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Research Paper

Expression and Purification of LigA Antigen, a Surface Lipoprotein in the Pathogenic *Leptospira interrogans*

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ABSTRACT

Introduction: Although considerable progress has been made in leptospiral vaccine development, their use is limited because of short-term and serovar-specific immunity. Thus far, many approaches have been used to identify heterologous and cost-effective antigen(s) against leptospirosis. Recent advances have identified leptospiral immunoglobulin-like (Lig) proteins as promising candidates for vaccine development.

Materials & Methods: Hence, in this study, the recombinant LigA subunit consists of the ligA9, ligA10, ligA11, and ligA12 domains, were selected as conserved regions of the LigA protein. Immunoinformatics approaches, including I-TASSER, ProSA, DiscoTope v2.0, and Molprobity were utilized to check the conformational accuracy. Furthermore, 10 of the most efficient peptides for MHC-I and II grooves were predicted by the ElliPro, NetMHCpan 4.1 EL and NetMHCIIpan 4.1 EL servers.

Results: The Ramachandran plot showed acceptable conformations of the selected recombinant protein amino acid residues. The results showed that selected epitopes elicit both humoral- and cell-mediated immune responses. Hence, the selected epitopes were constructed in the pET41a+ plasmid and synthesized by General Biosystems. Recombinant plasmids were transferred to *Escherichia coli* Top10-DH5α and BL21 StarTM (DE3) competent cells for cloning and expression, respectively. Plasmid transformation and purification were confirmed using polymerase chain reaction (PCR) and enzymatic digestion. Recombinant LigA (r-LigA) was expressed in the presence

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of 0.5 M IPTG at 30˚C for 16 hours. The sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) result revealed the production of 38-kDa protein, which accumulated mostly in inclusion bodies and was purified using the urea method and dialysis. Dot blotting of the r-LigA protein confirmed a high degree of accuracy of immunogenicity.

Conclusion: The present study revealed that r-LigA is a promising candidate for developing diagnostic and subunit leptospirosis vaccines

1. Introduction

Leptospirosis, also known as Weil's disease, is a zoonotic and a potentially serious bacterial disease caused by pathogenic spirochetes of the genus Leptospira. It is primarily transmitted to humans through contact with water or soil contaminated with the urine of infected animals, particularly rodents. Sporadic outbreaks have occurred worldwide, and it is particularly endemic in tropical and subtropical regions. Thus, leptospirosis is an important public health concern in many countries [1].

One of the most effective strategies for combating leptospirosis is vaccination, which stimulates the immune system by producing antibodies against the bacteria. The first successful vaccine against Leptospirosis was reported in 1919, which dramatically decreased the number of leptospirosis cases in Japan [2]. Heat-killed whole-cell vaccines (becterin) successfully protected coal miners against leptospirosis in Kyushu, where the disease was endemic in 1933. Live-attenuated and lipopolysaccharide (LPS) vaccine were also introduced as alternatives vaccine. However, major issues such as lack of immunological memory, incomplete immunity, reactogenicity, and serovar-restricted protection have led to a decrease in the use of these vaccine types. The highlight the necessity of novel vaccine strategies [2-4]. In addition, with recent advances in molecular techniques and the availability of the complete Leptospira genome sequence, the development of novel vaccines, such as recombinant proteins, using reverse vaccinology has shown promising outcomes. Recent studies have highlighted the key role of outer membrane proteins (OMPs) in pathogenesis through the communication between the leptospira and the host [5]. Highly immunogenic OMPs, such as Leptospira subsurface lipoproteins (LipL32, LipL41), transmembrane outer membrane protein L1 (OmpL1), leptospira immunoglobulin-like protein (LigA, LigB, and LigC), and leptospira OmpA-like protein (Loa22), serve as the main skeleton of recombinant vaccines and have played a major role in the development of new vaccines. Nevertheless, variation in serovar distribution across different regions hinders the development of effective leptospira vaccines [4-6].

Structurally, LigA contains 13 bacterial immunoglobulin (Big) -like fold domains, and numerous studies support its role in virulence. This protein promotes interactions with a range of specific host proteins that mediate adhesion of *Leptospira interrogans* to the extracellular matrix, inhibit hemostasis, and inactivate key complement proteins to establish a systemic infection [7].

The development of vaccines against bacterial pathogens can be complex and time-consuming, and there is always a risk of unforeseen complications or side effects. Several studies have employed in silico approaches to identify potential epitopes on the LigA protein for the development of vaccines or diagnostic tools for leptospirosis. Moreover, by providing targeted protection against specific serovars of Leptospira, such vaccine can reduce the incidence of leptospirosis and improve public health outcomes. Regarding successful vaccine production, recombinant-protein production and purification present significant challenges, particularly in terms of cost effectiveness, expression yield, and stability. On the other hand, Lig proteins have been identified as suitable markers for the serodiagnosis of acute leptospirosis; thus, they could be implemented in immunodiagnostic kits to address leptospirosis [8]. Accordingly, this study aimed to design and produce a recombinant LigA antigen in the prokaryotic system to develop a recombinant leptospirosis vaccine.

2. Materials and Methods

This study was conducted from March 2021 to December 2021 at Razi Serum and Vaccine Research Institute (Karaj, Iran).

