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ORIGINAL ARTICLE

Improvement the expression and purification of Loa22: a lipoprotein with OmpA domain from pathogenic *Leptospira* serovars

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ABSTRACT

Background and Objectives: One of the highly conserved outer membrane proteins expressed only by pathogenic Leptospires is Loa22. The study aims is to achieve the optimum conditions for high expression of recombinant Loa22 (rLoa22) protein.

Materials and Methods: Complete coding sequence of *loa22* gene sub-cloned into a pET32a (+) expression vector. BL21 competent *E. coli* (pLysS) used as expression host for transformation. The recombinant clones selected on ampicillin plates and subjected to PCR by using pET T7 primers. Then expression conditions optimized by adjusting parameters such as culture media, induction time, temperature, and IPTG concentration.

Results: SDS-PAGE analysis showed that high production of rLoa22 protein obtained when post induction incubation, IPTG concentration, and duration of induction were 37ºC, 0.1 M and 5 h in 2×TY medium respectively. The purification of rLoa22 protein under native conditions using Ni-NTA pull-down was optimum in one hour binding at 37°C, five times washing process and elution buffer with a pH 7.4 and a 0.3 M imidazole concentration.

Conclusion: The findings of the study led to high production of pure Loa22 protein, which can form the basis for future investigation on the design of rapid diagnostic tests and more effective vaccine candidates for leptospirosis.

Keywords: Leptospirosis; Outer membrane proteins; Gene expression; Recombinant protein Loa22

INTRODUCTION

Leptospirosis is a worldwide public health problem affecting both domestic animals and humans. The etiologic agent of the disease is pathogenic spirochetes of the genus *Leptospira* (1, 2). *Leptospira in-*

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terrogans constitute the major pathogenic *Leptospira* species that is responsible for human infection (3). *Leptospira* spreads through direct contact with urine or tissues of infected animals, or indirect exposure to contaminated water, soil, or vegetation (2, 4).

Leptospirosis has a broad geographical distribution but deployed in tropical and subtropical areas with high fall rain. In Iran, the areas along the Caspian Sea have the highest reported prevalence, but there also are case reports in some other parts of country (5, 6). Most common serovars in these areas that cause infection in humans include serovars Icterohaemorrhagiae, and Canicola, in cattle's serovars Hardjo-bovis, Canicola, and Grippotyphosa, in canines serovars Canicola, and Icterohaemorrhagiae, and in rodents serovars Icterohaemorrhagiae, and Grippotyphosa respectively (1, 7).

Accurate diagnosis of leptospirosis due to a wide diversity of clinical manifestations shared with many other febrile diseases depends on the type of diagnostic test (1, 3). Laboratory diagnosis of leptospirosis usually is accomplished by several different methods including; isolation of the causative leptospires, direct microscopy, molecular and serological methods. Although culture is a golden standard method, but is not effective for the early detection of the disease and also has low sensitivity (1, 5). Direct microscopy methods including using dark-field or phase contrast microscopy requires expertise and careful examination (8). Molecular methods employed have included several PCR-based procedures (9, 10). However, it also requires trained experts, and relatively elaborated laboratory outfits (11). Among serological methods, the microscopic agglutination test (MAT) is the gold standard test to diagnose leptospirosis using antigens from live leptospiral serovars to detect specific agglutination antibodies in serum but it is prone to contamination and less sensitive and specific (5). Among serology-based methods, ELISA is a simple, safe, and suitable assay for the examination of a large number of sera samples in diagnoses and seroepidemiological investigations using purified recombinant proteins (10).

The importance of control and prevention of leptospirosis, especially in endemic areas, emphasizes the need for the development of rapid and reliable laboratory tests and trying to achieve an effective vaccine. Commercially available leptospirosis vaccines including vaccines based on whole cell bacteria, and lipopolysaccharide (LPS), suffer from several limitations such as serovar-restricted protection, short-term immunity and usually fail to prevent the transmission of the disease. Currently, subunit vaccines based on recombinant proteins have the potential to overcome these limitations (12, 13).

Outer membrane proteins (OMPs) are the main surface antigens of the *Leptospira* spp. and in contrast to LPS are thought to be highly conserved. The OMPs play an important role in maintaining the cell structure, and attachment to various extra cellular matrix (ECM) components, and are protective immunogens in animal models of the disease (14, 15). Generally, these recombinant antigen-based tests are safe and better alternatives to live cultural antigens for the serodiagnosis of leptospirosis (16, 17).

The outer membrane protein A-like protein Loa22 from *Leptospira interrogans* is a 22 KDa lipoprotein with an OmpA domain in the C-terminus (16, 18). Several studies have shown that Loa22 is highly conserved and expressed only by pathogenic *Leptospira* during infection and is involved in pathogenesis at both acute and convalescent stages of infection (15, 16). In addition, experimental evidence has proven that Loa22 protein stimulated inflammatory responses and deletion of this protein from pathogenic *Leptospira* attenuated toxicity, while re-expression of the protein revives the virulence (18, 19).

