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Original article

HIGH EXPRESSION OF LIPL21 PROTEIN OF IRANIAN *LEPTOSPIRA INTERROGANS* IN *E. COLI*, APPLICABLE FOR DIAGNOSTIC ELISA

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Summary

Kamalzadeh, K., M. Esmaelizad, J. Ghalani, P. Khaki & M. Tebianian, 2023. High expression of LipL21 protein of Iranian *Leptospira interrogans* in *E. coli*, applicable for diagnostic ELISA. *Bulg. J. Vet. Med.,* **26**, No 3, 342−350.

Leptospirosis is an emerging infectious zoonotic disease caused by pathogenic *Leptospira*. The disease is more prevalent among farmers in hot and humid areas of Iran. Lack of clear clinical signs have impeded the diagnosis of leptospirosis. In this study, we attempted to produce a recombinant LipL21 protein of *Leptospira* based on a dominant pattern of Iranian isolates and to evaluate it in ELISA test. One hundred and sixty-two complete sequences of LipL21 available in GenBank until January 1, 2019 were compared. One dominant LipL21 protein pattern was selected. The codon optimised sequence was cloned into the pET32a+ expression vector. Trx-LipL21 fusion protein was induced, purified and confirmed by 10% SDS-PAGE followed Coomassie blue staining and immune blotting. For evaluation of effectiveness of rLip21 in ELISA test, 200 µg rLip21 with Montanide ISA70 adjuvant was injected subcutaneously in rabbits three times. Results showed that rLipL21 protein was highly expressed in 2YT media in presence of 0.1 mM IPTG after 16 hours incubation at 37 ^{o}C. Recombinant protein was purified 36 mg per liter using affinity batch formation method by Ni-NTA resin. ELISA with micro plate coated with 250 ng rLipL21 protein demonstrated prominently differences between test and control groups ($P<0.01$). The rLipL21 protein produced large amounts of antibodies in the rabbit. The protein was also able to detect high levels of antibody in animals immunised with *Leptospira* vaccine. The rLipL21 might be a good candidate for diagnosis and evaluation of antibody levels against *Leptospira*.

Key words: ELISA, *Leptospira*, LipL21, recombinant protein

INTRODUCTION

Leptospira belongs to spirochetes bacteria and causes leptospirosis, a global outbreak zoonotic disease. It is one of the most important public health anxieties (Bharti *et al.,* 2003). The infection in humans and animals occurs through direct or

indirect exposure with contaminated soil, water or urine (Barcellos & Sabroze, 2001; Sejvar *et al.,* 2003). The spread of this disease is high in tropical and subtropical areas. The prevalence of this disease in Iran has been reported in wide geographical area, such as Gilan (Abdollahpour, 2009), Mazandaran (Khaki *et al.,* 2013), Sari (Babamahmodi *et al.,* 2006), Lorestan (Maleki *et al.,* 2019), Zanjan (Soltani Majd *et al.,* 2012), Shiraz (Firouzi & Vandyousefi, 2000). Due to climatic conditions in north of Iran, spread of the disease in these areas is more significant *(*Asuthkar *et al.,* 2007). Leptospirosis is accepted as an occupational disease, and is more prevalent in occupations dealing with animals and surface water (Waitkins, 1986; Honarmand *et al.,* 2009), for example farmers made up about 86.1% of patients in Sari (Babamahmodi *et al.,* 2004) or 30% of patients were working on rice fields in Lorestan province (Abdollahpour *et al.,* 2017). Abattoir staff are 34.7% of sick people in Zanjan (Soltani Majd *et al.,* 2012).

Leptospirosis have different variants of clinical manifestations, from mild to acute (McBride *et al.*, 2005), like meningitis (de Souza *et al.*, 2006), nephritis (Schreiber *et al.*, 2005), hepatitis (Adamus *et al.*, 1997) and pancreatitis (Spichler *et al.*, 2007), it can eventually lead to death (Christova *et al.*, 2003). The fatality rate was up to 25% (Coudert *et al.*, 2007). An international survey has shown an annual estimation of 100,000 severe cases (WHO, 1999). Therefore, differential diagnosis is important for specific treatment because of the similar clinical signs of leptospirosis to other diseases.