2.1. Recombinant LigA (r-LigA) immunogenic epitope sequence and properties prediction and validations

Previously designed conserved amino acid regions of LigA, which stimulate the immune system, were used in this study (unpublished data). The sequence of the selected r- LigA protein consisted of amino acid positions 852-937 (ligA9: BID_2), 943-1029 (ligA10: Big_2), 1034-1119 (ligA11: Big_2), and 1125-1210 (ligA12: Big_2). The three-dimensional structure of the r-LigA protein was predicted by submitting the recombinant protein sequence to the I-TASSER (Iterative Threading ASSEmbly Refinement) [9]. The invasin protein of *Yersinia pseudotuberculosis* is a well-characterized protein, which belongs to immunoglobulin superfamily in structure and topology, and its Big domain shares common themes with those observed in LigA [10]. Hence, during threading for the conformational annotation, *Y. pseudotuberculosis* Invasin protein (PDB ID: 1CWV) was used as reference template for protein homology modeling [11].

The structure with the minimum score in the previous step was selected and downloaded in protein database (pdb) compatible file format, visualized by Pymol, and used for further analysis. The prediction model quality was assessed using ProSA [12]. The Ramachandran's plot was predicted using Molprobity web server [13].

2.2. Linear and conformational B-Cell epitopes presence in r-LigA prediction

To predict the linear (continuous) B-cell-epitopes present on r-LigA using the Ellipro module on the IEDB server, the threshold was adjusted to 0.5. Conformational (discontinuous) B-cell epitopes were predicted using DiscoTope 3.0 [14], with the input structure type set to the default (solved structure) and calibrated score set as default (Moderate confidence;0.90, recall up to ~50 %) [11].

2.3. MHC class I and II peptides t-cell epitopes presence in r-LigA prediction

T-cell epitopes were evaluated based on their binding capacity to major histocompatibility complex (MHC) I and II peptides on the iedb server [15], and their locations were mapped on structural models. The NetMHCpan 4.1 EL (recommended epitope predictor- 2023.09) method was used for MHC-I peptides. The human-specific alleles HLA-A01:01, HLA-A02:03, HLA-A03:01, HLA-A11:01, HLA-A23:01, HLA-B35:01, and HLA-B44:03 with a peptide length of 10, were selected [16]. MHC-II peptides were predicted using the NetMHCIIpan 4.1 EL (recommended epitope predictor- 2023.09) method. Human HLA-DR was selected for prediction with alleles DRB1*01:01, DQA1*01:01/DQB1*02:01, and DPA1*01:03/DPB1*04:01. A peptide length of 15 [11].

2.4. Cloning and expression of r-LigA

The r-LigA sequence synthesized between EcoRI and BamHI restriction sites of the pET41a+ plasmid by General Biosystems (Anhui, USA). This plasmid carries glutathione-S-transferase (GST-Tag), 6xHis, thrombin site, S-tag, enterokinase cleavage site and 8xHis tag coding sequence as a fusion partner, and kanamycin resistance gene. The recombinant r-LigA – pET41a + cloning vector transformed into Escherichia coliTop10-DH5α competent cells prepared using the conventional $MgCl_2$ protocol. Transformations were performed using either standard 42 ˚C heat shock protocol at 42 ˚C and cultured on 2xYT agar (Merck, Germany) with the addition of kanamycin (50 µg/mL, K1377 sigma). Random colonies were first screened by polymerase chain reaction (PCR) using reverse T7 universal (Gene Link, Cat# 26-3000-13) and forward T7 universal primers (Gene Link, Cat# 26-3000-13). Bacterial amplified plasmids were extracted using BioFact™ Plasmid Mini Prep Kit (Cat# PM105-100, South Korea) according to the manufacturer's instructions [17], followed by restriction enzyme digestion with EcoRI and BamHI (Fermentas Life Sciences, Thermo Fischer Scientific, Waltham, Massachusetts, USA) performed to confirm the presence of the insert *r-LigA* gene in the pET41a + -LigA cassette before downstream transformation. To achieve enhanced and better expression, extracted plasmids (500 ng) were transformed into *Escherichia coli* BL21 StarTM (DE3) competent cells were screened using the heat shock method described above.

Induction and expression analysis of recombinant *E. coli* was conducted in the presence of 0.5 mM IPTG in 2xYT broth at 30˚C for 16 hours with constant agitation. *E. coli* BL21 StarTM (DE3) without the plasmid was used as a control strain.

2.5. Purification of r-LigA and blotting analysis of the r-LigA protein

Transformed cells were harvested by centrifugation (5500 rpm for 5 min at 4 ˚C), and the resulting bacterial pellet was re-suspended in 10 mL of phosphate buffered saline (PBS 1X) containing 10 µL of phenylmethanesulfonyl fluoride (PMSF) and lysed using a sonicator (UP200St, Hielscher Ultrasonics, Germany). Sonication was conducted 12 times by 1 minute pulses with 1 minute intervals in an ice bath. The samples were then centrifuged at 15000 rpm for 15 minutes at 4 ˚C. Supernatants and pellets were collected separately and then subjected to sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) to compare their solubility and yield.

Since r-LigA was expressed as inclusion bodies, the protein was further purified under denaturation conditions using serial concentrations of 1–8 M urea solutions in PBS buffer at 37 ˚C. The pellets were suspended in 5 mL of 1 M urea solution, shaken for 1 hour, and centrifuged (5000 rpm and 10 minutes), after which supernatant was discarded. This process was repeated for 2, 3, 4, and 5 M urea solutions. For 6–8 M urea, the incubation time was extended to overnight, and the supernatant was collected. In addition, after each step, the purification efficiency at each step was assessed using 10% SDS-PAGE. Fractions containing the recombinant proteins were mixed and extensively dialyzed (MWCO 100 kDa, Thermo Fisher Scientific, MA) against PBS (1:1000) overnight for 24 h at 4˚C. Finally, the protein concentration was quantified using the Bradford assay [18].

