

Expression and purification of the LipI41, a surface-exposed lipoprotein antigen of pathogenic *Leptospira* spp.

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ABSTRACT

Pathogenic species of *Leptospira* lead to a zoonotic disease called leptospirosis, which is spread worldwide. A major topic of investigation is to detect the antigens that induce an immune response and to utilize them in diagnostic kits or vaccine development. The outer membrane proteins (OMPs) of *Leptospira* are potential candidates for this purpose. LipI41 is an OMP that is conserved among pathogenic *Leptospira*. The aim of this study was to express and purify the LipI41 recombinant protein in Iranian isolates. All collected LipI41 protein sequences were compared and analyzed using bioinformatics tools from NCBI databases. Complete codon sequences of the Iranian pattern of LipI41 recombinant protein were codon optimized and sub-cloned into a pET32a+ expression vector, and transformed into *Escherichia coli* BL21 (DE3). Optimal expression of recombinant LipI41 (47kDa) was achieved post-induction with IPTG within the inclusion body. It was then purified by denaturation using serial concentrations of urea, and the recombinant protein was confirmed by western blot. In this study, sufficient amounts of LipI41 were expressed and purified to be used for the development of a diagnostic kit and subunit vaccine.

Key words: recombinant protein LipI41; expression; purification; pathogenic *Leptospira*

Introduction

Leptospirosis is a global zoonotic disease caused by pathogenic *Leptospira*. It is regularly prevalent in tropical, subtropical and humid parts with high levels of rainfall (ADLER and MOCTEZUMA, 2010), mostly in poor urban areas in developing

countries (LOMAR et al., 2000). The clinical syndromes in humans are difficult to differentiate. The manifestations can range from a moderate fever similar to flu, to more serious illnesses presenting with hemorrhage, jaundice, myalgia,

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renal dysfunction, and aseptic meningitis, which can lead to death in some patients (SEIJO et al., 2002; KHAKI, 2016a). After infection, *Leptospira* can colonize the renal tubules of a number of wild and domestic animals for prolonged periods, and are discharged through urine into the environment (BURRIEL et al., 2003; MONAHAN et al., 2008). Humans are usually contaminated directly or indirectly by infected animal urine. (MCBRIDE et al., 2005; INANLOU et al., 2015). Due to the widespread animal reservoirs, eradication of leptospirosis is problematic (ZAKERI et al., 2010). Despite vaccination, the disease still survives (KOIZUMI and WATANABE, 2005). Although vaccine development is the main strategy to remove the illness, current drawbacks of whole-cell vaccines include short duration of immunity, serovar specificity and side effects (VEDHAGIRI et al., 2009; LIN et al., 2016). Thus, the production of an efficient vaccine with lower levels of toxicity is essential to control and eliminate this disease (DELLAGOSTIN et al., 2011). Many studies on the development of recombinant vaccines against leptospirosis have used conserved outer membrane proteins (*OMPs*) such as LipI21, LipI32, LipI41, Omp11, etc (VEDHAGIRI et al., 2009; MEGUDESWARAN et al., 2014; LIN et al., 2016; CHANDY et al., 2017). *OMPs* revealed on the surface are among the most impressive antigens that can affect immune responses throughout the course of the disease. (FLANNERY et al., 2001; OLIVEIRA et al., 2015). There are three classes of leptospiral OM proteins (*OMPs*) which consist of lipoproteins, trans membrane proteins and peripheral membrane proteins (SHANG et al., 1996; ADLER et al., 2011). Regarding abundance, LipI41 (41kDa) is the third among OM lipoproteins (CULLEN et al., 2005). This protein is an immunogenic antigen and expressed only in the pathogenic *Leptospira*, but notably absent in nonpathogenic species (KHAKI et al., 2016b), and has been considered as an indicator in serodiagnosis of the disease (HAAKE et al., 1999; MARIYA et al., 2006). Results of earlier studies have also shown that LipI41 is surface exposed and expressed during infection in mammalian hosts (SENTHILKUMAR et al., 2007; KING et al., 2013). Therefore, it may be considered

a potential subunit vaccine candidate against leptospirosis and, moreover, could be useful in a diagnostic kit. (SRIVASTAVA, 2006; SRIKRAM et al., 2011). Hence, in this present study, our aim was to express and purify Iranian isolates of the LipI41 r protein of pathogenic *Leptospira* to develop a recombinant antigen.