In the last decades, recombinant outer membrane proteins have investigated with two different approach; covering their potential in the development of serodiagnosis tests and overcoming restrictions of available leptospirosis vaccines (12, 20-22). Although there are some difficulties concerning to this matter, such as low levels of immune responses and in some cases, expression at only low levels or may not be expressed at all by host cells grown in artificial media (23, 24). Therefore the selection of protective leptospiral OMPs and investigation of their desirable expression, purification, and immune responses is essential.

MATERIALS AND METHODS

***Leptospira* serovars and culture conditions.** Six serovars including *L. interrogans* serovar Icterohaemorrhagiae, Canicola, Hardjo prajitno, and *L. Kirschneri* serovar Grippotyphosa and two nonpathogenic srovars of *L. bifelxa* obtained from reference laboratory of *Leptospira* of Razi Vaccine and Serum

Research Institute, Iran.

The bacteria were inoculated in Ellinghausen-Mc-Cullough-Johnson-Harris (EMJH) liquid medium (Difco, USA) supplemented with *Leptospira* Enrichment under aerobic conditions at 28°C. Bacterial growth within 7-10 days was confirmed by the dark field microscope (Nikon Eclipse, Japan) observation, and then pelleted through centrifugation at 17000 ×g for 20 min.

DNA extraction and PCR. Genomic DNA extracted from resuspended pellets by the phenol-chloroform method. The quality and quantity of extracted DNAs evaluated by nanodrop spectrometry system (Epoch, BioTek, USA). The presence of the *loa22* gene in the pathogenic and nonpathogenic serovars examined by PCR using the upstream primer: 5´- CGGCCTTTT-GAAAGATCGAATTG-3´ and the downstream primer: 5´- ACACTCTGATACCAAACCCCT-3´ that designed by Oligo 7 software to cover the CDs region of *loa22* gene (25). The PCR was performed with 50 ng of DNA template, 10 pM of each primer, and 1× of Taq 2× master mix (amplicon) in a 50 µl final reaction volume. The amplification was carried out with the initial heat denaturation at 95°C for 5 min followed by 35 cycles of 1 min at 94°C (denaturation), 1 min at 60°C (annealing), 1 min at 72°C (extension), and the final extension at 72°C for 10 min. The PCR products analyzed on 1% agarose gel electrophoresis and 100bp plus DNA Marker (Thermo scientific, Lithuania) was used to compare the size.

Sequencing and phylogenic studies. The amplified PCR products purified using the High Pure PCR product Purification Kit (Roche, Germany) and sequenced by Sanger dideoxy sequencing technology (ABI3730XL, Macrogen, Korea). DNASTAR Laser gene V.17 software used to assemble sequences generated from forward and reverse primers. In addition to our sequences, fourteen sequences of the *loa22* gene were obtained in a parallel study (accession No OP038304 - OP038317), and 17 sequences were derived from pathogenic *Leptospira* whole genomes registered in the NCBI Database (http://www.ncbi.nlm.nih.gov) by blasting with the only complete CDs sequence of corresponding *loa22* from Grippotyphosa (accession No. KC311551) as a reference (Table 1).

All sequences of the *loa22* gene in this study compared using the multiple sequence alignment program of MEGA software (version 7.0.26), where some sequences had to be trimmed to allow comparison. The phylogenic tree of the aligned sequences was drawn using the maximum-likelihood (ML) method, using the Tamura 3-parameter model and bootstrap analysis (n=1000) with MEGA7.0 software.

Bioinformatics study. Editseq™ software and Expasy-ProtParam used to predict the *loa22* gene-coding region, its amino acid sequence, the amino acid composition, and various other physiochemical properties of Loa22 protein, including theoretical pI, molecular weight, polarity, etc. Based on the evidence of phylogenetic and physiochemical studies, the complete coding sequence of the *loa22* gene was considered in a way to remove the signal peptide as predicted by the online server SMART (http://smart.embl-heidelberg.de/). The coding sequence optimized based on the codon usage chart of *E. coli* as a compatible host for the high-level expression.

The pET32a(+) vector was then designed by insertion of the optimized *loa22* sequence into the NcoI and XhoI restriction sites in the frame with histidine tag sequence at the N-terminal end.

Protein expression. The recombinant plasmid transformed into the *E. coli* BL21 (DE3) plysS using cold $CaCl_2$ treatment followed by heat shock method and was grown in LB medium containing 50µg/mL ampicillin. To confirmation of the positive recombinant clones selected on ampicillin plates, and related plasmids were isolated from bacterial cells using the Plasmid Mini extraction kit (Roche, Germany) as described by the manufacturer protocol. Extracted plasmids and positive clones subjected to PCR using pETT7 universal primers.

