Chimera of Heat-labile Enterotoxin B chain conjugate with LipL32 and or LipL21 proteins induces strong reactive antibodies in experimental mice

RawahFaraj*[1, 2], Ehsan Abdalla[2], GopalReddy[1], Temesgen Samuel[1], WoubitAbdela*[1]

[1]*College of Veterinary Medicine, Department of Pathobiology, Tuskegee University, Tuskegee, Alabama, USA.*
[2] *Middle Technical University, Institute of Medical Technology, Department of Community Health, Baghdad, Iraq.*
Corresponding auther: RawahFaraj

Abstract:*Current killed vaccines against Leptospira lack long-term protective ability and are mostly serovar-specific. Hence, there is an urgent need for a vaccine that can provide long-term and broad protection against the many existing Leptospiraserovars. LipL32 and LipL21 are important conserved lipoproteins among pathogenic Leptospira. As a precedent to vaccine development, we amplified and cloned LipL32, LipL21 and heat-labile enterotoxin B subunit (LTB) genes in E. coli by using the Gateway cloning system, followed by recombinant protein expression. The recombinant LipL21 and LipL32 proteins were then chemically conjugated to LTB. Mice were immunized with the LTB-conjugated LipL32 or LipL21 and the antibody responses against the corresponding proteins were compared with those from mice injected with LipL32 or LipL21 alone or mixed with Sigma Adjuvant. LTB-conjugated LipL32 or LipL21 induced strongly reactive antibodies as compared to the recombinant proteins alone or mixed with Sigma Adjuvant. These results indicated that LTB-conjugated LipL32 and LipL21 could serve as subunit vaccines capable of inducing strong protective immunity in humans and animals against a wide array of Leptospira infection.*
Keywords:*Leptospira, Leptospirosis, LipL32, LipL21,Outer membrane protein, Recombinant protein*

I.   Introduction

Leptospirosis is a severe febrile zoonotic disease in tropical and subtropical regions[1-5], however, in recent years, this disease has become an emerging public health concern in many areas of North and South America[6, 7]. The causative agent belongs to the genus Leptospira, a gram-negative bacterium. Wild and domesticated animals are the main sources of Leptospires, as they continuously excrete the bacteria in urine [8]. Humans become infected via direct contact with contaminated urine or indirect exposure through urine-contaminated water or soil [9]. Urban rodent reservoirs also contaminate the environment through their urine[5].

Leptospirosis affects animals, causing abortions, infertility, reduced milk production and death in livestock[5]. Current available veterinary vaccines based on inactivated whole cell or membrane preparations of pathogenic Leptospires are thought to confer protection through induction of antibodies against leptospiral lipopolysaccharide[10]. However, these vaccines fail to induce long-term protection against infection and do not provide cross-protective immunity against Leptospiralserovars not included in the vaccine preparation. A major limitation to the production of a multi-serovar component vaccine and to the development of immunization protocols based on whole cell or membrane preparations is the existence of > 250 pathogenic serovars[11].Efforts to develop recombinant vaccines against leptospirosis have focused on outer membrane proteins[12]. The most abundant, immunogenic, and conserved surface lipoprotein present in all pathogenic species of Leptospira is LipL32, an outer membrane protein of 32kDa molecular size[12]. In addition, LipL32 is expressed during mammalian leptospiral infection [13]and binds to extracellular matrix components, as shown by in vitro assays[13, 14]. LipL21 is the second major outer membrane protein in Leptospirainterrogansserovar Lai, which has exhibited potent immunogenic capabilities[15], indicating that LipL21 is also a potent candidate gene for the development of a vaccine. Different immunization protocols that have been tested with Lipl32 have shown some immune protection when administrated with naked-DNA[4], recombinant adenoviral[16], and Mycobacterium bovisBCG [17] delivery system. In spite of that, LipL32 recombinant subunit protein vaccination with either aluminum hydroxide or Freund adjuvants produced no protection[4, 18]. These data suggest that induction of protective immunity by LipL32 requires a more specific modulation of the immune

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system. The E. coli heat-labile enterotoxin (LT) consists of one A subunit with ADP-ribosyltransferaseactivity linked to five B subunits[19]. The B subunit of E. coli heat- labile enterotoxin (LTB) is highly immunogenic upon systemic[20, 21] and mucosal immunization[22]. LTB has a pentameric structure that binds to the GM1-ganglioside receptor on the surface of mammalian cells, and this binding is necessary for its adjuvant properties[19, 23].

The objective of the present study was to produce and characterize recombinant LipL32 and LipL21 proteins and perform chemical conjugation with LTB and study their efficacy as vaccines against a broad array of Leptospira.

II.   Materials and methods

2.1.Bacteria strain, media and oligonucleotide Primers

Leptospira reference serovarCopenhageni M-20 strain was purchased from National Veterinary Services Laboratories (NVSL) (Ames, IA) and E. coli strain ETEC H10407 was provided by our collaborator Dr. Terry D. Connell at the School of Medicine; University of Buffalo, USA.Other cells used in this study wereE. coli One Shot® OmniMAX™ 2 T1R competent cells (Thermofisher Scientific, Carlsbad, CA), and BL21 (DE3) pLysS (Novagen® EMD Millipore Sigma, Billerica, Massachusetts), which were used for cloning, transformation, and expression to purify the recombinant proteins. Lipl32 and LipL21 primers used in this study were designed based on Gateway Cloning System (Thermofisher Scientific, Carlsbad, CA). NCBI microbial genome-sequencing database (http://www.ncbi.nlm.nih.gov/genomes/lproks) was used to localize genes coding for Lipl32 and LipL21. Primers were designed from DNA sequences of these genes retrieved into Vector NTI Software (Thermofisher Scientific, Carlsbad, CA) (**Table 1**)