The microscopic agglutination test (MAT) is a standard diagnostic method for leptospirosis. Despite of high sensitivity of this method, it is not only a biohazard to staff because of using fresh *Leptospira* but also time-consuming (Faine, 1982; Levett, 2001) so, the preparation of a rapid and convenient serological diagnostic method that is safe, specific and sensitive is important for detection of leptospirosis.

Leptospira-specific IgM or IgG in patient sera could be detected by enzyme-linked immunosorbent assay (ELISA). It can detect anti-*Leptospira* IgM one week after infection. Anti-*Leptospira* IgG is usually detectable two weeks after infection and persists for several months (Alder *et al.*, 2010).

Although 300 serotypes, 24 serogroups and 25 species of *Leptospira* have been described (Picardeau, 2017), the most common serovars in Iran include Autumnalis, Pomona, Canicola, Grippotyphose, Icterohemorrhagiae, Sejro hardjo, Sejrosejro (Khaki *et al.*, 2014). The most common serovar in human samples was related to Sejrosejro, and in animal samples: to Sejro hardjo (Khaki *et al.*,2014). *Leptospira* is divided into 3 main pathotypes: pathogenic (*L. alstoni, L. weilli, L. santarosai*), intermediate (*L. broomi, L. wolffi*), non-pathogenic (*L. biflexa, L. meyeri, L. wolbachii*) (Djadid & Ganji, 2009).

Many virulence factors are encompassed in the pathogenesis and infection of *Leptospira* bacteria, such as haemolysin, membrane proteins, LPS and other superficial proteins and outer membrane proteins (Wang *et al.*, 2007). The outer membrane proteins of the bacterium are considered as antigenic or binding targets for antibacterial antibodies (Nally *et al.*, 2005; Reyes *et al.*, 2005). Outer membrane proteins (OM) are divided to lipoproteins (lip), transmembrane Omp (tm), peripheral membrane Omp (Nally *et al.*, 2005). The bioinformatics workflow and

total numbers of proteins identified by each bioinformatics algorithm (Grassmann *et al.*, 2017).

Leptospirae have a variety of surface lipoproteins, withhigh relative abundance on the cell surface in the following order: LipL32, LipL21, LipL41 (Cullen *et al.*, 2005). LipL21 is an OM lipoprotein, surface-exposed and expressed during infection. This protein is conserved among *Leptospira* species and temperature change has no variation in its expression. Pathogenic *Leptospira* loses virulence after numerous passages on laboratory media due to quantitative and qualitative changes in their outer membrane protein profile. However, the expression level of LipL21 did not change between different strains *of Leptospira* (Cullen *et al.*, 2002).

Today, utilising recombinant leptospiral OMP, such as rLipL21, rLipL41, or rLipl32 as the antigen, ELISA were developed (Theodoridis *et al.*, 2005; Dey *et al.*, 2008). Localisation of diagnostic methods is necessary due to differences in the pattern of antigens.

The aim of this study was to produce a recombinant LipL21 protein based on protein pattern of native Iranian *Leptospira* serovars and its evaluation in ELISA as a diagnostic method.

MATERIALS AND METHODS

Bioinformatic analysis

Total of 162 LipL21 sequences of *Leptospira* serovars available in GenBank at the National Center for Biotechnology Information (NCBI) website (ncbi.nlm.nih.gov) were collected until 1 January 2019.

Multiple alignments of nucleotide and protein sequences were done by MegAlign software and diversity between different sequences was determined. Some sequences of 162 non-redundant and polymorphic sequences including 15 Iranian isolates were selected and divided in two pathogenic and non-pathogenic groups. Based on multiple sequence alignment of LipL21 protein from different Iranian serovars, one dominant pattern (with KM507188.1 GenBank Number which belongs to Hardjo) with maximum coverage was considered for DNA construction (Hoseinpur *et al.*, 2014).

Chemical synthesis has been used as a gene source because codon optimisation can be done with high efficiency. Therefore, codon optimisation of rLipL21 was done based on codon table usage of *E. coli*. In addition, *EcoRI* and *XhoI* recognition sites were considered into the 5′ and 3′ ends of DNA for cloning into the pET32a+ expression vector (Fig. 1).

