Biolife
AN INTERNATIONAL QUARTERLY JOURNAL OF BIOLOGY & LIFE SCIENCES
4(4):678-686
ISSN (online): 2320-4257
www.biolifejournals.com
BIOLIFE RESEARCH ARTICLE

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

S. Senthil Kumar[1]*, M.Parthiban[2], and P. Agastian[3]

[1-2] *Department of Animal Biotechnology, Madras Veterinary College, Chennai-600007*
[3]*Department of Plant Biology and Biotechnology, Loyola College, Chennai, Tamil Nadu, India*

Email: senthilkumar9383@gmail.com

ABSTRACT

Major immunodominant proteins of *Leptospira interrogans* Icterohaemorrahagiae were cloned, expressed in prokaryotic system was purified and confirmed by western blot. The protective efficacy of purified recombinant proteins were assayed synergistically with adjuvant in hamster models. Three combination were assessed for homologous protection (i.e, r*Lip*L41, r*Omp*L1, r*Hap*I and r*Sph*H; r*Lip*L41, r*Omp*L1, r*Lig*A and r*Lig*B; r*Lig*A, r*Lig*B, r*Hap*I and r*Sph*H) each protein at 25 µg and 37.5 µg concentration. Among the combinations tested combination of immunogloblulins (r*Lig*A and r*Lig*B) with hemolysins (r*Hap*I and r*Sph*H) confered 100% protection in both the concentration tested, that is evident through the elevated elicited antibody levels. The combination of outer membrane proteins and the immunoglobulins stands next which is comparable with the former combination. The blend of recombinant outermembrane proteins and hemolysins did effect protection which is lesser than the coalescence of immunoglobulins 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 zooonosis 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 *Lip*L41, *Lip*L32, *Lip*L21 (Isogai *et al.,* 1986) and outer membrane glycoproteins *Omp*L1 (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.,*

How to Site This Article:
S. Senthil Kumar, M.Parthiban, and P. Agastian. (2016). Immunoprotection studies using Leptospiral recombinant proteins from *L. interrogans Icterohaemorrhagiae* in hamster models: A synergistic approach. *Biolife*. 4(4), pp 678-686.

DOI:10.5281/zenodo.7350368
Received: 3 October 2016;
Accepted; 26 November 2016;
Available online : 4 December 2016

1991), flagellins *fla*1, *fla*2 (Goldstein and Charon, 1990), Immunoglobulins, *Lig*A, *Lig*B and *Lig*C (Koizumi and Watanabe, 2004) and hemolysins like phospholipases and sphingomyelinases are expressed during *in-vivo* 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 origin are of great interest as they serve as markers for virulence of the spirochete bacteria (Palaniappan *et al.,* 2004)

Several studies revealed these surface exposed and sub-surface exposed proteins are conserved among the leptospires serves as potential immunogens (Matsunaga *et al.,* 2006). The lipoprotein *Lip*L41 and *Omp*L1 showed immunoefficacy was reported on a previous study. Although the purified *Lip*L41 and *Omp*L1 showed immunoefficacy the protective efficiency increased on synergistic studies (Haake *et al.,* 1999). The fusion protein of *Lip*L41 and *Omp*L1 showed enhanced homologous protection over individually tested r*Lip*L41 and r*Omp*L1 proteins was reported. (Parthiban *et al.,* 2015). Further, the *Lip*L32 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 *Lip*L32 expressing recombinant BCG showed partial protection over lethal challenge (Seixas *et al.,* 2007), as well the DNA based viral vector containing *Lip*L32 (*Hap*I) showed protective immunity (Branger *et al.,* 2005) and served as cross-protective immunogen 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 cytoloysis and enhanced expression during *in-vivo* pathogensis was reported (Lee *et al.,* 2002). Recent reports indicated that *Leptospiral* serovars expressing the sphingomyelinases (*Sph*A, *Sph*C) 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 *Sph*H 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 (r*Lig*A) 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 (r*Lig*B) as reported previously. (Croda *et al.,* 2008). The expression of these immunoglobulins are 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 sugeests that even purified recombinant conserved domains (Silva *et al.,* 2007) and the targeted domains of *Lig*A also confers protective immunity rather than the c*Omp*lete ORF of high molecular weight of r*Lig*A protein (Coutinho *et al.,* 2011). *E. coli* expressing lapidated form of r*Lig*A from *L. interrogans* of copenhageni showed protective immunity over hamsters models when administered orally was reported (Lourdault *et al.,* 2014). These immunoglobulins also serves 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 Icterohemorrhagiae 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). 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 mediumusing STET method (8% Sucrose, 50mM Tris-HCl pH-8.0, 50mM EDTApH-8.0,

