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ORIGINAL ARTICLE

# Recombinant outer membrane protein Lipl41 from *Leptospira interrogans* robust immune responses in mice model

**Narges Golab[1], Pejvak Khaki[2*], Majid Tebianian[3], Majid Esmaelizad[4], Naser Harzandi[1]**

*[1]Department of Microbiology, Karaj Branch, Islamic Azad University, Karaj, Iran*
*[2]Department of Microbiology, Razi Vaccine and Serum Research Institute, Agricultural Research, Education and Extension Organization (AREEO), Karaj, Iran*
*[3]Department of Immunology, Razi Vaccine and Serum Research Institute, Agricultural Research, Education and Extension Organization (AREEO), Karaj, Iran*
*[4]Department of Biotechnology, Razi Vaccine and Serum Research Institute, Agricultural Research, Education and Extension Organization (AREEO), Karaj, Iran*

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## ABSTRACT

**Background and Objectives:** Leptospirosis is an infectious zoonotic disease that can result in severe complications. It is widespread, especially in hot and humid climates such as the northern region of Iran. The immune responses to leptospirosis are multifaceted. Lipl41 is an outer membrane protein that is expressed during infection and is highly conserved among pathogenic species. This makes it a good candidate for diagnosis and induction of specific immune responses. The aim of the present study was to evaluate immune responses against recombinant Lipl41 in mice.

**Materials and Methods:** After immunizing of different groups of mice with recombinant Lipl41 (rLipl41), the levels of specific antibodies and cytokine profiles interferon-gamma/ interleukin-4 (IFN-γ/IL-4) were measured.

**Results:** The results revealed that rLipl41 showed a significant increase in antibody levels compared with the control groups ($P< 0.05$). Although the level of IL-4 in the groups that received Lipl41 was similar to that in the other control groups, the IFN-γ levels showed a significant increase ($P<0.05$).

**Conclusion:** It has been concluded that recombinant Lipl41 protein could strongly stimulate specific immune responses and be considered a potential candidate for vaccine development and diagnostic research.

**Keywords:** Leptospirosis; Immunization; Enzyme-linked immunosorbent assay; Humoral immunity; Cellular immunity

## INTRODUCTION

Leptospirosis is a major zoonotic disease caused by *Leptospira interrogans*, which is spread worldwide (1, 2). It is transmitted via direct or indirect contact with the urine of reservoir animals, including rodents, cattle and wild animals (3). The clinical presentations are varied and range from mild symptoms, to more severe cases (1, 3). The number of cases reported annually exceeds 500,000 (4). Despite the morbidity and mortality associated with infection, diagnosis remains a challenge and the global prevalence of this disease is probably higher than estimated (5, 6). *Leptospira* have the ability to persist in both animals and hu-

*Corresponding author: Pejvak Khaki, Ph.D, Department of Microbiology, Razi Vaccine and Serum Research Institute, Agricultural Research, Education and Extension Organization (AREEO), Karaj, Iran. Tel: +98-2634570038-46 Fax: +98-2634552194 Email: khakipejvak53@gmail.com; P.khaki@rvsri.ac.ir

