Original Article

Recombinant fusion protein LipL41-OmpL1 as a Potential Candidate for a Cost-Effective Vaccine against Leptospirosis

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ABSTRACT

Leptospirosis represents a significant threat to the health of both humans and animals. It is a disease caused by pathogenic Leptospira. The early detection of pathogenic Leptospira is an effective method for preventing a multitude of potential complications. The protected outer membrane protein (OMP) of pathogenic Leptospira, LipL41-OmpL1, was inserted into E. coli bacteria using different software for the amino acid sequence of OmpL1 and LipL41. This was done to design a recombinant fusion protein, which was then expressed to investigate immunogenicity. The selected genes were propagated and cloned as a fusion in a PET32a+ plasmid vector and expressed by Escherichia coli strain S (DE3) via a heat shock method. The evaluation was conducted using the BALB/c mouse as the laboratory animal model. The recombinant LipL41-OmpL1 protein was confirmed using the urea purification method and western blot, and its immunogenicity was evaluated by measuring the high humoral immune stimulation and antibody secretion in BALB/c mice by the ELISA method. The findings demonstrated that the animals that received both the OmpL1 and LipL41 proteins exhibited 85% immunogenicity, whereas the control group that did not receive the fusion protein demonstrated only 25% immunogenicity ($P<0.001$). Moreover, no evidence of infection was identified in recipients of the OmpL1-LipL41 fusion protein, indicating that this protein is safe for use. The protective effects of immunization with OmpL1 and LipL41 were synergistic, as no significant levels of protection were observed in animals immunized with OmpL1 or LipL41 alone. In conclusion, this study underscores the potential of a recombinant OmpL1 and LipL41 fusion protein as a promising avenue for research in the development of vaccines and ELISA diagnostic kits for the prevention and rapid diagnosis of leptospirosis.

Keywords: Expression; LipL41-OmpL1; Purification.

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1. Introduction

Leptospirosis is a significant zoonotic disease caused by the spirochete bacteria Leptospira (1, 2). The disease can manifest with a range of symptoms, from mild to severe, including Weil's disease and leptospirosis-associated pulmonary hemorrhage syndrome. The implementation of a vaccination program against animal leptospirosis has the potential to reduce the risk of disease transmission to humans. However, recent years have witnessed an increase in the prevalence of the disease. The presence of the pathogenic LipL41-OmpL1 antigen among all Leptospira species is an effective diagnostic tool and contributes to the development of recombinant vaccines. Additionally, antigenic proteins expressed during infection play a pivotal role in the diagnosis and production of new vaccine techniques. The complexity of the outer membrane proteins of Leptospira presents a challenge in identifying these structures. A number of proteins have been isolated from the Leptospira interrogans serovar Copen family, including Fla, OmpL, LipL, and Lig, which are further divided into subgroups (5).

Objectives

In this study, LipL41 and OmpL1, two proteins that are expressed as immunogenic antigens in pathogenic Leptospira, play an important role in protecting the structure of bacterial cells, in vaccine design, and in food entry mechanisms. Furthermore, they are pathogenic. These proteins were used as a fusion to design effective vaccines and ELISA diagnostic kits. The expression of a novel fusion protein comprising sequences from two distinct outer membrane proteins was investigated, and its safety and immunogenicity profiles in a laboratory mouse model are delineated. The results suggest the use of OmpL as a fusion protein in the development of a cost-effective recombinant vaccine against leptospirosis. The creation of recombinant vaccines using common pathogenic antigens for all Leptospira species may provide a promising solution in this regard (6-12).

2. Materials and Methods

2.1. Bioinformatics Analysis

The LipL41 and OmpL1 genes were selected from the NCBI database (accession number OQ941633) and compared with the genes of local isolated serovars sequenced using NCBI Blast software. The recombinant protein's physicochemical properties were analyzed using the ProtParam tool (13, 14). The restriction enzymes HindIII and BamHI were selected based on the structure of the plasmid multiple cloning site (MCS). The LipL41-OmpL1 fusion protein fragment was subjected to enzymatic digestion with the selected enzymes and subsequently inserted into the pet 32a+ vector. The synthesized plasmid was transformed into Escherichia coli BL21 (DE3) pLysS.

2.2. Cloning of Recombinant Protein

The chimeric gene inserts were obtained through the digestion of the commercial vector with XhoI and HindIII restriction enzymes, followed by gel purification of the fragment of interest. The inserts were ligated into the PET32+ expression vector, which was fully sequenced using an ABI 3100 automatic sequencer.