Materials and methods

Bioinformatics studies and generation of LipI41 construct. Twenty-seven complete coding sequences of LipI41 protein 1065bp (355aa) from six prevalent pathogenic *Leptospira* serovars, consisting of eleven from Iran, such as: serovar Canicola, serovar Autumnalis, serovar Hardjo, serovar Icterohaemorrhagiae, serovar Pomona, serovar Grippotyphosa, and sixteen lipI41 sequences from other countries available in NCBI database were collected from GenBank (<https://www.ncbi.nlm.nih.gov/GenBank>) by using the BLAST online software (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) (Table 1). All collected protein sequences were compared and analyzed using bioinformatics tools such as MegAlign software.

A partial sequence of LipI41, including coding sequences of amino acids number 21 to 355 (1005 bp) minus signal peptide (first 20aa), was synthesized by General Biosystems, Inc, USA. In addition, pET-32a+ was used to produce a 47kDa TRX-LipI41 fusion protein. EcoR I- Sal I restriction sites were used for sub cloning into the pET-32a+ vector and the His-Tag sequence was considered for affinity purification. Codon optimization of LipI41 was carried out for better expression in *Escherichia coli* with pET32a+ expression vector.

Expression. The recombinant plasmid of pET32a-LipI41 was transformed into *E. coli* BL21 (DE3) as the host strain, on LB Agar containing 50 µg/mL ampicillin by the heat shock method (SAMBROOK and RUSSEL, 2001). Then a matrix plate was sub-cultured, and one single colony of it was cultured in 5 mL LB broth medium, supplemented with 5 µg/mL Ampicillin at 37 °C overnight. One mL of this suspension was inoculated onto 50 mL 2YT/AMP broth medium for induction. When the optical density reached 0.6 A₆₀₀, the recombinant plasmid was induced with Isopropyl- β-D-Thio Galacto

Table 1. List of LipL41 sequences of *Leptospira interrogans* used in this study

Protein Id	Serovar	Strain	Country
AAT48499.1	<i>Australis</i>	65-9	China
BAE48274.1	<i>Australis</i>	Akiyami C	Japan
AAV88037.1	<i>Australis</i>	65-9	China
AHW80383.1	<i>Autumnalis</i>	RTCC2802	Iran
AAT48498.1	<i>Autumnalis</i>	Lin 4	China
BAE48272.1	<i>Autumnalis</i>	Akiyami A	Japan
AHW80387.1	<i>Canicola</i>	RTCC2824	Iran
AAT48495.1	<i>Canicola</i>	Lin	China
AHX26958.1	<i>Canicola</i>	RTCC2805	Iran
AAT65971.1	<i>Canicola</i>	-----	India
AHW80384.1	<i>Hardjo</i>	RTCC2810	Iran
AAT65970.1	<i>Hardjo</i>	-----	India
AHX26962.1	<i>Hardjo</i>	RTCC 2821	Iran
AHW80386.1	<i>Icterohaemorrhagiae</i>	RTCC2823	Iran
AHX26960.1	<i>Icterohaemorrhagiae</i>	RTCC2812	Iran
ACV53831.1	<i>Icterohaemorrhagiae</i>	-----	India
AAS21814.1	<i>Kremastos</i>	<i>Kremastos</i>	USA
AHW80385.1	<i>Pomona</i>	RTCC2822	Iran
AHX26961.1	<i>Pomona</i>	RTCC 2815	Iran
AAU04601.1	<i>Pomona</i>	Luo	China
AAV88038.1	<i>Pomona</i>	Luo	China
AAS21818.1	<i>Pomona</i>	PomonaRZ11	USA
AHA11954.1	<i>Pomona</i>	Pomona P	Argentina
AHW80388.1	<i>Grippotyphosa</i>	RTCC2825	Iran
AFH54361.1	<i>Grippotyphosa</i>	Moskva V	India
AAT48501.1	<i>Grippotyphosa</i>	Lin 6	China
AHX26959.1	<i>Grippotyphosa</i>	RTCC 2808	Iran

Pyranoside (IPTG). Sampling was performed before and after induction at different times (0 - 4 h and also 16 h). Different incubation temperatures (22 and 37 °C) and various concentrations (0.1 to 0.5mM) of IPTG were analyzed. The induced cells were harvested by centrifuge at 5000 g at 4 °C for 5 min. The pellets were suspended in 1XPBS (137 mMNaCl, 2 mM KH_2PO_4 , 10 mM Na_2HPO_4 , 2.7 mM KCl , pH 7.4) with sample buffer added (5× solution of 250 mMTris.HCl, pH 6.8, 10% SDS, 30% (v/v) Glycerol, 10 mM DTT, 0.05% (w/v) bromophenol blue, 2-Mercaptoethanol), boiled for 5 min, then analyzed by SDS-PAGE using a 10%

polyacrylamide gel along with Unstained protein markers (Thermo Scientific, USA). The gel was stained for 2 hr by standard Coomassie brilliant blue R-250 and destained with 45% methanol, 10% acetic acid glacial solution. Induced cells were resuspended in PBS with 10mM imidazole, and sonicated five times for 1 minute on ice with one minute intervals. After that, Phenyl Methyl Sulfonyl Fluoride (PMSF) was added to inhibit protease activity. The cell lysate was centrifuged at 15,000 g at 4 °C for 15 min. The supernatant and pellet analyzed by SDS-PAGE.