mans in spite of immune responses during the course of infection. Furthermore, it appears that *Leptospira* can evade innate and adaptive immunity, which makes it difficult to eliminate the pathogen (2, 7). It is noteworthy that the bulk of the immune response to this pathogen is humorally mediated and includes opsonization of the bacteria ensued by phagocytosis by macrophages and neutrophils (7, 8). Vaccination is considered to be the most effective preventive measure against Leptospirosis. Currently, the only available Leptospiral vaccine is the heat killed *Leptospira* (bacterins) which has been associated with variable side effects (9). In addition, these particular vaccines only provide serovar-specific protection (8, 9). For this reason, many researchers have been focused on the outer membrane proteins (OMPs) in recent years (10). OMPs play important roles in attachment and invasion during the preliminary stages of infection and have been considered suitable candidates for the development of numerous vaccines and diagnostic kits (9, 11). However, none of these proteins have proved the ability to confer cross-protection (10, 11). LipL41 (41kD), one of the major Leptospiral outer membrane proteins is highly conserved among pathogenic *Leptospira* and is absent in saprophytic species (12-14). It has been indicated that Lipl41 is expressed over the course of infection in mammalian hosts (15, 16) and found to be co-expressed with a chaperon protein, Lep, which helps its expression (17, 18). Notably, its high degree of immunogenicity, both humoral and cellular, have made it the target of a number of studies (13, 14). It may have the potential to be used in producing vaccines or diagnostic kits in the future (15, 16, 19). Regarding the epidemiology of Leptospirosis in Iran, it is a major health problem in the northern region of the country, which experiences heavy rainfall and where many of the people are farmers (5). Based on our previous study on the expression and purification of recombinant Lipl41(rLipl41) from local serovars of pathogenic *Leptospira* (20), the purpose of this study was to extend our studies on the immunogenicity of rLipl41 to establish whether this antigen could be used to generate immune responses in BALB/c mice.

## MATERIALS AND METHODS

**Design and construction of recombinant Lipl41.** The *Leptospira* Lipl41 r protein was made according to our previous study (20). Briefly, the Lipl41 protein sequences from prevalent local pathogenic *Leptospira* serovars and other countries were assessed and compared with bioinformatics tools from NCBI databases. Codon optimization was carried out on the sequences of the Iranian serovars. On the basis of polymorphisms, the Lipl41 protein sequence among local serovars was used to design the construct.

**Expression and purification of recombinant Lipl41.** Lipl41 protein was subcloned into a pET32a+ expression vector. After that, transformation into to *Escherichia coli* BL21 (DE3) was performed. In order to achieve optimal expression of 6×His-tagged rLipl41, different incubation temperatures and various concentrations of isopropyl Beta-D-thiogalacto pyranoside (IPTG, Sigma-Aldrich, Korea) were used. In the next phase, it was purified by denaturation using serial concentrations of urea (Merk, Germany); then, confirmation was performed using western blot (Paya Pajoohesh, Iran) and it was dialyzed against PBS overnight (1:1000) at 4°C, PH 7.4. The Protein concentration was quantified by the Bradford protein assay.

**Immunization of mice.** Fifty female BALB/c mice 7-8 weeks old weighing about 25 ± 5 g were provided by the Razi Vaccine and Serum Research Institute and randomly divided into five groups (n=10 per group). The immunization of animals was performed as depicted in Table 1. For immunological evaluation of rLipl41, the mice were immunized with rLipl41 subcutaneously three times at 2-week intervals with or without adjuvant. The vaccine group were immunized twice at 2-week intervals by subcutaneous injection of Razi inactivated trivalent *Leptospira* vaccine consisting of *Leptospira canicola, grippotyphosa, sejroe hardjo.* The control groups were inoculated with PBS and complete Freund's adjuvant (Razi Vaccine and Serum Research Institute, Iran) by the same procedure table. The mice were bled by phlebotomy of the ocular orbital vein for 6 times at 10-day intervals (10-60 days). The samples were centrifuged at 5000 rpm for 5 min and the sera were collected and stored at -20°C for further analysis.

**Ethics statement.** The study protocol was approved by the Animal Ethics Committee of the Razi Vaccine and Serum Research Institute, Karaj, Iran (Agreement number: 12-18-18-106-96045-961023).

