-isolation from Golden Syrian Chinese hamsters.

### Primer Design and Synthesis

The available nucleotide sequences of *leptospiral* experimental genes of *L. interrogans* were collected from the GenBank, NCBI ([www.ncbi.nih.gov/genbank](http://www.ncbi.nih.gov/genbank)). The retrieved sequences were aligned using ClustalX 2.0 and the gene primers were synthesized using Oligo 4.0 and DNA Star 7.0. Stop codon were excluded in the reverse primer for purification of recombinant proteins were synthesized from MWG Oligo synthesis (Bangalore).

### Genomic DNA Isolation and PCR

Isolation of genomic DNA was carried out from *L. interrogans* serovar *icterohaemorrhagiae* strain RGA, grown in EMJH medium using STET method (8% Sucrose, 50mM Tris-HCl pH-8.0, 50mM EDTA pH-8.0, 5% Triton-X-100) as per Bartlett and Stirling, 2004. Amplification of leptospiral experimental protein gene was carried out using gene specific primers that are designed with *NdeI* and *XhoI* recognition sites (Table-1). and the PCR was performed using polymerase chain

reaction (PCR) using ABI 9700 thermal cycler. PCR amplicons were analyzed in 1.5% agarose gel electrophoresis. The PCR amplicons of the amplified gene products was purified using Qiagen PCR product purification kit. Then, purified products were sequenced and analyzed using BLASTn analysis. The nucleotide sequences were edited through BIO EDIT software and aligned through CLUSTALX. The aligned sequence of *L. interrogans* Icterohaemorrhagiae RGA was submitted to NCBI, Genbank.

### Cloning and expression of experimental *Leptospiral* genes from *Icterohaemorrhagiae*

The vector (pET15b) plasmid was extracted using Qiagen miniprep kit and the plasmid was analyzed in 1% agarose gel electrophoresis. The isolated pET15b plasmid and the purified experimental gene amplicons were digested with *Nde*I and *Xho*I restriction enzymes (New England BioLabs) at 37 °C for 3 h and purified using Qiagen gel extraction kit. The digested pET15b plasmid and the PCR products were *Ligated* at 16 °C for overnight. The pET15b and the individually *Ligated* gene constructs (pET15b-*LipL41*, pET15b-*OmpL1*, pET15b-*Hapl*, pET15b-*SphH*, pET15b-*LigA*, pET15b-*LigB*) were transformed into *E. coli* DH5 $\alpha$  cells. The pET15b gene constructs were extracted from *E. coli* DH5 $\alpha$  cells and transformed into *E. coli* BL21 (DE3) codon plus RP cells.

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. *E. coli* BL21 (DE3) cells harboring recombinant plasmids were grown in LB medium supplemented with ampicillin at 50 mg/L at 37 °C for overnight. *E. coli* cells harboring the *rSphH* gene construct were induced with 1mM of isopropyl- beta-*D*-thiogalactopyranoside (IPTG) *E. coli* BL21 DE3 harboring the pET15b vector alone was induced simultaneously for 16 hrs as negative control. The protein expression was carried out at 28°C for optimal induction. The induced and uninduced *E. coli* cells were lysed by heat denaturation and resolved in 12% SDS-PAGE.

### Purification and western blot of recombinant proteins

The recombinant proteins were purified using Ni<sup>2+</sup> NTA affinity column chromatography as per the manufacturer's instructions (Invitrogen, USA). The purified recombinant proteins were dialyzed using dialysis membrane with a cut-off of 10 kDa (Sigma Aldrich, USA). The purified protein fractions collected and the gel eluted protein were analyzed by 12% SDS-PAGE and stained using coomassie brilliant stain R-250 (Merck, USA). The purified recombinant proteins were confirmed on 12% SDS-PAGE. SDS-PAGE analyses of purified protein from were transferred on to Polyvinylidene fluoride (PVDF) membrane (Whatman, UK). Western blot was carried out using BioRad western blotting system as per the procedure described by

Sambrook et al. with little modifications. The PVDF membrane was treated with hyper-immune serum (1:200) raised against virulent leptospires in hamster models to access the specificity of the expressed proteins.