Protein purification and immunoblotting. The rLip41 protein was purified from the post sonication pellets under denaturation conditions using serial concentrations of 1 to 8M urea in PBS buffer at 37 °C. The post sonication pellets were suspended in 5 mL 1M urea and shaken for 1h in 37 °C. Washing with 2 to 5 M urea was done for 1 hour, and the 6 to 8 M concentrations of urea were incubated overnight. Purified proteins were dialyzed in 1X PBS and assessed by 10% SDS-PAGE, then stored at -70 °C for further analysis.

Western-blotting was performed. The blocking step was performed by 1% BSA in PBS containing 0.1% Tween 20, then probed with a HRP conjugated anti-6x His-Tag antibodies (Abcam, USA) (diluted 1:5,000). 4-choloro-1-naphthol was used for developing the protein band.

Protein assay. Quantitation of recombinant protein was performed by Bradford protein assay. To achieve a standard curve, various concentrations of 0.25 mg/mL BSA (Bovine Serum Albumin) were utilized. Ten dilutions of BSA were prepared in range of 0 to 10 µg/µL protein as a standard lane in deionized water. 200 µL Bradford reagent was added to all dilutions containing BSA and recombinant protein up to 1 mL final volume.

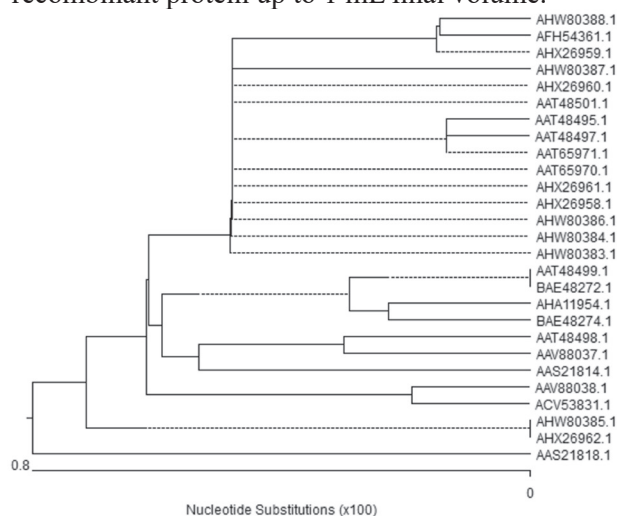


Fig. 1. Phylogenetic analysis of six *leptospira interrogans* serovars, including eleven from Iran and sixteen from other countries submitted to GenBank (protein Id). The phylogenetic tree based on LipL41 protein sequences used Megalign software.

Results

Phylogenetic analysis of full length LipL41 proteins (355aa) showed 0.8 percent divergence between six serovars of *leptospira interrogans* in Iran and the other countries (as shown in Fig. 1). Multiple Alignment of LipL41 protein sequences using MegAlign software identified significant amino acid substitutions had taken place among the serovars. Notably, 20 different amino acid substitutions were detected, 8 of which were observed among some Iranian isolates, as stipulated below: in Iranian *Leptospira* serovar Grippothyphosa (AHW80388.1) and (AHX26959.1) isolates, 3 amino acid substitutions were found in residues 146, 212 and 312 (I146→ V146, F212→ Y212 and T312→A312). Furthermore, in serovar Pomona (AHW80385.1) and serovar Hardjo (AHX26962.1) one amino acid substitution was observed in residue 204 (V204→ I204). On the basis of these polymorphisms, the local LipL41 protein pattern was used to design the construct.

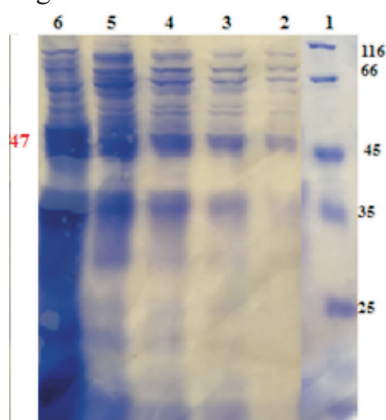


Fig. 2 . SDS-PAGE (12%) analysis of the expressed rLipL41 protein in BL21 (DE3) which was induced by 0.1mM IPTG at different times. Lane 1: Unstained protein molecular weight marker (kDa), (Thermo, scientific). Lane2: non-induced cell lysate. Lane 3: induced (1h) cell lysate. Lane 4: induced (3 h) cell lysate. Lane 5: induced (4 h) cell lysate. Lane 6: induced (over-night) cell lysate.