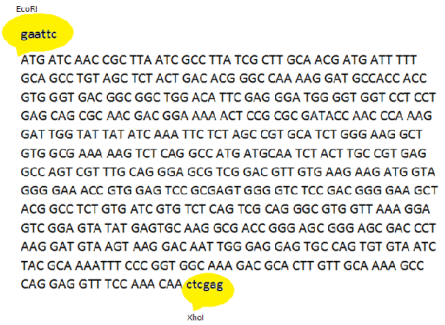

Fig. 1. Nucleotide coding sequences of LipL21 protein which was codon optimised for expression in *E. coli.*

Expression and purification of rLipL21 protein

pET32-LipL21 was transformed into the competent cells (DH5α) of *E. coli* strain BL21-DE3 in plasmid with heat shock method. Protein expression was induced in different densities of IPTG (isopropyl-β-D-thio galactopyranoside) in 50 mL 2YT media. Different incubation times (0

to 16 hours) and temperatures (22 °C to 37 °C) were examined. Then 10% SDS-PAGE followed by Coomassie blue staining was performed for evaluation of expression of recombinant protein. Sonication was done six times for 1 min with 1 min interval, at the final 1 mM PMSF (phenylmethyl-sulfonyl fluoride) was added. After centrifugation at 15,000 rpm for 15 min, inclusion body was resuspended by addition of 7M urea to the pellet.

Then, recombinant LipL21 protein in presence of urea was shacked with 100 µL Ni-NTA resin for an hour at 37 ^{o}C. The resin was washed three times with washing buffer (saline phosphate buffer and 25 mM imidazole); then protein was eluted tree times with 100 µL elution buffer (saline phosphate buffer and 250 mM imidazole).

Yield of purified rLipL21 protein was determined by Bradford assay. Standard curve prepared based on BSA (bovine serum albumin) serial dilutions from 0 to 10 µg/µL and absorptions read at wavelength of 595 nm.

Western blotting analysis

Western blotting was performed to detect the expression of recombinant LipL21 by HRP conjugated anti His-tag antibody. After electrophoresis of cell lysate on SDS-PAGE for 16 hours, the protein was transferred to the nitrocellulose membrane in transfer buffer 100 V for 2 hours. Blocking step was done by blocking buffer (BSA 1% added to PBST) for an hour and two washings by PBST buffer (0.1% Tween 20 in PBS 1×). The membrane was incubated in anti-His tag HRP conjugated at the dilution of 1:5000 for an hour. rLipL21 band was observed by 4-chloro-1-naphthol (4CN) and H_2O_2.

Study of immune response in rabbits

Four groups (n=2) of 1-kg female New Zealand rabbits from Razi Vaccine and Serum Research Institute were formed. Group 1 received recombinant protein LipL21, group 2 was immunised by *Leptospira* vaccine, group 3: with adjuvant montanide (ISA70) and group 4: injected with PBS) were considered for immunization. Sample volume in each injection was 1 mL per rabbit. Injections were performed three times, subcutaneously at two weeks interval. Blood samples (1 mL) were collected four times at 14 days interval from the central auricular ear artery using a 20-22 gauge butterfly needle. All experiments were executed according to Guide for the Care and Use of Laboratory Animals and approved by the institutional animal ethics committee.

Evaluation rLipL21 in ELISA test

The ELISA microplate was coated with different concentration of rLipL21 (100, 250, 500, 750 and 1000 ng) by carbonate coating buffer (pH=9.6) and incubated overnight at 4^{o}C. The plate was washed three times with PBS containing 0.05% Tween 20 (PBST). The sera of the four groups at a dilution of 1:100 were added to the wells in duplicate and incubated at 37 ^{o}C for 1 h and washed three times with PBST. Anti-rabbit antibody at 1:5000 was used as secondary antibody conjugate and incubated at 37 °C for 1 hour then washed with PBST. Fifty µL TMB-substrate was added and incubated at a dark place for 15 min and 50 µL 2N H_2SO_4 was added to stop reactions. The microplates were read at 450 nm wavelength.

Statistical analysis

Collected data from ELISA microplate was analysed by SPSS v. 22 software by paired sample t-test (n=4, P value=0.01).