2.3. Expression and Purification of Lipl41-Ompl1

The LipL41-OmpL1 recombinant protein was expressed and purified using a PET32a+ vector and an Escherichia coli strain designated BL21(DE3). The expression conditions were optimized by varying the concentration of IPTG (isopropyl β-d-1-thiogalactopyranoside), temperature, and time. The optimal conditions for the production of the LipL41-OmpL1 fusion protein were determined to be 22°C and 16 hours following the induction of protein expression with IPTG at a concentration of 0.4 mM. The molecular weight of the protein was confirmed to be 73 kDa, and expression was undetectable in the absence of IPTG. The induced cells were harvested by centrifugation. The bacterial pellet was then resuspended in the sonication buffer and lysed with the aid of the Q500 Sonicator probe. Subsequently, the insoluble fraction was suspended in a buffer containing urea and sodium dodecyl sulfate (SDS) and purified by nickel affinity chromatography. The purified protein was subsequently subjected to dialysis against PBS, followed by quantification using the Bradford method.

2.4. Investigating the Immunogenicity of LipL41-OmpL1 Recombinant Protein

The objective of this study was to investigate the immunogenicity of the LipL41-OmpL1 recombinant protein in a mouse model. Twenty-eight female BALB/c mice, aged 6 to 8 weeks and with a mean weight of 25 to 30 grams, were obtained from the Razi Institute. The animals were used in accordance with the ethical guidelines, including humane euthanasia.

2.5. Experimental Design

To evaluate the humoral immune response, the mice were randomly assigned to four groups, as outlined in Table 1.

2.6. Injection Protocol

The objective of the subcutaneous injection protocol was to assess the immunogenicity and potential protective effects of the LipL41-OmpL1 recombinant protein against leptospirosis. The same procedure was followed for the second and third injections, but incomplete Freund's adjuvant was utilized instead of complete Freund's adjuvant. The mice were administered the injections on days 0, 14, and 28. Blood samples were obtained from the orbital sinus via capillary tube on days 10, 20, 30, 40, 50, and 60 post-injections. The collected blood was transferred to microtubes and centrifuged at 5,000 rpm for five minutes to separate the serum, which was then stored at -20°C for subsequent analysis. This experimental design was devised to ensure the ethical treatment of the animals and to generate reliable scientific data on the immunogenicity of the LipL41-OmpL1 recombinant protein.

Table 1. Grouping of mice based on injection dose

Group	Injection Solution	Number of Mice	Injection Dose
Group 1	Antigen + Adjuvant	8	20 µg/mouse
Group 2	Antigen	8	20 µg/mouse
Group 3	PBS	5	-
Group 4	Adjuvant	5	-
	Injection Volume		100 µl

2.7. Evaluation of the Humoral Immune Response

To evaluate the humoral immune response, serum samples were obtained from the retro-orbital plexus of mice at two-week intervals following the final immunization and pooled. The serum was separated by centrifugation and stored at -20 °C until required for use. The levels of specific IgG antibodies against the recombinant protein were quantified by enzyme-linked immunosorbent assay (ELISA). ELISA was conducted by coating 96-well plates with 250 ng of each recombinant protein and blocking with PBS-T containing 10% non-fat dry milk. The serum samples were added at varying dilutions and subsequently incubated with horseradish peroxidase (HRP)-conjugated anti-mouse total IgG, anti-mouse IgG1, or anti-mouse IgG2/IgG3 antibodies. Subsequently, the plates were washed, and an o-phenylenediamine solution in citrate phosphate buffer (pH 5.0) with hydrogen peroxide was added. The reaction was terminated after 10 minutes by the addition of 2 M H_2SO_4, and the absorbance was measured at 492 nm using a microplate reader. The data were plotted as the mean absorbance values obtained at a serum dilution of 200-fold, and all experiments were conducted in triplicate. This methodology enabled the determination of total IgG antibody production.

2.8. Challenge Experiments

The protective efficacy of the LipL41-OmpL1 recombinant protein was evaluated by challenging mice with a lethal dose of Leptospira interrogans serovar Pomona. The mice that had been challenged were observed for any clinical signs of infection, and the survival rates were recorded for up to 21 days' post-challenge.

2.9. Statistical Analysis

The statistical analysis was conducted using GraphPad Prism software. The data were subjected to one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. The survival data were subjected to analysis using the log-rank test. A p-value of less than 0.05 was considered to indicate a statistically significant result.