### Determination of Lethal dose (LD<sub>50</sub>)

Virulent *L. interrogans* Icterohaemorrhagiae RGA obtained from infected hamsters were passaged twice in EMJH medium was serially diluted aseptically to 10 fold (10<sup>9</sup>-10<sup>5</sup>) were injected intraperitoneally in to 8-9 weeks old hamsters. The LD<sub>50</sub> was calculated by the method of Reed and Muench. The LD<sub>50</sub> value was calculated and found to be 10<sup>6</sup> dilutions.

### Immunization efficacy of *Leptospiral* recombinant proteins synergistically (100µg and 150µg Conc.) in Hamster models :

Hamsters of 4 weeks old were grouped into 5 groups with each group consisted of 5 animals.

- Group I: Control with PBS
- Group II: Membrane fractions of pET-15b harboring *E. coli* cells.  
Purified recombinant *rLipL41*, *rOmpL1*, *rHapl* and *rSphH* (*LOHaSp*) proteins.
- Group III: Purified recombinant *rLipL41*, *rOmpL1*, *rHapl* and *rSphH* proteins with Aluminum hydroxide adjuvant.
- Group IV: Purified recombinant *rLipL41*, *rOmpL1*, *rLigA* and *rLigB* (*LOLaLb*) proteins.  
Purified recombinant *rLipL41*, *rOmpL1*, *rLigA* and *rLigB* proteins with Aluminum hydroxide adjuvant
- Group V: Purified recombinant *rLigA*, *rLigB*, *rHapl* and *rSphH* (*LaLbHaSp*) proteins.  
Purified recombinant *rLigA*, *rLigB*, *rHapl* and *rSphH* proteins with Aluminum hydroxide adjuvant

The groups were immunized subcutaneously with recombinant proteins, 25 µg of each protein for 100 µg conc. and 37.5 µg of each protein for 150 µg conc. as per the given schedule. Hamsters maintained in the animal house were immunized from day 0 and subsequently boosted on day 21 for recombinant protein and day 42. Blood for serum separation was collected on the day 0 (pre vaccination), 21 and 42 directly from saphenous vein from all the groups.

### Enzyme linked immunosorbent assay with recombinant antigens

Sera samples collected on 0, 21, 41 and 71 days from all the groups of hamsters were analyzed by ELISA. Elicited antibody levels were found significant in hamsters receiving, especially in *rLaLbHaSp* when compared with *rLOHaSp* proteins and with adjuvant in comparison to controls treated with PBS (Table-1). On the other hand, antibody levels were not statistically significant in hamsters injected with membrane fractions of pET15b antigen when compared with recombinant proteins response. Increase in antibody levels in

hamster receiving rLigA and rLigB proteins indicates the effective surface binding of these virulent factors in turn elevates protective immunity.

### Challenge Experiment

The positive control groups were immunized with phosphate buffered saline and the placebo control groups received membrane fraction of pET15 in PBS. On day 42, the hamsters groups were challenged with approximately 1 LD<sub>50</sub> (6 x 10<sup>3</sup>) of *L. interrogans* serovar icterohaemorrhagiae RGA injected intraperitoneally. Hamsters were bled before the immunization (day 0), 1 day before the challenge (day 41) and at the time of killing (day 71). Antibody titers were determined as described above. After challenge (on day 71), the extant hamsters were sacrificed and the tissues (liver, kidney and blood) were collected aseptically from different vaccinated groups and cultured to assess the presence of *L. interrogans*. Liver and kidneys were homogenized aseptically in 0.5ml sterile EMJH medium

and transferred to 20mL EMJH medium and maintained at 30 °C for 4 weeks for growth. The growth of *Leptospira interrogans* was monitored using dark field microscopy (Haake *et al.*, 1999).

