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Methodologies for cloning and expression of immunodominant protein LipL32 of pathogenic Leptospira

TV Meenambigai, C Sworna Kumari and G Balakrishnan

Abstract
Pathogenic serovars of Leptospira have wide antigenic diversity, which is mainly attributed to the lipopolysaccharide which is present in the outer membrane. The most common surface exposed immunodominant lipopolysaccharide protein is LipL32. In the present study the highly conserved surface exposed LipL32 protein was cloned and expressed in pRSET B vector. The His-tagged protein was purified and characterized by western blot analysis. The developed LiPL32 recombinant protein will serve as a novel diagnostic candidate for effective diagnosis of pathogenic forms of Leptospira in animals.

Keywords: Cloning, immunodominant protein LipL32, pathogenic Leptospira

Introduction
Leptospirosis, a worldwide zoonotic disease with more than 250 pathogenic serovars, is a disease of public health concern. The leptospires are mainly recognized by their surface lipopolysaccharides (LPS) (Bulach *et al*., 2000) [1] The clinical symptoms of Leptospirosis are similar to dengue, Hepatitis, Typhoid, Fever, Meninigitis and influenza (Charlie *et al*., 2003). Diagnostic approaches are mainly based on the detection of antibodies that are generated by the dominant antigen. The leptospiral membranes are of three types viz.,outer membrane protein (lipoproteins), peripheral outer membrane proteins and transmembrane protein. (Claudia *et al*., 2007) The antigenic structure of leptospira is very complex with structural heterogeneity in the carbohydrate component of the lipopolysaccharides (LPS), keyplayer for serovar variation (Ko *et al*., 2009, moctezuma *et al* 2001) [4, 3]. Some outer membrane proteins are LipL32, LipL21 and LipL41 (Cullen *et al*., 2005) [2]. Among different outer membrane protein, LipL32 is described as one of the novel outer membrane lipoproteins, currently used as a biomarker in antigen detection, potential candidate for vaccine production and therapeutics. (Gomes *et al*., 2020). The most exclusive presence of proteins in pathogenic leptospira indicates that they are promising for developing diagnostic tools (Sriram *et al*., 2000 Wang *et al*., 2007)
The present research study embarks the methodology of cloning and expression of LipL32 protein and necessitates its novelty as a valuable marker in the diagnosis of leptospirosis in humans and animals.

Materials and Methods
Leptospira culturing and nucleic acid amplification
Leptospira cultures were maintained in Ellinghausen-McCullough-Johnson-Harris (EMJH) medium with a supplement of EMJH enrichment base, and incubated at 30˚c for 7 to 10 days. DNA was extracted by CTAB method (Boom., *et al*., 1990) and amplified with the forward primer F -5’ GTC GAC ATG AAA AAA CTT TCG ATT TTG -3’and reverse primer R-5’ CTG CAG TTA CTT AGT CGC GTC AGA AGC -3’.The PCR cyclic conditions were an initial denaturation of 94˚C for 4 minutes followed by 35 amplification cycles at 94˚C for 1minute, 58˚C for 1minute and 72˚C for 1 minute followed by a final extension at 72˚C for 10 minutes.

Cloning and Expression of LipL32

The amplicons of LipL32 protein was purified and ligated into pTZ57R/T vector and recloned in pRSET B (6XHis tag) expression vector with the restriction enzymes XhoI and EcoRI and transformed into *Escherichia coli* BL21 (DE3). The recombinant colonies were checked for the presence of the insert by colony PCR and restriction enzyme digestion to confirm insert presence and release. Plasmid was extracted from the confirmed clones ande induced with IPTG-100mM at 37 ˚C for 3 hours for protein expression with specific antibiotics (Sriram *et al*., 2014, Arivudainambi *et al*., 2014)

Purification Of rLipL32

pRSET B–LipL32 complex, confirmed by polyacrylamide gel electrophoresis (SDS-PAGE) was subjected to purification utilizing Ni-NTA resin purification system, for purification based on affinity chromatography. The eluate was dialyzed against phosphate-buffered saline (PBS) pH with glycine 0.1%, for approximately 16 hours at 4 °C.

