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Original Article

Expression of *Phlebotomus papatasi* Salivary Protein 15 (PpSP15) in COS-7 Cells

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Abstract

Background: Cutaneous leishmaniasis (CL) is a neglected tropical infection and the most prevalent vector-borne disease in Iran. There is no approved human vaccine and current treatments are restricted; some drugs are expensive and have notable side effects. Therefore, the need for the development of a safe and effective vaccine that can be produced at a low cost remains urgent. It has been shown that vaccinating animals with salivary gland homogenate or saliva components of sand flies protected against *Leishmania* infection. In this study, we aimed to prepare a mammalian expression vector encoding *Phlebotomus papatasi* salivary protein 15 (PpSP15) intended to be used as a DNA vaccine in our forthcoming studies.

Methods: In this study, we designed and constructed pcDNA3. 1, a constitutive mammalian expression vector, to encode the immunogenic protein PpSP15. The presence of the target gene was confirmed by enzymatic digestion and sequencing. The mammalian COS-7 cells were transfected with the pcDNA3.1 vector and the expression of PpSP15 protein was then examined in the cell line using Western Blotting analysis.

Results: Restriction enzyme digestion and sequencing revealed the correctly constructed pcDNA3.1-PpSP15. After the transfection of the COS-7 cell line with pcDNA3.1-PpSP15 using Linear Polyethylenimine, the PpSP15 protein expression was confirmed by western blot analysis using anti-His antibody.

Conclusion: A high expression level of PpSP15 protein in COS-7 cells was achieved after the transfection of COS-7 cells, using cationic Linear Polyethylenimine. In subsequent research, this recombinant plasmid is supposed to be utilized as a candidate DNA vaccine to find its immunity induction in susceptible animal models.

Keywords: *Leishmania major*; *Phlebotomus papatasi*; PpSP15; pcDNA 3.1; PEI

Introduction

Leishmaniasis is a vector-borne parasitic disease that is endemic in more than 100 countries worldwide (1) and transmitted by sand fly bites. Intracellular parasites of the genus *Leishmania* are the causative agents of the disease (2). Mucocutaneous leishmaniasis (MCL), visceral leishmaniasis (VL), and cutaneous leishmaniasis (CL) are the main clinical manifestations of the disease. Every year, 700000 to 1 million new cases are reported worldwide, putting an estimated 1 billion individuals at risk of the disease (3). Cutaneous leishmaniasis

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is more common and affects more people than VL (4). Algeria, Afghanistan, Brazil, Iran, Peru, Saudi Arabia, and Syria account for over 90% of CL cases (5). In Iran in 2008, there were 23202 cases of CL, however, in 2019, the cases decreased to 13124 due to control efforts (6).

Infected female sand flies inoculate saliva along with the *Leishmania* parasite into their host during blood-feeding. Interestingly, components of the saliva alter the feeding site's environment in favor of the parasite development (7, 8). However, it has been found that immunization with specific salivary proteins or prior exposure to uninfected sand flies bites elicit protective Th1 adaptive responses in animal models against subsequent *Leishmania* infection (9–12). Therefore, it is assumed that the induction of an early phase Th1 response, which inhibits the formation of permissive phagocytes and effectively contributes to disease control, is most likely related to salivary-mediated protective anti-*Leishmania* immunity (13, 14). Saliva-based cocktail vaccines have gained significant attention as they contain both parasitic antigens and immune-stimulating components of saliva. Despite the efforts of experts over the past few decades, there is still no appropriate specific treatment available for any form of human leishmaniasis, and there is no protective human vaccine either. Chemotherapy is the main treatment currently available, however, it has significant drawbacks including toxicity, high cost, challenging administration route, and ineffectiveness in endemic areas (15, 16). Therefore, a leishmaniasis vaccine that is both effective and safe is desperately needed.