### Statistical analysis

The data presented in the study were expressed as mean±SD. The difference between groups was evaluated by one-way analysis of variance (ANOVA), and multiple comparisons were carried out by Dunnett's test of Prism GraphPad version 5.0. The values were considered statistically significant where P<0.05.

## RESULTS

### PCR amplification and sequence analysis of *Leptospiral* immunogenic genes

In this study, the experimental *Leptospiral* gene (*LipL41*, *OmpL1*, *Hapl*, *SphH*, *LigA* and *LigB*) from *L. interrogans* Icterohaemorrhagiae RGA was amplified.

Table-1. List of primer used for PCR amplification of *Leptospiral* immunodominant protein genes

Gene details	Primer sequence 5'-.....-3'
<i>LipL41</i> FP	5'-GGG CAT ATG CTT CAG AAA TTC CTC GGA ACC ATT -3'
<i>LipL41</i> RP	5'- GGG CTC GAG TTA CTT TGC GTT GCT TTC GTC AAC- 3'
<i>OmpL1</i> FP	5'- CCG GGG CAT ATG CGT ATC ATA TGT ATG -3'
<i>OmpL1</i> RP	5'- CCC CTC GAG GAG TTC GTG TTT ATA TCC-3'
<i>Hapl</i> FP	5'- GCA GGA CAT ATG TCC ATT ACC GCT TGT GG -3'
<i>Hapl</i> RP	5'- TGC AAG CTC GAG CTT GGA AAT CAT ACG AAC -3'
<i>SphH</i> FP	5'- CCG AAA AAA GAA CAT ATG GAA TA -3'
<i>SphH</i> RP	5'- AA CTC GAG CTT TCC AAT CTC TTT C -3'
<i>LigA</i> FP	5' GGG TTT CAT ATG GCT GGC AAA AGA GGC - 3'
<i>LigA</i> RP	5'-GGG CTC GAG GTC TCT CCA GTT TTA CC -3'
<i>LigB</i> FP	5'-CAC AAG CTT CAT ATG TAC GCA TAT T-3'
<i>LigB</i> RP	5'- TTA CTC GAG CTT ATA CGA ATT AC -3'

Table-2.ELISA - Antibody titre levels of hamsters vaccinated with leptospiral recombinant protein in synergistically (Conc. 100 µg)

Conc. 100 µg	0th day	21st day	42nd day	Challenge
Control-PBS	0.0312 ± 0.003	0.0397 ± 0.001	0.0401 ± 0.009	0.4302 ± 0.0007
MFE-pET15b <sup>©</sup>	0.0398 ± 0.01	0.0626 ± 0.08 <sup>ns</sup>	0.1315 ± 0.07 <sup>©ns</sup>	0.4981 ± 0.06 <sup>ns</sup>
rLOHaSp <sup>a</sup>	0.0399 ± 0.001	0.3063 ± 0.03*	0.9244 ± 0.03 <sup>a*</sup>	1.6523 ± 0.007 <sup>a**</sup>
rLOHaSp + Adjuvant	0.0402 ± 0.002	0.3273 ± 0.003 <sup>a,©*</sup>	1.1491 ± 0.08 <sup>a,ns</sup>	1.9838 ± 0.001 <sup>a,©**</sup>
rLOLaLb protein <sup>b</sup>	0.0406 ± 0.03*	0.4921 ± 0.02 <sup>a,©*</sup>	1.5692 ± 0.03 <sup>a,©*</sup>	2.4212 ± 0.02 <sup>a,©*</sup>
rLOLaLb +Adjuvant	0.0401 ± 0.02*	0.5641 ± 0.001 <sup>a,b,©**</sup>	1.8766 ± 0.003 <sup>a,b,©**</sup>	2.5692 ± 0.002 <sup>a,b,©**</sup>
rLaLbHaSp <sup>c</sup>	0.0403 ± 0.02*	0.8342 ± 0.04 <sup>a,b,©*</sup>	2.1691 ± 0.005 <sup>a,b,©**</sup>	3.1261 ± 0.005 <sup>a,b,©**</sup>
rLaLbHaSp + Adjuvant	0.0408 ± 0.04*	0.9346 ± 0.001 <sup>a,b,©**</sup>	2.2523 ± 0.001 <sup>a,b,©**</sup>	3.4392 ± 0.004 <sup>a,b,©**</sup>

