ORIGINAL ARTICLE

Veterinary Research Forum. 2024; 15 (10): 565 - 573
doi: 10.30466/vrf.2024.2017700.4099
Journal Homepage: vrf.iranjournals.ir

Veterinary Research Forum

Immunological characterization of recombinant outer membrane Loa22 protein of local pathogenic *Leptospira* serovars

Mehdi Gharakhani[1], Mohammad Faezi Ghasemi[1], Pejvak Khaki[2]*, Majid Esmaelizad[3], Majid Tebianian[4]

[1] Department of Microbiology, Faculty of Basic Sciences, Lahijan Branch, Islamic Azad University, Lahijan, Iran; [2] Department of Microbiology, Razi Vaccine and Serum Research Institute, Agricultural Research, Education and Extension Organization (AREEO), Karaj, Iran; [3] Department of Research and Development, Razi Vaccine and Serum Research Institute, Agricultural Research, Education and Extension Organization (AREEO), Karaj, Iran; [4] Department of Immunology, Razi Vaccine and Serum Research Institute, Agricultural Research, Education and Extension Organization (AREEO), Karaj, Iran.

Article Info

Article history:

Received: 12 December 2023
Accepted: 05 June 2024
Available online: 15 October 2024

Keywords:

Immune responses
Leptospira
Leptospirosis
Recombinant Loa22 protein

Abstract

Leptospirosis is a worldwide zoonotic disease caused by pathogenic *Leptospira* spp., often occurring in tropical and subtropical regions. Focusing on development of rapid diagnostic methods to facilitate early diagnosis and a universal vaccine are the main critical issues to overcome the burden of leptospirosis. Here, we have studied the immunogenic potential of prepared recombinant Loa22 protein (rLoa22) of local pathogenic *Leptospira* species in mice and its ability to induce humoral and cellular immunity, being further evaluated by analyzing the immunoglobulin G (IgG) subclasses and cytokines produced through immunization. Based on the results, mice immunized with rLoa22/adjuvant and a trivalent vaccine, induced high titers of total IgG. All immunized groups increased IgG1 almost on the same level; but, IgG2a level was significantly higher in the vaccine and rLoa22/adjuvant groups than rLoa22 alone group. Animals immunized with the vaccine produced more interleukin 4 than rLoa22/ adjuvant group. The results of evaluating interferon gamma level showed that the rLoa22/adjuvant and vaccine groups had a significant increase compared to the rLoa22 alone group. The results also demonstrated that the rLoa22 protein, in indirect enzyme-linked immunosorbent assay, was able to detect the anti-*Leptospira* antibodies in mice serum that can be used as a marker in assessing the seroprevalence of leptospirosis and/or in combination with other leptospiral antigens in development of an effective vaccine against leptospirosis.

Introduction

Leptospirosis is a widespread bacterial zoonotic disease and a significant global human and animal health problem. Pathogenic *Leptospira* spp. are the etiological agents of leptospirosis encountered in all geographical areas, particularly in tropical and subtropical regions with high rainfall.[1] Epidemiological investigations have demonstrated that *Leptospira* serovars, including Ictero-haemorrhagiae, Canicola, Hardjo-bovis, Pomona, and Grippotyphosa, are the most prevalent causes of human and animal leptospirosis in Iran.[2]

The disease spreads by the contact of mucous membranes, lesions, or abrasions with water or soil being contaminated through the infected urine of animals or direct contact with the urine of carriers.[1] In humans, symptoms range from a mild influenza-like illness to a severe infection with fever, jaundice, and renal failure (Weil disease), leading to death in 5.00 - 10.00% of all patients or leptospirosis-associated pulmonary hemorrhage syndrome, with a fatality rate of more than 50.00%.[3]

Leptospirosis can be diagnosed using culture (isolation), direct microscopy, serology, and polymerase chain reaction. However, fast and reliable diagnosis of the causative agent is often difficult due to the low rate of isolation of microorganisms from cultures and non-specific clinical manifestations in the early stage of the disease. Therefore, serological diagnosis methods are of great importance.[4,5] Serological diagnosis of leptospirosis, like microscopic agglutination test (MAT) and enzyme-linked immune sorbent assay (ELISA), is the most suitable method for diagnosing leptospirosis.[5,6] The MAT is complicated and time-consuming and because uses live leptospires as antigens, antigen standardization may not

***Correspondence:**

Pejvak Khaki. PhD
Department of Microbiology, Razi Vaccine and Serum Research Institute, Agricultural Research, Education and Extension Organization, Karaj, Iran
E-mail: p.khaki@rvsri.ac.ir

be consistent, and requires biohazard considerations. In contrast, using the ELISA, several samples can be analyzed simultaneously with minimal time and training.[4-6]

Most available ELISA kits utilize antigens derived from a saprophyte serovar (*e.g.*, *Leptospira biflexa*) and have generally been found to have lower sensitivity than MAT because the ELISA antigens do not cover the detection of all infecting serovars and problems of cross-reactivity could not be resolved.[7] To overcome these problems, studies are focused on the development of recombinant outer membrane proteins (OMPs) of pathogenic *Leptospira* serovars that can be potentially useful for a cost-effective, safe, and efficient diagnosis of leptospirosis during the early stage of disease.[8,9] The OMPs are also potential targets capable of stimulating immune defense mechanisms and possess essential epitopes for binding to B and T lymphocytes.[10,11] Evaluating the immune response by measuring the level of antibodies and cytokines following stimulation with the surface antigens can render them ideal recombinant vaccine candidates or the target antigens for serological tests such as ELISA.[8] The immunity to leptospirosis is predominantly humoral when the host immune system triggers an inflammatory response, primarily through the production of cytokines. The T helper (Th) 1 cell-related cytokines like interferon gamma (IFN-γ) stimulate the production of immuno-globulin G (IgG)2a, having a role in the prevention of bacterial spread, and the Th2-dependent cytokines like interleukin 4 (IL-4) induce the expression of IgG1 antibodies, mainly inducing antibody production from B cells.[12,13] The leptospiral Loa22 lipoprotein, formerly known as Lp0222, is among the most abundant surface-exposed OMPs, playing an essential role in pathogenicity being conserved amongst the pathogenic *Leptospira* serovars.[14-16] Therefore, in this study, immunological responses against the prepared recombinant Loa22 (rLoa22) protein were detected, which could serve as a potential vaccine candidate and/or serodiagnostic marker to be tested for leptospirosis.