5% Triton-X-100) as per Bartlett and Stirling, 2004. Amplification of leptopsiral experimental protein gene was carried out using gene specifi primes that are designed with *Nde*I and *Xho*I 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* Icterohemorrhagiae 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 analysed 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 pET15bplasmid and the PCR products were *Lig*ated at 16 °C for overnight. The pET15b and the individually *Lig*ated gene constructs (pET15b-*Lip*L41, pET15b-*Omp*L1, pET15b-*Hap*I, pET15b-*Sph*H, pET15b-*Lig*A, pET15b-*Lig*B) were transformed into *E. coli* DH5α cells. The pET15b gene constructs were extracted from *E. coli* DH5α 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 r*Sph*H 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^{2+} 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 protein were confirmed on 12% SDS-PAGE. SDS-PAGE analysis 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 accesss the specificity of the expressed proteinas.

Determination of Lethal dose (LD_{50})

Virulent *L. interrogans* Icterohaemarrhagiae RGA obtained from infected hamsters were passaged twice in EMJH medium was serially diluted aseptically to 10 fold (10^9-10^5) were injected intraperitoneally in to 8-9 weeks old hamsters. The $LD_{50}$was calculated by the method of Reed and Muench. The LD_{50} value was calculated and found to be 10^6 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 r*Lip*L41, r*Omp*L1, r*Hap*I and r*Sph*H (*LOHaSp*) proteins.
Group III:	Purified recombinant r*Lip*L41, r*Omp*L1, r*Hap*I and r*Sph*H proteins with Aluminum hydroxide adjuvant.
Group IV:	Purified recombinant r*Lip*L41, r*Omp*L1, r*Lig*A and r*Lig*B (*LOLaLb*) proteins.
	Purified recombinant r*Lip*L41, r*Omp*L1, r*Lig*A and r*Lig*B proteins with Aluminum hydroxide adjuvant
Group V:	Purified recombinant r*Lig*A, r*Lig*B, r*Hap*I and r*Sph*H (*LaLbHaSp*) proteins.
	Purified recombinant r*Lig*A, r*Lig*B, r*Hap*I and r*Sph*H 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 r*LaLbHaSp* when compared with r*LOHaSp* 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 r*Lig*A and r*Lig*B 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_{50} (6 x 10^3) 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 were 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*,*Omp*L1, *Hap*I, *Sph*H, *Lig*A and *Lig*B) from *L. interrogans* Icterohaemorrhagiae RGA was amplified. The PCR conditions were optimized and the expected amplicon size of *LipL41* (1077bp at 52 °C), *Omp*L1 (921bp at 55 °C), *Hap*I (825 bp at 54 °C), *Sph*H (1665 bp at 55 °C), *Lig*A (691bp at 52 °C) and *Lig*B (2086bp at 56 °C) were observed in 1.5% agarose gel electrophoresis