Purified r-LigA protein (approximately 200 ng) was used to charge the polyvinylidene fluoride membrane (PVDF) (Hybond-P, Amersham Biosciences, UK) and allowed to dry. The membranes were then blocked with 5% BSA in Tris-Buffered Saline-Tween 20 (TBST) for 40 minutes at RT. Consequently, the membrane was washed in 1X TBST three times for 10 minutes, each with gentle rocking. The proteins were reacted with conjugated anti-His tags antibodies (Thermo Fisher Scientific, USA) at RT for 1.5 hours. Finally, after extensive washing with TBST once for 15 minutes and twice for 5 minutes, the reaction was visualized using 5-thio-2-nitrobenzoic acid (TNB).

3. Results

3.1. r-Liga immunogenic epitope structure and properties prediction and validations

The selected sequence of r-LigA consists of 359 amino acids (approximately 38 KDa). The 3D structure of the protein was predicted using the I-TASSER server, which is represented in Table 1.

The predicted three-dimensional structure of the r-LigA protein visualized using Chimera software (v1.13.1, University of California, San Francisco, CA, USA) is depicted in Figure 1A. The majority of the amino acid residues present in the protein may adopt β-sheet conformation. The overall quality of the predicted structure of r-LigA using ProSA had a z-score of −6.37 (Figure 1B), which is well within the range of native conformations, indicating that the predicted structure is reliable. According to MolProbity scores, 92.2% (329/357) of all residues were Ramachandran-favored (i.e. without any steric clashes) (Figure 1C).

3.2. Linear and conformational B-cell epitopes presence in R-Liga prediction

The graphical representation of amino acid scores as propensities for involvement in linear (continuous) and conformational (discontinuous) B-cell epitopes is presented in Figure 2. According to the DiscoTope calibrated score with the default threshold (0.9), only 44 amino acids were identified as functional discontinuous B-cell epitopes (Figures 2A and 2B). According to the Bepipred linear epitope prediction 2.0 module; there were 175 amino acid sequences above the threshold value (0.5) that are highlighted in yellow and considered continuous B-cell epitope residues (Figure 2C).

The ElliPro prediction server identified 13 linear and three conformational potential peptide sequences as B-cell-epitopes (Figures 3 and 4). The linear B-cell epitopes range in length from 5 to 31 amino acids, whereas

Table 1. Properties of modeled protein based on the ITASSER results

Parameters	Values
Number of amino acids	359
Molecular weight	37897.12= ˷38 kDa
C-score	-0.62
Estimated TM-score	0.63±0.13
Estimated RMSD	8.0±4.4Å

Table 2. MHC-I binding peptides predicted by the NetMHCpan EL 4.1.

No	Peptide Sequence	Allele	Position	Score
1	IVLNPTSSHK	HLA-A*03:01	5-14	0.892
2	ATYDSIKSNR	HLA-A*11:01	247-256	0.877
3	IVLNPTSSHK	HLA-A*11:01	5-14	0.758
4	FGDSEFTATY	HLA-A*01:01	240-249	0.747
5	SIEVTPNNFF	HLA-B*44:03	90-99	0.739
6	ATYDSIKSNR	HLA-A*03:01	247-256	0.738
7	TTALSVGSSK	HLA-A*11:01	321-330	0.732
8	VLSEGLTLQL	HLA-A*02:03	186-195	0.713
9	DSMSASTTLY	HLA-A*01:01	164-173	0.689
10	LTDKGSAQQF	HLA-A*01:01	272-261	0.892

Table 3. MHC-II binding peptides predicted by the IEDB recommended 2.22 method.

No.	Peptide Sequence	Allele	Position	Adjusted Rank *	Method Used
1	KSNRAWIFVNDEKLV	HLA-DQA1*01:01/DQB1*02:01	262-276	0.77	NetMHCIIpan
2	NRAWIFVNDEKLVNI	HLA-DQA1*01:01/DQB1*02:01	264-278	0.98	NetMHCIIpan
3	SNRAWIFVNDEKLVN	HLA-DQA1*01:01/DQB1*02:01	263-277	1.1	NetMHCIIpan
4	TTLYVTSAVLIDIEV	HLA-DQA1*01:01/DQB1*02:01	175-189	1.1	NetMHCIIpan
5	DNTFSLAGSATAIDD	HLA-DRB1*01:01	144-158	1.3	Consensus (comb.lib./smm/nn)
6	IDNTFSLAGSATAID	HLA-DRB1*01:01	143-157	1.3	Consensus (comb.lib./smm/nn)
7	NTFSLAGSATAIDDG	HLA-DRB1*01:01	145-159	1.3	Consensus (comb.lib./smm/nn)
8	TLYVTSAVLIDIEVK	HLA-DQA1*01:01/DQB1*02:01	176-190	1.4	NetMHCIIpan
9	IKSNRAWIFVNDEKL	HLA-DQA1*01:01/DQB1*02:01	261-275	1.6	NetMHCIIpan
10	SIDNTFSLAGSATAI	HLA-DRB1*01:01	142-156	1.8	Consensus (comb.lib./smm/nn)

*Lower adjusted rank indicates a better binding.