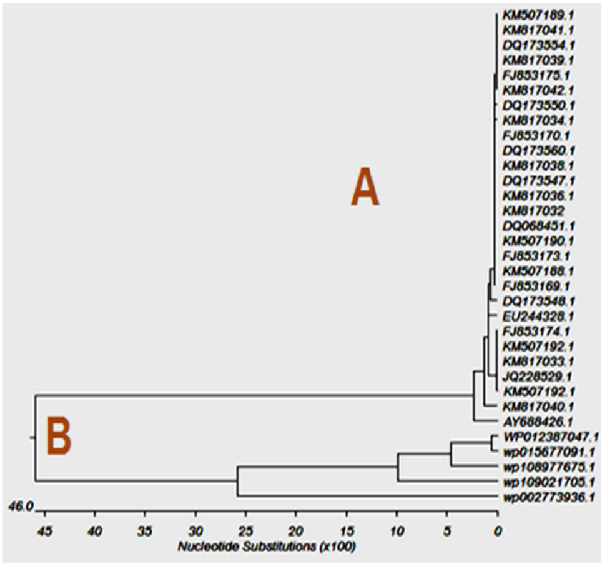

Fig. 2. Phylogeny tree of different serovars of *Leptospira* designed by MegAlign software based on LipL21 protein pattern. **A.** lineage of non pathogenic serovars; **B.** lineage of pathogenic serovars.

RESULTS

According to the analysis of 33 selected LipL21 protein sequences by MegAlign software, 46% divergence was identified between pathogenic (Pomona, Ballum, Canicola, Djasiman, Javanica, Australis, Bataviae, Icterohaemorrhajiae, Hardjo, Gripotiphase, Celledoni) and non-pathogenic (Biflexa, Yanagawae, YH101, sp. E30, illini) sequences. approximately <1.2% divergence was observed in LipL21 protein between different serovars located in pathogenic group (Fig. 2).

Expression of rLipL21 protein was optimised by 0.1 mM IPTG after 16 hours incubation at 37 ^{o}C. Over expression of rLipL21 was observed by 10% SDS-PAGE followed Coomassie blue staining (Fig. 3A). Expression of rLipL21 was confirmed by western blotting (Fig. 3C). The rLipL21 protein was expressed as inclusion bodies into the *E. coli* strain Bl21-DE3. The amount of rLipL21 (36 mg/L) was purified successfully from inclusion body by affinity batch formation method in presence of 7M urea (Fig. 3B).

The concentration of purified protein in three elution were calculated. The yield of maximum elution was 36 mg/L.

In enzyme-linked immunosorbent assay all data were compared in six paired groups. Results demonstrated that antibody level was increased prominently 10 days after last injection in rabbits immunised by rLipL21 compared to control groups (Table 1).

The results showed that rLipL21 can detect antibodies in rabbits immunised with *Leptospira* vaccine (Razi Vaccine and Serum Research Institute) (Fig. 3). The rLipL21 protein produced large amounts of antibodies in the immunised rabbits by recombinant LipL21. The protein was also able to detect high levels of antibody in immunised animals by *Leptospira* vaccine (Fig. 4).

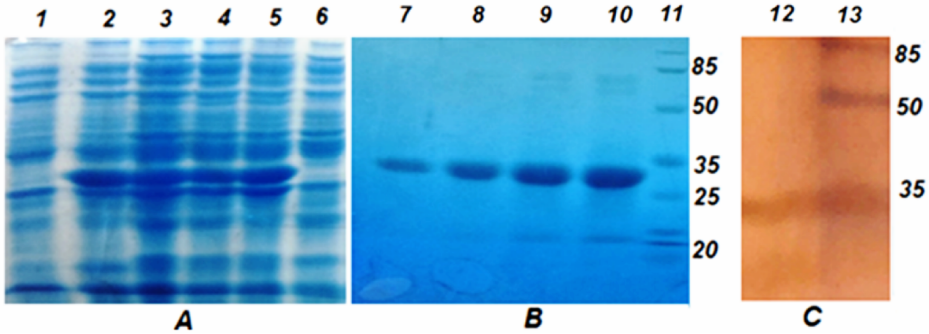

Fig. 3. Expression, purification and Western blotting of purified rLipL21 protein. **A.** lanes 1, 6: cell lysed before induction; lane 2 to 5 show t1, t3, t5, t16 after induction. **B.** lanes 7 to 10: purified proteins (elutions 4 to 1) by affinity method; lane 11: prestained protein MW marker (Thermo scientific); **C.** Western blotting: lane 12: purified rLipL21; lane 13: prestained protein ladder (Thermo Scientific).