The values are mean±SD, <sup>©</sup>Samples compared with Control-PBS; <sup>a</sup> Samples compared with Control-PBS and <sup>©</sup>; <sup>b</sup> Samples compared with Control-PBS and rLOHaSp; <sup>c</sup>Samples compared with Control PBS and a, b; \* Significant p<0.05; \*\*Significant p<0.01; <sup>ns</sup> Not Significant.

The PCR conditions were optimized and the expected amplicons size of *LipL41* (1077bp at 52 °C), *OmpL1* (921bp at 55 °C), *Hapl* (825 bp at 54 °C), *SphH* (1665 bp at 55 °C), *LigA* (691bp at 52 °C) and *LigB* (2086bp at 56 °C) were observed in 1.5% agarose gel electrophoresis with respective annealing temperature. The amplified and purified gene products were sequenced (Genetic analyzer 3130 (ABi systems)). BLAST analysis of the sequenced nucleotide revealed 97% to 98% homology for the sequenced experimental genes with other *Leptospira* sequences from the NCBI database. The nucleotide sequence edited through BIO-EDIT and aligned by CLUSTALW was submitted to NCBI, Genbank.. (Accession Number: GQ477369 (*Hapl*) and GU207476 (*SphH*) GQ477369 (*LigA*); GU552679 (*LigB*))

### Cloning, expression and Purification of experimental recombinant proteins

A precise band of respective base pair size as mentioned above was observed in colony PCR, whereas two bands with respective gene insert size and 5708bp plasmid vector (pET15b) were observed in insert release. The optimal expression of the experimental genes was standardized and obtained at 1.0mM concentration of IPTG at 28 °C and the expression of gene was noticed from the first hour of induction till fifth hour of post induction at a molecular weight (*LipL41*, 49kDa), (*OmpL1*, 39kDa), (*Hapl* 29kDa), (*SphH*, 65kDa), (*LigA*, 24kDa) and (*LigB*, 73kDa) respectively, in comparison to uninduced controls and tends to decrease after fifth hour. The induced, purified, eluted fractions of recombinant proteins were confirmed by Western blotting and were observed as precise bands with respective size on the PVDF membrane when developed using hyper immune serum raised in hamsters.

### Antibody response to recombinant antigens

The sera collected on 0, 21, 41 and 71 days from all the five different groups of hamsters were analyzed by ELISA. The results presented in Table-2 and Table-3

clearly indicates that, the levels of antibody elicited were significantly increased in animals treated with recombinant proteins with adjuvant when compared to controls treated with PBS. On the other hand, antibody levels were not detected significant in hamsters vaccinated against membrane fraction of *E. coli* cells harboring pET15b antigen and PBS which is incomparable with experimental recombinant proteins response.

### Challenge studies and *Leptospira* culture from tissues

The protective efficacies of recombinant proteins were presented in Table-4 and Table-5. Histopathological examination of controls injected with PBS revealed the evidence of acute interstitial nephritis inferred through interstitial inflammation with lymphocyte, macrophage and plasma cells infiltration in the kidney tissue. In the case of liver, the hepatocytes resembled fatty, cholestasis and with severe necrosis were observed. Similarly, these symptoms were observed in the case of rLOHS vaccinated groups with defending survival. However, the symptoms were least in the case of animals in the groups treated with rLOLaLb and no demonstrable lesions with rLaLbHaSp and adjuvant were observed (Results not shown). Tissues from all the control challenged animals showed growth in EMJH medium and presence of organism was observed on dark field microscopic examination. In contrast, tissues from survived animals vaccinated with rLOLaLb and rLaLbHaSp showed least to no growth were noticed in triplicate samples (1x10 counts / ml using petroff hauser counting chamber under Dark field microscopy). Among the tested three different combinations, the combination of rLigA, rLigB, rHapl and rSphH (LaLbHaSp) proteins in both the concentrations (100 µg, and 150 µg) conferred 100% protection with and without adjuvant when compared with the rLipL41, rOmpL1, rHapl, rSphH (LOHaSp) combination tested which is evident with the antibody levels elicited.