Characterization by western Blotting

The purified LipL32 protein was analyzed by 12% SDS-PAGE and electro transferred onto a Nitrocellulose membrane. The NC membrane was blocked with 3% BSA at 37˚C for 2 hours, followed by washing the membrane thrice with phosphate buffered saline with Tween-20 (PBST).The membrane was then incubated with primary antibody at 1:500 dilution for 1h, followed by series of washes with PBST. Conjugated secondary antibody was then added at a dilution of 1:2000 and incubated at 37˚C for 1h, followed by triple washes with PBST. The NC membrane was developed with diaminobenzidine (DAB) substrate. (Meenambigai *et al*., 2015) [7]

Results and Discussion:

Cloning and Expression of Lipl32

Polymerase chain reaction was performed with conserved primers. The amplicon size of LipL32 gene was 720bp.The product was analysed in 0.8% agarose gel (Fig - 1).

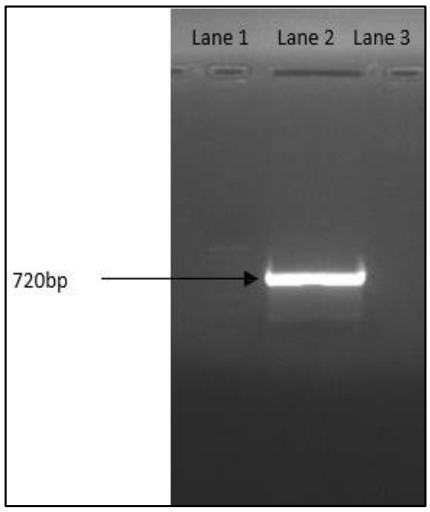

Fig 1: Amplified product of LipL32 on agarose gel

Legend: Lane 1: 100bp DNA Ladder, Lane 2: Amplified PCR Product, Lane 3 : Negative

Expression of Recombinant LipL32 gene

The LipL32 gene was cloned in TA (pTZ57R/T) vector initially and the plasmid was extracted and digested using the restriction Enzymes EcoRI and XhoI. The product from the Plasmid was cloned in expression vector pRSETB with an N-terminal His tag and the construct was transformed in the Expression host E.coli BL21 (Fig-2). Randomly, 7 positive clones were taken and confirmed by colony PCR using the LipL32 gene primers (Fig - 3). The insert release was confirmed by restriction enzyme digestion analysis (Fig - 4)

For expression of protein one positive clone was taken for the induction of Protein with IPTG. 0th hour, 1st hour, 2nd hour, 3rd hour, 4th hour, 5th hour and overnight sample were collected, confirmed in 12%SDS PAGE for standardizing the hour of protein expression (Fig-5).The His- tagged rLipL32 eluate was collected and dialyzed. The dialyzed eluate was analyzed initially in 12% SDS page (Fig - 6), and confirmed by western blot with Anti-his-HRP conjugate and then developed using DAB substrate. The dialyzed rLipL32 eluate was seen as 32kda size and compared with Prestained protein ladder (Fig - 7).

Confirmation of LipL32 insert release

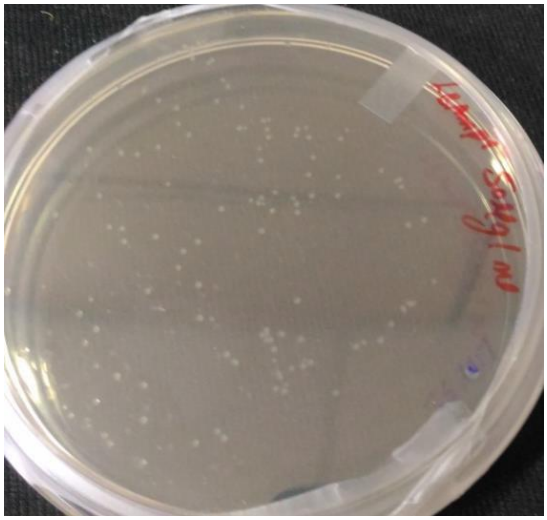

Fig 2: Recombinant LipL32 colonies

Fig 2: Ampicillin resistant colonies on luriabertoni plate with 50µg/ml ampicillin