The expression of recombinant protein optimized by adjusting parameters such as culture media, induction time, temperature, and isopropyl-β-d-thiogalactopyranoside (IPTG, Sigma-Aldrich, Korea) concentration. The best concentrations of inducer were determined by inducing the culture with different concentrations of IPTG. Recombinant colonies were cultured into 10 ml fresh sterile 2×TY (Tryptone 16 g/L, Yeast extract 10 g/L, NaCl containing 50 µg/mL ampicillin and incubated overnight at 37°C with constant shaking at 150 rpm. The following day 400 mL of 2×TY was inoculated with 1mL of preculture and when the growth achieved the mid-log phase (OD_{600}

Table 1. The *loa22* gene sequences of *Leptospira* serovars used in this study

No.	Accession No.	Serovar	Genome	Country	No.	Accession No.	Serovar	Genome	Country
1	OM913538	Grippotyphosa	CDs	Iran	20	CP022883.1	Canicola	Whole	Brazil
2	OM913536	Icterohaemorrhagiae	CDs	Iran	21	CP044513.1	Canicola	Whole	China
3	OM913537	Hardjo prajitno	CDs	Iran	22	CP012603.1	Hardjo	Whole	Brazil
4	OL689841	Canicola	CDs	Iran	23	MVAE01000002.1	Hardjo	Whole	Brazil
5	OP038304	Autumnalis	CDs	Iran	24	NZ_CP043041.1	Hardjo	Whole	Brazil
6	OP038305	Pomona	CDs	Iran	25	CP015046.1	Hardjo	Whole	Panama
7	OP038306	Serjae	CDs	Iran	26	CP000350.1	Hardjo-bovis	Whole	U.S.A
8	OP038307	Pyrogenes	CDs	Iran	27	CP000348.1	Hardjo-bovis	Whole	Australia
9	OP038308	Ballum	CDs	Iran	28	CP012029	Ballum	Whole	China
10	OP038309	Australis	CDs	Iran	29	MAC001000065	Icterohaemorrhagiae	Whole	Brazil
11	OP038310	Hardjo-bovis	CDs	Iran	30	AE016823	Copenhageni	Whole	Brazil
12	OP038311	Pomona	CDs	Iran	31	NZ_CP020414.2	Copenhageni	Whole	China
13	OP038312	Canicola	CDs	Iran	32	CP048830.1	Copenhageni	Whole	Panama
14	OP038313	Grrippotyphosa	CDs	Iran	33	NC_017551.1	Lai	Whole	China
15	OP038314	Pomona	CDs	Iran	34	AE010300	Lai	Whole	China
16	OP038315	Canicola	CDs	Iran	35	CP006723	Linhai	Whole	China
17	OP038316	Icterohaemorrhagiae	CDs	Iran	36	MAC001000065	Icterohaemorrhagiae	Whole	Brazil
18	OP038317	Javanica	CDs	Iran	37	NZ_CP020414.2	Copenhageni	Whole	China
19	KC311551	Grippotyphosa	CDs	India	38	CP022885.1	Canicola	Whole	Brazil

nm, 0.6) the culture was equally divided into four flasks. The cultures then induced by IPTG concentrations (0.1 M, 0.2 M, 0.3 M, and 0.5 M). One mL culture from each of the flasks were centrifuged at 10,000 ×g for 10 min to obtain the pellet.

To determine the optimum temperature and induction time, recombinant colonies were cultured into 100 mL fresh 2×TY media (3 flasks) containing 50 µg/mL ampicillin and incubated at 37, 30 and 22ºC with constant agitation (150 rpm) until cells reached OD_{600} nm, 0.6. The cultures induced for up to 20 hours with 0.1M of IPTG. An equal amount of Samples collected in situ at the following time points: 0, 1, 2, 3, 5, and 20 h and stored at -20ºC.

To achieve the high yield of the Loa22 protein in *E. coli*, we evaluated three different media formulations; 2×TY medium (16 g/L tryptone, 10 g/L yeast extract, 5 g/L NaCl), Terrific broth (TB) complex cultivation medium (12 g/L tryptone, 24 g/L yeast extract 0.04 g/L glycerol, 12.54 g/L KH_2PO_4, 15 g/L K_2HPO_4) and LB (10 g/L tryptone and 5 g/L yeast extract, 5 g/L NaCl). The pH of all media adjusted to 7.4 and supplemented with the corresponding antibiotic (ampicillin 50 µg/mL). Overnight culture of *E. coli* BL21 (DE3) pLysS harboring pET32-*loa22* was grown overnight at 37°C with shaking at 150 rpm. Then 1 mL of the starting culture inoculated into 100 mL of each of the three media. When the growth in OD_{600} reached 0.6, cultures induced with 0.1M IPTG and incubated at 37°C with shaking at 150 rpm. After 5h of incubation, the cell suspensions harvested by centrifugation, washed with ice-cold 1×PBS and the bacterial pellets were stored at -20°C until further processing.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE 12%) performed to evaluate protein expression for each sample collected.