Materials and Methods

Analysis of the *loa22* sequences. The *loa22* gene sequences were retrieved from 18 Iranian pathogenic serovars of *Leptospira* (GenBank Accession No. OL689841, OM913536-38, and OP038304-17) and compared with the sequences available in NCBI (Accession No. KC311551). The multiple sequence alignment program of MEGA Software (version 7.0; Biodesign Institute, Tempe, USA) was used for the conservation, amino acid substitutions, and other polymorphisms among the serovars. The SignalP 5.0 Server (http://www. cbs.dtu.dk/services/ SignalP/) was used to predict the signal peptides.[17] According to phylogeny and bioinformatics studies of the local sequences, a single-stranded oligonucleotide encoding Loa22 protein with maximum coverage having all pathogenic serovars was considered for *rloa22* gene construction using Gene Designer software (V 2.0; ATUM; Newark, USA).

Prediction of B cell epitopes. Linear B cell epitopes of the Loa22 protein were predicted by BepiPred Linear Epitope Prediction 3.0 running under DTU Health Teach (https://www.healthtech.dtu.dk/) web server using the LM method for prediction of full-length epitopes.[18]

Prediction of T cell epitopes. The prediction of murine T cell epitopes of the Loa22 protein was analyzed by Immune Epitope Database (https://www.iedb.org/) server. For prediction of major histocompatibility complex (MHC)-I epitopes, six different MHC-I alleles (H-2-Db, H-2-Dd, H-2-Kb, H-2-Kd, H-2-Kk, and H-2-Ld), being H-2 super-type representatives, were utilized in the analysis.

To predict the binding of 15-mer epitopes in the binding groove of MHC-II, three different alleles (H2-IAb, H2-IAd, and H2-IEd) were used in the analysis by SMM method. Epitopes with half maximal inhibitory concentration (IC_{50}) value of MHC-I lower than 200 nM and percentile rank of MHC-II >1 were chosen.[19]

Codon-optimized *loa22* gene synthesis. The synthetic gene, lacking signal peptide, codon-optimized for *Escherichia coli* expression (https://www.genscript.com/ gensmart-free-gene-codon-optimization) was then inserted into *NcoI* and *XhoI* restriction sites of pET-32a (+) generating *loa22*-pET32.

Expression and purification of rLoa22 protein. To achieve a high level of the purified recombinant antigen, the expression and purification of the rLoa22 protein were performed as previously described.[20] The BL21 (pLysS) was used as an expression host for transformation. The transformed clones were incubated at 37.00 ˚C, with shacking (200 rpm). Isopropyl β-D-thiogalactopyronoside (IPTG; Sigma-Aldrich, St. Louis, USA) was added when the culture reached an absorbance (Epoch2; BioTek, Winooski, USA) of 0.70 ± 0.10 at 600 nm for expression of 6His fusion protein at 37.00 ˚C. After 5 hr of induction, the bacterial cell pellet was harvested and re-suspended in 1.00 mL of 1X phosphate-buffered saline (PBS) *per* 1.00 g pellet. The suspension was sonicated (Hielscher Ultra-sound Technology, Brandenburg, Germany) on ice five times for 1 min at intervals of 1 min in the presence of phenyl methyl sulfonyl fluoride (Merck, Darmstadt, Germany) to inhibit protease activity. Production of the recombinant protein, either in solubilized or insolubilized form, was analyzed by 12.00% sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE; Hercules, USA). The 6×His-tagged rLoa22 protein was purified from the soluble fraction by Ni-NTA affinity chromatography under native conditions according to the manufacturer's protocol.[21] To confirm the correct expression, the cell lysates and purified rLoa22 protein were subjected to western blot analysis with a horseradish peroxidase (HRP) conjugated anti-His antibody (Sigma-Aldrich).

Purified protein concentration determination. The concentration of purified rLoa22 protein was measured at 595 nm (25.00 ˚C) using the colorimetric Bradford assay. The bovine serum albumin (BSA; Sigma-Aldrich) standard curve was constructed with a serial dilution from 0.00 to 20.00 mg mL^{-1} on GraphPad online server regression tools (www.graphpad.com) by linear regression calculator and plotted *versus* BSA.

Mice immunization. The 6- to 8-week-old (20.00 - 25.00 g) female inbred BALB/c mice were obtained from the Razi Vaccine and Serum Research Institute, Karaj, Iran, randomly divided into five groups of 8 mice each, and given a week to adapt to the new housing conditions. The Research Ethics Committees of Islamic Azad University, Rasht Branch, Iran, approved (ID: IR.IAU.RASHT.REC. 1401.042) the animal use. To evaluate rLoa22 immunogenicity, the mice were subcutaneously immunized with 10.00 µg rLoa22 protein formulated with and without Freund's incomplete adjuvant. Immunization of mice was performed in three administrations at 2-week intervals. As a positive control, a group of mice was received Trivalent Leptospiral Vaccine (Razi) adjuvanted with alum, containing inactivated local pathogenic *Leptospira* serovars (Canicola, Icterohaemorrhagiae, and Grippotyphosa), with one booster dose. The negative control animals were inoculated with an equal amount of the adjuvant and PBS. The blood samples were collected *via* the retro-orbital venous sinus on day 0 (prior to immunization) and every ten days for two months. The sera were separated by centrifugation (Sigma, Osterode am Harz, Germany) in 4.00 ˚C (5,000 *g* for 10 min) and stored in small aliquots at – 20.00 ˚C.