Table (1) PCR primer sequences used to amplify LipL32, LipL21 and LTB genes

Name of primers	Oligonucleotide Sequences (5'-3')
Forward LipL32	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCTTCGGTGGTCTGCCAAGCCT
Reverse LipL32	GGGGACCACTTTGTACAAGAAAGCTGGGTAGATTACTTAGTCGCGTCAGAAGC
Forward LipL21	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCTGTTCCAGTACTGACACAGGAC
Reverse LipL21	GGGGACCACTTTGTACAAGAAAGCTGGGTGAATTATTGTTTGGAAACCTCTTG
Forward LTB	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGAATAAAGTAAAATGTTATGTT
Reverse LTB	GGGGACCACTTTGTACAAGAAAGCTGGGTACTAGTTTTTCATACTGATTGC

2.2. Bacterial culture, microscopy and DNA extraction

LeptospiraCopenhageni strain M-20 was grown in the Ellinghausen, McCullough, Johnson and Harris media (EMJH) (Difco®–Detroit, MI) with the addition of enrichment supplements and incubated in aerobic condition at 30ºC for 3 to 4 weeks.E. coli strain ETEC H10407 was cultured in (Luria-Bertani) medium with ampicillin (100μg/ml) and kanamycin (50μg/ml) at 37°C overnight to mid-log phase for LTB plasmid extraction using the Miniprep (Qiagen, Valencia, CA). TheE. coli competent cells were grown in LB medium at 37°C with ampicillin (100μg/ml), kanamycin (50μg/ml) and chloramphenicol (34μg/ml).After growth turbidity, Leptospiral cultureswere stained with Basic Fuchsinand examined under bright field microscopy [24]. Genomic DNA was extracted from 1×10^8 cells using the DNeasy® kit (Qiagen, Valencia, CA).

2.3. PCR protocol for LipL32, LipL21 and LTB genes

The PCR was conducted using a 25 µl reaction volume consisting of 11 µl of the PWO master mix (DNA Polymerase, reaction buffer with 4 mM MgCl$_2$ and 0.4 mM each of PCR-grade dNTPs; Roche, Mannheim, Germany), 11 µl of the PCR water, 2 µl (25 pmol) of forward and reverse primers and 1 µl (100 ng) of a plasmid template. LipL32, LipL21 and LTB specific PCR was performed with thermocycling conditions of initial denaturation at 95°C for 5 min followed by 35 cycles each of denaturation at 95°C 1 min, annealing at 65°C for 1 min, extension at 72°C for 1 min followed by a final extension at 72°C for 10 min. The PCR product of the expected 816bp, 573bp and 477bp, for LipL32, LipL21 and LTB respectively were checked for successful amplification by electrophoresis using 1.5% agarose gel and ethidium bromide staining using Alpha-Imager (Alpha Innotech Corporation, San Leandro, CA). Annotation of the gel pictures were performed using AlphaView® Software (Alpha Innotech Corporation, San Leandro, CA).

2.4. Creating LipL32, LipL21 and LTB Entry clones

The resulting PCR amplicons were purified by QIAquick® PCR purification Kit (Qiagen, Valencia, CA). The purified amplicons of LipL32 (150 ng/µl), LipL21 (150 ng/µl) and LTB (150 ng/µl) each were inserted separately into the pDONR™221 vector (150 ng/µl) using BP reaction as described in Gateway

Cloning technology instructions (Thermofisher Scientific, Carlsbad, CA). After overnight incubation at 25°C, the BP reaction was stopped by adding 1 μl (2 μg/μl) proteinase K. The pDONR™221 vector–LipL32 and pDONR™221 vector–LipL21 entry clones were transformed into E. coli competent cells using One Shot® OmniMAX™ 2 T1R (Thermofisher Scientific, Carlsbad, CA). Competent cells were then cultured on LB agar supplemented with 50 μg/ml kanamycin and 20 mg/ml X-gal and incubated overnight at 37°C. PCR was performed on white colonies to confirm positive transformants. A single positive white colony of E. coli competent cells containing the right insert was cultured into LB media (10 ml) supplemented with 50 μg/ml of kanamycin and incubated overnightin a shaker incubator at 37°C/ 200 rpm. After overnight incubation, plasmid was extracted using QIAprep® Miniprep kit (Qiagen, Valencia, CA). The fidelity of the entry clone plasmid construct was confirmed by PCR and sequencing using the M13 F/R primers. Sequencing of all plasmid samples were done at ACGT (Wheeling, IL).

2.5. Construction of LipL32, LipL21 and LTB expression plasmids

The purified plasmids containing LipL32 (150 ng/μl), LipL21 (150 ng/μl) and LTB (150 ng/μl) were mixed independently with Champion™ pET 300/NT-DEST Gateway® vector (150 ng/μl) (Thermofisher Scientific, Carlsbad, CA) for LR reaction to take place following the Gateway cloning instructions (Thermofisher Scientific, Carlsbad, CA). After overnight incubation at 25°C, the reaction was stopped by the addition of 1 μl proteinase K (2μg /μl) at 37°C for 10 min. The final expression clones pET 300 /NT-DEST– LipL32, pET 300 /NT-DEST-LipL21 and pET 300 /NT-DEST–LTB were transformed into One Shot® OmniMAX™ 2 T1R competent cells and cultured on LB agar supplemented with ampicillin (100 μg /ml) and X-gal (20 mg/ml) and incubated overnight at 37°C. Single positive white colonies of E. coli competent cells, One Shot® OmniMAX™ 2 T1R carrying the pET 300 /NT-DEST vector–LipL32 gene, or pET 300 /NT-DEST vector − LipL21 or pET 300 /NT-DEST vector–LTB gene were grown in 10 ml LB media with ampicillin (100 μg /ml) and incubated overnight in a shaker incubator at 37°C/ 200 rpm. Plasmid was extracted using QIAprep® Miniprep kit (Qiagen, Valencia, CA), and the expression clone plasmid construct was confirmed by PCR with LipL32 or LipL21 and LTB primers, followed by sequencing with T7 promoter and T7 reverse primers. Multiple sequence alignments were performed using Uniport and Geneious Pro version 5.4 (http://www.geneious.com) with sequences of these genes with other Leptospiraserovarsretrieved from the NCBI database.