Optimization of purification using Ni-NTA resin. The solubility or insolubility of the expressed recombinant protein was determined by resuspending the bacterial pellet (5h post-induction) in 1×PBS and disrupted by sonication (6 cycles, 1 min each with 1 min of intervals). Phenyl Methyl Sulfonyl Fluoride (PMSF) added to inhibit probable protease activity. The lysate was centrifuged at 10000 ×g at 4°C for 20 min and the production of recombinant protein, either in solubilized or insolubilized form, was analyzed by SDS-PAGE.

Purification of the recombinant protein was performed by Ni-NTA chromatography under the native buffer system, according to Thermo Scientific protocol (26). Optimization of the protein purification

process carried out at the stage of binding, washing, and elution. Therefore, after sedimentation, the supernatant was loaded onto 500 µl Ni-NTA resin and shacked for an hour at 37ºC with constant agitation (200 rpm) to more interaction between His-tag in recombinant proteins and Ni-NTA resin. After the protein was bound, impurities washed using wash buffer (50 mM NaH_2PO_4, 0.3 M NaCl, 0.25 M imidazole, adjust pH to 7.4) with repeated variations two, three, and five times. The protein, in the next step, eluted in three steps by gradient concentration of imidazole ranging from 0.05-0.3 M in potassium phosphate buffer (50 mM NaH_2PO_4, 300 mM NaCl). Depending on the structure and the isoelectric point (pI) of the protein, the elution buffer then optimized in terms of pH (4, 6, and 7.4). The Purity of Loa22 protein in each step checked on 12% SDS-PAGE.

Measurement of purified recombinant protein. Total concentration of protein measured using Spectrophotometer (Epoch, BioTek, USA) and colorimetric assay according to Bradford standard method (27).

Linear standard BSA (bovine serum albumin) curve is created using Microsoft Excel to find out the equation of the line obtained, so that the sample concentration can be calculated. The standard concentrations used in range of 0 to 20 mg/mL.

Dot-blot and western-blotting analysis. Overall, 2µg/mL of 6X his-tagged rLoa22 the protein, the *E. coli* total protein extract not induced with IPTG as a negative control, the extract of the transformed bacteria, and purified recombinant protein were dotted on the nitrocellulose membrane. It was immersed in 5% skim milk and shaken (30 minutes) and washed with PBST (137 Mm NaCl, 2 mM KH_2PO_4, 10 mM Na2HPO4, 2.7 mM KCl, pH 7.4 with 500 µL Tween20) and incubated for 1 hour with HRP (Horseradish Peroxidase) conjugated anti-6x His-Tag antibodies (Abcam, USA). The membrane washed with PBST. Color development was detected by Tetramethyl Benzidine (TMB-blotting substrate solution, Sigma Aldrich).

For western blot assay the samples, along with the marker, were run on 12% SDS-PAGE using a discontinuous buffer system and subsequently transferred (0.5 V/cm2 for 2 hour) to the nitrocellulose membrane (Bio-Rad) by the wet blot transfer method in transfer buffer (0.2 M glycine; 24 mM Tris; 10% methanol, pH 8.3). The membrane immediately placed in blocking buffer (5% BSA in PBS containing 500 µL Tween20) for 1 hour at 4°C to block non-specific binding sites and the membrane washed with PBST, three times for 5 min per wash. Afterwards the blot again incubated at 37°C for 2 hour with a HRP conjugated anti-6x His-Tag antibodies, in the blocking buffer. The membrane washed as described above, and Anti-His tagged antibody was probed by adding TMB.

RESULTS

DNA isolation and PCR amplification. The amplified fragments by PCR viewed at 1% agarose gel and obtained a single band of 671 bp; while it was, absent in saprophyte serovars such as *L. biflexa* as shown in Fig. 1.

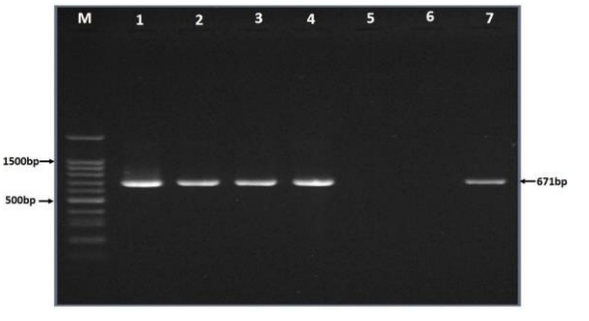

Fig. 1. Agarose gel (1%) electrophoresis showing PCR product of 671 bp encoding for *loa22* gene in the pathogenic *Leptospira*. Lane 1. *L. interrogans* serovar Canicola, Lane 2. *L. interrogans* serovar Hardjo Lane 3. *L. kirschneri* serovar Grrippotyphosa, Lane 4. *L. interrogans* serovar Icterohaemorrhagiae, lane 5 and 6: *L. biflexa*, Lane 7. Positive control. M. 100 bp plus DNA Marker (Thermo scientific, Lithuania).