3. Results

3.1. Bioinformatics Analysis

The recombinant protein LipL41-OmpL1 has a length of 2081 base pairs (bp), which is located on plasmid PET32a+. The insertion is 5900 bp, resulting in a molecular weight of 73 kDa (Figure 1). The recombinant plasmids LipL41 and OmpL1 were successfully transformed into αDH5 and BL21 plysS competent cells, respectively. Following a 24-hour incubation period at 37°C, the growth of colonies on ampicillin-containing media served to confirm the successful transformation, as evidenced by the presence of colonies carrying ampicillin resistance genes (Figure 2). The protein induction procedure was conducted in accordance with the prescribed protocol. Once the optical density (OD) of the culture medium reached 0.6, 0.2 mM of IPTG was added, and the culture was incubated in a shaker at 37°C for 16 hours. Subsequently, the culture was subjected to centrifugation in order to separate the supernatant from the pellet. Subsequently, both fractions underwent SDS-PAGE analysis. The results of the SDS-PAGE analysis demonstrated that the target protein, with a molecular weight of 73 kDa, was predominantly present in the insoluble fraction. Optimization experiments considering various temperatures, incubation times, and IPTG concentrations demonstrated that the optimal conditions for protein expression were 16 hours of induction at 22°C with 0.4 mM IPTG. The optimized conditions were subsequently employed in a one-liter culture for further experimentation.

3.2 Expression and Purification of LipL41-OmpL1

Ultrasonic waves were employed to lyse the bacterial cell wall, and the resulting supernatant and sediment were subjected to examination. The recombinant protein was identified in the sediment, and its purification in 6 M urea was confirmed by SDS-PAGE (Figure 3). In the blotting experiments, the anti-His tag antibody, which exhibits ligand-receptor properties with the expressed antigen, was employed for the detection of the target protein. The antibody binds specifically to the His-tagged protein, and the addition of a substrate allows for the location of the target protein to be determined. Samples A, B, and C were loaded in volumes of 4, 2, and 1 µl, respectively. Upon the addition of the substrate, the desired antigen was clearly visible, as illustrated in the accompanying figure. Furthermore, a sediment sample from untransfected E. coli bacteria was employed as a negative control in conjunction with the transformed sample (D) on the membrane. The results clearly demonstrated a distinction between the transformed and untransformed samples, confirming the presence of the target antigen in the transformed samples. The visual contrast between the two samples serves to illustrate the successful expression of the antigen under investigation (Figure 4). To assess the solubility of the expressed protein, cell lysis was conducted using

Figure 1. Bioinformatic analysis and 3D structure: The fusion protein LipL41-OmpL1 molecularly modeled by I-TASSER web server and visualized by PyMoL.