2.6. Expression of recombinant LipL32, LipL21 and LTB in E. coli BL21 (DE3) PlysS host

For protein expression, plasmids pET300/NT–DEST–LipL32 and pET300/NT-DEST–LipL21 were each transformed into E. coli strain BL21 (DE3) plysS (Novagen® EMD Millipore Sigma, Billerica, Massachusetts). Transformed bacteria were cultured in LB agar with ampicillin (100 μg /ml), chloramphenicol (34 μg /ml) and X–gal (20 mg/ml) overnight at 37°C.White colonies were selected for starter culture in 5 ml LB media with ampicillin (100 μg /ml) in a shaker incubator at 37°C/ 200 rpm. Next day starter culture was added to 300 ml of Magic Media–expression medium (Thermofisher Scientific, Carlsbad, CA) with ampicillin (100 μg/ml) for 24 hrin a shaker incubator at 37°C/ 250 rpm.

2.7.Harvesting and analysis of recombinant LipL32, LipL21 and LTB proteins by SDS – PAGE

The bacterial culture for recombinant protein expression was spun down for 20 min at 4°C / 4000 rpm. The supernatant was removed and the pellet weighed, 5 ml of chilled PBS (pH 7.4) was added to 500 mg pellet and re-suspended on ice. The cell suspension was then sonicated with 10 short bursts of 30 sec followed by intervals of 30 sec for cooling. The lysed bacterial cell matrix was then spun down for 20 min at 4°C / 4000 rpm. The supernatant, which contained the soluble Histidine–tagged proteins, was carefully collected and loaded on to histidine–tag column (Takara bio, San Francisco, CA). The purification was performed according to the manufacturer's instructions. The presence of each of the recombinant LipL32, recombinant LipL21 and recombinant LTB proteins in the purified fractions were monitored by measuring absorbance at 280 nm, and by SDS-PAGE resolution on 12 % gels.

2.8. Western blot analysis of recombinant LTB protein with rabbit anti-cholera toxin and Hist-Tag antibody

Purified recombinant LTB protein resolved by SDS-PAGE in 12 % gel was electro-transferred onto nitrocellulose membrane (EMD Millipore Sigma, Billerica, Massachusetts). The membrane was blocked with 3 % bovine serum albumin (BSA) (Sigma-Aldrich, St. Louis, MO) in TBS (500 mMNaCl, 50mM Tris-HCl, pH 7.4) on a shaker at room temperature for 1hr. TBS was discarded and the membrane was incubated overnight at 4°C with the primary antibody rabbitanti-cholera toxin antibody (Sigma-Aldrich, St. Louis, MO) diluted 1/20000 in TTBS containing 1% BSA and 0.05% Tween 20. To detect Histidine-tag, the membrane was incubated overnight at 4°C with primary rabbit polyclonal anti-His antibody (Cell Signaling Technology,

Danvers, MA) diluted 1/1000 in TBS containing 1% BSA and 0.05% Tween 20. After 24 hrmembranes were washed twice with 10 ml TTBS and incubated for 1hr at room temperature with alkaline phosphataseconjugated rabbit anti-mouse antibody (Sigma-Aldrich, St. Louis, MO), it was diluted 1/30,000 in TBS containing 1% BSA / 0.05% Tween 20 for LTB protein detection. The blot was then washed twice with TTBS and once with TBS for 10 min each. The last washing buffer was discarded and 1 ml of Alkaline Phosphatase Substrate (BCIP/ NTB the 5-bromo-4-chlor-3-indolyl phosphate / nitro blue tetrazolium) (Sigma-Aldrich, St. Louis, MO) was added to the membrane and incubated for 30 min at room temperature. The specific protein band was detected as dark blue colored line of the expected molecular size. Membrane was washed with Millie-Q water to stop the enzymatic reaction.

2.9.Western blot analysis of recombinant LipL32 and Lipl21proteins with anti-Leptospira antibody and Histidine–tag antibody
For this assay the procedure followed was same as mentioned previouslyexcept that anti-Leptospira mouse monoclonal antibody (My BioSource, San Diego, CA) or primary rabbit polyclonal anti-His-tag antibody (Cell Signaling Technology, Danvers, MA) was used instead of anti-cholera toxin antibody.

2.10. Chemical Conjugation of recombinant LipL32 and LipL21 proteins with LTB
1 mg/ml of recombinant proteins LipL32 or LipL21 were subsequently modified to incorporate a 4FB-moiety by treatment with S-4FB (Solulink Biosciences, San Diego, CA). 1 mg/ml recombinant LTB protein was modified to incorporate HyNic moieties on lysine residues using S-HyNic (Solulink Biosciences, San Diego, CA). Synthesis of the conjugate was accomplished by mixing the 4FB-modified LipL32 or LipL21 protein with the HyNic-modified LTB protein according to the manufacturer protocol (Solulink Biosciences, San Diego, CA), followed by purification of conjugate proteins by size exclusion chromatography (BIO-RAD, Hercules, CA). Purity of the conjugated proteins was confirmed by running the sample on 12% resolution SDS-PAGE.

2.11. Animals
Eight week-oldMale BALB/c mice were purchased from Envigo (Indianapolis, IN) and were housed at Tuskegee University animal facility. Tuskegee University Animal Care and Use Committee approved the animal protocol before the experiment.

2.12.Animal Immunization protocol and collection of serum samples
To evaluate the immunogenicity of recombinant proteins LipL32, LipL21, LTB, or their chemical conjugates, eight groups consisting of 4 mice each were injected with 16 μg of LTB, LipL32 or LipL21 recombinant proteins alone, or, LipL32 or LipL21 mixed with 1:1 ratio of sigma adjuvant (Sigma-Aldrich, St. Louis, MO), or conjugates of LTB with LipL32 or LTB with LipL21 based on the manufacturers protocol (Solulink Biosciences, San Diego, CA). Control group mice were injected with PBS. All treatments were administered through intramuscular route. Each animal received three doses administrated at 0, 14, and 21 days of the experiment. Blood was harvested from all mice 7 days after the last injection, by heart puncture bleeding; serum was isolated and stored at -20 °C until further analysis.