**Table-3. ELISA - Antibody titre levels of hamsters vaccinated with leptospiral recombinant protein in synergistically (Conc. 150 µg)**

Conc. 150 µg	0th day	21st day	42nd day	Challenge
Control-PBS	0.0414 ± 0.001	0.0437 ± 0.001	0.0437 ± 0.0009	0.4831 ± 0.0007
MFE-pET15b <sup>©</sup>	0.0419 ± 0.001	0.0711 ± 0.06 <sup>ns</sup>	0.1412 ± 0.003 <sup>ns</sup>	0.5024 ± 0.006 <sup>ns</sup>
rLOHaSp <sup>a</sup>	0.0421 ± 0.001	0.4563 ± 0.03*	0.9989 ± 0.03 <sup>a*</sup>	1.7015 ± 0.007 <sup>a,©**</sup>
rLOHaSp + Adjuvant	0.0418 ± 0.001	0.6027 ± 0.0009 <sup>a,©**</sup>	1.1549 ± 0.005 <sup>a, ab*</sup>	1.9998 ± 0.001 <sup>a,b,©**</sup>
rLOLaLb protein <sup>b</sup>	0.0420 ± 0.001	0.6941 ± 0.001 <sup>a, ab*</sup>	1.8762 ± 0.002 <sup>a, ab*</sup>	2.5214 ± 0.002 <sup>a,b,©**</sup>
rLOLaLb + Adjuvant	0.0429 ± 0.001	0.7341 ± 0.005 <sup>a, ab*</sup>	1.9961 ± 0.003 <sup>a, ab*</sup>	2.7465 ± 0.001 <sup>a,b,©**</sup>
rLaLbHaSp <sup>c</sup>	0.0402 ± 0.001	0.8417 ± 0.003 <sup>a, ab*</sup>	2.2763 ± 0.001 <sup>a, ab*</sup>	3.2212 ± 0.002 <sup>a,b,©**</sup>
rLaLbHaSp + Adjuvant	0.0399 ± 0.001	0.9346 ± 0.002 <sup>a, ab*</sup>	2.8764 ± 0.006 <sup>a, ab*</sup>	3.5235 ± 0.007 <sup>a,b,©**</sup>

The values are mean±SD, <sup>©</sup>Samples compared with Control-PBS; <sup>a</sup>Samples compared with Control-PBS and <sup>©</sup>; <sup>b</sup>Samples compared with Control-PBS and rLOHaSp; <sup>c</sup>Samples compared with Control PBS and a, b; \* Significant p<0.05; \*\*Significant p<0.01; <sup>ns</sup> Not Significant.

Table-4. Protective efficiency of leptospiral recombinant protein in synergistically (Conc. 100 µg)