Leishmania vaccines can be classified into three generations. First-generation vaccines consisted of whole-killed *Leishmania*, live-attenuated parasites, or fractions of the pathogen with or without adjuvants that demonstrated lackluster efficacy in trials. Although the first-generation vaccines are still being evaluated, the focus is now on the second-generation vaccines. Second-generation vaccines are composed of DNA vaccines, recombinant proteins, or a combination of them, which are relatively easy to produce at a low cost. This type of vaccine is covered in greater depth below.

Finally, third-generation vaccines, which include cDNA encoding protective *Leishmania* antigen, cloned into a eukaryotic vector for protein expression are the most recent approach (16).

DNA vaccines are a safer type of vaccination to administer (17, 18) and can stimulate the host immune system's humoral and cellular responses against various diseases, notably infectious ones (19, 20). In this type of vaccine, a specific antigen-encoding gene is inserted into a vector (19). The gene should be expressed in a selected vector, to prompt a cellular and adaptive immune response, such as the T-cell response that is required to create an effective leishmaniasis vaccine (8). The pcDNA3.1 is a 5.4 kb vector that is derived from pcDNA3 and has been designed for both high-level stable and transient expression in mammalian hosts and is frequently used to express recombinant proteins (21). The Kozak sequence preceding the start codon (ATG) enhances the expression of recombinant proteins. As most eukaryotic mRNA sequences contain adenine in position-3, the GCCACC motif is considered ideal for initiation, rather than GCCGCC (22, 23).

In addition to choosing a suitable vector, specific antigens that can trigger an immune response are required to construct a DNA vaccine (17, 24). Recent studies demonstrated that immunization with the PpSP15 salivary protein of *Phlebotomus papatasi* can protect BALB/c mice against *Leishmania major* infection, and it was determined that PpSP15 is an immunogenic protein and considered a vaccine candidate (25). Here, the pcDNA3.1 vector was transferred to the mammalian COS-7 cells and the expression of PpSP15 protein was then examined in the cell line using West-

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ern Blotting analysis. COS cell-based mammalian expression systems are widely used because they are easily obtainable, easy to maintain in culture, and transfect. These plasmids are replicated at a high rate in transfected COS cells. The recombinant plasmid can be subsequently tested in an animal model to analyze its ability to induce immunity and be considered for use as a DNA vaccine (26). This study aims to clone the SP15 gene from *Ph. papatasi* into the pcDNA3.1 vector and to evaluate its expression in the COS-7 cells at a low-cost to produce recombinant SP15 protein.

Materials and Methods

Gene cloning

The Iranian *Ph. papatasi* saliva, PpSP15 gene (NCBI accession number: MN938856.1), was codon optimized for expression in mice with a hexa-histidine tag at the C-terminus. The HindIII and EcoRI restriction enzyme cutting sites are at the 5′ and 3′ of PpSP15. This fragment is 426 bp in length, and the Kozak translation initiation sequence (GCC ACC) has been placed before the start codon to enhance eukaryotic transcription (Fig. 1). pcDNA3.1 (+) encoding PpSP15 was synthesized by Gene Universal Company, United States. pcDNA-GFP plasmid was also obtained from this company.

Transformation of recombinant plasmid

The *Escherichia coli* strain Top 10 colony was obtained from the Pathobiology Department, School of Public Health, Tehran University of Medical Sciences. The cloned vector pcDNA3.1-PpSP15 was transformed into competent *E. coli* strain Top10 by $CaCl_2$ and heat shock protocol described by Sambrook (27). Briefly, 50 ng of DNA plasmid was added to 200 µl $CaCl_2$-treated cells, mixed gently, and incubated on ice for 30 min. The transformation mixture was heat shocked in a 42 °C water bath for 90 seconds. After that 800 µl of Luria broth medium (Ibresco Life Science, Iran) was added to the tube, incubated for 45 min at 37 °C at 200 RPM in a shaker, and then about 200 µl of transformed competent cells were plated on Luria–Bertani (LB) agar containing 100 µg/ml ampicillin incubated overnight at 37 ℃ to pick the recombinant colonies.