2.13.Antibody responses of mice by ELISA
Polystyrene 96-well microtiter plates were coated with recombinant proteins LipL32, LipL21 and LTB (10 μg/ ml in 50 mM sodium bicarbonate buffer, pH 9.6) overnight at 4°C. Next day plates were washed 3 times with TBS/00.5% Tween 20 and then blocked with 200 μl of 1% BSA in TBS/ 0.05% Tween 20 by incubating at 37°C for 1 hour. Serum samples serially diluted 1:10, 1:20, and 1:40 in 1% BSA/TBS/0.05% Tween 20 were added to triplicate wells (100 μl/well). The plate was incubated for 3 hours at room temperature, and then washed with 1 % BSA/TBS/0.05% Tween 20 twice. Goat anti-mouse IgG alkaline phosphatase conjugate diluted at 1/30,000 (Sigma-Aldrich, St. Louis, MO) in 1% BSA/TBS and 0.05% Tween 20 was added and incubated for 1 hr at room temperature. Following washing, wells were incubated with alkaline phosphatase substrate (p-Nitrophenyl Phosphate Substrate Systems, (pNPP); Sigma-Aldrich, St. Louis, MO) for 30 minutes after which absorbance was read in an ELISA plate reader (Gen5 microplate reader and Imager software, BioTek, Winooski, VT).

2.14.Antibody response of mice by Dot immunoblot assay
To evaluate antibody responses of mice to each of the recombinant proteins LipL32, LipL21 and LTB, 10 μl (20 μg/ml) of purified recombinant proteins LipL32, LipL21 and LTB were spotted onto a polyvinylidene difluoride (PVDF) Immobilon-P membranes (Sigma-Aldrich, St. Louis, MO) using a dot blot apparatus (Bio-Rad Laboratories, Richmond, CA). The membranes were blocked with 3 % BSA in TBS at room temperature for 1 hr. Mice serum samples diluted 1:10 in 1% BSA/TBS/0.05 % Tween 20 were spotted on the membrane

and incubated for 2 hr. at room temperature. Membranes were then washed twice with TBS containing 1% BSA/0.05% Tween 20 and incubated for 1 hr at room temperature with goat anti- mouse alkaline phosphatase conjugate antibody diluted at 1/30,000 (Sigma-Aldrich, St. Louis, MO) in 1% BSA/TBS and 0.05 % Tween 20. The blots were washed twice with TTBS and once with TBS for 10 min each. The last wash buffer was discarded and 1 ml of Alkaline Phosphatase Substrate (BCIP/ NTB the 5-bromo-4-chlor-3-indolyl phosphate/nitro blue tetrazolium) (Sigma-Aldrich, St. Louis, MO) was added to the membrane and incubated for 30 min at room temperature. The specific protein dots were detected as dark blue colored dots.

2.15. Statistical analysis
To determine if the treatment means of the experimental groups were significantly different, SAS software, version 9.4 (SAS Institute Inc., Cary, NC, USA) was used. One-way analysis of variance (ANOVA) followed by post hoc Tukey multiple comparison were performed to compare and gather more information about mean differences of multiple treatments. A probability value of $p < 0.05$ was considered statistically significant.

III. Results
3.1. Amplification of LipL32, LipL21 and LTB genes
We successfully amplified 816bp of LipL32 and 573bp of LipL21 fragments from the pathogenic LeptospiraCopenhageni M-20 strain. Similarly,a477bp fragment of LTB gene from E. coli strain ETEC H10407 was successfully amplified(**Fig. 1A, 1B and 1C).**

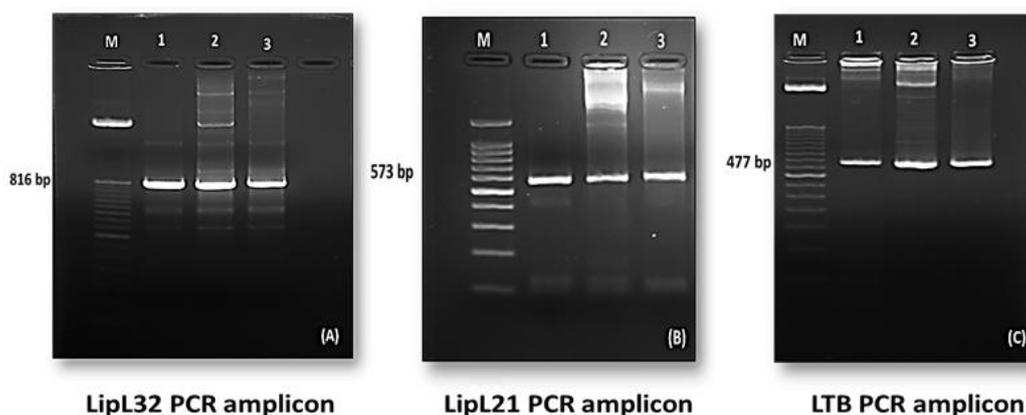**Figure 1: PCR amplification of LipL32, LipL21 and LTB genes segments.**