Table 1. Comparison of immunised groups by paired sample t-test. Pair 1: PBS and Adjuvant groups, Pair 2: PBS and vaccine groups, Pair 3: PBS and rLipL21groups. Pair 4: *Leptospira* vaccine and adjuvant groups. Pair 5: *Leptospira* vaccine and rLipL21 groups. Pair 6: Adjuvant and rLipL21 groups

	Paired differences				Two-tailed P value
	Mean	SD	SEM	95% CI	
Pair 1 PBS-ADJ	–.26650	.05369	.02685	–.35194÷ –.18106	.002
Pair 2 PBC-VAC	–1.12900	.31754	.15877	–1.63428÷ .62372	.006
Pair 3 PBS-LipL21	–1.60775	.12702	.06351	–1.80987÷–1.40563	.000
Pair 4 VAC-ADJ	.86250	.26385	.13192	.442661÷.28234	.007
Pair 5 VAC-LipL21	–.47875	.40823	.20411	–1.12833÷.17083	.101
Pair 6 ADJ-LipL21	–1.34125	.16558	.08279	–1.60473÷–1.07777	.001

DISCUSSION

Bioinformatics studies showed that the pattern of pathogenic and non-pathogenic serovars have 46% divergence, while Paul *et al.* (2003) stated in their studies on southern hybridisation analysis that LipL21 was present in all pathogenic strains but not in saprophytic strains. Comparison of the patterns of Iranian isolates from several serovars showed little difference between the various serovars (Autumnalis, Pomona, Canicola, Grippotyphosa, Sejrohardjo, Sejrosejro, Icterohaemorrhagiae). Comparison of LipL21 sequences among Iranian isolates indicated that LipL21 was a conserved antigen that showed less than 1.2% variation. On the other hand up to 4% diver-

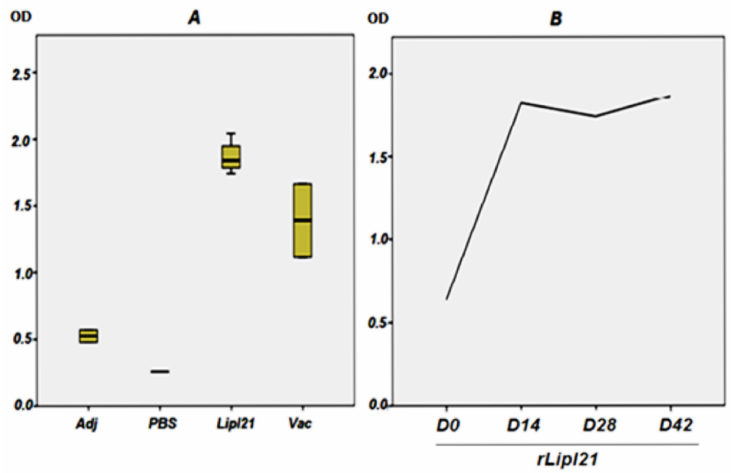

Fig. 4. A. Antibody levels in different groups of immunised rabbits by ELISA test (OD values are given as mean±SD). **B.** Antibody levels at 0, 14, 28, 42 days after immunisation of rabbits by rLipL21 protein).

gence was observed in LipL21 protein among pathogenic *Leptospira* isolates from different countries. Therefore, we choose the dominant pattern of LipL21 protein for construction of recombinant plasmid based on common serovars in Iran to have high coverage and less false negative results.

Fortunately, high amount of rLipL21 was expressed in *E. coli* and purified simply by Ni-NTA resin. Purification of the recombinant protein resulted in a yield of 36 mg/L, approximately four times higher than previous similar studies (Kumari *et al.*, 2018).

The results of ELISA showed high antibody titres in rabbits immunised by LipL21 indicating that this antigen was well immunogenic and could stimulate the immune system being significantly different from control groups. Hence, rLipL21 which was coated in the ELISA plate can detect the antibody raised by rabbits immunised with *Leptospira* vaccine (group vac), so it might be a good candidate for designing a diagnostic ELISA test (Table 1, Fig. 4).

CONCLUSION

This recombinant protein was a candidate for developing diagnostic rELISA kit that might be useful for developing recombinant vaccine against leptospirosis. Further study is needed to evaluate the function of rLipL21 for detection of antibody against *Leptospira* in the main host.

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