Analyze transformants for the presence intended gene

The presence of PpSP15 in the recombinant plasmid was assessed by enzymatic digestion using EcoRI (Fermentas, Germany) and HindIII (Thermo Scientific, USA). Plasmids were purified from transformants cultured in LB broth containing ampicillin according to the Plasmid maxi kit protocol (FavorPrep Plasmid DNA extraction maxi kit, Favorgen, Taiwan). This reaction was performed in a sterile microtube in a final volume of 20 µl containing 1 µg of DNA plasmid encoding the PpSP15 gene, 3 µl of 10x buffer, 1 unit of each digestion enzyme, and incubated for 3 hours at 37 °C. The product was then electrophoresed on 0.8% safe-stained agarose gel and photographed. Also, sanger sequencing with universal primers (CMV and BGH as forward and reverse primers) was performed to verify the integrity and lack of any mutations in the PpSP15 gene insert (Pishgam Biotech Co., Iran).

COS-7 cell transfection using linear polyethylenimine (PEI)

To confirm the PpSP15 protein expression potency in pcDNA(+)-PpSP15 construct, the COS-7 cell line (ATCC CRL-1651), an African green monkey (*Cercopithecus aethiops*) kidney fibroblast-like cell line, was obtained from the Pasteur Institute of Iran and was used as an expression host. Briefly, COS-7 cells were counted with a hemocytometer and cultured into each well of a six-well plate, a total of 2×10^5 COS-7 cells per well in complete RPMI medium (Roswell Park Memorial

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Institute) supplemented with 10% FCS (Gibco, New York), gentamicin (50mg/ml), additional NaCl (180 mg/ml), and L- glutamine (292 mg/ml) at 37 °C and in the presence of 5% CO_2 overnight. After 24 h, the cells become adherent and reach a confluency of approximately 80%. pcDNA-PpSP15, pcDNA-GFP, and pcDNA3.1 plasmids were transfected into COS-7 cells using PEI 25 kDa as a cationic transfection reagent. Briefly, PEI/DNA complexes were prepared by mixing Linear PEI (LINPEI 10 µM, 25 kDa, NrE= 10) with 5 µg of recombinant plasmid (pcDNA(+)-PpSP15), pcDNA-GFP (to check the transfection efficiency) or pcDNA3.1 (as the vector control) in a final volume of 300 µl HBS buffer (NaCl 0.15 M and HEPES 0.02 M, pH 7.4) and incubating for one hour at room temperature. After washing cells with serum- and antibiotic-free medium, PEI/DNA complexes were introduced into each well for 6 hours. Fresh complete 10% heat-inactivated FCS medium was added to each well. The transfection efficiency of pcDNA-GFP was checked by fluorescence microscopy at 24 and 48 hours after transfection. After two days, pcDNA(+)-PpSP15 and pcDNA3.1 transfected cells were detached using cold PBS and then used for blotting.

Protein expression confirmation using western blot analysis

The expression of PpSP15 in transfected COS-7 cells was verified by Western blot analysis. Briefly, 48 hours following transfection, the transfected COS-7 cells were collected, mixed with SDS-PAGE sample loading buffer, boiled for 5 minutes, and then run on a 15% SDS-PAGE gel. Subsequently, proteins were electrotransferred from the gel onto the polyvinylidene difluoride (PVDF) membrane.

Free binding sites on the PVDF membrane were blocked using a blocking solution (Phosphate-buffered saline containing 2.5% Bovine serum albumin and 0.05% Tween 20) for 2 hours. Following the three washing steps (TBS_Tween 0.1%), the membrane was incubated for 2 hours at room temperature with mouse anti-His antibody. After washing, the membrane was incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (1:2000) for 2 hours at room temperature, and the chromatic reaction was detected using a 3, 30-diaminobenzidine substrate (DAB). The molecular weight of PpSP15 was predicted by www.aatbio.com

Results

Confirmation of pcDNA-PpSP15 construct

As shown in Fig. 1, the digestion result was validated on 0.8% safe-stained agarose gel. A DNA band at 459 bp, showed that the SP15 gene had been successfully inserted into the plasmid vector.