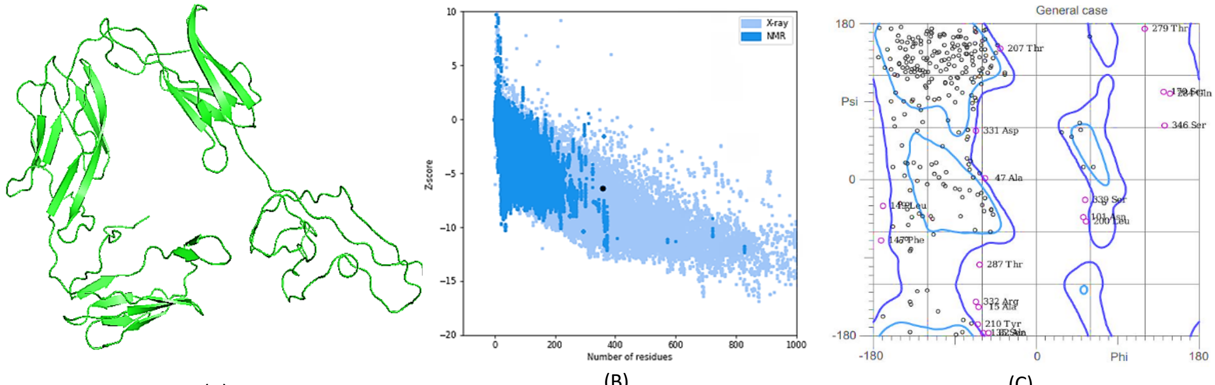
the three potential conformational B-cell epitopes comprise 57 and 87 amino acids, respectively.

3.3. MHC class I and II peptides T-cell epitopes presence in r-LigA prediction

Based on the IEDB analysis prediction results, there were various amino acid residues in the shape of the MHC-I and MHC-II grooves, and their most efficient peptides are presented with their scores in Tables 2 and 3, respectively.

3.4. Cloning and expression of r-LigA protein

Transformation of the r-LigA pET41a+ plasmid into *E. coli* Top10-DH5α (cloning vector) and *E. coli* BL21 StarTM (DE3) (expression vector) produced several colonies in the presence of ampicillin. This was confirmed by amplification of a 2000-bp segment in PCR of the target gene using T7 universal primers on multiple random clones (Figure 5A). As shown in Figure 5B, EcoRI and BamHI digestion of the purified r-LigA - pET41a + plasmid yielded 5.9-kb and 1.1-kb fragments that can be attributed to the plasmid and r-LigA, respectively.

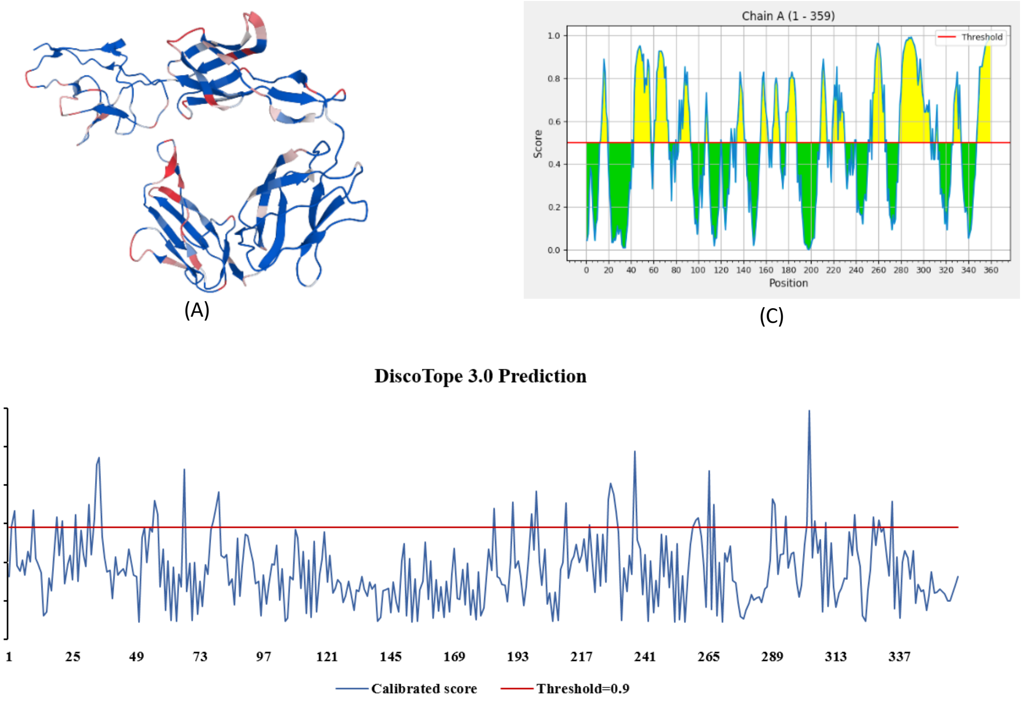


(A) (B) (C)

Figure 1. Prediction and validation results of r-LigA protein

A) Predicted 3D structure of r-LigA protein visualized by Pymol software; B) Overall model quality of the predicted structure given in a graphical form where X & Y axes represents residue position & predicted