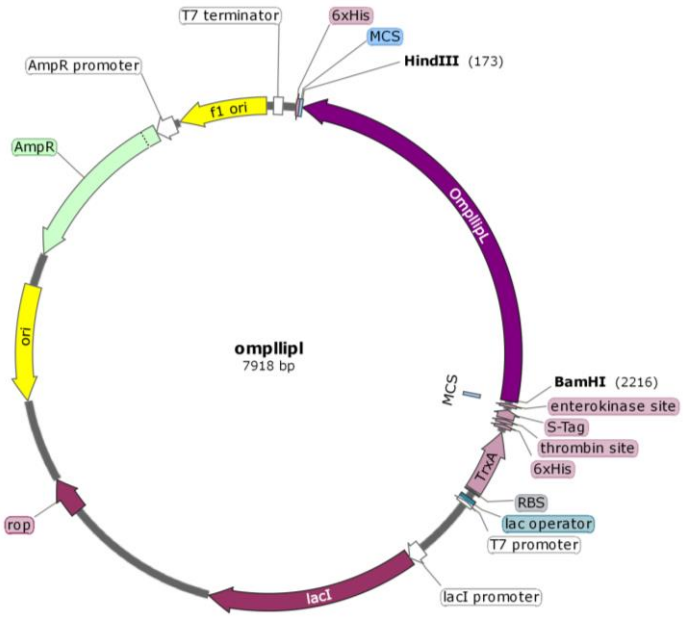


Figure 2. The structure of the fusion protein: The PET 32 a + vector

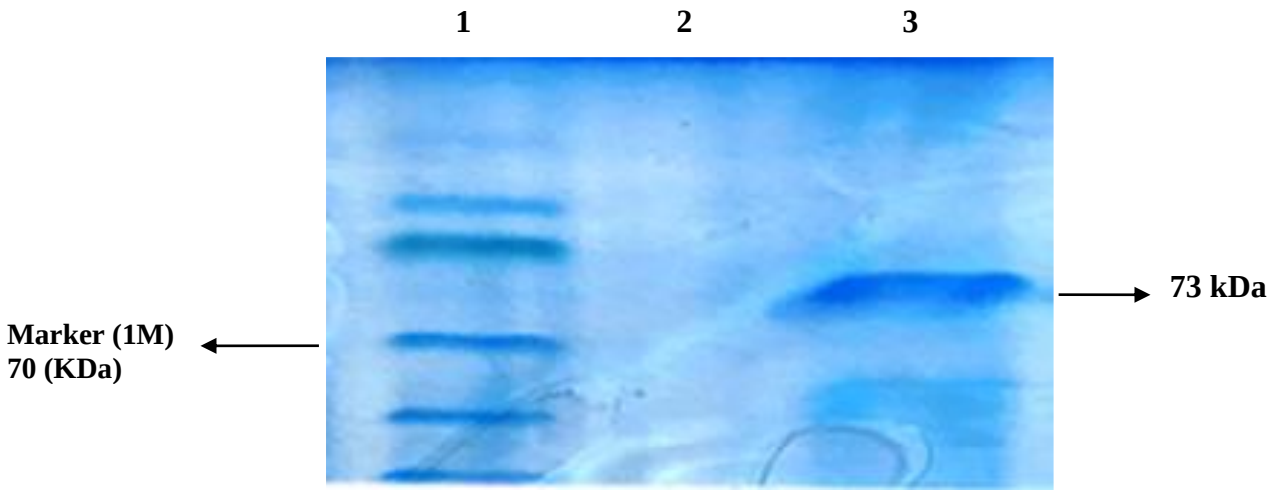


Figure 3. Lane 1; 10 bp protein molecular Thermo Scientific(1M). Lane 2; supernatant of the resulting suspension (which indicated the absence of our recombinant protein). Lane 3; the 73 kDa protein present in the sediment of the resulting suspension was confirmed in the third well.

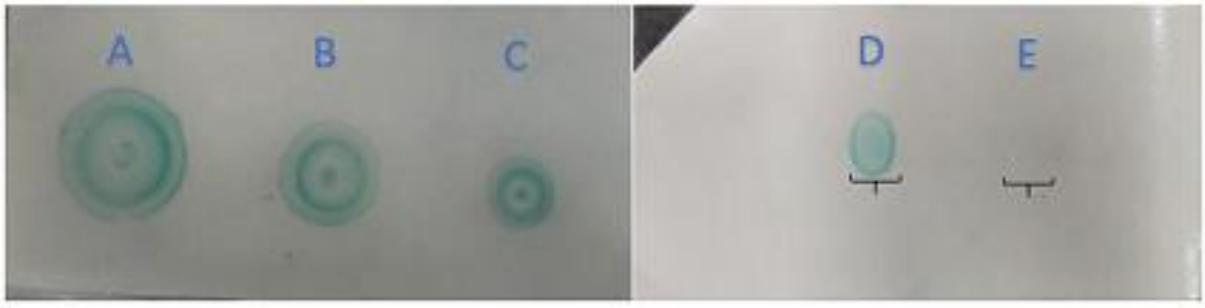


Figure 4. The analysis of the samples after adding the substrate was evaluated using the dot blot method, in volumes of **1(A)**, **2(B)** and **3(C)** microliters, sample **(E)** non-transferable bacteria, sample **(D)** negative control.

sonication. Immediately following the application of ultrasound, the sediment and supernatant samples were separated by centrifugation. Both fractions were subjected to SDS-PAGE analysis to assess protein solubility. The SDS-PAGE results demonstrated that the gel electrophoresis of the supernatant and sediment samples was conducted at the optimal temperature of 22°C and the optimal induction period of 16 hours. This indicated that some of the proteins were soluble, while a significant portion of the protein remained insoluble (Figure 5). To further substantiate the presence of the recombinant proteins, Western blot analysis was conducted. In this method, the protein bands obtained from the SDS-PAGE were transferred onto nitrocellulose paper in conjunction with a protein marker (prestained, Thermo Scientific). As illustrated in the accompanying figure, the Western blot results corroborated the presence of the recombinant proteins, as evidenced by the specific bands aligning with the molecular weight marker. The combination of SDS-PAGE and Western blot analyses provided compelling evidence for the successful purification and presence of the LipL41 and OmpL1 recombinant proteins (Figure 6).

3.3. Evaluation of Protein Concentration Using the Bradford Method

To ascertain the concentration of the extracted LipL41 and OmpL1 recombinant proteins, the Bradford method was utilized. The absorbance of the standard sample at varying concentrations was quantified using a spectrophotometer, and the results are presented in Table 2. The standard curve was constructed using the aforementioned values, with absorbance (A595) on the y-axis and protein concentration (µg/ml) on the x-axis. In order to calculate the concentration of the unknown protein sample, it is necessary to absorb the measured protein sample and then insert the absorbance value into the equation of the standard curve. The resulting concentration was divided by the sample volume (20 microliters) to determine the recombinant protein concentration in micrograms per milliliter. The Bradford method was employed to ensure accurate quantification of the extracted recombinant proteins, thereby providing essential data for subsequent analyses and experiments.

$$0.518 = 0.0329X + 0.056$$

$$X = 14.04 \div 20 = 0.7\ \mu g/ml$$

which is based on the formation of a complex between Coomassie Brilliant Blue G-250 and the LipL41-OmpL1 protein. The interpretation of the results is presented in the form of a table in (Figure 7).

