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Cloning and Expression of Partial CDS of Leptospiral Beta Propeller Repeat Protein in Prokaryotic System

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ABSTRACT

Leptospirosis is the wide spread neglected zoonotic disease among domestic and pet animals and poses major threat to human public health. Although it is treatable with antibiotics, vaccination is the prime strategy to control the disease. However, the current vaccines are ineffective, lack of cross reactivity and require booster doses. Therefore, it is inevitable to identify conserved protein candidates that can provide immunity against the majority of serovars. In this context, the present study was aimed to clone and express the lipoprotein Beta Propeller repeat (BPR) of *L. canicola*. In this study, 435 base pair of partial coding sequences between 195 to 340 aa region of beta propeller gene was amplified by PCR. The fragment of 435 bp BPR gene was inserted into the pET 32(a) vector and expressed in *E. coli* Bl21(DE3) cells. SDS-PAGE revealed an expected size of 36 kDa and the immunoblot with anti sera raised against the whole cell lysate of *L. canicola*, confirmed the specificity of the protein expressed in *E. coli* system. NCBI BLAST analysis showed that 435 nucleotides are flanking the coding region of 422164 to 422598 positions of *L. interrogans* sps chromosome number 1 and having 95 percent identities with the published sequences. Phylogenetic analysis revealed that the BPR gene of this study occupied the same clade in the phylogenetic tree as other *L. canicola* serovars. From the findings of this study, it may be concluded that the Beta Propeller Repeat protein gene was conserved in the genus Leptospira and which could be a potential vaccine candidate for subunit vaccines.

HIGHLIGHTS

- Beta propeller repeat protein of *L. canicola* was amplified, sequenced partially and expressed in prokaryotic system.
- BLAST analysis showed that 435 nucleotides are flanking the coding region of 422164 to 422598 positions of *L. interrogans* spp.
- Immuno blot and *in-silico* analysis confirmed its specificity and conservancy among other serovars of *Leptospira* sps

Keywords: Beta Propeller Repeat gene, Canine Leptospirosis, PCR, Cloning and expression

Leptospirosis is the most neglected zoonotic disease of global public and animal health concern particularly in tropical and subtropical regions, with an estimated global burden of 500,000 cases per year (Torgerson *et al*., 2015) In the past century, several epidemics have been reported worldwide including India, where the disease has been endemic since the 20th century (Adler 2015). Leptospirosis is caused by a Spirochaete of the genus, Leptospira, which comprises about 22 genome species, further divided into about 300 antigenically different serovars (Lehmann *et al*., 2014). Infection with pathogenic *Leptospira* spp. can

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result in a large range of clinical manifestations, including fever, renal failure, jaundice, hemorrhages, meningitis, and death. It affects rodents, domestic and wild animals and human beings. Although it is treatable with antibiotics, early diagnosis is essential for effective treatment.

Presently, the vaccination with inactivated whole cell preparations (bacterins) has limited efficacy due to wide antigenic variation and no cross-protection against the different serovars of pathogenic Leptospira. The drawbacks of commercially available conventional vaccines highlight the need for new vaccine strategies in the prevention of leptospirosis. Consequently, for the last two decades, the classical research approach is being used to identify protein targets for the development of subunit and recombinant vaccines against leptospirosis (Dellagostin *et al.,* 2017). Genus-specific leptospiral proteins that are conserved throughout different serovars of Leptospira which are immunogenic and uniquely expressed during acute infection may help in the development of an effective vaccine for leptospirosis as well as aid in studies on its pathogenesis. Therefore, it is highly essential to identify conserved protein candidates that can stimulate both humoral and cell-mediated immunity which is crucial for the development of an effective vaccine to provide immunity against the majority of serovars (Grassmann *et al.,* 2017). In recent years, the use of leptospira recombinant proteins has shown promise for developing diagnostic tests and vaccines for this disease. Therefore, the present study was aimed to clone and express the highly conserved Beta Propeller repeat (BPR) which is lipoprotein (Locus tag LIC12209 Putative lipoprotein) in nature and responsible for the virulence and pathogenesis of leptospirosis.

MATERIALS AND METHODS

Ethical approval

All the procedures have been carried out in accordance with the guidelines laid down by the Institutional Ethics Committee and with local laws and regulations.