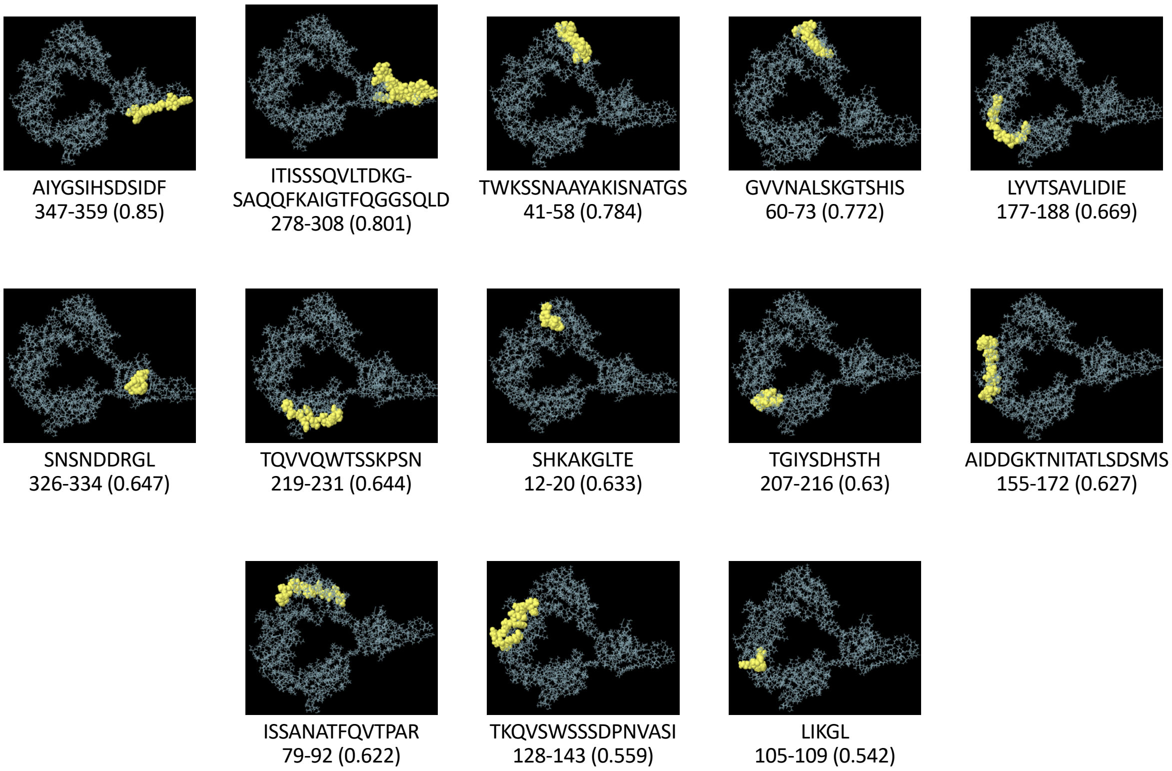
To achieve high-level expression of the peptide plasmid transformed into the *E. coli* BL21 StarTM (DE3) induced with 0.5 mM IPTG at 30˚C for 16 hours, which led to the expression of recombinant r-LigA protein with approximately 71 KDa confirmed on SDS-PAGE. *E. coli* BL21 StarTM (DE3) without the plasmid was subjected to the same experiment and did not express the protein under any conditions.



(A) (C)

(B)

Figure 2. A) Continuous epitope by DiscoTope: Residues with higher epitope propensity are colored in a deeper red, while residues with lower epitope propensity are colored in a deeper blue.; B) Residues interacting as continuous epitope calculated by DiscoTope; C) Linear epitope: Amino acid residue with a score above the threshold Bepipred Linear Epitope Prediction is considered to be part of an epitope & coloured in yellow



AIYGSIHSDSIDF
347-359 (0.85)

ITISSSQVLTDKG-SAQQFKAIGTFQGGSQLD
278-308 (0.801)

TWKSSNAAYAKISNATGS
41-58 (0.784)

GVVNALSKGTSHIS
60-73 (0.772)

LYVTSAVLIDIE
177-188 (0.669)

SNSNDDRGL
326-334 (0.647)

TQVVQWTSSKPSN
219-231 (0.644)

SHKAKGLTE
12-20 (0.633)

TGIYSDHSTH
207-216 (0.63)

AIDDGKTNITATLSDSMS
155-172 (0.627)

ISSANATFQVTPAR
79-92 (0.622)

TKQVSWSSSDPNVASI
128-143 (0.559)

LIKGL
105-109 (0.542)

Figure 3. Linear B cell epitopes predicted using Ellipro prediction server

Note: Yellow highlight regions capable to stimulate B-cells. Peptide sequences, residue positions and predicted scores (in parenthesis) are given are given above each pictures.

3.5. Purification and blotting analysis of the r-LigA protein

Analysis of the cell lysate post-sonication revealed that the r-LigA r protein expressed using the pET41a+ vector in *E. coli* BL21 system mostly accumulates as an insoluble form in the pellet, probably in inclusion bodies. The molecular weight of r-LigA is approximately 38 KDa, whereas the different fused partner proteins of the pET41a+ plasmid are approximately 32.29 KDa. Thus, it is speculated that the molecular weight of the expressed protein of this construct is approximately 71 KDa. There was an obvious band at 71 KDa in the purified protein after washing in serial concentrations of urea in PBS (1 to 5 M) followed by solubilization (6 to 8 M) at 37 ˚C. The outcome of this procedure on SDS-PAGE revealed that r-LigA was successfully induced, expressed, and purified. The protein content gradually increased and then decreased. The highest level of r-LigA gained in 6-M urea (Figure 6A). To ensure high confidence, the Dot blot-binding assay was performed, and its result confirmed the interaction of r-LigA with conjugated anti-His tags antibodies (Figure 6B).

The quantity of the yield of purified recombinant protein was estimated to be 834 µg/mL in Bradford assay.

4. Discussion

The global distribution of leptospirosis and its association with autoimmune disease have motivated researchers to develop effective and safe vaccines. Commercial Leptospiral vaccines are available in many countries for use in dogs, pigs, and livestock, most of these have limited efficacy because of a lack of serovar specificity and their adverse effects, such as pain and fever [19, 20].