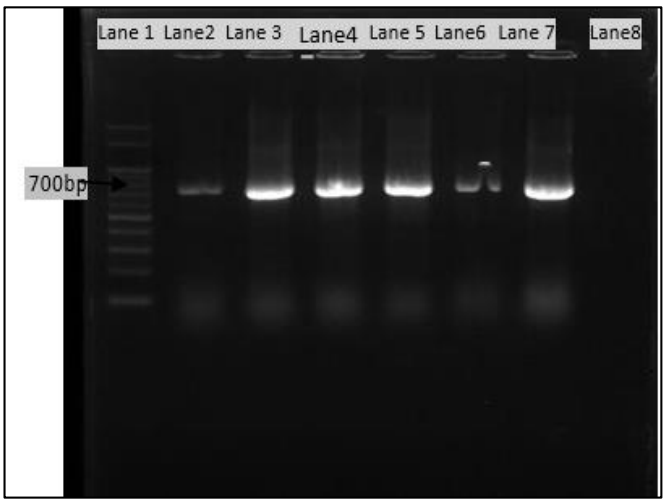

Fig 3: Colony PCR Lane 1 : 100bp DNA Ladder Lane 2 to 7: positive clones from Colony PCR- LipL32 Lane 8 : Negative sample

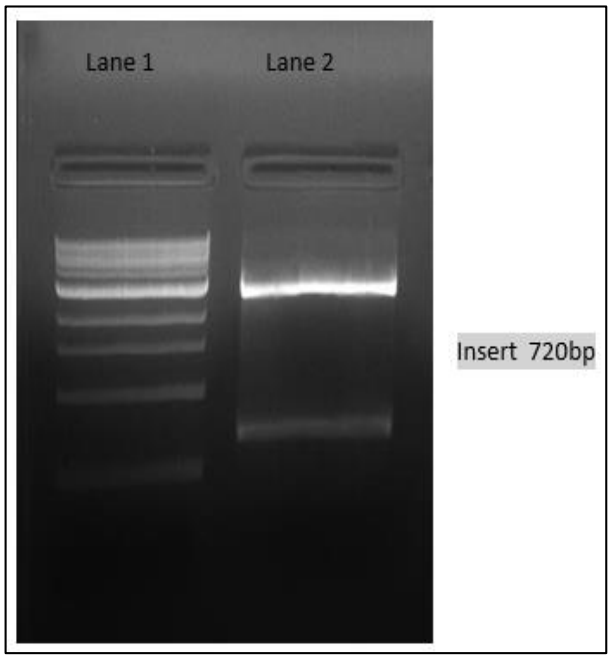

Fig 4: Restriction Digestion **Legend:** Lane 1 -1000 bp ladder Lane 2– Double digested plasmid with Xho I and EcoR I enzyme,