Sequencing and phylogenic analysis. The sequences analysis, showed 97-100% similarity among all serovars examined in this study. Based on the results, the *loa22* gene of local *L. interrogans* serovars Hardjo prajitno and Conicola had 100% homology. Local isolates of *L. kirschneri* serovar Grippotyphosa were in a close relationship (99.7% identity) with Grippotyphosa isolated in India (KC311551). Similarity between *L. interrogans* and *L. krischneri* serovars were up to 97.3%. The alignment of the deduced amino acid sequences of the *loa22* genes using MEGA software revealed amino acid substitutions at positions 37 (Ala→Thr), 87 (Ala→Pro), and 165 (Val→Ala), were the only consistent differences between the *L. krischneri* and *L. interrogans* serovars respectively.

The phylogenetic tree presentation showed two dis-

tinctive clusters. A cluster consisting of three Grippotyphosa serovars belongs to the *L. kirschneri* serogroup and 33 serovars belong to other serogroups, which formed a separate cluster (Fig. 2).

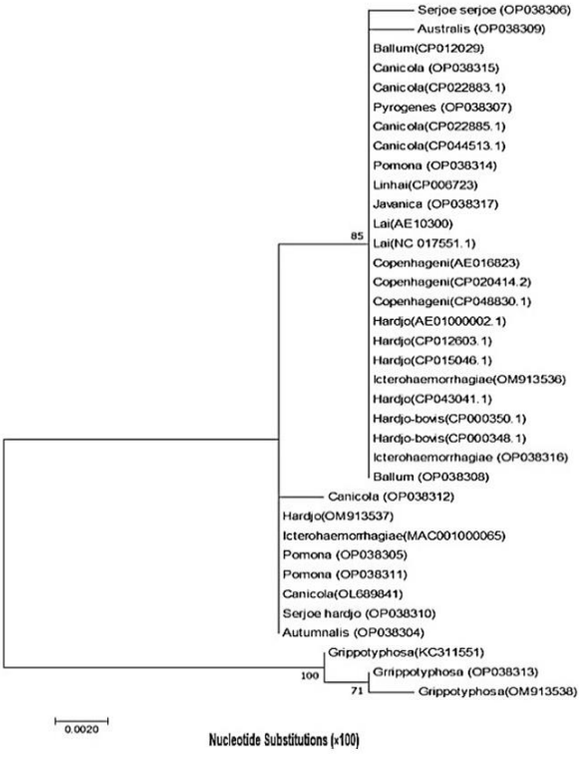

Fig. 2. The phylogenic tree was constructed with nucleotide of the aligned *loa22* gene sequences using the maximum-likelihood (ML) method, with the Tamura 3-parameter model and bootstrap analysis (n=1000) within MEGA7.

Bioinformatics study. The Loa22 protein sequence contained 199 amino acids with a molecular weight of 21.6 KDa, pI of 8.8, and maximum activity at pH.7 as predicted using the Expasy-translate online application and Editseq software. Bioinformatics analysis predicts that *loa22* encodes membrane protein; has a signal peptide with a cleavage site between amino acids 24/25 and has no assigned function.

Finally, based on the physiochemical properties and findings from the phylogenetic studies, the local Loa22 protein pattern was used to design the recombinant vector *loa22*-pET32a(+) by insertion of the optimized *loa22* sequence into the NcoI and XhoI restriction sites in frame with histidine tag sequence at N-terminal end. General Biosystems, Inc, USA, synthesized the designed gene construct.

Transformation. For over-expression and production, *loa22*+pET32a construct transformed into *E. coli* BL21 (DE3) pLysS under the control of the T7 promoter.

The recombinant plasmid purified from the transformed cell and identified based on plasmid size (Fig. 3a). Eventually, the results of colony PCR assay with T7 universal primer and growth in presence of ampicillin revealed that recombinant plasmid correctly transformed into the host (Fig. 3b).

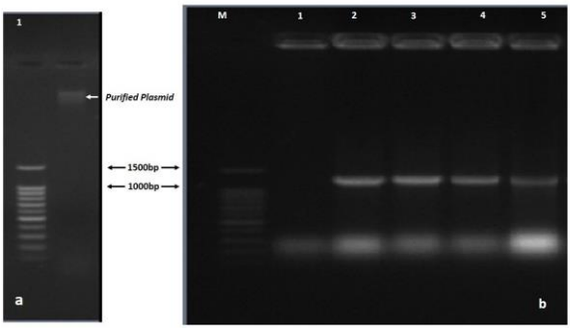

Fig. 3. (a) Purified plasmid from transferred cells. (b) Clony-PCR amplification of *loa22* from pE32-*loa22* plasmid with pET T7 primers, Lane 1. Untransferred cell, Lane 2-5. Transferred cells. M. 100 bp plus DNA Marker (Thermo scientific, Lithuania).