Recombinant protein was induced at different times, temperatures and various concentrations of IPTG. The protein electrophoresis by SDS-PAGE analysis revealed a distinct band that corresponded to successful induction of full-length 47 kDa LipL41 protein. However, maximum expression of the LipL41 recombinant protein was observed at 4 h

post induction incubation at 37 °C in the presence of 0.1 mM IPTG (Fig. 2).

Analysis of the cell lysate post-sonication demonstrated that the recombinant protein was mostly found in the pellet, not in the supernatant.

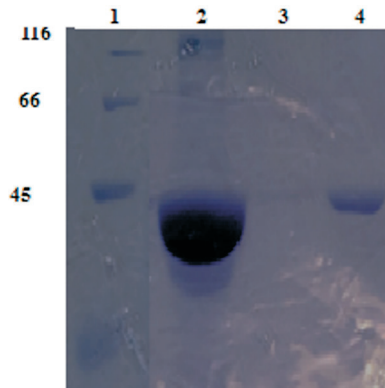


Fig. 3. Analysis of the purified LipI41 recombinant protein (47kDa) with SDS PAGE followed by Coomassie blue staining. Lane 1: unstained protein molecular weight marker (kDa), (Thermo, scientific).

Lane 2: purified LipI41 protein with 6M urea concentration. Lane 3: purified LipI41 protein with 8M urea concentration. Lane 4: purified LipI41 protein with 7M urea concentration.



Fig. 4. Western blot analysis of the purified LipI41 recombinant protein using HRP-conjugated Anti His-Tag antibody. Lane 1: LipI41 Purified recombinant protein (47kDa) in SDS PAGE. Lane 2: Prestained Protein Molecular Weight ladder (Thermo, scientific).

The protein was purified after washing in serial concentrations of urea in PBS (1 to 5M) and then solubilized in (6 to 8M) at 37 °C. The results of protein purification in all three concentrations of urea (6 to 8M) showed a sharp single band (at 47 KDa). Moreover, the protein obtained from purification with an urea concentration of 6M had the maximum protein concentration (Fig. 3). The yield of purified recombinant protein was estimated to be 28.3 mg/mL.

Western blot confirmed the distinct 47 kDa band (Fig. 4).

Discussion

In recent years many studies have been conducted on the identification, expression, and characterization of the leptospiral *OMPs*, such as OmpL1, Omp52, LipL21, LipL36, LipL41, etc (HAAKE and MATSUNAGA, 2002; SENTHILKUMAR et al., 2007; DEZHBORD et al., 2014; HOSEINPUR et al., 2015; SOLTANI et al., 2015). Some other studies revealed that, unlike leptospiral LPS, *OMPs* are generally well conserved, not serovar specific and able to induce cross-protective immunity (KOIZUMI and WATANABE, 2005; OLIVEIRA et al., 2015). In addition, leptospiral *OMPs* are commonly exposed on the surface and are among the first antigens that interact with host immune cells (CULLEN et al., 2002; NALLY et al., 2007).

One of the most important *OMPs* expressed during human infection is LipI41, a 41kDa lipoprotein that is highly conserved in pathogenic *Leptospira* species. (MEGUDESWARAN et al., 2014) Furthermore, LipI41 has been determined as a hemin-binding protein (LIN et al., 2013), and is one of the key virulence factors involved in host-pathogen contact. Thus, it appears that LipI41 may play an important role in inducing immunological responses in the host (GUERREIRO et al., 2001).

According to previous reports, the use of LipI41 of pathogenic *Leptospira* in a multi-serovar ELISA enhanced the sensitivity of the test (THEODORIDIS et al., 2005; NATARAJASEENIVASAN et al., 2008). Another investigation conducted by MARIYA et al in 2006 demonstrated that LipI41 antigen in ELISA

is a possible candidate for serodiagnosis of bovine leptospiral infection. Moreover, earlier studies indicated that this protein is expressed throughout acute infection (KING et al., 2013), and may be susceptible to the host immune defenses, either by itself or in combination with OmpL1 (HAAKE et al., 1999; LIN et al., 2011).

Consequently, LipL41 could be considered a genus-specific antigen for improvement of new broad spectrum vaccines and serodiagnostic techniques against leptospirosis.