In fact, most surface proteins of pathogens are involved in virulence and even host immune response stimulation, making them good targets for vaccine development [2]. Matsunaga et al. (2003) expressed surface-exposed proteins belonging to the bacterial immunoglobulin superfamily by pathogenic *Leptospira* species and reported a new family of Big domain proteins called immunoglobulin-like proteins (Lig) in pathogenic *Leptospira* [10]. LigA, as a member of the Leptospiral OMPs, possess highly conserved domains, which make it a proper candidate in antigen-based vaccine development [2, 21].

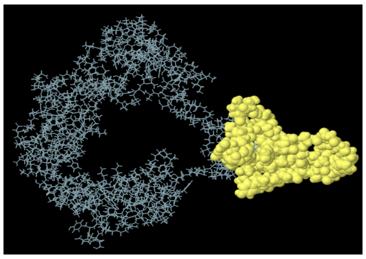
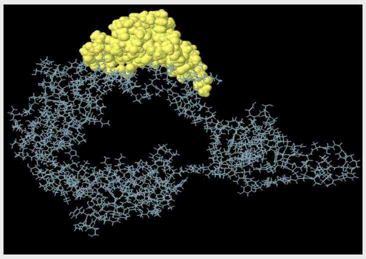
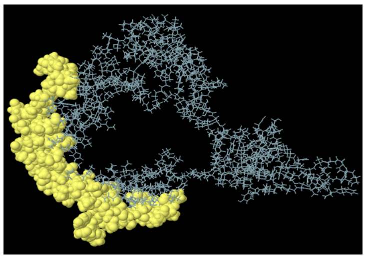
Residues	No of residues	Score	3-D structure
I278, T279, I280, S281, S282, S283, Q284, V285, L286, T287, D288, K289, G290, S291, A292, Q293, Q294, F295, K296, A297, I298, G299, T300, F301, Q302, G303, G304, S305, Q306, L307, D308, D311, L312, V313, S326, N327, S328, N329, D330, D331, R332, G333, L334, T335, A347, I348, Y349, G350, S351, I352, H353, S354, D355, S356, I357, D358, F359	57	0.766	
S11, S12, H13, K14, A15, K16, G17, L18, T19, E20, T41, K43, S44, S45, N46, A47, A48, Y49, A50, K51, I52, S53, N54, A55, T56, G57, S58, G60, V61, V62, N63, A64, L65, S66, K67, G68, T69, S70, H71, I72, S73, I79, S80, S81, A82, N83, A84, T85, F86, Q87, V88, T89, P90, A91, R92, T120, D121	57	0.704	
F104, L105, I106, K107, G108, L109, Q130, V131, S132, S134, S135, S136, D137, P138, N139, V140, A141, S142, I143, A155, I156, D157, D158, G159, K160, T161, N162, T164, T166, L167, S168, D169, S170, M171, S172, L177, Y178, V179, T180, S181, A182, V183, L184, I185, D186, I187, V189, A206, T207, G208, I209, Y210, S211, D212, H213, S214, H216, T219, Q220, V221, V222, Q223, W224, T225, S226, S227, K228, P229, S230, N231, G239, K240, A248, D251, E253, T255, A256, T257, Y258, D259, S260, I261, K262, S263, N264, R265, W267	87	0.647	

Figure 4. Conformational B cell epitopes predicted using Ellipro prediction server

Note: Yellow highlight regions capable to stimulate B cells.

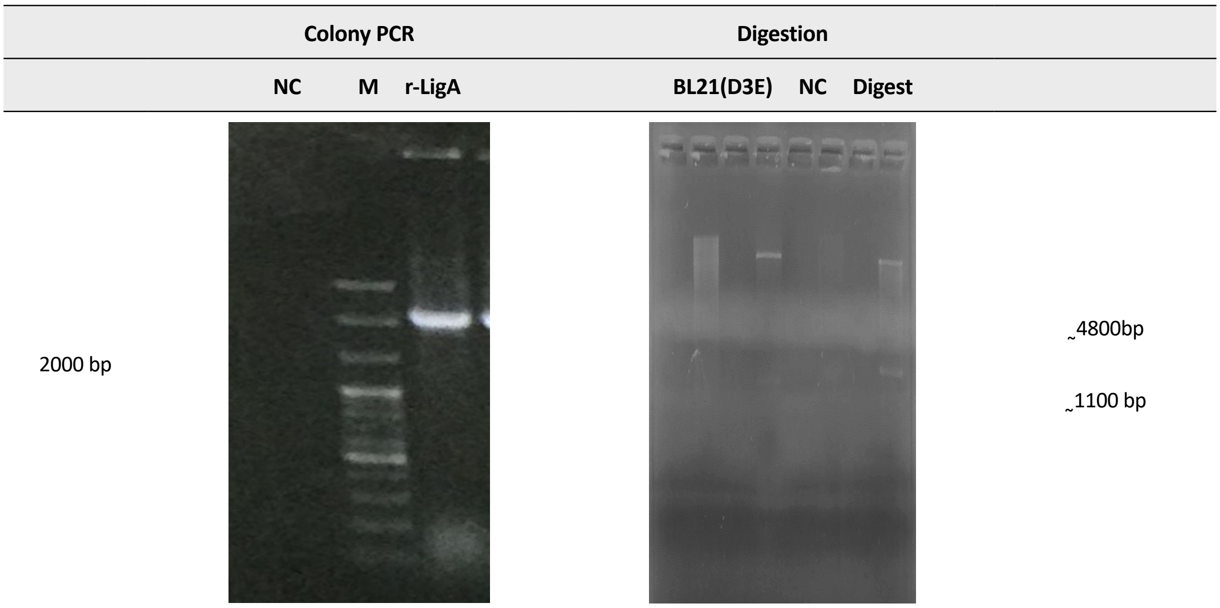


Figure 5. Transformation confirmation electrophoresis on 1% agarose gel

M: Marker, NC: Negative control.