Protein expression. To examine the effect of media on the production of rLoa22 protein, the relative level of expression analyzed using the three different media by SDS-PAGE. The induced culture in the TB medium expressing Loa22 showed the lowest volumetric yield relative to LB and 2×TY medium, although there was no significant difference in growth rate in LB and 2×TY medium. However, the 2×TY medium was found to support the best growth rate and produce more recombinant protein than other media under induced culture conditions (Fig. 4b).

The best conditions for the high-level Loa22 expression were achieved at 0.1 M IPTG concentration, 37°C (Fig. 4c), and 5 hours induction (Fig. 4a), although other IPTG concentrations also demonstrated sufficient results (Fig. 4d). The cells harboring recombinant lipoproteins in optimum conditions expressed a specific band of approximately 38 KDa (pET32; 18.5 KDa +Loa22; 19.3 KDa) compared with the cultures without IPTG as the control and Protein Size Marker (Thermo scientific, Lithuania) on 12% SDS-PAGE electrophoresis after staining.

Optimization of purification using Ni-NTA resin. SDS-PAGE analysis after disrupting the cells with ultrasound showed that the amount of protein in the

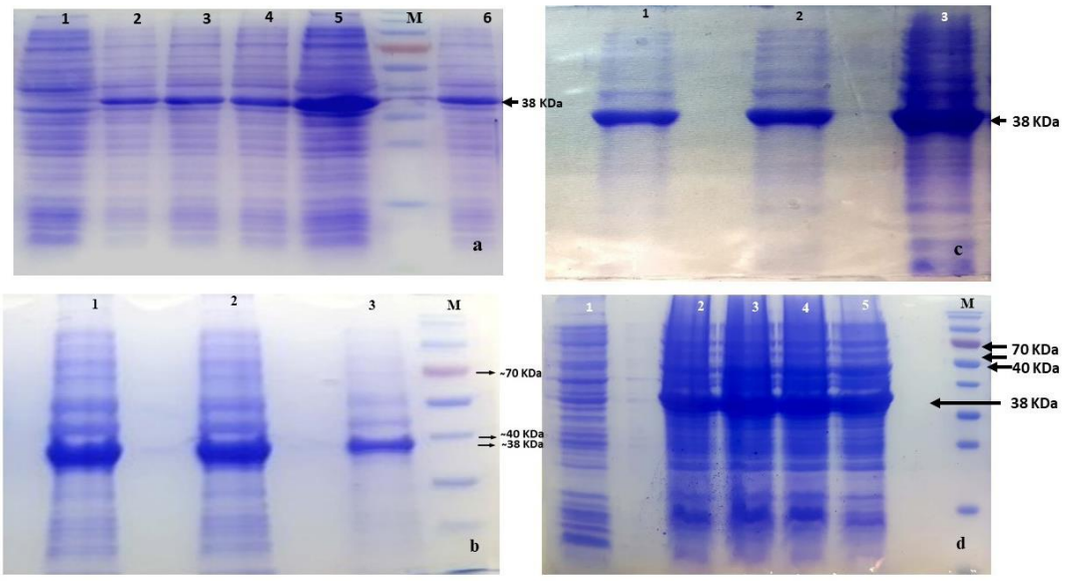

Fig. 4. (a) SDS-PAGE analysis of expressed Loa22 protein in *E. coli* (plysS) after incubation at 37°C. Lane 1. Non-induced cell lysate, lanes 2 to 5. Cells induced with 0.1 mM IPTG at 1, 2, 3, and 5 hours after induction, respectively. Lane 6. Cells induced after 20 hours. (b) SDS-PAGE analysis of Loa22 expression in three different culture media. Lane1. LB Broth Lane2. 2xTY medium Lane3. BT Broth. (c) The effect of temperature on Loa22 expression. Lane1. (21°C), Lane2. (30°C) lane 3. (37°C). (d) Lane 1. Non-induced cells, lanes 2 to 5. Cells induced with IPTG at 0.1 to 0.4 mM concentrations. M. Protein Size Marker (Thermo scientific, Lithuania).

soluble part was significantly higher. After sonication, the contents centrifuged at 5000×g for 15 min and the recombinant protein in the supernatant purified by Ni-NTA resin as a chromatographic procedure.

The SDS PAGE characterization process indicated that more washing carried out by the wash buffer containing 0.025 M imidazole, caused non-specific proteins to be released from the resin. In washing twice there were still some impurities in the presence of non-target protein bands, and washing five times has almost no impurities, although, some protein waste was also observed (Fig. 5b). The band thickness and high protein purity increased with the increase in the concentration of imidazole and elution at a concentration of 0.3 M gave maximum results (Fig. 5a). In addition, the pH of solutions was 7.4, which is a mimic biological condition, and most proteins are stable at this pH (Fig. 5c).

Measurement of purified rLoa22 protein. The standard BSA concentration used in the range of 0 to 30 mg/mL. The concentration of the recombinant protein in the optimal conditions of purification (0.3 M Imidazole. pH 7.4) was about 3.3 mg/mL by Bradford assay, almost consistent with the results of the Nanodrop spectrophotometer (3.1 mg/mL).