A. LipL32 PCR Lane M: DNA Marker 50bp, Lane 1: LipL32 PCR positive control from Leptospira M-20 strain, Lane 2: LipL32 PCR entry clone pDONR™221 + LipL32 gene, Lane 3: LipL32 PCR expression clone pET300/NT-DEST vector +LipL32 gene. **B.** LipL21 PCR. Lane M: DNA Marker, Lane 1: LipL21 PCR positive control from Leptospira M-20 strain, Lane 2: LipL21 PCR entry clone pDONR™221 + LipL21 gene, Lane 3: LipL21 PCR expression clone pET300/NT-DEST vector +LipL21 gene. **C.**LTB PCR product Lane M: DNA Marker, Lane 1: LTB PCR positive control from,E.coli strain ETEC strain H10407 Lane 2: LTB PCR entry clone pDONR™221 + LTB gene, Lane 3: LTB PCR expression clone pET300/NT-DEST vector + LTB gene.BLAST analysis using Uniprot and Geneious software program showed that LipL32 and LipL21 proteins are very well conserved among Leptospira species. LipL32 sequences were 100 % similar at nucleotide level and 98 % similar at the predicted amino acid level to annotated sequences from most pathogenic Leptospira species (**Fig.2A**). Similarly, LipL21 sequences were 100 and 96 % similar at the nucleotide and predicted amino acid levelsrespectively (**Fig.2B**)
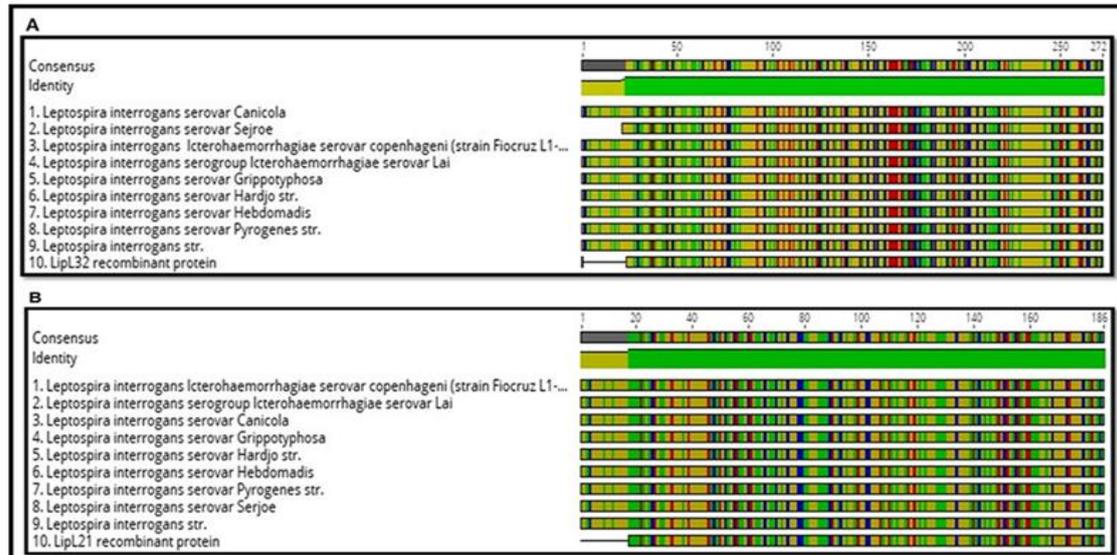
Figure 2: Sequence Alignment of recombinant LipL32 and LipL21 proteins with LipL32 and LipL21 outer membrane proteins from the most common Leptospira strains.

A. Multiple sequence alignment of recombinant LipL32 protein with the homologous gene sequences of LipL32 proteins found in different strains of Leptospira. The recombinant protein comprises amino acids 24–272 of the full LipL32 protein. **B**. Multiple sequence alignment of recombinant LipL21 protein with the homologous gene sequences of LipL21 protein found in different strains of Leptospira. The recombinant protein comprises amino acids (18-186) of the full LipL32 protein.

3.2.Confirmation of expressed and purified Histidine tagged LipL32, LipL21 and LTB Proteins
Western blot analysis of the purified proteins indicated the presence of corresponding band confirming successful expression of recombinant LipL32, LipL21and LTB proteins **(Fig.3 (A, B, C),Fig.4(A, B),and Fig.5 (A, B, C).**
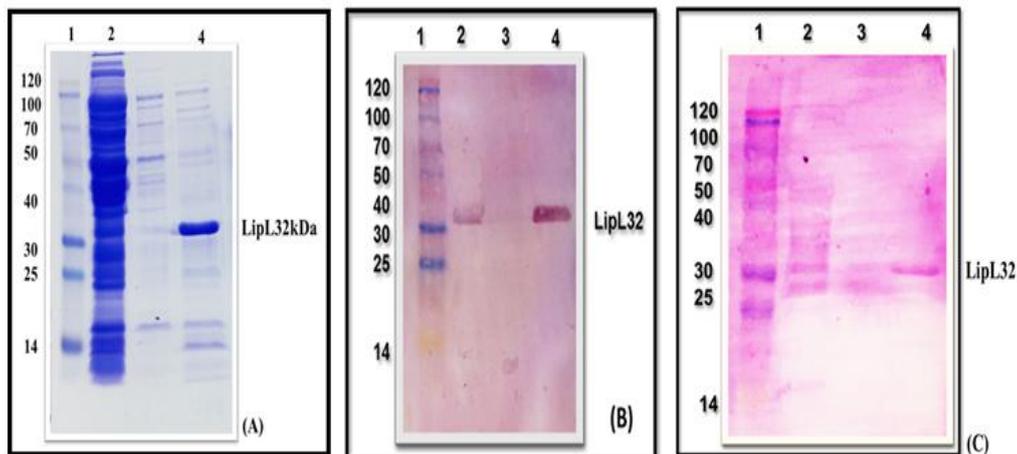

Figure 3:Protein expression and Western Blot analysis of purified recombinant LipL32 proteins.

A. Histidine-tag column- purified protein **B.** Detection of recombinant LipL32 protein with antibody against Histidine tag by Western Blot. **C.** Detection of recombinant LipL32 protein with a monoclonal antibody against Leptospira. Lane 1: protein Marker, Lane 2: cleared bacterial filtrate, Lane 3: first flow through, Lane 4: purified recombinant protein LipL32.
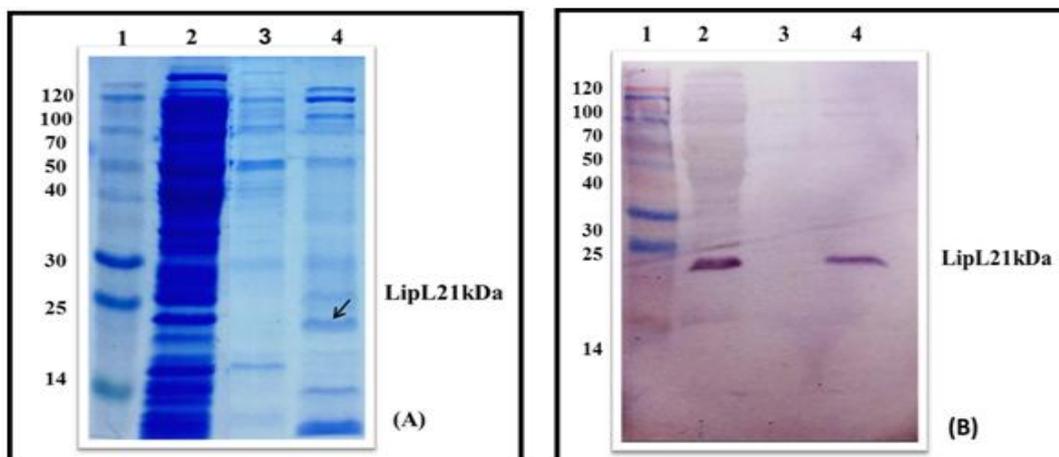
Figure 4: Protein expression and Western Blot analysis of purified recombinant LipL21 protein.