**Table 1.** Time table of immunization with Lipl41 recombinant protein in mice.

| Group | Number | Dose | Prime (0 Day) | Boost 1(14 Day) | Boost 2 (28 Day) |
|---|---|---|---|---|---|
| Neg. control | n=10 | 100 µl | PBS | PBS | PBS |
| Adj. control | n=10 | 50 µl | CFA | CFA | CFA |
| Lipl41+ Adj. | n=10 | 20 µg | rLipl41+ CFA (50:50) | rLipl41+ IFA (50:50) | rLipl41+ IFA (50:50) |
| Lipl41 | n=10 | 20 µg | rLipl41 | rLipl41 | rLipl41 |
| Vaccine | n=10 | 50 µl | *Leptospira* vaccine | *Leptospira* vaccine | ---------- |

CFA= Complete Freund's Adjuvant IFA= Incomplete Freund's Adjuvant

**Evaluation of specific antibodies against LipL41 by Indirect ELISA.** The levels of specific antibodies were evaluated by indirect ELISA and rlipl41 protein were used as capture antigens. The ELISA was optimized with different concentrations of capture antigens, sera and conjugated antibodies. A 96- well plate (Jet Biofil, China) was coated with 100 µL of rLipl41 dissolved in the coating buffer, 0.05 M bicarbonate (Merk, Germany) with pH 9.6 and left overnight at 4°C. The free-binding sites were blocked by 3% bovine serum album (BSA (for one hour in 37°C. The wells were washed three times with PBST (PBS with 0.05% Tween 20) and samples were added at a 1:50 dilution and incubated for 1 h at 37°C. Following three washing phases, 100 µl of rabbit anti-mouse HRP conjugated IgG antibody (Sigma. Germany) (1:15, 000) was added to each well and incubated for 1 h in darkness at 37°C. Then, plates were washed five times. 100 µL of substrate solution of tetra methyl benzydene (TMB, Sigma, USA) was added into each well. The reaction was stopped with 100 µl of 1M $H_2SO_4$, and the absorbance was read at 450 nm by an ELISA reader (Bio Tek, Elx 800).

**Cytokine measurement.** Two weeks after the last immunization, 5 mice from each group were sacrificed and their spleens were aseptically removed. After erythrocyte lysis, the single-cell suspensions of splenocytes were prepared and $3\times10^6$ cells were cultured in RPMI-1640 (Sigma, USA) containing 10% fetal calf serum in the presence of 5µg of r-LipL41 and incubated at 37°C in an atmosphere of 5% $CO_2$ for 60 h. Then, the culture supernatant was aspirated and the concentrations of IFN-γ and IL-4 were measured by commercial ELISA kits (Karmania Pars Gene, Iran) according to the manufacturer's instruction.

**Statistical analysis.** The statistical analyses were performed using Prism® software (GraphPad Software, version 6.0). The means of two independent groups were compared with the Independent Samples t Test. Data were expressed as mean ± SEM. The threshold of significance was set at (P values <0.05).

## RESULTS

**LipL41 recombinant protein design and construction.** Phylogenetic analysis showed 0.8 percent divergence between serovars in Iran and the other countries. Multiple Alignment of LipL41 protein sequences identified 20 different amino acid substitutions, 8 of which were observed among some local serovars.

**Expression and purification.** A high expression of Lipl41 r protein was achieved using the pET32a+ vector and *E. coli* BL21 in an insoluble form, as an inclusion body, and was purified after solubilisation in 8 M urea. The yield of purified recombinant protein was estimated to be 107.78 mg/L.

**Humoral immunity response assessment.** In order to assess of humeral immune response, the mice sera were collected on days 0 and 10, 20 to 60 and the levels of specific antibody against rLipL41 were measured by indirect ELISA, (Fig. 1). The data revealed a gradual elevation in the specific antibody levels within the group had immunized with the Lipl41 alongside of adjuvant. This increase commenced on the 10th day, peaked on the 40$^{th}$ day, and maintained a relatively steady state for the subsequent 60 days. The same pattern was observed, to a lesser extent, in the mice that had immunized with rLipl41 in the absence of adjuvant, but the difference between these two groups was completely significant from the 20th day onwards ($p<0.05$). The group injected by the heat killed vaccine had a minimum level of specific antibody against