Recombinant Loa22 protein-specific IgG and its subclasses. The collected sera were evaluated for recombinant Loa22 protein-specific total IgG and its isotypes (IgG1 and IgG2a) using an indirect ELISA. The optimal dilution for both the antigen coating and anti-serum was determined by checkerboard titration. The ELISA microplate (Jet Biofil, Guangzhou, China) was coated with 10.00 mg mL^{-1} of the rLoa22 protein diluted in coating buffer including 0.05 M carbonate-bicarbonate (pH: 9.60; Merck) and incubated at 4.00 ˚C overnight. After three times washing with 1.00x PBS containing 0.05% Tween20 (PBST; Merck), the non-specific sites were blocked with 5.00% BSA at 37.00 ˚C for 1 hr. A volume of 100 µL of diluted sera (1:50 dilution) were added to wells, incubated at 37.00 ˚C for 1 hr, and washed three times with PBST. Horseradish peroxidase-conjugated anti-mouse IgG (Sigma), at a dilution of 1:5,000, was subsequently added to incubate at 37.00 ˚C for 1 hr. For measuring IgG isotypes, the detection of bound antibodies was determined using either anti-mouse IgG1-HRP or IgG2a-HRP conjugates (1:1,000 dilution; Abcam, Waltham, USA). After three times additional washing of wells with PBST, 50.00 µL of the tetramethylbenzidine substrate (Sigma Aldrich) was added to each well and incubated for 10 - 15 min in the dark at room temperature. The reaction was stopped by adding 50.00 µL of 2.00 M H_2So_4 (Merck), and the optical absorbance of samples was read using an ELISA plate reader (BioTek) at a wavelength of 450 nm. Biological replicates of samples from each experimental group were performed at least in triplicates.

Lymphocyte proliferation and cytokine assays. Two weeks after the last immunization, three mice from each group were euthanized, and their spleens were isolated aseptically. A single-cell suspension of splenocytes was prepared by pressing the homogenized spleens through a fine nylon mesh, washed in complete RPMI 1640 (Merck) having 10.00% fetal bovine serum, 0.10% 0.05 M 2-mercaptoethanol, and 2.00% 0.20 M glutamine without antibiotics, and harvested at 300 *g* for 3 min at laboratory temperature. The supernatant fluid was discarded by centrifugation, and the pellet was re-suspended in 1.00 mL of CRPMI containing 10.00 mg mL^{-1} of streptomycin penicillin (Hayyan Pharmaceutical Co., Tehran, Iran) and 100 U mL^{-1} of penicillin (Hayyan Pharmaceutical Co.) and treated for 5 min with 5.00 mL of erythrocyte lysing buffer (0.15 M NH_4Cl, 10.00 mM $KHCO_3$, and 0.10 mM Na_2EDTA) purchased from Merck. The cells were then washed twice before being re-suspended in CRPMI and counted. The number of each cell suspension was adjusted to 3×10^6 cells mL^{-1} and dispensed in 24-well plates (200 µL per well). During the process, three wells were set up for each sample. The antigen (2.00 µg per well) was added in two wells, and another well was used as a control treated with an equal volume of CRPMI, being incubated in a humidified 37.00 ˚C, 5.00% CO_2 incubator. The supernatants were aspirated after 60-hr culture and tested for IL-4 and IFN-γ concentrations using commercial ELISA kits according to the manufacturer's instructions (R&D Systems Inc., Minneapolis, USA).[22]

Statistical analysis. Statistical analysis was performed using GraphPad Prism (version 7.0; GraphPad Software Inc., San Diego, USA). One-way ANOVA was used to evaluate differences between variations in antibody titers. Analysis of variance and Tukey's test were used to compare cytokine production levels. A *p* value less than 0.05 was considered statistically significant.

Results

Sequence analysis. After a comprehensive study of the sequences of *loa22* gene, a dominant pattern with maximum coverage of high-prevalence pathogenic serovars in Iran was selected as a prime sequence. The reference sequence was optimized to the preferred codon usage in *E. coli* and its signal peptide sequence was subtracted. The redesigned sequence (539 bp) was submitted to GenBank® with the accession number of OR134515.

B cell epitopes. Five top linear B cell epitopes and their residue candidates were predicted by the BepiPred default threshold (0.1512) for the complete coding sequence of Loa22 protein in several different positions (Fig. 1).

T cell epitopes. Several MHC-I epitopes were predicted as probable antigens. Among all predicted epitopes by the Immune Epitope Database server, AEGAKKGNI, PEAIADSLN, and AANRNVDVN epitopes had lower IC_{50} (> 200) and percentile rank (> 1.00) values, indicating a solid binding among the epitope and murine MHC-I alleles (Table 1). Similarly, among all predicted core peptides (9-mer) interacted with MHC-II alleles in the complete coding sequence of Loa22 protein, the VKKILNLIL core peptide with four, FRFATSAPQ core peptide with three, and VTFRFATSA and IFYSELRAN core peptides with one potential primary anchor, based on the least percentile rank (> 1.00), had the highest probability of binding to the murine MHC-II alleles (Table 2).

Expression vector construction. The designed sequence was used to construct an expression vector, *loa22*-pET32a (+), by insertion into the NcoI and XhoI restriction sites in a frame with Trx and six-His Tags at the N-terminal end. A protein with a molecular weight of about 38.00 kDa (200 residues) consisting of Trx and six-His Tags (18.50 kDa) and rLoa22 (19.30 kDa) was expected to be expressed. General Biosystems Company (Durham, USA) synthesized the designed gene construct.