Protective efficiency of <i>Leptospiral</i> experimental recombinant proteins synergistic effect with adjuvant in hamster models (100µg conc.) (Each protein @ 25 µg conc.)				
Groups	No. (%) of Surviving animals / groups <sup>a</sup>			
	Expt1	Expt2	Expt3	Total
MFE-pET15b	1/5 (20)	0/5 (0)	2/5 (40)	3/15 (20)
Control <sup>a</sup>	0/5 (0)	1/5 (20)	0/5 (0)	1/15 (6)
Significance	NS	NS	P<0.004	NS
rLOHaSp	3/5 (60)	3/5 (60)	4/5 (80)	10/15 (66)
rLOHaSp + Adjuvant	4/5 (80)	4/5 (80)	3/5 (60)	11/15 (73)
Significance	P<0.002	P<0.001	P<0.001	P<0.003
rLOLaLb protein	4/5 (80)	5/5 (100)	5/5 (100)	14/15 (93)
rLOLaLb + Adjuvant	5/5 (100)	5/5 (100)	5/5 (100)	15/15 (100)
Significance	P<0.003	P<0.001	P<0.001	P<0.003
rLaLbHaSp <sup>ab</sup>	5/5 (100)	5/5 (100)	5/5 (100)	15/15 (100)
rLaLbHaSp + Adjuvant	5/5 (100)	5/5 (100)	5/5 (100)	15/15 (100)
Significance	P<0.001	P<0.001	P<0.001	P<0.001

<sup>a</sup> Number of surviving animals after challenged. Statistical analysis was performed using Student's T test- two independent means (Significance, P<0.05); NS, Not significant.

Table-5. Protective efficiency of leptospiral recombinant protein in synergistically (Conc. 150 µg)

Protective efficiency of <i>Leptospiral</i> experimental recombinant proteins synergistic effect with adjuvant in hamster models (150µg conc.) (Each protein @ 37.5 µg conc.)				
Groups	No. (%) of Surviving animals / groups <sup>a</sup>			
	Expt1	Expt2	Expt3	Total
MFE-pET15b	1/5 (20)	0/5 (0)	1/5 (20)	2/15 (20)
Control	0/5 (0)	1/5 (20)	0/5 (0)	1/15 (6)
Significance	P<0.004	NS	P<0.003	NS
rLOHaSp	3/5 (60)	4/5 (80)	4/5 (80)	11/15 (73)
rLOHaSp + Adjuvant	4/5 (80)	5/5 (100)	4/5 (80)	13/15 (86)
Significance	P<0.001	P<0.001	P<0.001	P<0.001
rLOLaLb protein	5/5 (100)	5/5 (100)	5/5 (100)	15/15 (100)
rLOLaLb + Adjuvant	5/5 (100)	5/5 (100)	5/5 (100)	15/15 (100)
Significance	P<0.002	P<0.001	P<0.004	P<0.003
rLaLbHaSp	5/5 (100)	5/5 (100)	5/5 (100)	15/15 (100)
rLaLbHaSp + Adjuvant	5/5 (100)	5/5 (100)	5/5 (100)	15/15 (100)
Significance	P<0.001	P<0.001	P<0.001	P<0.001

<sup>a</sup> Number of surviving animals after challenged. Statistical analysis was performed using Student's T test- two independent means (Significance, P<0.05); NS, Not significant.

## DISCUSSION

In the present study, the *Leptospiral* rLipL41, rOmpL1, rHapl, rSphH, rLigA and rLigB proteins from *L. interrogans* Icterohaemorrhagiae were cloned, expressed and purified by Ni<sup>2+</sup> NTA affinity column

chromatography. Proteins purified were dialyzed and confirmed by western blot through the hyper immune serum raised against the virulent leptospires in hamster. Protective efficacies of the recombinant proteins were assessed in hamster models. Recombinant LOHaSp showed 66% and 73% of protection with 100 µg of protein with adjuvant respectively. Whereas with 150 µg

of protein showed 73% and 86% of protection. In contrast, the antigenic combination with *rLaLbHaSp* conferred 100% protection in both the experiments. This in accordance with the previous experiments reported with hamsters receiving *rLigA* *L. interrogans* serovar Pomona (Palaniappan *et al.*, 2002) alone in combination with *rLigB* of *L. interrogans* Copenhageni (Choy *et al.*, 2007) from showed 100% protection. *rLipL41* (29%) and *rOmpL1* (100%) tested alone and synergistically gave (100%) in the experiment conducted over hamster models is reported. However, the results was not in accordance in the consecutive experiments was reported (Haake *et al.*, 1999).