In addition, according to an analysis of the Sanger sequencing results, there are no mutations in the DNA insert, and the integrity of the sequence was confirmed to be similar to the reference gene.

Transfection efficiency of pcDNA-GFP transfected COS-7 cells using PEI

In vitro GFP expression was determined by fluorescence microscopy at 24 and 48 h after transfecting COS-7 cells. The best conditions were achieved using PEI at NrE: 10 at 48 h after transfection. Fig. 2A and 2B shows before and after glinting of fluorescence, respectively.

Confirmation of PpSP15 protein expression in COS-7 cells

Protein PpSP15 expression in transfected COS-7 cells was confirmed by western blot analysis using an anti-His antibody (Fig. 3). In COS-7 cells transfected with pcDNA(+)-PpSP15, a 17.5 kDa protein band was observed that related to PpSP15 protein expression.

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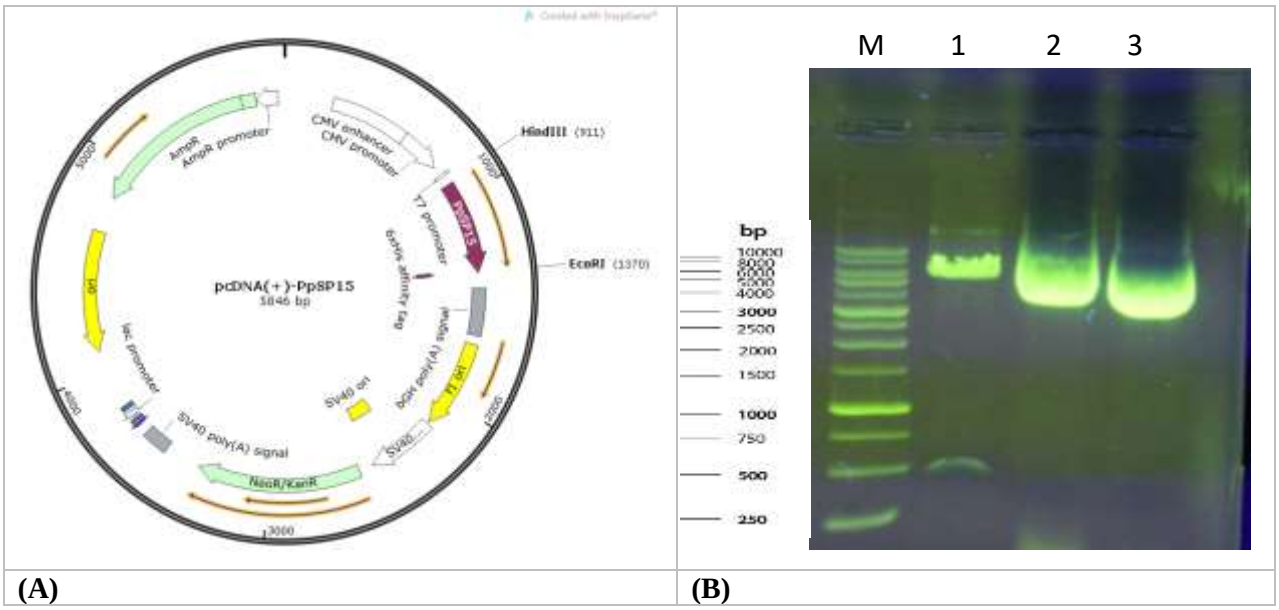

Fig. 1. pcDNA3.1-PpSP15 construct. (A) pcDNA3.1-PpSP15 plasmid map. (B) Confirmation of pcDNA3.1-PpSP15 construct: M: 1 kb DNA ladder (SMOBIO). Lane 1: EcoRI and HindIII digested pcDNA3.1-PpSP15 (Arrow Shows the Band related to the PpSP15 gene with 459bp). Lane 2: Supercoiled undigested pcDNA 3.1-PpSP15. Lane 3: Supercoiled undigested pcDNA 3.1