Bacterial strain and Culturing:

Leptospira cultures maintained at State Level Diagnostic laboratory, SVVU, Tirupati was used in the present study. Leptospira interrogans serovar canicola (Hond Utrecht IV strain) was maintained with EMJH (Difco, USA) and serum free medium used in ICMR-RMRC, Portblair,Andaman.

Amplification of partial CDS of BPR gene of *L.canicola*

Nucleotide sequences of beta-propeller repeat protein (Leptospira interrogans serovar Canicola str. LT1962) GenBank: EMF72505.1 was retrieved from NCBI genomic database. Part of the gene 345bp (196-340; 145 aa) was selected, primers were designed for selected region using NCBI primer blast without changing reading frame. Finally restriction sites (ECoRI and SacI) and extra bases were added to 5" end.

Table 1: Primers targeting the BPR gene used in the study

Sl. No.	Primer Used	Sequence
1	Forward	GCA**GAATTC** TAA CAC CGA TGG CAC CA
2	Reverse	GG**GAGCTC**TCG CGA TAC AGG TTG GTC

Genomic DNA was extracted from *al*. four ml of well grown leptospiral cultures and subjected to centrifugation at 13,000 rpm for 15 min. The pellet was washed twice and re-suspended in 500 ul of solution I. To the above suspension, 50 µl of lysozyme (5 mg/ml dissolved in solution I) was added and incubated at 37ºC for 15 min. Later, 50 µl of 10% SDS, 5 µl of proteinase K (10 mg/ml) were added and incubated at 65º C for 30 min (Appendix). After that 40 µl of 5M NaCl and 32 µl of CTAB NaCI were added and incubated at 65ºC for 30 min. After incubation, equal volumes of chloroform isoamyl alcohol (24:1) (approximately 677 ul) were added. Then the contents were vortexed and centrifuged at 13,000 rpm for 15 min. The supernatant (300 ul approximately) was collected and 180 ul of chilled ethanol was added. After gentle mixing, the contents were kept at -40^0 C for 6hrs. Finally centrifugation was done at 10,000 rpm for 30 min to pellet down the DNA. Contents were decanted and the pellet was air dried and reconstituted in 50 ul of TE buffer and stored at -20ºC for further use

In brief PCR was carried out in a gradient thermal cycler (Eppendorf) for 35 cycles. It involves , Initial denaturation at 95ºC for 3 min, Cyclic denaturation at 95ºC for 45 sec, Annealing at 57ºC for 1 min, Extension at 72ºC for l min, followed by final extension for 10 min at 72ºC and hold

at 4ºC. Each 25 ul of reaction mixture consists of 25 mM MgCl (1.5 µl), 10 mm dNTPs (0.5 µl), 10x Taq buffer (2.5 µl), 0.5 ul of Taq DNA polymerase, 20 p moles of each primer(1 µl each) and 2 µl of template DNA . The amplified products were analyzed in 1.5% agarose gel with 100 bp ladder and stained with ethidium bromide.

Cloning of BPR gene of *L.interrogans canicola*

The PCR product was eluted from the gel using Qiagen gel extraction kit as per manufacturer instructions. Later, it was cloned into with pGEMT-Easy vector. Then it was digested with EcoRI and SacI enzyme and ligated into pET32(a) vector. The product was transformed into calcium chloride treated DH5 (*E. coli*) competent cells and plated on lysogeny broth (LB) agar medium containing ampicillin (50 g/ml), IPTG (100 mM/ml) and X-gal (20 g/ml) solutions. The recombinant positive clones were screened by colony touch PCR, restriction enzyme digestion and sequencing methods.

Sequencing and Sequence analysis of Beta propeller gene

Sequencing of pGEMT recombinant plasmid carrying BPR gene of *L. canicola* was carried out with vector specific universal M13 forward and reverse primers and BPR gene specific internal forward and reverse primers. The reaction was carried out using one µg of plasmid DNA and 20 pm of gene specific primers. Sequence data was analyzed using BLAST N software (http:// www.ncbi.n/n.nih.gov/BLAST/cgi) with non-redundant database. The nucleotide sequences obtained were further subjected to multiple sequence alignment.

Phylogenetic analysis

Phylogenetic analysis of leptospira isolate was compared using deduced amino acid sequences. The sequence alignment was done by Clustal W method in the BioEdit software. Evolutionary analyses were conducted in MEGA 6.06 software. The evolutionary history was inferred using the maximum likelihood method based on the Tamura–Nei model.