Similar experiments conducted with the hamster models receiving the fusion protein approach of *rLipL41* and *rOmpL1* showed 83% protection was reported (Parthiban *et al.*, 2015). The *OmpL1* and *LipL41* are membrane associated in specific the *OmpL1* is a transmembrane OMP's with amino acids having beta conformation with hydrophobic interior of lipid bilayer and the porin channel which functions as porin in the *Leptospiral* outer membrane (Haake *et al.*, 1999). Similarly, the *Hapl* of 31-34kDa protein fraction from serovar *L. interrogans* autumnalis showed cross protective immunogenicity (Branger *et al.*, 2001) and host inducible immunogenic *Sphingomyelinases* like protein of 80 kDa confirmed protective immunity and invoke strong immune response was reported (Artiushin *et al.*, 2004). Although the protein is pore forming (*Sphingomyelinase*) in *in-vitro* studies at 100µg/mL (Lee *et al.*, 2002) its showed increased protection in combination with the outer membrane proteins and immunoglobulins is reported in our study. The antibody elicited by the combination of *rLaLbHaSp* protein at booster dosage is highly comparable with the *rLOHaSp* protein tested. These results were corroborated with the previously reported studies of the antibody elicited of *rGroEL* protein from *L. interrogans* serovar Autumnalis (Natarajaseenivasan *et al.*, 2011). In a previous report, hamsters receiving 50 µg of *rSph2* and *rSphH* alone and with adjuvant of *L. interrogans* pomona showed less efficient in protective against challenging with leptospires (Carvalho *et al.*, 2010) whereas in apparent contradiction, hamsters receiving the recombinant proteins of *L. interrogans* icterohaemorrhagiae produced protective efficacy with *LOHaSp* (66%-86%) and *LaLbHaSp* (100%) while challenging against virulent leptospires that are evident through the elevated levels of antibody in the surviving animals and the least lesions observed in histopathology. This is due to the activity of *rLigA* and *rLigB* which has conserved regions with repeated variable tandem repeats that imposes hydrophobicity in the protein sequence and confers maximum protection. In a previous report it has been revealed that *rLigA* and *rLigB* when tested alone conferred 100% protection against lethal infection over hamster models. (Palaniappan *et al.*, 2006; Silva *et al.*, 2007). In addition, the combination of *rHapl* and *rSphH* impacted protective immunity in hamster models were inferred in our study. This is in correlation with the

previous reports with *rHapl* (Branger *et al.*, 2001) and *rSphH* (Artiushin *et al.*, 2004).

Control hamsters receiving PBS and membrane fractions of *E. coli* showed extravasated blood vessels with interstitial inflammatory infiltration and severe interstitial nephritis, composed of immune cells followed by fatty hepatocytes, cholestasis and other hepatic abnormalities. The lesions that were observed with the controls and hamsters receiving *E. coli* membrane fractions were less evident or not at demonstrable levels among the hamster groups treated with *rLOLaLb* and *rLaLbHaSp* with adjuvant in both the experiments were observed. The observed pathological conditions after challenged was in comparison with the previous report in control hamster models with the indications of severe chronic and acute degenerative lesions in liver, kidney and lungs when challenged with *L. borgpetersenii* Hardjo (Zuerner *et al.*, 2011). These results suggests that the experimental recombinant proteins mediated vaccination method would be effective as it is tested with the combination of outer membrane proteins, virulent factors and immunoglobulins and are expressed in significant levels in virulent leptospires. Since, pathogenicity of the bacteria is mediated by the microbial adhesion to the host and its colonization or its removal from the host is critical for its pathogenesis (Patti *et al.*, 1994), targeting these virulent factors and immunoglobulins would be an effective tool in vaccine development. Identification of structural motifs of these experimental proteins of *L. interrogans* possibly helps to understand the protein that could be used as potential diagnostic and vaccine candidate.

## Conflict of Interests

Authors declare that there is no conflict of interests regarding the publication of this paper.

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