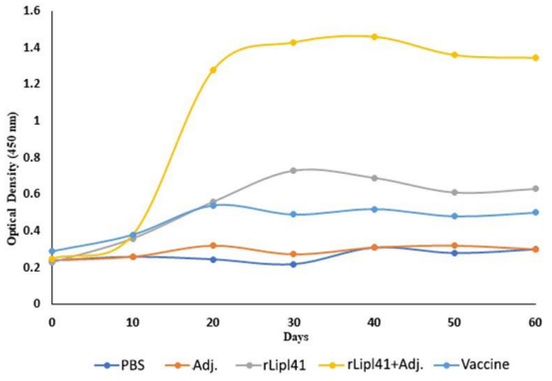


**Fig. 1.** Levels of LipL41 antibody production in BALB/c mice were detected by indirect ELISA.

Lip 41 and after a weak increase on the tenth day, it remained in an almost static state from the 20th day onwards.

The mice were immunized subcutaneously using PBS and Freund's. adj (controls) and the inactivated Trivalent *Leptospira* vaccine (Vaccine control) and Lipl41 (target). Animals were bled from the eyes prior to each immunization.

**Evaluation of cytokine pattern.** To evaluate the cellular immune response, the levels of IL-4 and INF-γ were measured in cultured supernatants after re-stimulation of splenocytes with recombinant antigen. As illustrated in Fig. 2, the IL-4 level in mice that had immunized rLipl41with or without adjuvant increased very slightly, but there was no significant difference in contrast to other groups ($p>0.05$). However, the levels of IFN-γ increased extensively in mice immunized with rLipl41 protein in the presence of adjuvant in comparison with the vaccinated and control groups ($P < 0.05$) in Fig. 3.

Splenocytes from immunized mice were isolated 14 days after the last administration. The production of IL-4 and IFN-γ in splencoyte cultures of immunized

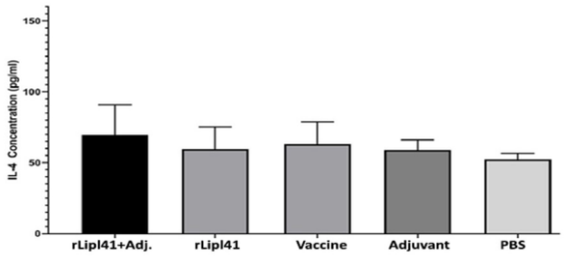


**Fig. 2.** Evaluation of IL-4 levels produced by splenocytes from different immunized mice.

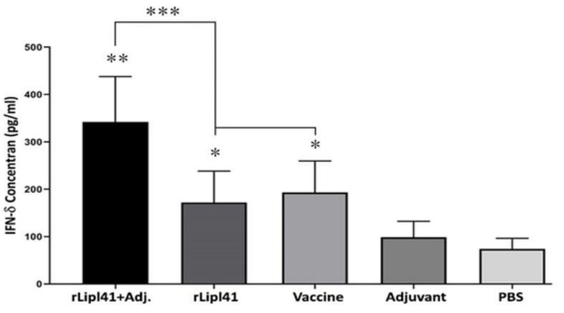


**Fig. 3.** Evaluation of IFN-γ levels produced by splenocytes from different immunized mice.
*, ** indicates significant increases in IFN-δ levels compared to adjuvant and PBS control groups $p<0.05$ and $p<0.001$ respectively). *** indicates significant change ($p < 0.001$) compared to antigen and vaccine groups.

animals were assessed after stimulation with Lipl41 recombinant protein. All mentioned experiments were repeated at least twice and data presented were the mean measurements ± SD. (statistical significances: $p < 0.05$), as shown in Table 1.