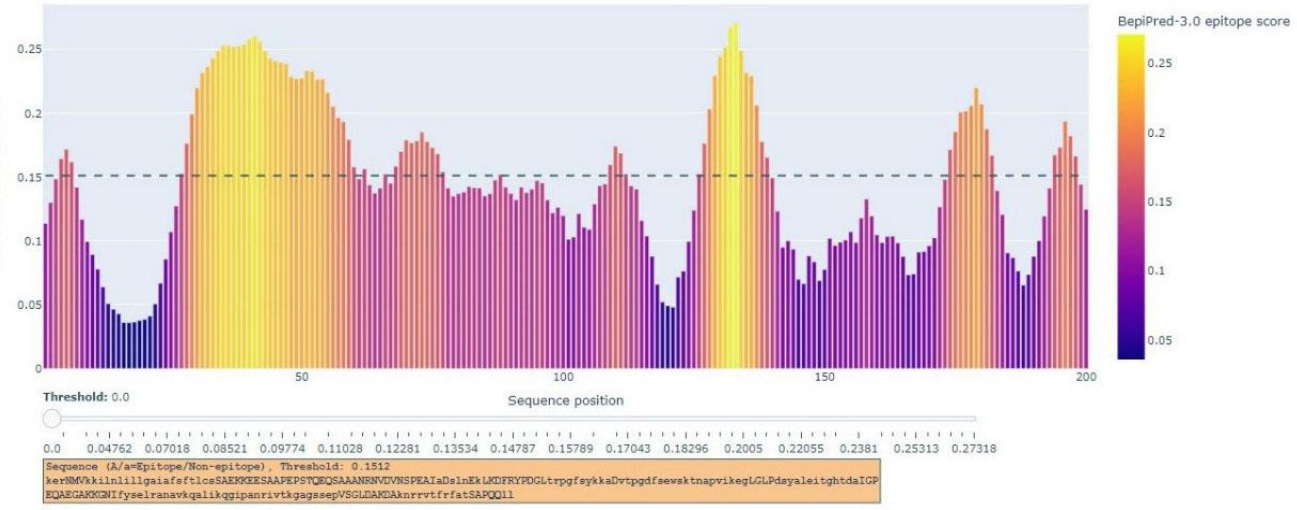

Fig. 1. Graphical results of predicted linear Bcell epitopes of the full lengths Loa22 protein predicted by BepiPred Linear Epitope Prediction 3.0 running using the LM method. The X-axis and Y-axis show antigen sequence position and orientation, respectively. The threshold value is 0.1512. Areas above the threshold (dashed line) predicted as B cell epitopes. Residues with a higher score are shown in yellow.

Table 1. Predicted MHC-I epitopes for Loa22 using Immune Epitope Database server (IC_{50} < 200).

Alleles	Start	End	Length	Peptide	IC_{50} (nM)	Percentile rank
H-2-Kk	111	119	9	AEGAKKGNI	89.83	0.90
H-2-Kk	36	44	9	PEAIADSLN	92.14	0.90
H-2-Kk	74	82	9	SEWSKTNAP	155.04	1.40
H-2-Kk	122	130	9	SELRANAVK	176.78	1.60
H-2-Kk	11	19	9	EESAAPEPS	186.39	1.70
H-2-Db	26	34	9	AANRNVDVN	190.38	0.30

IC_{50}: Half maximal inhibitory concentration.

Table 2. Predicted MHC-II epitopes for Loa22 using Immune Epitope Database server (adjusted rank < 1).

Alleles	Start	End	Length	Core sequence	Peptide sequence	IC_{50} (nM)	Percentile rank
H2-IAb	165	179	15	FRFATSAPQ	RRVTFRFATSAPQQL	189.00	0.55
H2-IAb	166	180	15		RVTFRFATSAPQQLE	195.00	0.64
H2-IAb	164	178	15		NRRVTFRFATSAPQQ	201.00	0.65
H2-IAb	116	130	15	YSELRANAV	KGNIFYSELRANAVK	230.00	0.81
H2-IAb	117	131	15		GNIFYSELRANAVKQ	231.00	0.83
H2-IAb	119	133	15		IFYSELRANAVKQAL	231.00	0.83
H2-IAb	118	132	15		NIFYSELRANAVKQA	236.00	0.86
H2-IAb	163	177	15	VTFRFATSA	KNRRVTFRFATSAPQ	207.00	0.66
H2-IAb	115	129	15	IFYSELRAN	KKGNIFYSELRANAV	232.00	0.83

IC_{50}: Half maximal inhibitory concentration.

Expression and purification of rLoa22 protein. The cells induced with 0.10 mM IPTG for 5 hr at 37.00 ˚C expressed a significant fraction of the protein in the soluble form rather than sonicated precipitates (Figs. 2A and 2B). The best conditions for purification of the rLoa22 protein by affinity chromatography with Ni-NTA agarose included a three-time binding process, five times washing in the presence of 25.00 mM imidazole to remove non-specific contaminants, and elution buffer with a pH of 7.40 and a 0.30 M imidazole concentration for maximum efficiency.

Purified rLoa22 protein concentration. The known line equation based on the BSA standard solution curve was y = 0.0375x + 0.0161 with a regression of 0.9911. The concentration of purified rLoa22 protein in the best conditions was approximately 1.00 mg mL^{-1} by Bradford assay.

Western blotting analysis and SDS-PAGE. The results of SDS-PAGE showed a distinct band at the expected position in the induced cells and purified rLoa22 (Fig. 2C). The result of western blotting with anti-His tag HRP conjugated antibody also showed that the rLoa22-*His-tag* fusion protein had a clear band of approximately 38 kDa (Fig. 2D).

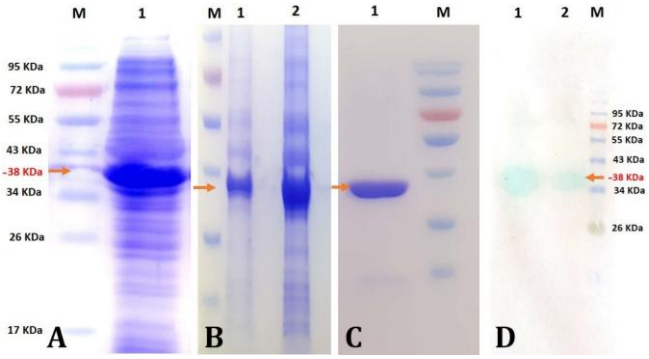

Fig. 2. Expression and purification of rLoa22 protein, sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting analysis. **A)** Expression of recombinant Loa22 (rLoa22) protein using optimized expression parameters such as 0.10 M IPTG, 37.00 ˚C pre- and post-induction temperatures and 5 hr post-induction incubations. Lane 1: Over-expressed rLoa22 band was at the expected size (~38.00 kDa); M: Pre-stained protein size marker (Thermo Fisher Scientific, Vilnius, Lithuania). **B)** The SDS-PAGE analysis of the total cell lysate. Lane 1: Pellet; Lane 2: Supernatant; M: Pre-stained protein size marker (Thermo Fisher Scientific, Vilnius, Lithuania). **C)** The SDS-PAGE analysis of affinity purification of the His-tagged protein from *Escherichia coli* cell lysates under optimized conditions. Lane 1: High pure rLoa22 eluted with 0.30 M imidazole (pH: 7.40); M: Pre-stained protein size marker (Thermo Fisher Scientific, Vilnius, Lithuania). **D)** Western blot analysis. Lane 1: The *E. coli* total protein extract after induction; Lane 2: Specific band of purified *r*Loa22 protein; M: Pre-stained protein size marker (Thermo Fisher Scientific, Vilnius, Lithuania). The arrows indicate the position of 6xHis-rLoa22.