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

Gene details	Primer sequence 5'-…..-3'
***LipL41* FP**	**5'-**GGG **CAT ATG** CTT CAG AAA TTC CTC GGA ACC ATT **-3'**
***LipL41* RP**	**5'-** GGG **CTC GAG** TTA CTT TGC GTT GCT TTC GTC AAC- **3'**
***Omp*L1 FP**	**5'-** CCG GGG **CAT ATG** CGT ATC ATA TGT ATG **-3'**
***Omp*L1 RP**	**5'-** CCC **CTC GAG** GAG TTC GTG TTT ATA TCC**-3'**
***HapI* FP**	**5'**- GCA GGA **CAT ATG** TCC ATT ACC GCT TGT GG **-3'**
***HapI* RP**	**5'-** TGC AAG **CTC GAG** CTT GGA AAT CAT ACG AAC **-3'**
***Sph*H FP**	**5'**- CCG AAA AAA GAA **CAT ATG** GAA TA **-3'**
***Sph*H RP**	**5'**- AA **CTC GAG** CTT TCC AAT CTC TTT C **-3'**
***Lig*A FP**	**5'** GGG TTT **CAT ATG** GCT GGC AAA AGA GGC - **3'**
***Lig*A RP**	**5'**-GGG **CTC GAG** GTC TCT CCA GTT TTA CC **-3'**
***Lig*B FP**	**5'**-CAC AAG CTT **CAT ATG** TAC GCA TAT T**-3'**
***Lig*B 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[©]	0.0398 ± 0.01	0.0626 ± 0.08[ns]	0.1315 ± 0.07[©ns]	0.4981 ± 0.06[ns]
rLOHaSp[a]	0.0399 ± 0.001	0.3063 ± 0.03*	0.9244 ± 0.03[a*]	1.6523 ± 0.007[a**]
rLOHaSp + Adjuvant	0.0402 ± 0.002	0.3273 ± 0.003 [a,©*]	1.1491 ± 0.08 [a,ns]	1.9838 ± 0.001 [a,©**]
rLOLaLb protein[b]	0.0406 ± 0.03*	0.4921 ± 0.02 [a,©*]	1.5692 ± 0.03 [a,©*]	2.4212 ± 0.02 [a,©*]
rLOLaLb +Adjuvant	0.0401 ± 0.02*	0.5641 ± 0.001[a,b,©**]	1.8766 ± 0.003 a,b,©**	2.5692 ± 0.002 a,b,©**
rLaLbHaSp[c]	0.0403 ± 0.02*	0.8342 ± 0.04[a,b,©*]	2.1691 ± 0.005 a,b,©**	3.1261 ± 0.005 a,b,©**
rLaLbHaSp + Adjuvant	0.0408 ± 0.04*	0.9346 ± 0.001[a,b,©**]	2.2523 ± 0.001 a,b,©**	3.4392 ± 0.004 a,b,©**

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

with respective annhealing 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.. (Accesssion Number: GQ477369 (*Hap*I) and GU207476 (*Sph*H) GQ477369 (*Lig*A); GU552679 (*Lig*B))

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 concenrataion of IPTG at 28 ^{o}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), (HapI 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 was 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 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 efficacy 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 heptocytes resembled fatty, cholestasis and with severe necrosis were observed. Similarly, these symptoms were observed in the case of r*LOHS* vaccinated groups with defending survival. However, the symptoms were least in the case of animals in the groups treated with r*LOLaLb* and no demonstrable lesions with r*LaLbHaSp* 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 r*LOLaLb* and r *LaLbHaSp* 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 r*Lig*A, r*Lig*B, r*Hap*I and r*Sph*H (*LaLbHaSp*) proteins in both the concentrations (100 µg, and 150 µg) conferred 100% protection with and without adjuvant when compared with the r*Lip*L41, r*Omp*L1, r*Hap*I, r*Sph*H