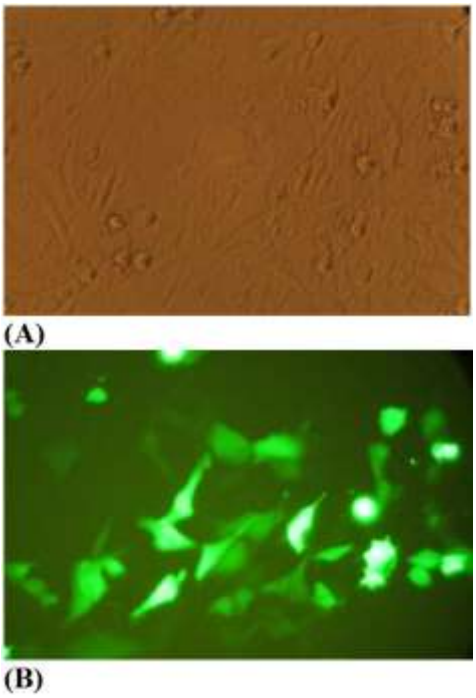

Fig. 2. Transfection efficiency of pcDNA-GFP (green fluorescent protein) using Polyethylenimine in COS-7 cell line before (A) and after 48 hrs (B) glinting of fluorescence. The COS-7 cells were treated with 5 µg of pcDNA-GFP plasmid combined with PEI 25 kDa in serum- and antibiotic-free medium. The in vitro GFP expression was assessed by fluorescence microscopy at 24 h after transfection

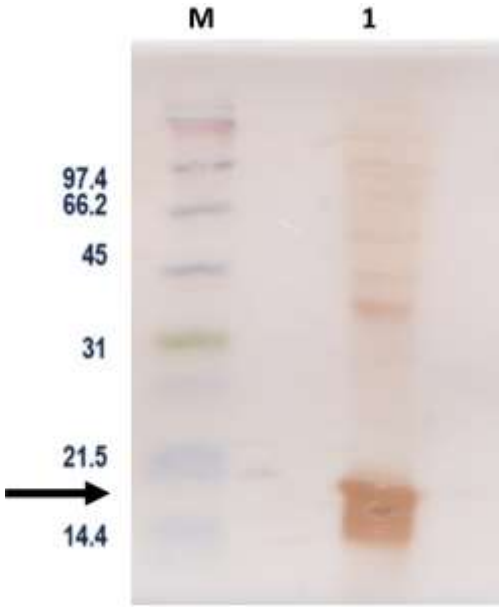

Fig. 3. Confirmation of *Phlebotomus papatasi* salivary protein PpSP15 expression in transfected COS-7 cells using western blot analysis with anti-His antibody. The COS-7 cell line was transiently transfected with pcDNA(+)-PpSP15. Forty-eight hours after transfection, the cells were subjected to western blot analysis using anti-His antibody. M: pre-stained protein ladder (Thermo Fisher), Lane1: PcDNA3.1-PpSP15 transfected COS-7, Lane2: pcDNA3.1(+) transfected COS-7 as a control. The existence of a 17.5 kDa band for PpSP15 is shown

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Discussion

Every attempt to develop a vaccine against human leishmaniasis has failed so far (28). It is therefore necessary to develop new vaccines with ideally novel mechanisms of action that are safe, reliable, and effective. One of the reasons for the development of leishmaniasis vaccines is that most people previously diagnosed with active ZCL can resist subsequent clinical infection. Also, there is strong evidence that the vector's saliva plays an essential role in the development of the disease (29). Several studies have demonstrated that the immune response to a single protein in sand fly saliva or exposure to the bites of uninfected sand flies can protect against *Leishmania* infection (11, 13). Consequently, it seems prudent to investigate the potential of these components in creating potent anti-*Leishmania* vaccines.