A. Histidine-tag column- purified protein. **B.** Detection of recombinant LipL21 protein with antibody against Histidine tag by Western Blot. Lane1: Protein Marker, Lane 2: cleared bacterial filtrate, Lane 3: first flow through, Lane 4: purified recombinant protein LipL21.
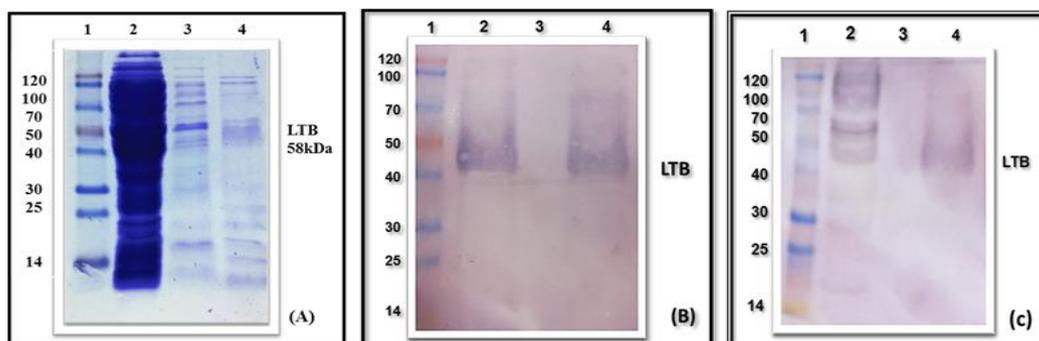

Figure 5: Protein expression and Western Blot analysis of purified recombinant LTB protein.

A. Histidine-tag column- purified protein **B.** Detection of recombinant LTB protein with antibody against Histidine tag by Western Blot. **C.** Detection of recombinant LTB protein with a monoclonal antibody against E.coli Lane 1: protein Marker, Lane 2: cleared bacterial filtrate, Lane 3: first flow through, Lane 4: purified recombinant protein LTB.

3.3.Chemical conjugations of recombinant proteins LipL32 +LTB or LipL21+LTB

Purified conjugated proteins LipL32+ LTB and LipL21+LTB were analyzed by12 % acrylamide SDS-PAGE, confirming the expected molecular weight of conjugated proteins LipL32+LTB (90kDa) and LipL21+LTB (79kDa) (**Fig. 6 A &B).**
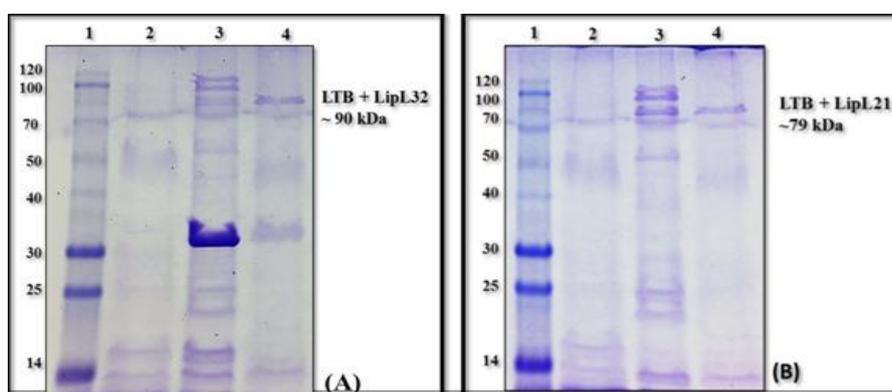

Figure 6: Chemical conjugation of recombinant LipL32 or LipL21 proteins with LTB.

A. Purified conjugated proteins LipL32+LTB. Lane1: Protein Marker, Lane 2: LTB recombinant protein, Lane 3: LipL32 recombinant protein, Lane 4: purified conjugated proteins LipL32+LTB. **B.** Purified conjugated proteins LipL21+LTB. Lane1: Protein Marker, Lane 2: LTB recombinant protein, Lane 3: LipL21 recombinant protein, Lane 4: purified conjugated proteins LipL21+LTB