3.4. ELISA Evaluation of Specific IgG Antibody Against Recombinant Antigen in Mice

An enzyme-linked immunosorbent assay (ELISA) was employed to evaluate the humoral immune response in BALB/c mice. The specific IgG antibody against LipL41 and OmpL1 recombinant proteins was quantified using the indirect ELISA method. In this method, the recombinant proteins were conjugated with anti-mouse rabbit horseradish peroxidase (HRP) antibody in the well of the enzyme-linked immunosorbent assay (ELISA) kit. The optical absorption of the samples was quantified using an ELISA device at a wavelength of 450 nm, and the results were compared with those of the control groups. Notable observations include: 1) The antibody titer in the antigen group with adjuvant remained consistently elevated until the conclusion of the blood collection period. 2) The presence of the adjuvant resulted in a significantly enhanced antibody response in comparison to that observed in the recombinant protein alone. At day 50, the OMPL-1-LipL41 with adjuvant group exhibited sustained elevated antibody levels, whereas the OMPL-1-LipL41 alone group demonstrated a decline until day 60. 4) Antibody production was not significantly elevated in the adjuvant and PBS groups. In contrast, the antigen-only group demonstrated a notable decline in antibody levels on the 50th and 60th days of blood sampling. These findings indicate that the incorporation of an adjuvant enhances the stability and longevity of the immune response, resulting in a more consistent antibody production profile compared to immunization with the recombinant antigen alone. The data underscore the necessity of incorporating an adjuvant into vaccination strategies to achieve a robust and sustained immune response.

3.5. ELISA Analysis of Antibody Titers Against Recombinant Antigen

The antibody titers against the recombinant OMPL-1-LipL41 protein were quantified at various time points using enzyme-linked immunosorbent assay (ELISA). The data for each group are presented in tabular form below. The ELISA results substantiate the assertion that the

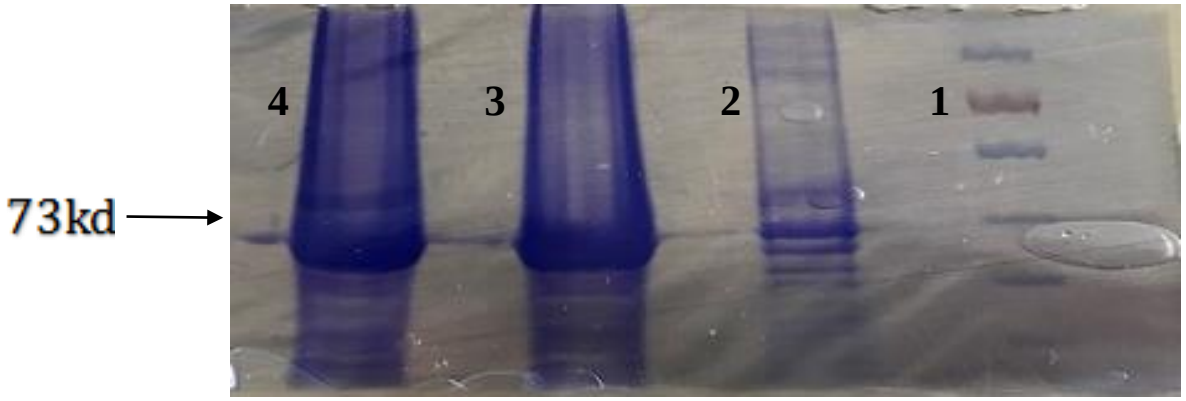


Figure 5: SDS-PAGE results of checking protein solubility after cell lysis with a sonicator at 22 degrees. Well 1: molecular weight marker, Well 2: Lipl41, OMPL1 soup after centrifugation, after cell lysis, Well 3: Sedimentation of Lipl41, OMPL1 after centrifugation, after cell lysis, Well 4: Sedimentation of Lipl41, OMPL1 after centrifugation, after cell lysis.