Expression and purification of recombinant VSG

The orientation of the target gene sequence in recombinant plasmid was screened by using vector-specific and insert-specific primers and sequenced to confirm its orientation. Further, the plasmid DNA was transformed into *E. coli* BL21 (DE3) (Invitrogen) expression host system. Single colony of recombinant plasmid was grown in 10 ml LB broth with selective antibiotic (Ampicillin 100mg/ml) for overnight at 37 ºC with shaking. Two hundred fifty microliters of overnight culture was inoculated into 25 ml of LB medium containing Ampicillin and was allowed to grow in shaking incubator at 37 ◦C for 3 h. Then the protein expression was induced by adding IPTG (isopropyl beta thiogalacto pyranoside) to a final concentration of 1 mM at 30 ºC for 6 h (optimized for maximum expression level) and the cells were pelleted and resuspended in 1 ml of TE-PMSF and stored at −80 ºC till further use. The histidine tagged recombinant BPR was purified using NiNTA agarose column (Qiagen, USA) as specified by the manufacturer. The purified protein was dialyzed against PBS, pH7.2 and its concentration was determined and stored at −20 ºC till further.

Detection of recombinant BPR by SDS-PAGE and Immunoblot

Bacterial cell lysates (100 g/well) and purified recombinant protein (50 g/well) were added with electrophoresis sample buffer (50 mM Tris pH 6.8, 10% glycerol, 5% mercaptoethanol, 2% SDS and 0.1% bromophenol blue) and boiled for 5 min prior to loading into the 12% polyacrylamide gel. The gel was stained with PAGE blue staining solution (Fermentas, USA) overnight with shaking. Immunoblot analysis was also performed to test the specificity of the purified protein and its reactivity with the antisera raised in rabbit against the inactivated whole cell lysate antigen of *L. canicola*. To develop the immuno blot, rabbit sera were diluted to 1:100 and tested. The goat anti rabbit antibody conjugated with horse radish peroxidase (Sigma, USA) was used as permanufacturer's instruction and diaminobenzidene tetrahydrochloride (Sigma) was used as a chromogen.

RESULTS AND DISCUSSION

The central mechanism in pathogenesis of leptospirosis is the ability of the pathogens to disseminate widely within the host during the early stage of infection. Number of virulence factors has been identified at cellular and molecular level for the mechanism of pathogenesis. Genus-specific leptospiral proteins that are conserved throughout different serovars of Leptospira which are immunogenic and uniquely expressed during acute infection may help in the development of an effective vaccine for leptospirosis as well as aid in studies on its pathogenesis. Development of vaccines against leptospirosis is mainly targeted on the extracellular outer membrane proteins of leptospires. The beta propeller repeat was identified as putative extracellular protein using three bio-informatics sub cellular localization tools and grouped in the SBBP domain (seven beta blade propeller proteins, Pfam PF06739) with the locus tag of LIC12099 including nine hypothetical proteins Hence the present study was conducted to amplify clone and express the partial portion of beta propeller repeat protein of *L. canicola.*

Polymerase chain reaction

Bacterial outer membrane proteins (OMPs) are the major target of the immune system in a variety of infectious diseases and they have been suggested as candidates for diagnosis and immunization (Lin *et al*., 2016). In case of leptospira, three classes of these OMPs have been identified viz., lipoproteins, transmembrane proteins and peripheral membrane proteins. The current research for developing recombinant and subunit vaccines are mostly focused on leptospiral motility, outer-membrane proteins (OMPs), lipoproteins, lipopolysaccharides (LPSs) and virulence factors (Grassmann *et al*., 2017; Dellagostin *et al*., 2017). In this study, the genomic DNA isolated from the 5-6 days old culture of *L. canicola* was subjected to Polymerase Chain Reaction. A 35 cycle PCR at annealing temperature of 57ºC with 1.75mM concentration of $MgCl_2$ was found to be optimum for the amplification of 435 bp product of beta propeller gene (BPR). The size of the amplified product was analyzed by agarose gel electrophoresis using standard 1kb DNA molecular size markers. The DNA amplified was 435 bp which is the size of the amplicon defined by the selected primers to amplify between 195 to 340 aa region of beta propeller gene. No amplification was observed in negative control indicating that newly designed primers were specific for BPR gene of *L. canicola* (Fig. 1).