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[©]	0.0419 ± 0.001	0.0711 ± 0.06[ns]	0.1412 ± 0.003 [ns]	0.5024 ± 0.006[ns]
rLOHaSp[a]	0.0421 ± 0.001	0.4563 ± 0.03*	0.9989 ± 0.03[a]*	1.7015 ± 0.007 [a,©]**
rLOHaSp + Adjuvant	0.0418 ± 0.001	0.6027 ± 0.0009 [a,©]**	1.1549 ± 0.005 [a, ab]*	1.9998 ± 0.001[a,b,©]**
rLOLaLb protein[b]	0.0420 ± 0.001	0.6941 ± 0.001 [a, ab]*	1.8762 ± 0.002 [a, ab]*	2.5214 ± 0.002 [a,b,©]**
rLOLaLb +Adjuvant	0.0429 ± 0.001	0.7341 ± 0.005 [a, ab]*	1.9961 ± 0.003 [a, ab]*	2.7465 ± 0.001 [a,b,©]**
rLaLbHaSp[c]	0.0402 ± 0.001	0.8417 ± 0.003 [a, ab]*	2.2763 ± 0.001 [a, ab]*	3.2212 ± 0.002 [a,b,©]**
rLaLbHaSp + Adjuvant	0.0399 ± 0.001	0.9346 ± 0.002 [a, ab]*	2.8764 ± 0.006 [a, ab]*	3.5235 ± 0.007 [a,b,©]**

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

clearly indicate that, the levels of antibody elicited were significantly increased in animals treated with (*LOHaSp*) combination tested which is evident with the antibody levels elicited.

Table-3. Protective efficiency of leptospiral recombinant protein in synergistically (Conc. 100 µg)

Protective efficiency of *Leptospiral* experimental recombinant proteins synergistic effect with adjuvant in hamster models (100µg conc.) (Each protein @ 25 µg conc.)				
Groups	No. (%) of Surviving animals / groups[a]			
	Expt1	Expt2	Expt3	Total
MFE-pET15b	1/5 (20)	0/5 (0)	2/5 (40)	3/15 (20)
Control [a]	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 (80)	4/5 (80)	10/15 (66)
rLOHaSp + Adjuvant	4/5 (80)	4/5 (80)	3/5 (80)	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[ab]	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

[a] Number of surving animals after challenged. Statisitical analyisis 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 *Leptospiral* experimental recombinant proteins synergistic effect with adjuvant in hamster models (150µg conc.) (Each protein @ 37.5 µg conc.)				
Groups	No. (%) of Surviving animals / groups[a]			
	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

[a] Number of surving animals after challenged. Statisitical analyisis was performed using Student's T test- two independent means (Significance, P<0.05); NS, Not significant.

DISCUSSION

In the present study, the *Leptospiral* r*Lip*L41, r*Omp*L1, r*Hap*I, r*Sph*H, r*Lig*A and r*Lig*B proteins from *L. interrogans* Icterohaemorrhagiae were cloned, expressed and purified by Ni^{2+} NTA affinity column chromatography. Proteins purified were dialyzed and confirmed by western blot through the hyperimmune serum raised against the virulent leptospires in hamster. Protective efficacy 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 r*Lig*A *L. interrogans* serovar Pomona (Palaniappan *et al.,* 2002) alone in combination with r*Lig*B of *L. interrogans* Copenhageni (Choy *et al.,* 2007) from showed 100% protection. r*LipL41* (29%) and r*Omp*L1 (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 receving the fusion protein approach of r*LipL41* and r*Omp*L1 showed 83% protection was reported (Parthiban *et al.,* 2015). The *Omp*L1 and *LipL41* are membrane associated in specific the *Omp*L1 is a transmembrane *OMP* 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 *Hap*I of 31-34kDa protein fraction from serovar *L. interrogans* autumnalis showed cross protective immunogenicity (Branger *et al.,* 2001) and host inducible immunogenic *Sphingo*myelinases 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 (Sphingomyleinase) in *in-vitro* studies at 100µg/mL (Lee *et al.,* 2002) its showed increased protection in combination with the outermembrane proteins and immunoglobulins is reported in our study. The antibody elicited by the combination of r*LaLbHaSp* protein at booster dosage is highly comparable with the r*LOHaSp* protein tested. These results were corroborated with the previously reported studies of the antibody elicited of r*Gro*EL protein from *L. interrogans* serovar Autumnalis (Natarajaseenivasan *et al.,* 2011). In a previous report, hamsters receiving 50 µg of r*Sph*2 and r*Sph*H alone and with adjuvant of *L. interogans* 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 animlas 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 hydrophocity in the protein sequence and confers maximum protection. In a previous report it has been revealed that r*Lig*A and r*Lig*B 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 r*Hap*I and r*Sph*H impacted protective immunity in hamster models were inferred in our study. This is in correlation with the previous reports with r*Hap*I (Branger et al., 2001) and r*Sph*H (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, choletasis and other hepatic abnormalities. The lesions that was 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 r*LOLaLb* and r*LaLbHaSp* with adjuvant in both the experiments were observed. The observed pathological conditions after challenged was in comparsion 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.

