FULL LENGTH	Iranian Biomedical Journal 29 (4): 206-215 July 2025

Apyrase Protein (PsSP42) Expression in DNA Construct Transfected COS-7 Cells

Samira Hosseinpour Jahednia[1,2], Hossein Rezvan[1*], Alireza Nourian[1], Tahereh Taheri[2], Negar Seyed[2], Zahra Eghbali[2] Elham Gholami[2], Sima Rafati[2*]

[1]Department of Pathobiology, Faculty of Veterinary Medicine, Bu-Ali Sina University, Hamedan, Iran;
[2]Department of Immunotherapy and Leishmania Vaccine Research, Pasteur Institute of Iran, Tehran, Iran

OPEN ACCESS

Article type: Research Article
Received: May 28, 2025
Revised: June 23, 2025
Accepted: June 29, 2025
Published online: June 25, 2025

How to cite:
Hosseinpour Jahednia S, Rezvan H, Nourian A, Taheri T, Seyed N, Eghbali A, Gholami D, Rafati S. Apyrase Protein (PsSP42) Expression in DNA Construct Transfected COS-7 Cells. *Iran. Biomed. J.* 2025; 29(4): 206-215.

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ABSTRACT

Background: DNA vaccines offer dual humoral and cellular immunity, showing potential effectiveness against intracellular parasites, including *Leishmania*. However, challenges in delivering large DNA constructs has been already existed, which influence protein expression and immune efficacy. This study evaluated the expression of *P. sergenti* salivary protein PsSP42 in COS-7 cells. Confirming the expression of PsSP42 is crucial before its use as a DNA vaccine in preclinical experiments.

Methods: The expression of the PsSP42 protein was achieved using two plasmids: the small, antibiotic-free plasmid NTC9385R and the conventional plasmid VR1020. Both recombinant vectors were transfected into COS-7 cells using electroporation and PEI-mediated transfection methods. Ni-NTA beads were utilized to enrich proteins from the supernatants collected from transfected cells, and expression was confirmed by Western blotting.

Results: Successful expression of the PsSP42 protein was affirmed from both VR1020-PsSP42 and NTC-PsSP42 constructs in the transfected COS-7 cells, regardless of the transfection method employed (PEI or electroporation). A 39.6 kDa band corresponding to the PsSP42 protein was detected, indicating its secretion into the supernatants of COS-7 cells transfected with both plasmids.

Conclusion: Plasmids VR1020-PsSP42 and NTC-PsSP42 demonstrated similar protein expression levels in vitro, regardless of the transfection method used. Performing these evaluations is recommended to thoroughly assess construct expression levels before conducting in vivo studies.
DOI: 10.61882/ibj.5055

Keywords: Electroporation, Gene expression, *Leishmania tropica*, Polyethylenimine

Corresponding Authors:
Hossein Rezvan
Department of Pathobiology, Faculty of Veterinary Medicine Bu-Ali University, Hamedan, Iran; E-mail: h.rezvan@basu.ac.ir;
ORCID ID: 0000-0002-9328-0245

Sima Rafati
Department of Immunotherapy and Leishmania Vaccine Research, Pasteur Institute of Iran, Tehran, Iran; E-mail: s_rafati@yahoo.com;
ORCID ID: 0000-0002-9328-0245

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[DOI: 10.61882/ibj.5055]

List of Abbreviations:
CMV: cytomegalovirus; **EGFP:** enhanced green fluorescent protein; **FCS:** fetal calf serum; **HBS:** hepes buffered saline; **Histidine-tag:** His-tag; **NTC:** nanoplasmid; ***P. sergenti***: *Phlebotomus sergenti*; **PEI:** polyethylenimine; **pI:** isoelectric point; **RPMI:** RPMI-1640; **TPA:** tissue plasminogen activator

INTRODUCTION

DNA vaccines have shown advantages due to their unique characteristics, such as simplicity and cost-effectiveness in large-scale production. They effectively activate both humoral and cellular immune responses and do not require a cold chain for storage and distribution, a limitation of many vaccine platforms[1,2]. These attributes make DNA vaccines promising candidates for combating infectious diseases. To fully understand their potential, selecting an appropriate vector and an effective delivery method is crucial to ensure the successful transfection of the DNA construct into host cells. Successful transfection is necessary for eliciting a robust immune response against the target pathogen. A major challenge in this process is the low transfection efficiency that is often associated with large DNA constructs, which can significantly impair protein expression. Since the effective intracellular expression of DNA constructs is critical for triggering strong immune responses in experimental models, in vitro evaluation of expression levels is fundamental before conducting in vivo studies.