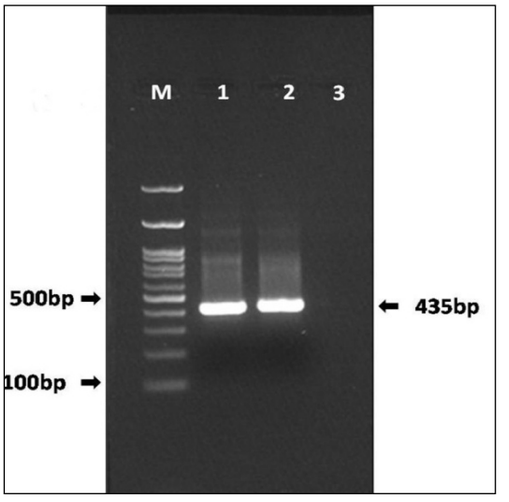

Fig. 1: PCR ampilification of partial fragment of Beta propeller gene (BPR) of *L. canicola*

Lane M: 1 Kb DNA ladder
Lane 1&2: 435 bp amplicon
Lane 3: PCR control

Sequence analysis

In the present study genomic approach was done to identify the conserved potential vaccine candidate using PCR and sequence analysis. Our findings confirmed the gene specific amplification of 435bp product. NCBI BLAST analysis of 435 nucleotides showed that they are flanking the region between 422164 to 422598 positions of *L. interrogans* sps chromosome no. 1. The partially sequenced gene was having 95 percent identities with the published sequences and confirmed it's homology with other leptopira serovars (Fig. 2). The multiple alignments of amino acid sequences of selected region showed highly conserved residues among the nine serovars compared. The recombinant BPR clone has an open reading frame for a protein of 145 amino acids. The results of the study were in accordance with the earlier studies using the genome approach (Eshghi *et al*., 2015) for identifying the lipoproteins, cell surface proteins and exoproteins of leptospira.

Fig 2: Multiple sequence alignment of BPR gene with published Sequences

Expression, purification and characterization of recombinant BPR

The fragment of BPR gene was inserted into the pET 32(a) vector at EcoRI and SacI site and confirmed by EcoRI and SacI digestion (Fig. 3).

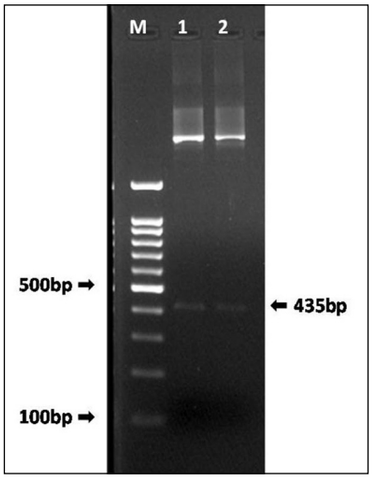

Fig. 3: Showing the release of 435bp product from pGEMTvector by restriction enzyme digestion with EcoRI and SacI

Lane M: 1 Kb DNA ladder

Lane 1&2: RE digested clones

The sequence was in frame with the ATG codon of pET 32(a) vector thereby resulting in the production of recombinant protein carrying N-terminus fusion including the histidine (His) tag, thioredoxin protein at Nterminal and some amino acids (aa) at C-terminal end of the vector. The calculated molecular weight of the expressed recombinant VSG fused protein was 36 kDa approximately. Cloning and expression of outer membrane and lipoproteins was reported in previous studies which includes, a 31 kDa surface protein OmpL1 OmpL1, lipoprotein LipL41, hemolysis-associated protein 1 (Hap1) and immunoglobulin-like (Lig) protein).

The gene cloned and expressed in the present study encodes the N-terminal part of the Beta Propeller repeat protein *L. canicola*. The recombinant BPR was expressed in *E. coli* BL21 (DE3) expression host system as a fusion protein with histidine residues at N-terminal end. The yield of the expressed purified protein ranged 96 mg of /liter of bacterial culture. SDS-PAGE analysis of the supernatant from the induced cell lysate and purified protein indicates that the recombinant protein (36kDa) was present in the lysate and purified sample (Fig. 4).