The dot blot and western blotting analysis. The band (38 KDa) of interaction with His-tagged recombinant protein and anti-His tagged antibodies in 1:2000 dilution corresponding to pre-stained protein ladder were detected by western blot TMB (Figs. 6a and 6b). The dot blot results showed no detectable blot in negative control while rLoa22 observed both in the supernatant fraction of induced *E. coli* cell lysates and purified recombinant protein (Fig. 6c).

DISCUSSION

Leptospirosis is one of the most widespread zoonosis and public health concerns, particularly in tropical and sub-tropical areas (28). Leptospirosis is frequently underreported due to a lack of reliable diagnostic tests, variable symptoms, and a wide variety of pathogenic serovars. On the other hand its initial symptoms can easily be mistaken for other infectious diseases (3, 9). Therefore, it is required to develop rapid and effective tests and vaccines for prevention and treatment of leptospirosis. The leptospiral highly conserved OMPs which are expressed on the surface of pathogenic serovars and expressed during infection may have potential immunoprotective capabili-

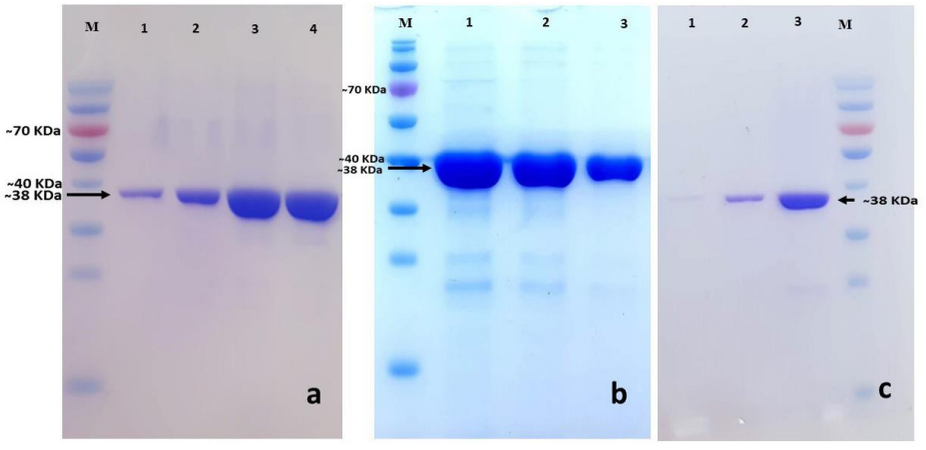

Fig. 5. (a) Washing step: Lane 1-3; Washing 2, 3, and 5 times, respectively, (b) Effect of concentration of imidazole in 1×PBS with a pH close to a neutral range (7.4); Lane 1-4. Elution with 0.05, 0.1, 0.2, and 0.3 M concentration of imidazole respectively, (c) Effect of buffer pH on the elution; Lane 1 to 3, solution with pH 4, 6, and 7.4 respectively. M. Prestained Protein Size Marker (Thermo scientific, Lithuania).

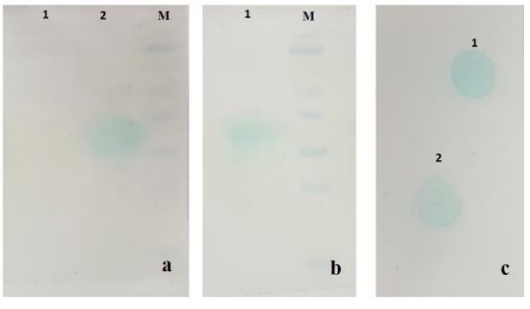

Fig. 6. Immunoblotting of recombinant proteins using HRP conjugated anti-6× His-Tag antibodies. (a) Lane 1: The *E. coli* total protein extract not induced with IPTG, Lane 2: The transformed cell lysate, (b) Lane 1: Purified recombinant protein, (c) Dot-blot analysis of the Loa22 protein; 1: Purified protein, 2: Transformed cell lysate, M: Protein Size Marker (Thermo scientific, Lithuania).

ties and a beneficial tool for serodiagnosis and vaccine development for leptospirosis (23, 29).

Three most abundant known lipoproteins on the surface of *Leptospira* are LipL32, Loa22, and Lip41, respectively (29). The outer membrane protein Loa22 is also the first genetically described virulence factor in *Leptospira* that confirmed by mutagenesis studies (18). It is a lipoprotein with the C-terminal OmpA domain located in the outer membrane (15).