References

1. Alves, V.A., Gayotto, L.C., Brito, T.De., Santos, R.T., Wakamatsu, A., Vianna, M.R. and Sakata, E.E. (1992). *Leptospiral* antigens in the liver of experimentally infected guinea pig and their relation to the morphogenesis of liver damage. Exp. Toxicol. Pathol. 44(7): 425-434.
2. Artiushin, S., Timoney, J.F., Nally, J. and Verma. A. (2004). Host inducibile immunogenic *Sphingo*myelinases like protein, Lk73.5, of *Leptospira interrogans*. Infect. Immun. (72): 742-749.
3. Bartlett, J.M.S. and Stirling, D. (2004). DNA Extraction form Fungi, Yeast and Bacteria. PCR Protocols Second Edition, Methods in Molecular Biology, Humana Press, (226): 53-54.
4. Bernheimer , A.W., and Bey, R.F. 1986. Copurification of *Leptospira interrogans* serovar pomona hemolysin and *Sphingo*myelinase C. Infect. Immun. 54(1): 262-264.

5. Bolin CA, Cassells, J.A., Zuerner, R.L. and Trueba, G. (1991). Effect of vaccination with a monovalent *Leptospira interrogans* serovar hardjo type hardjo-bovis vaccine on type hardjo-bovis infection of cattle. Am. J. Vet. Res. (52): 1639-1643.
6. Branger, C., Sonrier, C., Chatrenet, B., Klonjkowski, B. and Ruvoen-Clouet, N. (2001). Identification of the hemolysis-associated protein I as a cross-protective immunogen of *Leptospira interrogans* by adenovirus-mediated vaccination. Infect. Immun. (69): 6831-6838.
7. Branger, C., Chatrenet, B., Gauvrit, A., Aviat, F. and Aubert, A. (2005). Protection against *Leptospira interrogans* sensu lato challenge by DNA immunization with the gene encoding hemolysin-associated protein I. Infect. Immun. (73): 4062-4069.
8. Carvalho, E., Barbosa, A.S, Gomez, R.M., Oliveira, M.L.S., Romero, E.C., Goncales, A.P., Morais, Z.M., Vasconcellos, S.A. and Ho, P.L. (2010). Evaluation of the Expression and Protective Potential of *Leptospiral Sphingo*myelinases. Curr. Microbiol. (60): 134-142.
9. Choy, H.A., Kelley, M.M., Chen, T.L., Moller,A.K., Matsunaga, J. and Haake, D.A. (2007). Physiological osmotic induction of *Leptospira interrogans* adhesion: *Lig*A and *Lig*B bind extracellular matrix proteins and fibrinogen. Infect. Immun. (75): 2441-2450.
10. Coutinho, M.L., Choy, H.A., Kelley, M.M., Matsunaga, J., Babbitt, J.T., Lewis, M.S., Aleixo, J.G. and Haake, D.A. (2011). A *Lig*A three domain region protects hamsters from lethal infection by *Leptospira interrogans*. PLoS Negl. Trop. Dis. 5(12): e1422.
11. Porika Raju and Estari Mamidala (2015). Anti-diabetic activity of compound isolated from *Physalis angulata* fruit extracts in alloxan induced diabetic rats. The Ame J Sci & Med Res, 2015,1(1); Pages 1 -6. doi:10.17812/ajsmr2015.11.1.
12. Croda, J., Ramos, J.G.R., Matsunaga, J., Queiroz, A., Homma, A., Riley, L.W., Haake, D.A., Reis, M.G. and Ko, A.I. (2007). *Leptospira* immunoglobulin like proteins as a serodiagnostic marker for acute Leptospirosis. J. Clin. Microbiol. 45(5): 1528-1534.