Evaluation of specific IgG and its subclasses against rLoa22 protein. Using checkerboard titration tests, the final concentration of rLoa22 based on antigen capture ELISA was adjusted on 0.850 mg mL^{-1}, and optimum dilutions of the serum and HRP IgG conjugates were calculated as 1:50 and 1:5000, respectively. For all immunized groups, there was no detectable antigen-specific IgG antibody before the 1st immunization. Also, the levels of specific antibodies of rLoa22/adjuvant immunized mice were recorded higher than the group immunized with rLoa22 alone. All animals had no anti-rLoa22 specific antibody at the beginning of the study. Nevertheless, after antigen administration, the rLoa22 specific antibody response was enhanced in the respective groups and, as expected, reached its highest levels in the animals immunized with rLoa22/adjuvant, having a significant difference with the others immunized with rLoa22 alone ($p < 0.05$). However, the results showed that the rLoa22 could detect high levels of antibodies in immunized mice by trivalent vaccine. Because there were no differences in any immune response between the adjuvant and PBS groups at all times, data from the adjuvant group are not shown ($p > 0.05$). The level of anti-Loa22 antibodies increased after the administration of recombinant antigen. This was greater in the group receiving antigen with adjuvant compared to ones received antigen without adjuvant. High levels of antibodies were also seen in the group received the trivalent vaccine (Fig. 3).

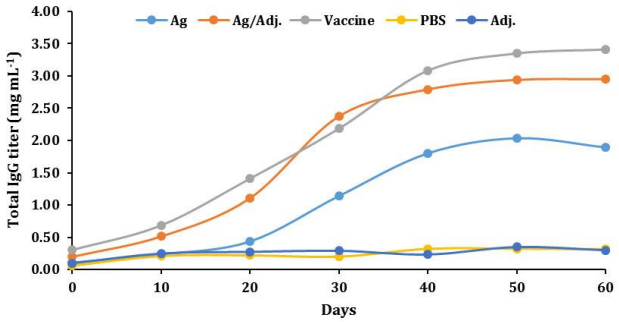

Fig. 3. Indirect enzyme-linked immunosorbent assay detected levels of anti-rLoa22 antibody production in BALB/c mice. The mice were immunized subcutaneously using phosphate-buffered saline (control), Freund adjuvant (adj.; control), inactivated trivalent *Leptospira* vaccine and the recombinant Loa22 with and without adjuvant. The blood samples were collected *via* retro-orbital venous sinus on day 0 (prior to immunization) and every ten days for two months to determine the antigen (Ag)-specific total response in the sera of control and vaccinated groups.

The IgG response in immunized mice was further characterized to determine the IgG1 and IgG2a subclasses. The administration of the recombinant antigen resulted in an increase in anti-Loa22 specific IgG1 levels. This increase was more pronounced in the group that received the antigen with adjuvant compared to the group that received the antigen without adjuvant ($p < 0.05$).

Additionally, high levels of anti-Loa22 specific IgG1 were observed in the group that received the trivalent vaccine. However, in the IgG2a subclass, the antibody levels were significantly higher in rLoa22/adjuvant group followed by trivalent vaccine group in comparison with the mice received rLoa22 without adjuvant ($p < 0.01$ and $p < 0.05$, respectively; Fig. 4).

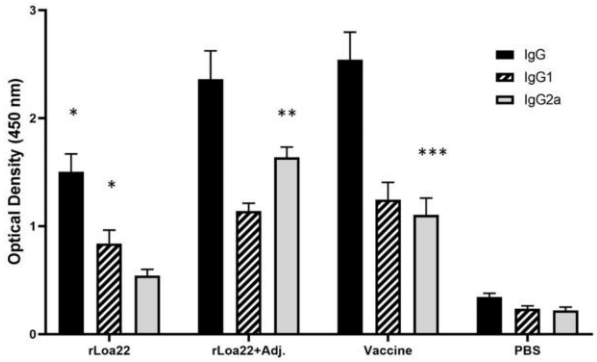

Fig. 4. The levels of recombinant Loa22 (rLoa22)-specific total immunoglobulin G (IgG) and subclasses. Sera were tested for the rLoa22-specific total IgG and rLoa22-specific IgG subclasses (IgG1 and IgG2a) 2 weeks after the 2nd immunization antibodies by indirect enzyme-linked immunosorbent assay using rLoa22 as a coating antigen. Antibody titers are presented as optical density. All experiments were conducted at least twice, and the data are presented as mean ± SD. PBS: Phosphate-buffered saline; Adj: Adjuvant.
Statistically significant differences ($p < 0.05$) are indicated by * (compared with rLoa22/Adj. and Vaccine groups), ** (compared with rLoa22 and Vaccine groups), and *** (compared with rLoa22 groups).