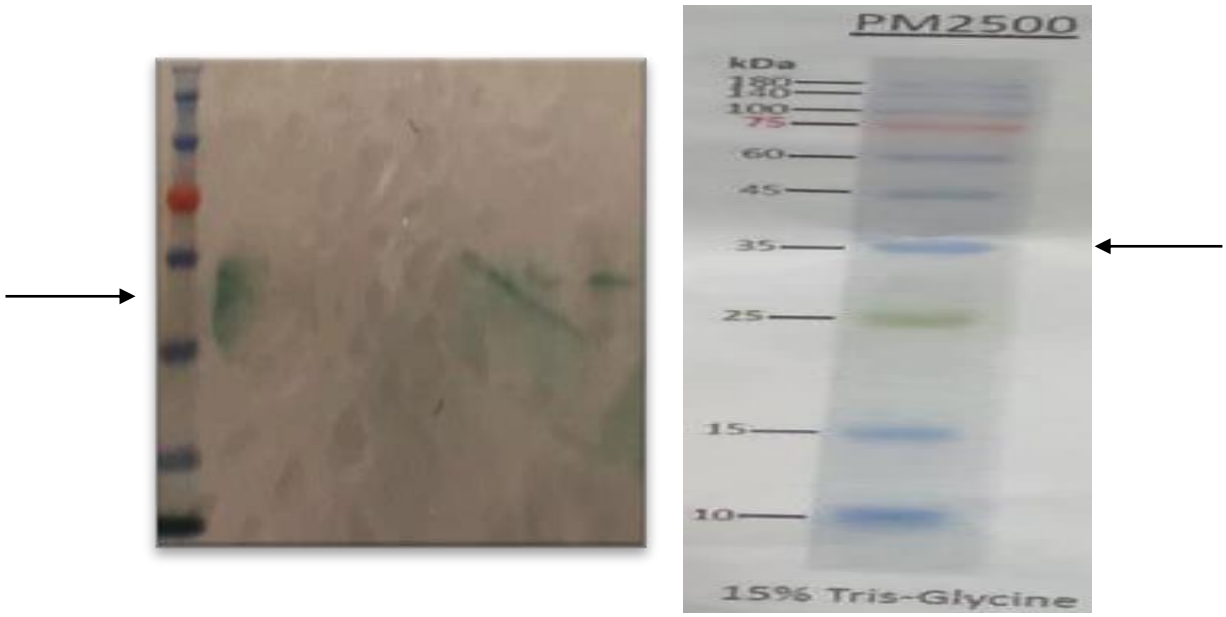


Figure 6. Western blotting results and confirmation of LipL41-OmpL1 recombinant protein.

Table 2. Absorbance of Standard Protein Concentrations

Concentration (µg/ml)	Absorbance (A595)
0	0.000
10	0.125
20	0.250
40	0.500
60	0.750
80	1.000
100	1.250

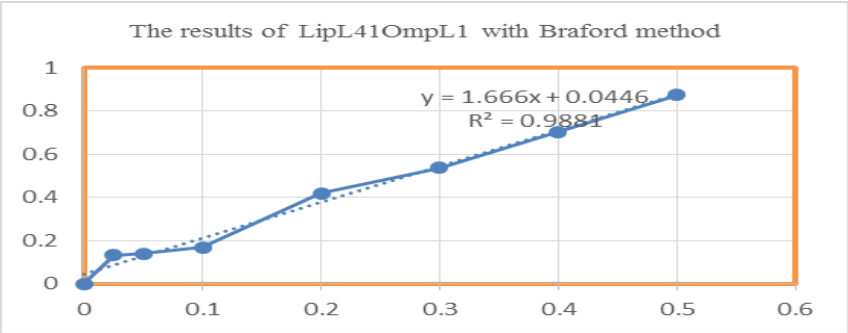
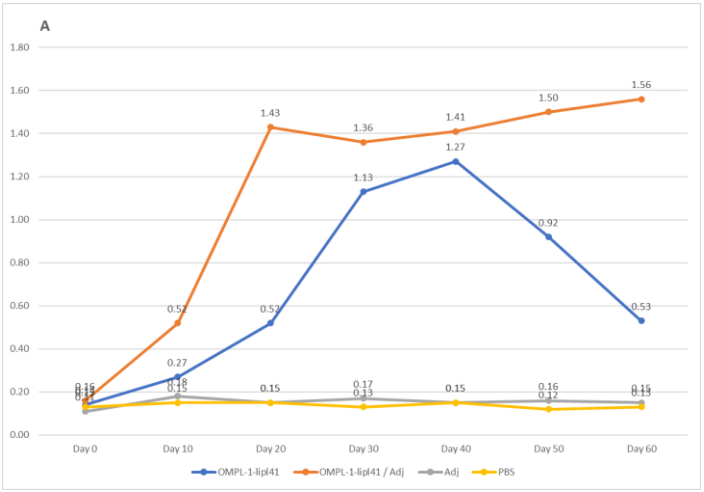
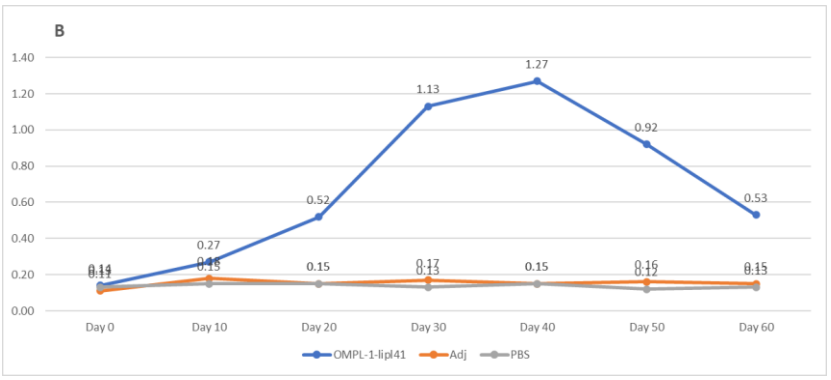
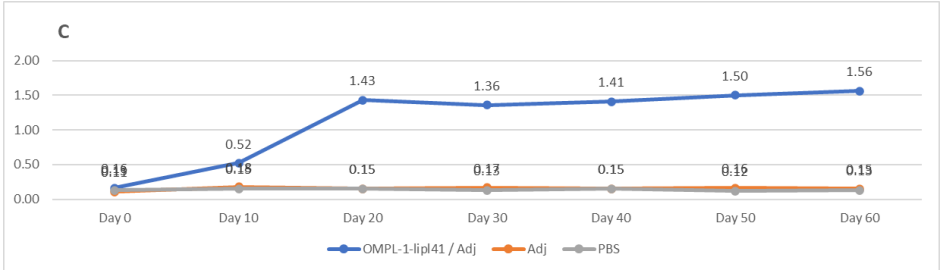