13. Croda, J., Figueira, C.P., Wunder Jr., A.E., Santos, C.S., Reis, M.G., Ko, A.I. and Picardeau, M. (2008). Targeted Mutagenesis in pathogenic *Leptospira* species: Disruption of the *Lig*B gene does not affect virulence in animal models of leptospirosis. Infect. Immun. 76(12): 5826-5833.
14. Faine, S. (1994). *Leptospira* and leptospirosis, 1st ed. CRC press, Boca Raton, Fla.
15. Faucher, J.F, Hoen, B. and Estavoyer, J.M. (2004). The management of Leptospirosis. Expert Opin. Pharmacother. 5(4): 819-827..
16. Goldstein, S.F. and Charon, N.W. (1990). Multiple exposure photographic analysis of motile spirochetes. Proc Natl Acad Sci, (87): 4895-4899.
17. Haake, D.A., Mazel, M.K., McCoy, A.M., Milward, F., Chao, G., Matsunga, J. and Wager, E.A., (1999). *Leptospiral* outer membrane proteins *Omp*L1 and *LipL41* exhibit synergistic immune protection. Infect Immun. (67): 6572-6582.
18. Isogai, E., Isogai, H., Kurebayashi, Y. and Ito, N. (1986). Biological activities of *Leptospiral* lipopolysaccharide. Zentralbl. Bakteriol. Mikrobiol. Hyg. A. (261): 53-64.
19. Koizumi, N. and Watanabe, H. (2004). Leptopsira immunoglobulin-like protein elicit protective immunity. Vaccine (22): 1545-1552.
20. Lee, S.H., Sangduk, K., Seung, C.P. and Min, J.K. (2002). Cytotoxic Activities of *Leptospira interrogans* Hemolysin *Sph*H as a Pore-Forming Protein on Mammalian Cells. Infect. Immun. (70): 315-322.
21. Levett, P.N. (2001). Leptospirosis. Clin. Microbiol. Rev. (14): 296-326.
22. Lourdault, K., Wang, L.C., Vieira, A., Matsunaga, J., Melo, R., Lewis, M.S., Haake, D.A. and Solecki, M.G. (2014). Oral immunization with *Escherichia coli* expressing a lapidated form of *Lig*A protects hamsters against challenge with *Leptospira interrogans* serovar copenhageni. Infect. Immun. 82(2): 893-902.
23. Lucas, D.S., Cullen, P.A., Lo, M., Srikram, A. and Sermswan, R.W. (2011). Recombinant *Lip*L32 and *Lig*A from *Leptospira* are unable to stimulate protective immunity against leptospirosis in the hamster model. Vaccine. (29): 3413-3418.
24. Matsunaga, J., Barocchi, M.A., Croda, J., Young, T.A., Sanchez, Y., Siqueira, I., Bolin, C.A., Reis, M.G., Riley, L.W., Haake, D.A. and Ko, A.I. 2003. Pathogenic *Leptospira* species express surface exposed proteins belonging to the bacterial immunoglobulin superfamily. Mol. Microbiol. (49): 929-945.
25. Matsunaga, J., Werneid, K., Zuerner, R.L., Frank, A. and Haake, D.A. (2006). *Lip*L46 is a novel surface exposed lipoprotein expressed during *Leptospiral* dissemination in the mammalian host. Microbiology. (152): 3777-3786.
26. Merino, S., Rubires, X., Knoche, S. and Tomas, J.M. (1995). Emerging pathogens: *Aeromonas* spp. Int. J. Food. Microbiol. (28): 157-168.
27. Natarajaseenivasan, K., Shanmughapriya, S., Velineni, S., Artiushin, S.C. and Timoney, J.F. (2011). Cloning, Expression, and Homology Modeling of *Gro*EL Protein from *Leptospira interrogans* Serovar *Autumnalis* Strain N2. Genomics Proteomics Bioinform. 9(4-5): 151.
28. Palaniappan, R.U., Chang, Y.F., Jusuf, S.S.D., Artiushin, S., Timoney, J.F., McDonough, S.P., Barr, S.C., Divers, T.J., Simpson, K.W.,