Lymphocyte proliferation and cytokine assays. To evaluate the cytokine profile, the production of IFN-γ and IL-4 was measured in the culture supernatants of spleen cells stimulated with the rLoa22 protein and trivalent vaccine (Fig. 5). Production of IL-4 from stimulated splenocytes was detectable for all groups compared to the control group. However, the mice immunized with the trivalent vaccine produced more IL-4 than recombinant groups ($p < 0.05$). On the other hand, no significant difference was observed between the rLoa22 with and without adjuvant groups regarding the induction of IL-4 expression ($p > 0.05$). The comparison between the levels of IFN-γ produced from the spleen cells of immunized animals showed that the administration of rLoa22 antigen stimulated the production of IFN-γ. However, this increase was substantially higher in the presence of adjuvant ($p < 0.01$). Interestingly, the animals received vaccine were also able to produce a large amount of IFN-γ; although, this was also lower than rLoa22/ adjuvant group ($p < 0.05$).

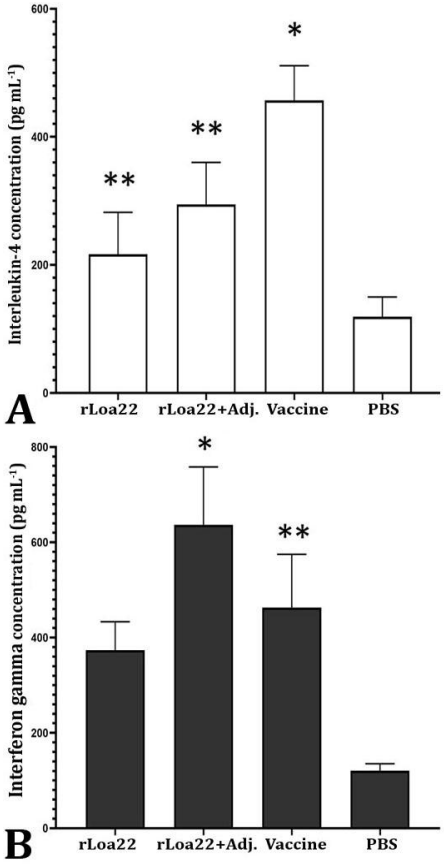

Fig. 5. Evaluation of **A)** interleukin 4, and **B)** interferon gamma levels produced by splenocytes from different immunized mice. After the 14th day of the last immunization, the splenocytes were isolated from mice and cultured in either the absence (unstimulated) or presence of recombinant Loa22 for 60 hr in Co_2 incubator at 37.00 ˚C. The absorbance was measured by indirect enzyme-linked immunosorbent assay at 450 nm. All mentioned experiments were performed at least in triplicates, and the data presented were the mean measurements ± SD. Statistically significant differences are indicated by * (compared with rLoa22 group), ** (compared with rLoa22 and rLoa22/Adj groups).

Discussion

Leptospirosis is believed to be the most prevalent zoonosis in the world.[4,23] Currently, *Leptospira* vaccines provide only short-term immunity and afford little cross-protection against different *Leptospira* serovars.[24] In addition, vaccination with the multi-valent whole-cell leptospiral vaccine mainly results in serious side effects being considered to be caused by lipopolysaccharide.[25] Serological tests based on serogroup and serovar-specific antigens with low cross-reactivity complicate prevention and laboratory diagnosis of leptospirosis.[26] Due to rapid advances in genomic technologies and bioinformatics,

studies made it possible to explore protective antigens for bacterial pathogens. Hence, the identification of leptospiral antigens being exclusively expressed during infection may not only contribute to the development of an ideal vaccine but also helps studies on the rapid and reliable diagnostic methods of leptospirosis.[27,28] Therefore, the efforts to develop recombinant vaccines based on the OMPs of bacteria, given their exposed location at the interface between pathogen and host, have been promising.[8,16,29] It was predicted that potential protective B-cell epitopes can also be found exclusively among portions located in the outer loops, being involved in dynamic interactions with the host immune system.[10]

The OMPs and lipoproteins are the main components of the leptospiral surface.[9] These proteins have been focused with utmost interest due to their distribution, conservation, functional relevance, and decisive role in virulence.[8-25] The OMPs of bacteria can trigger a strong antibody response.[28]

The protein antigens LipL32, LipL21, Lipl41, OmpL1, and Loa22 are generally found in all pathogenic *Leptospira* spp. Of these, leptospiral immunoglobulin-like domain proteins, OmpL1, LipL41, and LipL32 are the most protein-based antigens evaluated for their potential use as vaccine candidates.[10-22]

The *loa22* gene encodes a protein of 195 amino acid residues that could induce a protective response against broad serovars because it is conserved among pathogenic serovars of *Leptospira*.[30,31] It exhibits a bipartite structure with a C-terminus OmpA domain, including a predicted peptidoglycan-binding motif.[30] It is confirmed by a mutagenesis study to be a known virulence factor and expressed at high levels both during cultivation and infection.[30] Therefore, the protein appears to play an essential role in pathogenesis and host immune response.[32]

The Loa22 protein was previously identified as an adhesion associated with plasma fibronectin and collagen-binding protein in an *in vitro* assay.[32] A recent study by Hsu *et al.*[32], has suggested that Loa22, like LipL32, is a potential toll-like receptor 2 (TLR2) binding candidate in pathogenic *Leptospira* serovars.[15,33] The TLR2, in association with TLR1 or TLR6, is essential for sensing bacterial lipopeptides and lipoproteins, and it has proven that the interactions of the innate immune system cell receptors with Loa22, can induce inflammatory responses.[32]

Serological methods based on Loa22 recombinant antigen have been used in several studies for diagnosing *Leptospira* infection and epidemic analysis due to their convenience, sensitivity, and relatively low cost. In the study by Balamurugan *et al*., ELISAs based on the rLoa22 protein to detect anti-*Leptospira* antibodies in the sera of animals resulted in high sensitivity and accuracy (94.30 and 93.30%, respectively).[27]

The other studies demonstrated that the rLoa22, alone or in combination with other antigens, especially rLipL32, had an acceptable sensitivity and specificity in diagnosing leptospirosis in equine and canine, in agreement with the MAT results.[34,35] However, there is no report relating to the use of synthetic genes encoding the Loa22 protein to evaluate the efficiency of the expression of the protein and its immunogenicity. In the present study, the immunogenic potential of prepared rLoa22 protein of local pathogenic *Leptospira* species in a murine model was studied. The protein of approximately 38.00 kDa purified from the soluble fraction, and its ability to induce humoral and cellular immunity were further evaluated by the IgG1 and IgG2a subclasses produced through immunization.