The first step in this study was to design a recombinant *loa22* gene construct based on phylogenetic evidence and homology studies using bioinformatics tools. The resultant nucleotide and amino acid sequences of *loa22* analyzed using the BLAST online program of NCBI, which revealed more than 99% similarity at both levels among pathogenic *Leptospira* serovars. The percentage of homology at the nucleotide and amino acid levels among *L. krischneri* Grippotyphosa serovars were 97.3 and 98.3% respectively with *L. interrogans* serovars. Difference in three amino acids was the reason for the difference in identity *L. krischneri* Grippotyphosa serovars with *L. interrogans* serovars. Generally, the results of multiple sequence alignment for the deduced nucleotide and amino acid sequences of all serovars in the study for *loa22*, in congruent with the results of Kaur et al. (2014) and Balamurugan et al. (2021), conferred a high degree of homology and present only in pathogenic serovars of *Leptospira* (20, 30).

The production of a pure protein in sufficient amounts is the key to its study or use as an antigen for diagnostic or immunization purposes. Today, recombinant protein production in large volumes and their application in genetic engineering have increased. However, high-level production of prokaryotic recombinant proteins in *E. coli* may not be a routine matter; sometimes it is quite challenging (23, 24, 31).

Here, *loa22* gene encoded an approximately 38 KDa recombinant protein which was confirmed with immune-blotting analysis. Based on our results, deletion of the signal sequence of Loa22 protein located in the transmembrane region resulted in a soluble mature protein, which similar to other studies supported the theory that the mature protein, without the signal peptide, retained in the soluble fraction (16, 20).

The high percentage of similarity in the Loa22

amino acid sequences among different serovars from Iran and other countries considered to design the recombinant plasmid according a pattern consisting of common pathogenic serovars. Codon optimization strategies attempt to increase the protein of interest expression by altering the codon usage of the gene. Based on the optimization results, the high yield of purified Loa22 protein showed that the optimized gene construct according to the genomic codon usage in the *E. coli*, is probably one of the reasons for the higher yield compared to restriction cloning with wild-type sequences. Previously, expression of LipL41 using a codon optimization approaches have been conducted and have obtained considerable amounts of recombinant protein, which is useful for academic studies and preclinical work (32).

In this study, using the pET32a vector to express the Loa22 along with other parameters, led to the production of a high amount of the recombinant protein (3.3 mg/mL) after purification. However, a study by Xin Zhao et al. (2015) were unable to successfully express the rLoa22 (33) . In two other studies, Loa22 produced through different vectors including pETite with the yield of 5-6mg/L, and pGEX-4T with the yield of 16 mg/L of culture (16, 20).

Since the high concentration of IPTG may have toxic effects and cause the expressing of the protein in an insoluble form, we considered the minimum required concentration for induction. And also reduction of the temperature down to 30ºC along with incubation time of 16 h decreased the protein yield compared to the 5 hours post induction at 37ºC, which was consistent with findings of Fraser et al (2017) regarding the reduction of Loa22 expression at 30ºC (34). The represented experimental data showed that the production of rLoa22 in the native state was at the highest level when post-induction incubation, IPTG concentration, and duration of induction were 37ºC, 0.5 mM, and 5 h in a 2xTY medium respectively.

According to our results, this protein expressed in native form and found in the flow-through. These results were in agreement with the findings of the other researchers that corroborated that the *E. coli* BL21 (DE) pLysS expression vector was able to express Loa22 protein in a soluble form (5, 16).

Protein purification is a set of processes that usually involves one or more chromatographic steps, depending on the size, physicochemical properties, binding affinity, and biological activity (26). The presence of the N-terminal His-tag facilitated an efficient Ni-NTA affinity purification, which resulted in a proper yield of soluble purified recombinant protein without contaminants. The optimization of the purification process conducted at the stage of binding, washing, and elution. Increasing the time and repetition of the binding process was expected to increase the binding target protein to Ni-NTA resin. The more washing also there will be produced proteins that have fewer impurities. Imidazole in low concentration in washing solution (25 mM) can reduce nonspecific binding of resin.

Concentration of imidazole, as a competitive agent for elution of histidine-Tagged proteins due to having a structure similar to the histidine, influences the elution process. In the elution step, it found that increasing the concentration of imidazole and the pH of the elution buffer around the pI of the protein lead to an increase in both concentration and purity of the protein. In addition, pH adjustment is necessary to keep the protein soluble and not degraded.

Hence, following the thorough utilization of optimization parameters, the purification of the Loa22 recombinant protein under native conditions using Ni-NTA pull-down was optimum in a one-hour binding at 37°C, five times washing process, and elution buffer with a pH a near- neutral range (7.4) and the 0.3 M imidazole concentration.

CONCLUSION

Based on the results, the high percentage of *loa22* gene sequence similarity between Iranian serovars and serovars from other countries proves that this gene is conserved. We used this conservation to design a gene construct based on the *loa22* gene that can cover all serovars. Then we optimized the expression and purification process for over production of Loa22 protein. The large quantity of pure recombinant antigen produced by these methods forms the basis for future investigation on the design of rapid diagnostic tests and more effective vaccine for leptospirosis based on Loa22 protein in prompt treatments and prevention and control of leptospirosis.

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