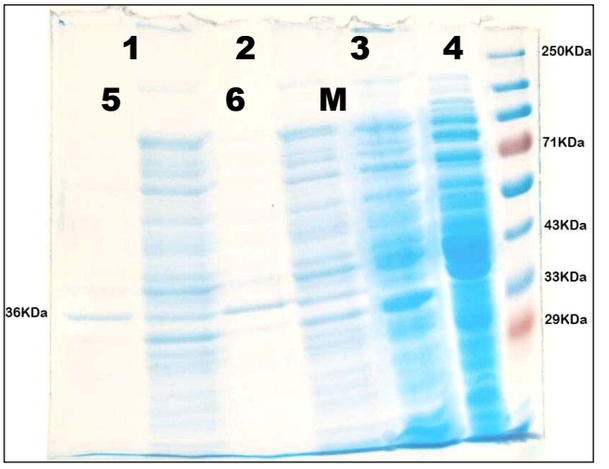

Fig. 4: SDS –PAGE analysis of recombinant BPR protein

Lane M: Prestained protein ladder

Lane 1,3: showing expression of 36KDa protein

Lane 2,4&,5 Negative for expression

Lane 6: Uninduced cell control

However such a protein was not observed in the bacterial cell lysate or purified sample of pET 32a vector without

insert or uninduced clone with insert. Immunoblot analysis (Fig. 5) has shown that the expressed purified protein reacted with rabbit hyper immune sera raised against the inactivated whole cell lysate of *L. canicola*. However the expressed protein was not recognized by healthy rabbit serum.

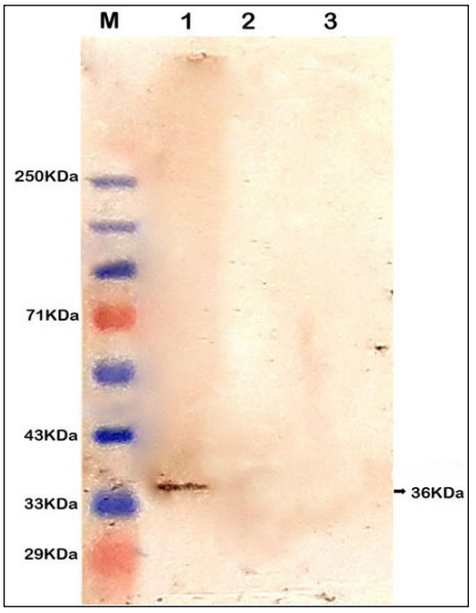

Fig. 5: Immuno Blot analysis of recombinant BPR protein Leptospira interrogans serovar canicola

Our findings were corroborate with the earlier studies on cloning and expression of recombinant LIC12209 (encoding a Leptospira beta-propeller protein of 52 kDa (LP52) (Pretre *et al.,* 2013; Eshghi *et al*., 2015), outer membrane OmpL1 and LipL41 (Parthiban *et al.,* 2015) and Cloning and sequence analysis of a partial CDS of leptospiral ligA gene of *L. canicola* in pET-32a – *Escherichia coli* system (Soman *et al*., 2018). The amplified DNA sequence was cloned on to pET-32a expression vector using host *E.coli* DH5α. The pET vectors are considered to be one of the most powerful vector systems developed for cloning and expression of bacterial genes in E. coli. In this study, the pET-32a BPR construct was transformed into E. coli BL21-(DE3), an expression host. *E. coli* is the most preferred host for recombinant DNA technology because its genome is well studied, relatively cheap and has short generation time.

Phylogenetic analysis

The phylogenetic analysis revealed that the BPR gene of the tested strain of Leptospira species occupied the same position in the phylogenetic tree (Fig. 6) as other reference leptospiral strains of the *L. canicola* serovars. The findings confirmed that the BPR gene was conserved in the genus Leptospira and suggested that the Beta Propeller Repeat protein could be a potential vaccine candidate for subunit vaccines.

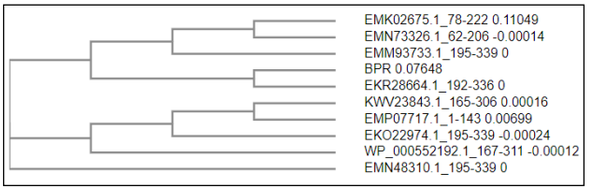

Fig. 6: Phylogenetic tree of Leptospira interrogans serovar Canicola strain Hond Utrecht IV BPR gene, partial CDS sequence

CONCLUSION

It may be concluded that the present study confirmed that the BPR gene was conserved among the genus Leptospira by molecular and immnuno blot studies and suggested that the Beta Propeller Repeat protein could be a potential vaccine candidate for subunit vaccines. However, the immunogenicity trials in lab animals and natural host could be conducted to validate the BPR gene as promising vaccine candidate for preparation of recombinant vaccines against canine leptospirosis

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