3.4. Analysis of Serum antibody responses of mice immunized with recombinant proteins by ELISA and Dot-Blot assay

Serum antibody (IgG) levels as measured by ELISA (optical density at 450 nm) showed significantly higher levels in mice that received immunization with LipL32+LTB or LipL21+LTB, compared to mice that received LipL32 or LipL21 alone or with sigma adjuvant. One-way ANOVA and post hoc Tukey multiple comparison tests of two different treatments showed that antibody levels with LipL32 + LTB and LipL21 + LTB at all the three serum dilutions of 1:10, 1:20 and 1:40 were significantly higher ($p < 0.05$) compared to corresponding serum dilutions of other treatment groups. There were no significant differences between antibody levels in all the three dilutions of sera of mice treated with recombinant proteins LipL32 or LipL21 alone and in those treated with Sigma adjuvant mix**(Fig. 7A and B).**
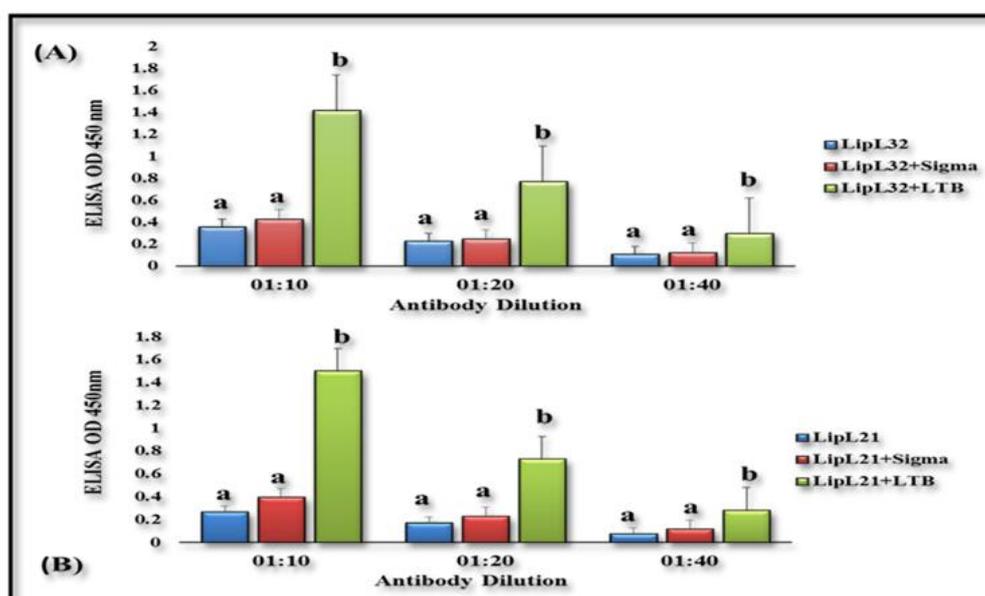

Figure 7 (A and B): Antibody responses of mice by ELISA. Group of 4 mice each were immunized with recombinant protein (16 μg / mouse) and PBS as negative control   on day 1, days 14, 21 and bled on day 28. Mice antibodies were tested byELISA for reactivity against recombinantproteins and conjugates (a, b means with alphabets differ significantly ($P < 0.05$)

Integrated densities of Dot-blots generated by sera at 1:10 dilution werequantitated using ImageJNIH software (imagej.nih.gov). The density values of sera from mice immunized with LTB–adjuvant LipL32 or LipL21 were significantly higher as confirmed by one-way ANOVA $p < 0.0095$ and $p < 0.0001$, respectively. The results of dot-blot figures **Fig. 8 (A, B) and Fig. 9** are supporting the ELISA results. One-way ANOVA and post hoc Tukey multiple comparison tests showed no significant differences between density values obtained with sera from mice treated with recombinant proteins alone and sera from mice treated with recombinant proteins administered along with Sigma adjuvant ($p > 0.05$).
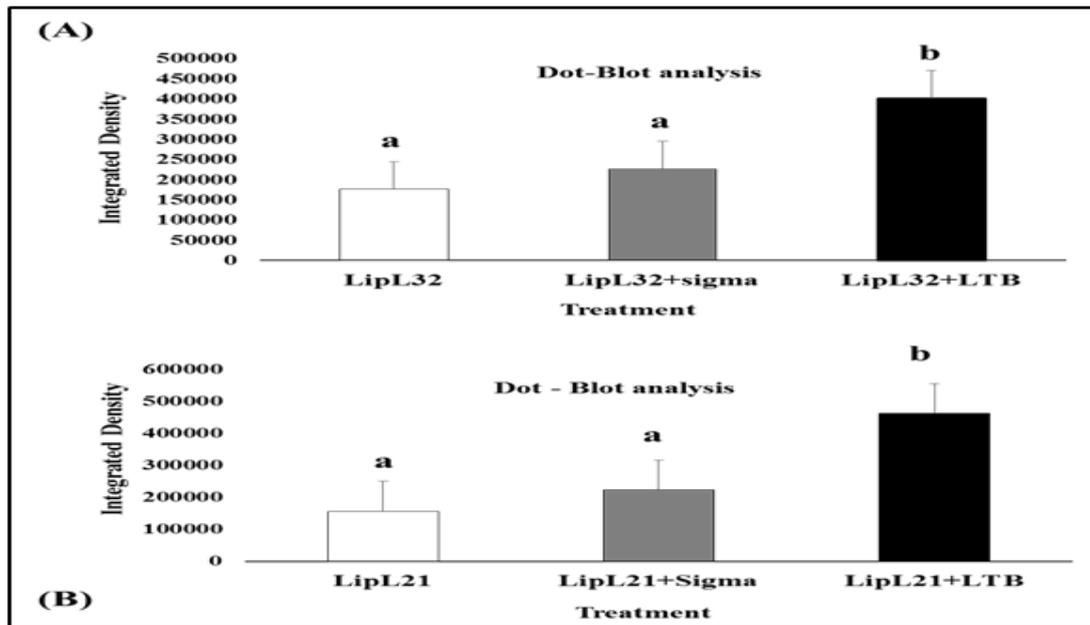
Figure 8 (A and B): Integrated density of Dot- Blot for testing reactivity of mice antibodies with purified recombinant LipL32, LipL21 and LTB proteins. Group of 4 mice each were immunized with recombinant protein (16 µg / mouse) and PBS as negative control on day 1, days 14, 21 and bled on day 28. Mice antibodies were tested byDot- Blot for reactivity against recombinantproteins and conjugates (a, b means with alphabets differ significantly ($P < 0.0001$)
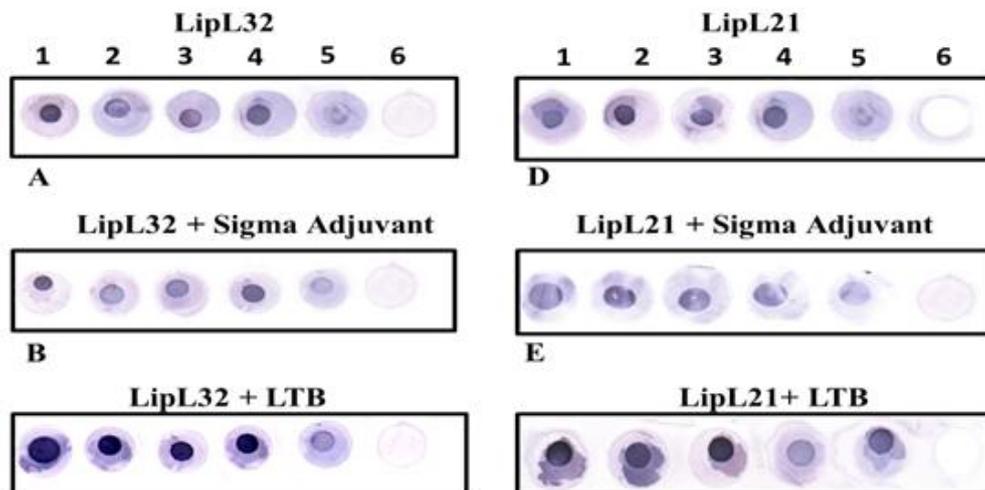