Figure 7: The results of evaluating the concentration of extracted proteins with the Bradford method

recombinant LipL41 and OmpL1 proteins are highly antigenic and capable of eliciting a robust and sustained immune response, particularly when formulated with an adjuvant and at elevated protein-specific antibody levels. This is a recombinant process. The data were subsequently entered into an Excel spreadsheet. Subsequently, the pertinent graph was constructed and subjected to analysis. Based on the graphical representation and examination of light absorption of the samples, as well as comparison with the control groups, it was concluded that the LipL41-OmpL1 recombinant protein has high antigenicity and has the ability to stimulate the immune system (Figure 8).







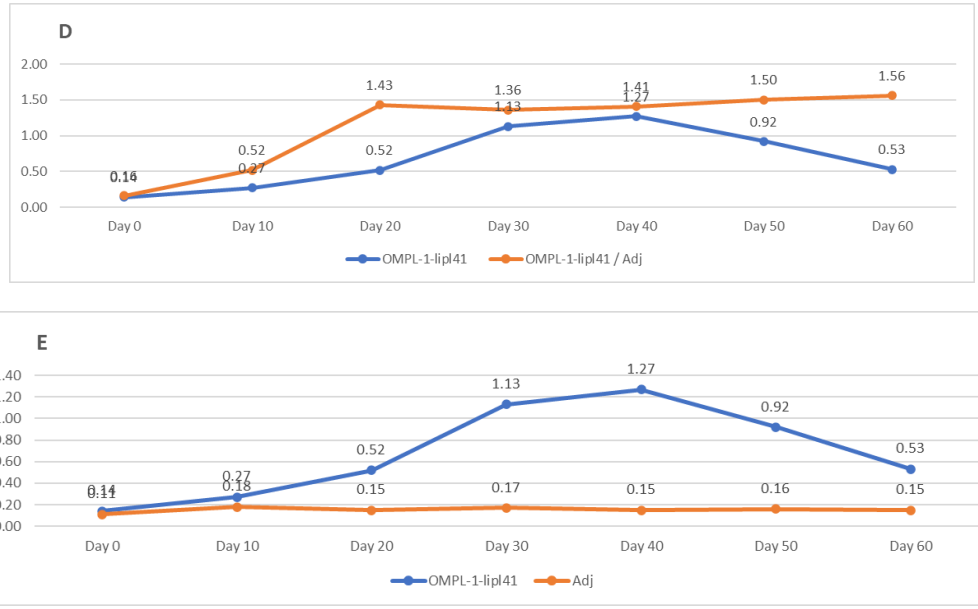


Figure 8. Diagram related to immune system stimulation: **(A)** Based on the graphic display and the examination of the light absorption of the samples as well as the comparison with the control groups, it was shown that the antigenicity stimulation was high based on the injection of the recombinant protein LipL41-OmpL1 with adjuvant. **(B)** Comparing the injection of LipL41-Ompl1 with adjuvant and PBS shows the stimulation of the LipL41-Ompl1. **(C)** High stimulation of the immune system after injection with LipL41-Ompl1/Adj compared to adjuvant and PBS. **(D)** Stimulation of the immune system after injection with LipL41-Ompl1 compared to injection with LipL41-Ompl1/Adj. **(E)** Comparing the injection of LipL41-Ompl1 and Adj.