Based on the results, mice immunized with rLoa22 induced high titers of total IgG antibodies, indicating that the protein had good immunogenic attributes. High levels of IgG1, in this study, confirmed previous findings based on the role of humoral immune response in clearing the *Leptospira via* production of antibodies.[36] However, immunizations with rLoa22 emulsified in Freund adjuvant, induced strong IgG1 production followed by IgG2a, indicating that Loa22 could induce both Th1 and Th2 types of immune responses, according to the study by Yan *et al*., and Umthong *et al*. [29,37] These results confirmed by evaluating the production of IL-4 and IFN-γ by spleen cells of mice immunized with the antigen, compared to control. The level of IL-4 was only slightly higher in the group received the rLoa22/adjuvant than rLoa22 group. However, it was significantly higher in the group receiving the trivalent vaccine. The levels of IFN-γ showed a significant increase in the groups immunized with rLoa22/adjuvant compared to the other groups. However, the IFN-γ level in the group immunized with the vaccine was also high and insignificant. The relatively high level of IFN-γ in the vaccine group is probably due to the synergistic effects of other antigens in its composition.

Conversely, in addition to the effect of the type of adjuvant in the redirection of IgG subclass response, BALB/c mice typically respond to protein as an antigen with a Th1-type immune response.[38] However, the principal antibody response to soluble protein antigens and membrane proteins is IgG2a isotype.[12-13] Based on the immunoinformatics studies, we predicted a combination of T and B cell epitopes from the conserved Loa22 protein with high binding affinity. Hence, demonstrating the high capacity to induce Th1 responses in BALB/C mice, caused by the combined epitopes of B and T cells in the conserved regions of LipL41 and OmpL1 proteins, could be another evidence to justify the significant IgG2a production in our study.[39,40]

Furthermore, the ELISA results indicated that the rLoa22 protein could recognize *Leptospira* antibody in serum of mice immunized with trivalent vaccine, confirming the presence of protein in the structure of

serovars used in the formulation of this vaccine. Similar results have been observed in the studies conducted by Ghalani *et al.*, and Lin *et al.*[41,42]

Therefore, the rLoa22 protein can be used as a promising antigen to develop a reliable test for diagnosis of leptospirosis or potential vaccine candidate to overcome the drawbacks of whole-cell vaccines.

Acknowledgments

The researchers would like to thank the managers and heads of the Razi Vaccine and Serum Research Institute, Agricultural Research, Education and Extension Organization, Karaj, Iran.

Conflict of interest

The authors declare that they have no conflict of interest and agree to publish the current version of the manuscript.

References

1. Bierque E, Thibeaux R, Girault D, et al. A systematic review of *Leptospira* in water and soil environments. PloS One 2020;15(1): e0227055. doi: 10.1371/journal.pone.0227055.
2. Esfandiari B, Nahrevanian H, Pourshafie MR, et al. Association of prevalent *Leptospira* species with different rodents of three northern provinces in Iran using microscopic agglutination test. Adv Stud Biol 2016; 8(2): 53-63.
3. de Oliveira NR, Santos FDS, Dos Santos VAC, et al. Challenges and strategies for developing recombinant vaccines against leptospirosis: role of expression platforms and adjuvants in achieving protective efficacy. Pathogens 2023; 12(6): 787. doi: 10.3390/pathogens12060787.
4. Khaki P. Clinical laboratory diagnosis of human leptospirosis. Int J Enteric Pathog 2016; 4(1): e31859. doi: 10.17795/ijep31859.
5. Widiasih DA, Isozumi R, Yoshimatsu K, et al. Serological and molecular biological diagnosis for leptospirosis. In: Widiasih DA (Ed). International conference on tropical agriculture. 1st ed. California, USA; Springer International Publishing 2017; 533-547.
6. Pinto GV, Senthilkumar K, Rai P, et al. Current methods for the diagnosis of leptospirosis: issues and challenges. J Microbiol Methods 2022; 195: 106438. doi: 10.1016/j.mimet.2022.106438.
7. Chalayon P, Chanket P, Boonchawalit T, et al. Leptospirosis serodiagnosis by ELISA based on recombinant outer membrane protein. Trans R Soc Trop Med Hyg 2011; 105(5): 289-297.
8. Raja V, Natarajaseenivasan K. Pathogenic, diagnostic and vaccine potential of leptospiral outer membrane proteins (OMPs). Crit Rev Microbiol 2015; 41(1): 1-17.
9. Haake DA, Zückert WR. The leptospiral outer membrane. Curr Top Microbiol Immunol 2015; 387: 187-221.
10. Hsu SH, Yang CW. Insight into the structure, functions, and dynamics of the *Leptospira* outer membrane proteins with the pathogenicity. Membranes (Basel) 2022; 12(3): 300. doi: 10.3390/membranes 12030300.
11. Adler B. Vaccines against leptospirosis. Curr Top Microbiol Immunol 2015; 387: 251-272.
12. Gor DO, Rose NR, Greenspan NS. TH1-TH2: a procrustean paradigm. Nat Immunol 2003; 4(6): 503-505.
13. Snapper CM, Paul WE. Interferon-gamma and B cell stimulatory factor-1 reciprocally regulate Ig isotype production. Science 1987; 236(4804): 944-947.
14. Koizumi N, Watanabe H. Molecular cloning and characterization of a novel leptospiral lipoprotein with OmpA domain. FEMS Microbiol Lett 2003; 226(2): 215-219.
15. Zhang Y, Bao L, Zhu H, et al. OmpA-like protein Loa22 from *Leptospira interrogans* serovar Lai is cytotoxic to cultured rat renal cells and promotes inflammatory responses. Acta Biochim Biophys Sin (Shanghai) 2010; 42(1): 70-79.
16. Pinne M, Haake DA. A comprehensive approach to identification of surface-exposed, outer membrane-spanning proteins of *Leptospira interrogans*. PloS One 2009; 4(6): e6071. doi: 10.1371/journal.pone. 0006071.
17. Almagro Armenteros JJ, Tsirigos KD, Sønderby CK, et al. SignalP 5.0 improves signal peptide predictions using deep neural networks. Nat Biotechnol 2019; 37(4): 420-423.
18. Clifford JN, Høie MH, Deleuran S, et al. BepiPred-3.0: improved B-cell epitope prediction using protein language models. Protein Sci 2022; 31(12): e4497. doi: 10.1002/pro.4497.
19. Vita R, Mahajan S, Overton JA, et al. The immune epitope database (IEDB): 2018 update. Nucleic Acids Res 2019; 47(D1): D339-D343.
20. Gharakhani M, Faezi Ghasemi M, Khaki P, et al. Improvement the expression and purification of Loa22: a Lipoprotein with OmpA domain from pathogenic Leptospira serovars. Iran J Microbiol 2023; 15(5): 674-684.
21. Qiagen A. A handbook for high-level expression and purification of 6xhis-tagged proteins. 5th ed., Chatsworth, USA: Qiagen 2003; 1-125.
22. Golab N, Khaki P, Tebianian M, et al. Recombinant outer membrane protein Lipl41 from *Leptospira interrogans* robust immune responses in mice model. Iran J Microbiol. 2024; 16(4): 545-552.