Therefore, our study focused on the expression and purification of LipL41 protein of Iranian pathogenic *Leptospira* in *E. coli*. We used BLAST software to gather all the protein sequences of LipL41 of pathogenic *Leptospira* from different serovars from Iran and other countries available in the NCBI database. A significant finding in our study was the detection of 8 amino acid substitutions in some Iranian isolates, which, to our knowledge, have not been reported in the literature. Gene construct was then designed according the pattern of local LipL41 protein prevalent pathogenic *Leptospira* isolates.

Induction of the recombinant protein using various concentrations of IPTG was performed at different times and temperatures, which demonstrated the maximum expression of LipL41 recombinant protein in BL21 (DE3) at 37 °C and 0.1mM IPTG at 4 hours post induction. A high level of expression of LipL41 was observed (28.38 mg/mL). Hence, under these conditions, optimal recombinant protein purification was achieved as one distinct band, with an approximate molecular weight of 47kDa. On the basis of our results, the blotted protein (using HRP-conjugated Anti anti-6x His-Tag Ab) verified the presence of the recombinant LipL41 protein as illustrated in Fig. 4.

Some researchers have obtained high expression rates of this protein using either pET series (MEGUDESWARAN et al., 2014; LIN et al., 2016) or other similar expression vectors (MARIYA et al., 2006; KING et al., 2013; LIN et al., 2013). Solubility is an important factor for producing recombinant proteins in heterologous expression systems. Due to differences in folding structure, soluble recombinant proteins are usually considerably easier to purify

compared with insoluble ones (COSTA et al., 2014). Despite the use of the TRX tag sequence during gene construction to enhance solubility, a significant amount of the recombinant protein was found in the inclusion body, while lesser quantities were detected in the supernatant, and expressed in the insoluble form. These results agreed with other studies where the over expressed LipL41 was observed mostly in the inclusion body, and purified under denaturing conditions (MARIYA et al., 2006; KING et al., 2013; LIN et al., 2013). Although in one report this protein was solubilized with 6M guanidine, (HAAKE et al., 1999), in this study we used 1-8 M urea according to the manual protocol (to be under published). This provided an efficient and cost-effective method which is applicable for purification of LipL41 insoluble proteins.

Conclusion

In conclusion, this study demonstrated 0.8 percent divergence between LipL41 protein sequences of Iranian and foreign *Leptospira interrogans* serovars. 8 amino acid substitutions were identified among local serovars which were used to design the gene construct. It was successfully expressed in *E. coli* BL 21(DE3) and purified by denaturation, using serial concentrations of urea.

The presence of purified protein was confirmed with SDS-PAGE analysis and western blotting.

Therefore, the possible immunogenicity of this recombinant protein was demonstrated, and it may have the potential to induce effective protective immunity against leptospirosis in the future. However, the serodiagnostic ability of this recombinant protein as a capture antigen in the ELISA method in animal and human subjects requires further investigation.

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SAŽETAK

Patogene vrste *Leptospira* uzrokuju zoonotsku bolest leptospirozu koja je raširena u cijelom svijetu. Cilj istraživanja bio je dokazati antigene koji potiču imunosni odgovor i mogu se primijeniti u dijagnostičkim kompletima ili za razvoj cjepiva. Vanjska proteinska membrana (OMPs) leptospira potencijalni je kandidat za tu svrhu, a gen Lip141 je dobro očuvan među patogenim leptospirama. Ekspresija i pročišćavanje rekombinantnog proteina Lip141 provedeno je u izolatima iz Irana. Uspoređene su sve prikupljene sekvencije proteina Lip141 i analizirane bioinformatičkim alatom iz baze podataka NCBI. Kompletne sekvencije kodona rekombinantnog proteina Lip141 u izolatima su optimizirane, zatim subklonirane u pET32a+ vektor i pretvorene u bakteriju *Escherichia coli* BL21 (DE3). Optimalna ekspresija rekombinantnog Lip141 (47kDa) postignuta je post-indukcijski pomoću isopropyl β -d-1-thiogalactopyranoside (IPTG). Zatim je pročišćena denaturacijom što je uključilo primjenu serijskih koncentracija ureje. Rekombinantni protein je potvrđen metodom western blot. Istraživanje potvrđuje mogućnost ostvarenja dostatne količine i ekspresije pročišćenog Lip141 da se može upotrijebiti za razvoj dijagnostičkih kompleta i subjediničnih cjepiva.

Ključne riječi: rekombinirani protein Lip141; ekspresija; pročišćavanje; patogena *Leptospira*