Figure 9: Dot-Blot for testing reactivity of mice antibodies with purified recombinant LipL32, LipL21 and LTB proteins. Mice (four per group) were immunized with recombinant proteins (16 µg / mouse) in PBS on day 1, days 14, 21 and bled on day 28 to collect sera. Purified recombinant LipL32, LipL21 and LTB were dotted on nitrocellulose membrane. and probed with mouse sera as sources for polyclonal antibodies. **A-C**. Dot blot testing of antibodies against recombinant proteins LipL32, LipL32+sigma and LipL32+LTB. **D-F** Dot-blot testing of antibodies produced by injection of recombinant proteins LipL21 and LipL21 + sigma adjuvants and LipL21+LTB. **1,2,3 and 4** are mouse sera (as polyclonal antibodies), **5** positive control Histidine -Tag antibody and **6** negative control.

IV. Discussion

Current vaccines against leptospirosis are multivalent inactivated whole-cell vaccines. However, efficacies of such vaccines are limited due to the LPS-based adverse reactions and the weak cross-immunogenicity among different Leptospira species[25].

As a step towards development of cross protective vaccine, we chose to develop recombinant Leptospira conjugated proteins. Accordingly, we amplified, cloned, expressed and purified LTB, LipL21, and LipL32 genes,LipL32 and LipL21 proteins are conserved and abundant lipoproteins in most pathogenic

Leptospira. In this study, PCR was used to amplify LipL32 and LipL21 genes from pathogenic LeptospiraCopenhageni M-20 strain, and LTB gene fromE. coli strain ETEC H10407 using specific primers. Sequence comparison of the amplified product of LipL32 and LipL21 from LeptospiraCopenhageni M-20 showed 96-100 % similarity with other pathogenic Leptospira spp.

To avoid misfolding and aggregation of the recombinant protein the signal peptide amino acids 1-23 of Lipl32 and 1-17 of Lipl21 were omitted during the cloning step[26]. Therefore, the recombinant proteins comprise of amino acids 24-272 of the full LipL32 protein, and amino acids 18-186 of the full length LipL21 protein were selected for recombinant protein synthesis **(Fig.2)**. The entire LipL21 and LipL32 amino acid sequences did not have any significant similarity with the sequences of proteins from other organisms [25, 27].Such conservation confirms that these proteins could be good candidates for developing diagnostic tools [28].

The results of our study show the potential for conjugated recombinant proteins LipL32 and LipL21 in producing subunit vaccine with a broad-protective immunity. Importantly in this study, we used E. coli Heat-labile Enterotoxin B Subunit (LTB) as an adjuvant for LipL32 or LipL21, which offers many advantages, these includes LTB is non-toxic, has a pentameric structure and can be a potent adjuvant for conferring protective immune response against huge types of pathogens [29].With LTB conjugation, our results showed that recombinant LTB can be a potent systemic adjuvant, as observed with twice as high IgG levels in mice immunized with LipL32+LTB or LipL21+LTB than those induced by sigma adjuvant or recombinant proteins alone. This could have resulted from LTB influencing the maturation and activation of dendritic cells [19], enhancing antigen presentation, activating selective differentiation of lymphocytes, and increasing the expression levels of activation markers on B lymphocytes. Chemical conjugations of recombinant proteins LTB+LipL32 or LTB+LipL21 were based on the formation of a stable bis-arlhydrazone from an aromatic hydrazine and an aromatic aldehyde. Succinimidyl 6-hydrazinonicotinate acetone hydrazine (S-HyNic) is used to incorporate aromatic hydrazine moieties on biomolecules. S-HyNic is an amino reactive reagent that directly converts amino groups on biomolecules and surfaces to HyNic groups. Succinimidyl 4-formylbenzoate (S-4FB) is used to convert amino groups to aromatic aldehydes such as 4-formylbenzamide. Addition of HyNic-modified biomolecules to a 4FB-modified biomolecules or surface directly leads to the formation of the conjugate. The bis-arylhydrazone bond is stable at up to 92°C and in a wide pH (2.0-10.0) range and is probably involved in the stability of the LTB-conjugated LipL32 and LipL21 proteins.

In future experiments we will study the efficacy of a combination of both the LTB conjugated proteins as a single vaccine candidate to elicit a stronger immune response. To prove the protective immunity with the LTB-conjugated proteins, we will challenge the immunized animals with live bacteria. Compared to the current traditional vaccines, such conjugated recombinant subunit vaccines are expected to be free of contaminations, stable and safe, and therefore easy to transport and store. The recombinant proteins produced in this study may also serve as antigens for developing sensitive diagnostic assays.

V. Conclusions

We have successfully amplified, cloned, expressed and chemically conjugated recombinant proteins LipL32 and LipL21 with LTB using Gateway-cloning system and protein-protein conjugation approaches. The results suggest that these conserved recombinant proteins of Leptospira outer membrane proteins have great potential for recombinant subunit vaccine production and also could be useful as reagents for the sensitive and specific diagnosis of leptospirosis. Importantly, our results suggest that rLTB is a more powerful systemic adjuvant compared to a commercial adjuvant we tested, as it induced higher antibody titers. Therefore, we propose that rLTB be an alternative adjuvant for immunization using recombinant LipL32 and LipL21.

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