23. Bharti AR, Nally JE, Ricaldi JN, et al. Leptospirosis: a zoonotic disease of global importance. Lancet Infect Dis 2003; 3(12): 757-771.
24. Felix SR, Silva ÉF, Jouglard SDD, et al. Leptospirosis vaccine: search for subunit candidates. Procedia Vaccinol 2009; 1(1): 110-114.
25. Grassmann AA, Souza JD, McBride AJ. A universal vaccine against leptospirosis: are we going in the right direction? Front Immunol 2017; 8: 256. doi: 10.3389/fimmu.2017.00256.
26. Sonrier C, Branger C, Michel V, et al. Evidence of cross-protection within *Leptospira* interrogans in an experimental model. Vaccine 2000; 19(1): 86-94.
27. Balamurugan V, Thirumalesh SRA, Alamuri A, et al. Evaluation of the diagnostic potential of recombinant leptospiral OMP A-like protein (Loa22) and transmembrane (OmpL37) protein in latex agglutination test for serodiagnosis of leptospirosis in animals. Lett Appl Microbiol 2021; 72(6): 730-740.
28. Guerreiro H, Croda J, Flannery B, et al. Leptospiral proteins recognized during the humoral immune response to leptospirosis in humans. Infect Immun 2001; 69(8): 4958-4968.
29. Yan W, Faisal SM, McDonough SP, et al. Identification and characterization of OmpA-like proteins as novel vaccine candidates for leptospirosis. Vaccine 2010; 28(11): 2277-2283.
30. Ristow P, Bourhy P, da Cruz McBride FW, et al. The OmpA-like protein Loa22 is essential for leptospiral virulence. PLoS Pathog 2007; 3(7): e97. doi: 10.1371/journal.ppat.0030097.
31. Malek Mohammadi Y, Khaki P, Moradi Bidhendi S, et al. Evaluation of the presence of gene encoding Loa22 in pathogenic *Leptospira* serovars. Iran J Med Microbiol 2022; 16(5): 392-398.
32. Hsu SH, Chang MY, Lin SM, et al. Peptidoglycan mediates *Leptospira* outer membrane protein Loa22 to toll-like receptor 2 for inflammatory interaction: a novel innate immune recognition. Sci Rep 2021; 11(1): 1064. doi: 10.1038/s41598-020-79662-8.
33. Lo YY, Hsu SH, Ko YC, et al. Essential calcium-binding cluster of *Leptospira* LipL32 protein for inflammatory responses through the Toll-like receptor 2 pathway. J Biol Chem 2013; 288(17): 12335-12344.
34. Ye C, Yan W, Xiang H, et al. Recombinant antigens rLipL21, rLoa22, rLipL32 and rLigACon4-8 for serological diagnosis of leptospirosis by enzyme-linked immunosorbent assays in dogs. PLoS One 2014; 9(12): e111367. doi: 10.1371/journal.pone.0111367.
35. Ye C, Yan W, McDonough PL, et al. Serodiagnosis of equine leptospirosis by enzyme-linked immunosorbent assay using four recombinant protein markers. Clin Vaccine Immunol 2014; 21(4): 478-483.
36. Adler B, Faine S. Host immunological mechanisms in the resistance of mice to leptospiral infections. Infect Immun 1977; 17(1): 67-72.
37. Umthong S, Buaklin A, Jacquet A, et al. Immunogenicity of a DNA and recombinant protein vaccine combining LipL32 and Loa22 for leptospirosis using chitosan as a delivery system. J Microbiol Biotechnol 2015; 25(4): 526-536.
38. Teixeira AF, Fernandes LGV, Cavenague MF, et al. Adjuvanted leptospiral vaccines: challenges and future development of new leptospirosis vaccines. Vaccine 2019; 37(30): 3961-3973.
39. Lin X, Sun A, Ruan P, et al. Characterization of conserved combined T and B cell epitopes in Leptospira interrogans major outer membrane proteins OmpL1 and LipL41. BMC Microbiol 2011; 11(1): 21. doi: 10.1186/1471-2180-11-21.
40. Dong H, Hu Y, Xue F, et al. Characterization of the ompL1 gene of pathogenic *Leptospira* species in China and cross-immunogenicity of the OmpL1 protein. BMC Microbiol 2008; 8:223. doi: 10.1186/1471-2180-8-223.
41. Ghalani J, Esmaelizad M, Kamalzadeh K. Highly expression and purification of an antigenic region (D259-R462) of adenylate guanylate cyclase protein, applicable for ELISA test. Braz Arch Biol Technol 2021; 64: e21200476. doi: 10.1590/1678-4324-2021200476.
42. Lin X, Xiao G, Luo D, et al. Chimeric epitope vaccine against *Leptospira* interrogans infection and induced specific immunity in guinea pigs. BMC Microbiol 2016;16: 241. doi: 10.1186/s12866-016-0852-y.