## DISCUSSION

The surface proteins of pathogenic strains are often involved in significant aspects of virulence and host immune responses to the organism (21). Therefore, in addition to enabling the pathogen to attack or evade the host, surface proteins could also facilitate the development of host immunity if used as vaccines (22, 23). Thus, an immunogenic surface protein could be considered as a potential target to produce antibodies that could protect against infection (23). The Leptospiral outer membrane proteins, particularly highly conserved antigens, may possess significant advantages over current vaccinal antigens (8, 11). Moreover, these antigens can be considered as attractive candidates in the development of serological methods such as ELISA (15, 24, 25). Recent research has enhanced our understanding of LipL41, one of the key leptospiral OMPs, which only presents in pathogenic *Leptospira* species. It could play an important role in virulence and pathogenesis (14, 19). Given that potential cross-protective immunogens usually hold conserved sequences. In our previous study, for the first time the local sequences of this antigen were selected and used for expression and purification of Lipl41 recombinant protein (20).

Considering that no document has been conducted to evaluate the Lipl41 antigen from local serovars in Iran, the objective of this study was the assessment of immune responses against LipL41 recombinant protein in mice. Both humoral and cell mediated immunity (CMI) are involved in the immune response to leptospiral infection (26, 27). Several studies have revealed the humoral immune response induced by certain leptospiral surface exposed OMPs (2, 13). In general, antibodies play a pivotal role in the phagocytosis of pathogenic variants of *Leptospira* (8, 28). As well as humoral responses, cell-mediated immunity is known to play an important role in the control or clearance of intracellular bacterial infections (26). Several studies have demonstrated that one of the most effective ways for the assessment of cellular immune responses is to evaluate cytokines released by T helpers (Th1 and Th2) cells (27, 29, 30). Th1 cells commonly secrete IFN-γ, whereas Th2 cells usually secrete IL-4, and these particular cytokines can be measured in the immune response to a specific antigen by stimulated splenocytes (31-33). According to our results, a group of mice that had received rLipl41 antigen with adjuvant produced a significant amount of specific antibodies. However, a similar pattern of antibody increase was seen to a smaller extent in the group that had received the antigen without adjuvant. This showed that the Lipl41 recombinant protein could stimulate humoral immune responses. Our study findings were in agreement with those conducted by others (12, 13, 17). Here, in this experiment, splenocytes from rLipL41-immunized mice released a large amount of IFN- γ compared to other control groups. The slight increase in the levels of IFN- γ in the vaccine group indicated that the expression of Lipl41 in vaccinal strains was not as high as the level that would be expected from injecting the pure form. Due to the use of the inactivated vaccines, certain antigens, such as Lipl41, might have been present on the surface or could have been lost due to the production processes. However, we did not find any significant differences in levels of IL-4 production between rLipL41 immunized animals with other groups. These findings were consistent with previous studies that had reviewed that the immune response against Leptospiral antigens was mainly related with Th1-mediated immunity and IFN- γ production (13). Also, Lin et al. in 2011 confirmed that LipL41-immunized mice released large amounts of IFN- γ, but not IL-4, compared to control cells (12). In addition to that, several leptospiral OMPs have been investigated as possible vaccine candidates because they can elicit immunity in animal models; for instance, a study on vaccines used LipL32 and Loa22 as antigens in combination and alone in mice. The cytokine profiles in response to restimulation with LipL32 or Loa22 alone showed a significant increase in the levels of IFN-γ, but the levels of Th2 cytokines IL-4 and IL-10, produced by stimulated splenocytes, were extremely low. These results were in agreement with ours (34), whereas another study showed all the OmpA-like domain outer proteins tasted induced significantly higher levels of both Th1 (IFN- γ) and Th2 (IL-4 and IL-10) cytokines in immunized animals (35). Taken together, based on our findings, the immunogenicity induced by LipL41 revealed a Th1 response, through the production of INF-γ, and also a Th2 response, as demonstrated by significant antibody secretion, so it indicated a mixed Th1 and Th2 responses.

## CONCLUSION

In general, according to these results, it can be concluded that rLpl41 from local *Leptospira* serovars may be able to stimulate secretion of IFN- γ in addition to a significant humoral immune response, both of which are very effective in immune responses against leptospiral infection. Thus, we believe that this antigen could be used in studies related to vaccine design or the development of diagnostic methods for this disease.

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