4. Discussion

Leptospirosis represents a significant public health concern in humid regions of Iran, underscoring the need for research aimed at the development of vaccines and rapid diagnostic tools. The bioinformatics software analysis of the fusion protein comprising LipL41 and OmpL1 recombinant proteins revealed that these two proteins are stable and protective across all generations of pathogenic Leptospira. The LipL41-OmpL1 fusion protein has been demonstrated to elicit cross-immunity and is not serovar-specific. Prior research has also indicated the efficacy of LipL41 and OmpL1 in stimulating the immune system and their potential as diagnostic tools (9, 15-18). In this study, the structure of the LipL41-OmpL1 fusion protein was designed using bioinformatics software, and the optimal conditions for its induction were determined. The purification of the protein was accomplished through the use of a cost-effective method, namely dialysis in the presence of 1-8 M urea (19). Western blot analysis confirmed that the purified protein was a 73 kDa single band, which was consistent with the expected size of the recombinant protein. It has been demonstrated that LipL41 can induce high levels of humoral immunity and antibody secretion capacity (21, 22). Furthermore, the conservation of the LipL41 gene among different strains of Leptospira indicates its potential for use in diagnostic methods such as ELISA and diagnostic PCR. Additionally, LipL41 has been identified as a membrane-binding protein. The combination of LipL41 and OmpL1 has been demonstrated to elicit a synergistic effect on immune system stimulation (23, 24). Similarly, the presence of the LipL32, LipL41, and OmpL1 genes has been evaluated and found to be exclusive to pathogenic serovars. These three antigens are the primary response to hemorrhagic immunity. With regard to the antigenicity of the recombinant protein, the study demonstrated that LipL41-OmpL1 induces robust humoral immunity and antibody secretion in BALB/c mice. The results of the indirect ELISA method indicated that LipL41-OmpL1 is a highly antigenic protein capable of stimulating the immune system. In conclusion, our investigation into the fusion protein LipL41-OmpL1 presents compelling evidence for its potential as a candidate for the treatment of leptospirosis. Through bioinformatics analysis and experimental validation, we have demonstrated the stability and protective attributes of LipL41-OmpL1 across various generations of pathogenic Leptospira. It is noteworthy that our findings indicate that LipL41-OmpL1 induces cross-immunity, rendering it effective against multiple serovars and demonstrating its versatility as a vaccine candidate. Moreover, our findings illustrate the immunogenicity of LipL41-OmpL1, as evidenced by its capacity to elicit robust humoral immunity and antibody secretion in experimental models. In the study conducted by Haji et al. in 2016, the results indicated the widespread transmission of Leptospira among the entire herd (27). The findings of Maliki et al. (2013) indicated that cows may play a significant role in maintaining the Pomona strain. In the study conducted by Abdullah Pour et al. in 2009 on

traditional dairy farms in Gilan, the most prevalent Leptospira serovar was identified as Canicola through the MAT serological test (29). The antigenic potency of LipL41-OmpL1, when considered alongside its stability under optimal induction and purification conditions, positions the recombinant protein as a promising tool for both vaccination and diagnostic purposes (23, 25, 26). The observed synergy between LipL41 and OmpL1 in stimulating the immune system further underscores the potential of LipL41-OmpL1 as a pivotal element in the development of leptospirosis prevention strategies. Nevertheless, while our findings are encouraging, further research is necessary to assess the effectiveness of LipL41-OmpL1-based vaccines in preventing leptospirosis in real-world scenarios and to investigate its potential for other diagnostic and therapeutic applications. This study provides a robust basis for further investigation of LipL41-OmpL1 as a potentially valuable asset in the ongoing efforts to combat leptospirosis. It also highlights the continued need for research in this field to address the significant public health challenges posed by this disease.

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Authors' Contribution
Study concept and design: P. KH and M. E.
Acquisition of data: MO. T, M. E and P. KH.
Analysis and interpretation of data: MO. T, MA. T and P. KH.
Drafting of the manuscript: MO. T, F. GH, M. E and P. KH.
Critical revision of the manuscript: M. E and MO. T.
Statistical analysis: MO.T.

Ethics
It is hereby stated that all ethical considerations were taken into account in the preparation of the submitted manuscript.

Conflict of Interest
The authors certify that they have no conflicts of interest.

Data Availability
The data that support the findings of this study are available on request from the corresponding author.

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