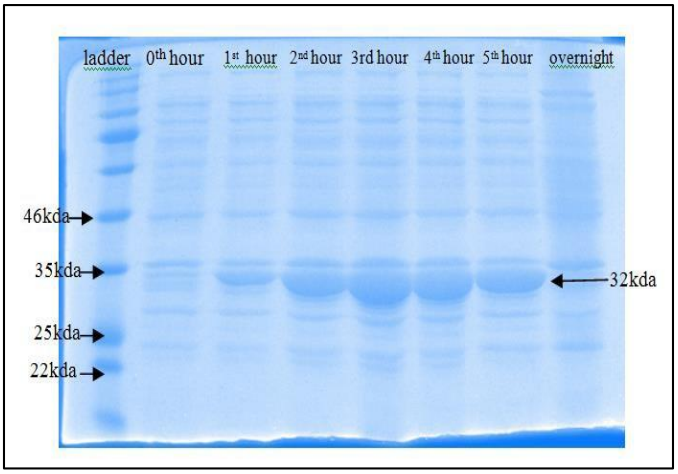

Fig 5: Induction analysis of LipL32 protein Expression

Legend- Lane 0th hour to overnight- induced Lipl32 Protein with IPTG,Lane- ladder broad range protein marker

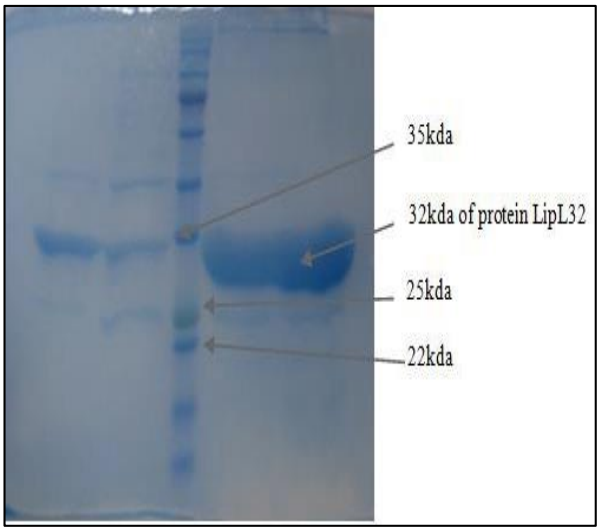

Fig 6: SDS – PAGE Analysis of LipL32 Purified protein

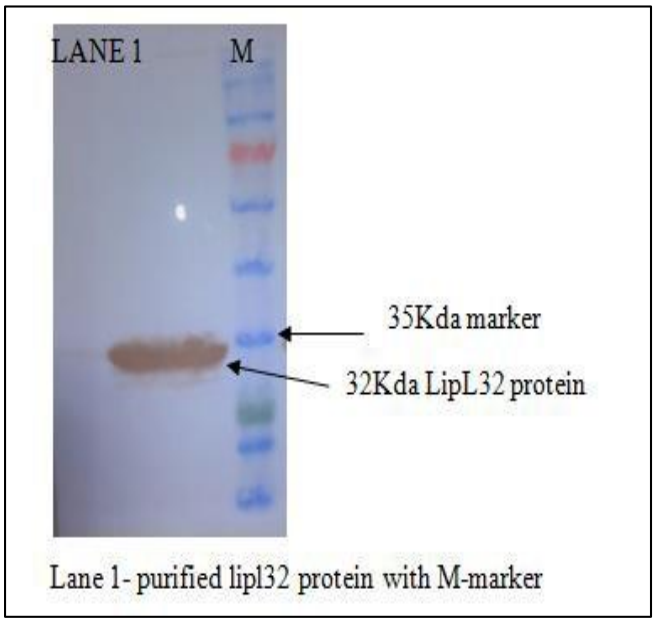

Fig 7: Western Blot Analysis for LipL32 protein Characterization

Pathogenic Leptospira outer membrane may determine virulence and be the main target for immunity. The most abundant proteins in the spirochetal outer membrane are lipoproteins, which may lead to a possible mechanism for pathogenesis. LipL32 is expressed during mammalian infection and is highly conserved among pathogenic Leptospira species. The novelty of this conserved nature has made LipL32 as an important diagnostic marker. LipL32 has been shown to induce inflammatory cytokines and stimulates extracellular matrix production in cultured renal tubular epithelial cells. In the present research study the conserved region of the immunodominant protein LipL32 was cloned and expressed in pRSET B vector. The methodologies for the same has being remarked in a sequential manner.

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