Leishmaniasis is a neglected tropical disease caused by motile, unicellular protozoan parasites of the genus *Leishmania*[3]. Clinically, it presents in three distinct forms: cutaneous, mucocutaneous, and visceral, depending on the vector type and the host's immune response[4]. Current drug treatments are significantly hindered by toxicity, high costs, and resistance[5,6]. Hence, vaccination has emerged as a vital and promising strategy for the effective prevention and control of leishmaniasis.

Vaccine development against leishmaniasis has progressed through several generations. First-generation vaccines include killed, live-attenuated, and purified fraction vaccines derived from *Leishmania* parasites[7], serving as foundational approaches. Second-generation vaccine utilizes synthetic or recombinant subunit antigens[8,9], while third-generation such as DNA vaccines, represent the cutting-edge advancements[10]. The effectiveness of these vaccines is influenced by various critical factors, including the selection of appropriate antigens, the use of effective adjuvants, and the optimization of delivery methods[11].

In recent years, growing evidence has highlighted the potential of sand fly salivary proteins as vaccine candidates against leishmaniasis[12,13]. When feeding, infected female sand flies inject saliva together with *Leishmania* parasites into their hosts. The components of saliva significantly alter the bite site, facilitating parasite transmission and potentially increasing pathogenicity[14]. Research has demonstrated that immunization with specific salivary proteins, or prior exposure to bites from uninfected sand flies, can activate a protective Th1 adaptive immune response in animal models. This immune response appears to play a vital role in protecting against future infections with *Leishmania* species[15]. The immunity provided by saliva may arise from its ability to induce an early Th1 response, which is vital for minimizing the development of permissive phagocytes and regulating disease progression[16].

Given the demonstrated efficacy of DNA vaccines against *Leishmania* infection[17-19] and the advantages of using novel antibiotic-free and small-sized NTC plasmids, we evaluated the expression of PsSP42, an apyrase enzyme derived from *P. sergenti*, in COS-7 cells transfected with the NTC-PsSP42 construct. Transfection was conducted using two different methods: electroporation and PEI. This expression analysis is important for the subsequent immunization studies involving experimental BALB/c mice.

MATERIALS AND METHODS

Preparation and confirmation of constructs encoding *PsSP42*

The *PsSP42* gene (NCBI accession number: HM560861), which encodes an apyrase enzyme, was codon-optimized for expression in mammalian cells. The gene, kindly provided by Dr. Jesus Valenzuela (Vector Molecular Biology Section, Laboratory of Malaria and Vector Research, National Institute of Allergy and Infectious Diseases, NIH, MD, USA), was cloned into a modified mammalian expression plasmid (VR1020-TOPO). The 1058-bp insert included a signal secretory peptide derived from TPA at the N-terminus, which was replaced by the native signal peptide of PsSP42 found in sand flies. Additionally, a hexa-His tag was located at the C-terminus. PCR amplification of the *TPA-PsSP42-6×His* fragment was performed using the VR1020-PsSP42 construct as a template, along with specific primers that introduced *Sal*I and *Not*I restriction sites (Table 1). The amplified fragment, measuring 1100 bp, was subcloned into the *Sal*I and *Not*I restriction sites of a predigested and gel-purified NTC9385R vector. To confirm the successful insertion of the fragment into the vector, the VR1020-PsSP42 and NTC-PsSP42 constructs were digested with *Kpn*I, along with *Sal*I and *Not*I restriction enzymes, respectively (Thermo Scientific, USA). The integrity of the cloned gene sequence was then verified by submitting the NTC-PsSP42 recombinant vector for Sanger sequencing analysis. The NTC-EGFP construct (720 bp) had been previously prepared and fully validated[20]. Recombinant plasmids (VR1020-PsSP42, NTC-PsSP42, and NTC-EGFP) were purified using the