McDonough, P.L. and Mohammed, H.O. (2002). Cloning and Molecular Characterization of an Immunogenic *Lig*A Protein of *Leptospira interrogans*. Infect. Immun. 70(11): 5924-5930.

29. Palaniappan, R.U., Chang, Y.F., Hassan, F., McDonough, S.P., Pough, M., Barr, S.C., Simpson, K.W., Mohammed, H.O., Shin, S., McDonough, P.L., Zuerner, R.L., Qu, J. and Roe, B. 2004. Expression of *Leptospiral* immunoglobulin like protein by *Leptospira interrogans* and evaluation of its diagnostic potential in a kinetic ELISA. J. Med. Microbiol. (53): 975-984.
30. Palaniappan, R.U., McDonough, S.P., Divers, T.J., Chen, C.S., Pan, M.J., Matsumoto, M. and Chang, Y.F. (2006). Immunoprotection of recombinant *Leptospiral* immunoglobulin like protein A against *Leptospira interrogans* serovar Pomona infection. Infect. Immun. (74(3)): 1745-1750.
31. Parthiban, M., Senthil Kumar, S., Balachandran, C., Kumanan, K., Aarthi, K.S. and Nireesha, G. 2015. Comparison of Immunoprotection of *Leptospira* recombinant proteins with conventional vaccine in experimental animals. Ind. J. Exp. Biol. (53): 779-785.
32. Patti, J.M., Allen, B.L., McGavin, M.J. and Hook, M. (1994). MSCRAMM-mediated adherence of microorganisms to host tissues. Ann. Rev. Microbiol. (48): 585-617.
33. Picardeau, M.D., Bulach, D.M. and Bouchier., C. (2008). Genome sequence of the saprophyte *Leptospira biflexa* provides insightsinto the evolution of *Leptospira* and the pathogenesis of leptospirosis. PLoS ONE 3: 1607
34. Pinnie, M. and Haake, D.A. (2009). A comprehensive approach to identification of surface-exposed, outer membrane spanning proteins of *Leptospira interrogans*. PLoS One (4): e6071.
35. Seixas, F.K., da Silva, E.F., Hartwig, D.D., Cerqueira, G.M., Amaral, M. (2007). Recombinant Mycobacterium bovis BCG expressing the LipL32 antigen of *Leptospira interrogans* protects hamster from challenge. Vaccine. 26: 88-95.
36. Silva, E.F., Medeiros, M.A., McBride, A.J., Matsunaga, J., Esteves, G.S., Ramos, J.G., Santos, C.S., Croda, J., Homma, A. and Dellgostin, O.A. (2007). The terminal portion of *Leptospiral* immunoglobulin-like protein *Lig*A confers protective immunity against lethal infection in the hamster model of leptospirosis. Vaccine. 25(33): 6277-6286.
37. Trowbridge, A.A., Green, J.B., Bonnet, J.D., Shohet, S.B., Ponnappa, B.D. and Mccombs, W.B. 1981. Hemolytic anemia associated with leptospirosis morphologic and lipid studies. Am. J. Clin. Pathol. (76): 493-498.
38. Zuerner, R.L., Alt, D.P., Palmer, M.V., Thacker, T.C. and Olsen, S.C. (2011). A *Leptospira borgpetersenii* serovar Hardjo vaccine induces a Th1 response, activates NK cells, and reduces renal colonization. Clin. Vaccine. Immunol. (18): 684-691.