pcDNA3 has been extensively used in numerous studies, including vaccination for the treatment of different diseases (30–32). Some of the characteristics of pcDNA3.1 plasmid which indicate that this vector could be the right choice in this study, include a) a human cytomegalovirus immediate-early promoter for high-level expression in many mammalian cell lines. Since this promoter can drive foreign gene expression in mammalian cells, transfection with this vector is the ideal strategy for expressing desired genes in mammalian cells (33), b) a bovine growth hormone (BGH) polyadenylation signal region that is a specialized termination sequence to boost mRNA stability (32, 33), c) SV40 promotor (promoter of the simian virus 40 to enhance translation, d) pUC origin that facilitates *E. coli* cell replication (33), e) multiple cloning sites that are oriented in both the forward (+) and reverse (-) directions for facilitating cloning.

In addition to the selection of an appropriate vector, the choice of an immunogenic molecule is another major challenge. Recently, several sand fly salivary proteins have been investigated as potential candidates for vector-based vaccines against cutaneous leishmaniasis, such as PpSP15 (25), PpSP28 (34), PdSP 15 (35), PsSP9(36), Maxadilan (37), Linb 11 (38), and SALO (39, 40). *Phlebotomus papatasi* salivary contains approximately 49 different proteins (41). PpSP15 (*Ph. papatasi* Salivary Protein 15), is one of these compounds that has been thoroughly investigated because it is highly immunogenic and has been shown to protect animal models against *L. major* infection (25). So, in this study, we generated a recombinant pcDNA-PpSP15 construct for PpSP15 protein production.

Escherichia coli, a gram-negative bacterium, was used for transformation because, unlike gram-positive bacteria, gram-negative bacteria preferentially interact with and transfer double-stranded DNA, although the former transfer only one strand into the cell (42). Other characteristics of *E. coli* that have led to it being used more than other prokaryotic organisms to produce recombinant proteins include its short generation time, ease of culture, and cost-effectiveness (43). The selection of the Top 10 strain, was also based on its suitability for cloning (44).

Before using DNA constructs as a DNA vaccine, it is necessary to in vitro check protein expression in mammalian cells. Utilizing the mammalian cell line expression systems has rendered them an optimal platform for protein production due to their ability to ensure accurate protein folding and facilitate essential post-translational modifications like phosphorylation, glycosylation, and acetylation. These processes are crucial for the proper functioning of proteins in biological systems (45). Protein expression has been facilitated by the utilization of various mammalian cell lines, including HEK 293 (44), CHO (45), HeLa (46), Vero(47), NS0 (48), and COS (26). COS-7 cells represent one of the three categories of COS cells that are extensively utilized as host cell lines for the expression of recombinant proteins (46). There are numer-

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ous techniques for transfection, which can be classified into three categories: biological, chemical, and physical methods (47). An optimal approach should possess low cytotoxicity, negligible impact on normal physiology, superior transfection efficiency, user-friendly operation, and cost-effectiveness (49, 50). Linear polyethylenimine, a cationic chemical method employed in this investigation, encompasses most of the attributes above.

Considering the significant importance of the PpSP15 protein as a potential candidate for an anti-*Leishmania* vaccine, it is crucial to achieve a high level of expression of this protein in eukaryotic cells to stimulate the immune system which this study allowed us to accomplish.

Conclusion

We succeeded in constructing the pcDNA 3.1 vector encoding SP15 derived from the *Ph. papatasi* Iranian strain and its transfection in COS-7 cells. Considering that this recombinant plasmid has a very high expression of PpSP15 in eukaryotic cells, in the next phase, the protective immunity of the constructed plasmid as a candidate anti-*Leishmania* DNA vaccine should be evaluated in the murine model. This study is a promising first step toward a candidate vaccine.

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Ethical considerations

This project was approved by the Ethical Committee of the Tehran University of Medical Sciences (Code No: IR.TUMS.SPH.REC. 1400.024).

Conflict of interests

The authors have no conflict of interest to declare.

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