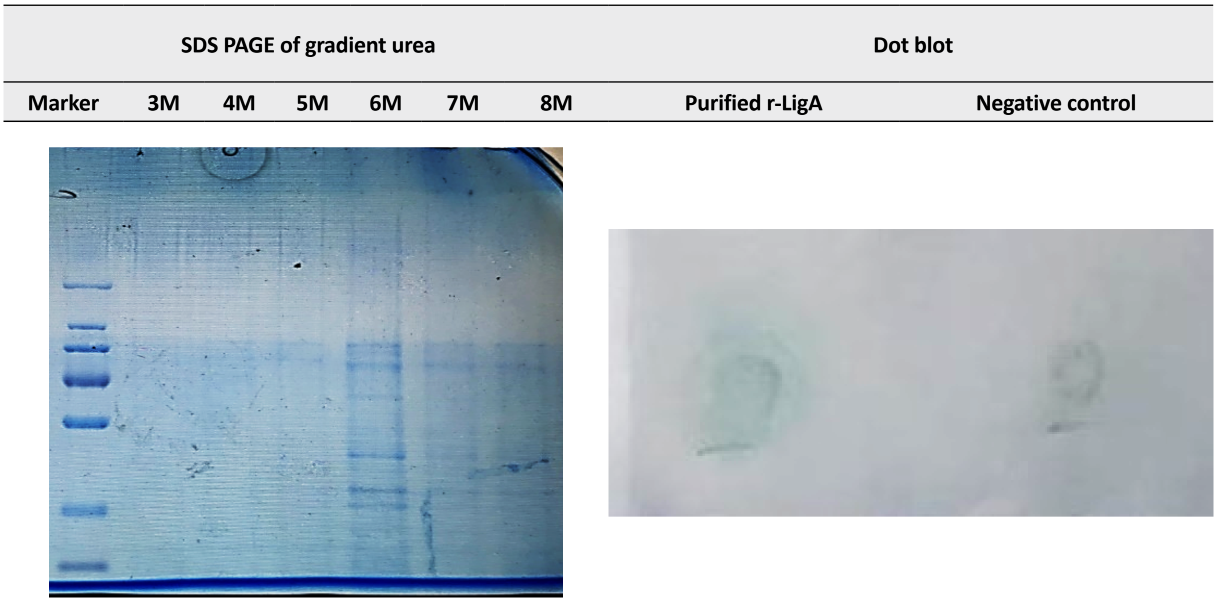


Figure 6. A) SDS-PAGE of expressed r-LigA in *E. coli* (BL21 StarTM [DE3]) under different concentration of urea after dialyze (Marker: PM2500); B) Confirmation dot blot after purification with urea

Koizumi et al. determined that recombinant full-length LigA and truncated LigB proteins can induce immunoprotection against Leptospiral lethal infection in C3H/HeJ mice [22]. Silva et al. expressed the unique carboxy-terminal regions of LigA (LigANI), LigB (LigBNI), and LigBrep from *L. interrogans serovar* Copenhageni in *E. coli* BL21(DE3)Star as recombinant proteins. Evaluation in the hamster infection model revealed strong immunoprotective activity, resulting in protection against mortality [23]. As it has been suggested that the presence of at least three Big domains (especially ligA11, and ligA12 specific Big) is critical, a construct of r-LigA protein carrying ligA9, 10, 11, and 12 domains was inserted in pET41a + plasmid transformed into the *E. coli* Top10-DH5α and *E. coli* StarTM (DE3) cells in this study [24].

I-TASSER is an automated server for protein tertiary structure prediction that uses PSSpred for second structure prediction, LOMETS for template detection, replica-exchange Monte Carlo simulation for fragment structure assembly, SPICKER for model selection by clustering structure decoys, fragment-guided molecular dynamics simulation (FG-MD) or ModRefiner for atomic-level structure refinement, and COACH for structure-based biology function annotation [9, 25]. The I-TASSER server calculates the confidence score (C-score), which estimates the quality of the predicted models based on the significance of threading template alignments and the convergence parameters of the structure assembly simulations, revealing high confidence in prediction. Normally, C-score values lie between [−5 to 2], where a higher value denotes a model with a higher confidence and correct topology [9]. Then, a C-score value of -0.62 indicates good topology of the modeled structure. The topological similarity of protein structures is reported as TM-score in I-TASSER. TM-score <0.17 indicates a random similarity, while a TM-score >0.5 indicates a model of correct topology (9). Based on these results, the TM-score 0.63±0.13 confirms a proper topology.

As a structural validation, the statistical distribution of the combinations of the backbone dihedral angles ϕ and ψ of a protein can be determine using the Ramachandran plot. In good-quality homology models, very few dihedral angles are found in forbidden regions [11]. In this study, the three-dimensional structure predicted for the r-LigA protein was reliable because only 7.8% of the residues were Ramachandran outliers.

Epitopes are small specific segments of antigens that usually affect the specificity of cellular and humoral immune responses. In linear epitopes, the primary amino acid sequence is recognized by the antibodies, whereas in conformational epitopes, amino acids are not neighboring and are brought into close proximity in the folded protein, directly binding to a receptor of the immune system [26, 27]. Interestingly, the linear epitopes with high scores as follows: AIYGSIHSDSIDF (347-359), ITISSSQVLTDKGSAQQFKAIGTFQGGSQLD (278-308), and SNSNDDRGL (326-334), which compose a conformational epitope with the highest score as well.

Binding to MHC class I molecules (MHC-I) plays a pivotal role in antigen presentation and the induction of cytotoxic T-cell responses. MHC class I and II genes in humans are called "Human leukocyte antigen" (HLA). NetMHCpan 4.1 uses artificial neural networks (ANNs) and has been trained on a combination of more than 850,000 quantitative binding affinity (BA) and mass spectrometry, eluted ligands (EL) peptides making it one of the most reliable prediction servers [11, 28]. The limited amount of data available for most MHC class II molecules and considerable differences in sequence polymorphism and corresponding details in the molecular structures across different MHC class II loci hinder the development of cross-species training strategies and is known as main limitation of pan-specific or cross species approach for MHC class II prediction [11]. The results for MHC-I binding peptides showed that epitope binding with HLA-A*03:01 allele had the highest binding affinity scores at 0.892.

Behera et al. applied similar approaches and found that three out of the most efficient peptides for MHC-I grooves predicted by NetMHCpan 4.1 server were presented by MHC Class II molecules, indicating that both CMI and humoral immune response can be induced by those peptides [11].

The NetMHCIIpan 4.0 server was used to determine helper T lymphocyte (HTL) epitopes [23]. The number of epitope binding with the HLA-DQA1*01:01/DQB1*02:01 allele was higher than other alleles in MHC-II binding peptides. Interestingly, NRAWIFVNDEKLV core sequence binding with the HLA-DQA1*01:01/DQB1*02:01 allele was present in top three NetMHCIIpan 4.0 prediction server, followed by the NTFSLAGSATAIDD sequence binding with HLA-DRB1*01:01 in consensus method. For subunit vaccines, several expression systems were used, including bacteria, yeast, mammalian or plant cells [29]. In this study, the r-LigA construct transferred into *E. coli* Top10-DH5α and *E. coli* BL21 StarTM (DE3). r-LigA was expressed in *E. coli* BL21 StarTM (DE3), and it was observed that it is mostly in the form of inclusion bodies. It has been reported that LigA and LigB normally contain a lipobox, whereas LigA encodes a lipoprotein signal peptide distinct from the motifs of *E. coli* and other gram-negative bacteria. Due to the presence of the lipobox, there is a sec-dependent export pathway for LigA exists across the cytoplasmic membrane. Expression of LigA in *Leptospira* sp. results in a surface protein with a signal peptide subject to proteolytic removal of its lipid anchor. Therefore, a portion of LigA protein can be recovered from the culture supernatant in the normal system [30]. Palaniappan et al. demonstrated that intact ligA, without its signal sequence expressed in *E. coli* was toxic to *E. coli*, whereas the expression of a 90-kDa truncated LigA was not toxic to *E. coli* cells. They found that complete LigA protein can be expressed only in leptospira-infected hamster kidney [31]. Interestingly, in a study LigA lipoproteins were expressed and exposed on the surface of the saprophyte *L. biflexa* cells, and it was suggested that *L. biflexa* is an appropriate surrogate host for the expression of at least some *L. interrogans* OMPs [32].

Due to differences in leptospiral lipobox sequences, it is anticipated that leptospiral lipoproteins are processed correctly in *E. coli* [32]. To minimize protein inclusion bodies formation, it is recommended to take some strategies such as changing vectors, host strains, production of endogenous chaperones or /chaperone co-expression, low temperature induction, and using the target protein to soluble protein or peptide tags [33]. High levels of r-ligA (834 µg/mL) were purified from inclusion bodies with high efficiency by serial washing with 6–8 molar urea. It can be concluded that r-LigA protein could be recovered from inclusion bodies using the urea method, which is a simple and low-cost technique. Hartwig et al., (2010) expressed rLigANI (61 kDa) in the eukaryotic expression system of Pichia pastoris, resulting in a significantly lower protein yield [29]. In this study, the r-LigA construct produced an approximately 38-kDa recombinant protein, and a high yield of protein was obtained at 6 m urea solution. The urea purification method is a low-cost purification strategy in comparison to Ni-NTA affinity chromatography purification. The optimum urea concentration in the purification procedure of this study were in accordance with a study focused on Lipl41 recombinant protein expression in *E. coli* BL21 (DE3) carrying pET32a+ expression vector [18].

r-LigA protein production was confirmed by immunoblotting analysis with HRP-conjugated Anti-6x His-Tag antibodies. The performed dot-blot technique showed promising results in discriminating between positive and negative serum samples. Although expression of r-LigA was successful, due to the limited commercial availability of immunological reagents for use in hamsters, we were not able to determine the cell-mediated immune response of hamsters to r-LigA protein immunizations. Although this study focused on a comprehensive immunoinformatics, the identified epitopes have not yet been assessed as vaccine candidates; and hence,they could be worthy of further investigation as novel vaccine candidates.

5. Conclusion

In conclusion, these findings suggest that this immunoinformatics study represents novel vaccine candidates that will further aid in the development of improved vaccines for leptospirosis. However, further refinement of this technique is required before it can be used for leptospirosiss diagnosis or vaccine development.

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Compliance with ethical guidelines

This study was approved by the Research Ethics Committees of North Tehran Branch, Islamic Azad University, Tehran, Iran (Code: IR.IAU.TNB.REC.1401.049). All the procedures for animal experiments were performed in accordance with the ethical principles and the national norms and standards for conducting Medical Research in Iran guidelines.

Data availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author upon reasonable request.

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Authors' contributions

Conceptualization, methodology, and supervision: Pejvak Khaki; Formal analysis, investigation, review, editing, and Final approval: All authors; Writing the original draft: Aida Chalesh; Funding acquisition: Pejvak Khaki and Sohaila Moradi Bidhendi.

Conflict of interest

The authors declared no conflict of interest.

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