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Table 1. Primer sequences designed for subcloning

Forward primer	5′-GCGTCGACATGGATGCAATGAAGAGAGGGCT-3′	*Sal*I
Reverse primer	5′-TAGCGGCCGCACAGCAGATCTGGATCGA-3′	*Not*I

The underlines indicate the site of enzyme digestion.

Endo-Free Plasmid Mega Kit (Qiagen, Germany) according to the manufacturer's protocol. The integrity of the constructs was confirmed through restriction digestion and PCR analysis as explained above.

COS-7 cell culture

The COS-7 cell line (ATCC CRL-1651) was derived from kidney fibroblast-like cell lines of the African green monkey (*Cercopithecus aethiops*). The cell line was obtained from the National Cell Bank of the Pasteur Institute of Iran (Tehran, Iran). The cells were cultured in complete RPMI medium (Sigma, Germany), which was supplemented with 5% heat-inactivated FCS (hiFCS, Gibco, Germany), 10 mM of HEPES (Sigma), 10 mM of L-glutamine (Sigma), and 50 µg/mL of gentamicin (Sigma). The cultures were maintained in a humidified incubator containing 5% CO_2 at 37 °C. The cells were counted using a hemocytometer, and 2×10^5 cells were seeded into each well of a six-well plate. Following overnight incubation, the cells reached approximately 80% confluency within 24 hours and were subsequently used for transfection.

COS-7 cell transfection using PEI

Cationic polymers such as PEI are effective delivery systems for nucleic acids due to their positive charge and amino group composition. The strong electrostatic interactions between these nucleic acids and the cationic polymers significantly enhance cellular uptake, facilitating the efficient entry of molecules into cells[21]. To prepare PEI/DNA complexes, we mixed 5 µg of each DNA construct VR1020-PsSP42 (used as a positive control[15]), NTC-PsSP42, and NTC-EGFP (used to assess transfection efficiency) with 26 µL of linear PEI (10 µM; 25 kDa; NrE = 10) in HBS buffer (0.15 M of NaCl and 0.02 M of HEPES, pH 7.4). The mixture was adjusted to a final volume of 200 µL and incubated at room temperature for 1 hour. After washing the cells with serum- and antibiotic-free medium, we added the PEI/DNA complexes to each well and incubated them in a 5% CO_2 environment at 37 °C for 6 hours. Following the incubation, we replaced the medium with fresh complete RPMI medium supplemented with 5% FCS. EGFP expression was monitored using a fluorescent microscope 24 hours post-transfection. After 48 hours, supernatants from COS-7 cells were transfected with VR1020-PsSP42 and NTC-PsSP42 for Western blot analysis.

COS-7 cell transfection with electroporation

Electroporation is a technique that uses brief electric impulses to temporarily disrupt the cell membrane, facilitating the entry of macromolecules such as DNA into the cells[22]. On the day of the procedure, we carefully harvested adherent COS-7 cells using a pre-warmed 0.25% trypsin solution. After neutralizing the trypsin with complete RPMI medium supplemented with 5% FCS, we centrifuged the cell suspension at 250 ×g for 5 minutes. The resultant cell pellet was resuspended in RPMI medium adjusted to pH 7.2, without any additives, and then counted. We proceeded to resuspend a total of 2.5×10^5 COS-7 cells in 100 µL of RPMI and mixed them with 10 µg of either VR1020-PsSP42, NTC-PsSP42, or NTC-EGFP vector in 1.5 mL microtubes. This mixture was then carefully transferred to 2 mm electroporation cuvettes. Electroporation was conducted using the GenePulser Xcell™ (Bio-Rad, California, USA), configured to the wave-square program at a voltage of 180 mV. The procedure delivered two waves, with a 0.1-second interval and a pulse length of 5 ms. Immediately following the electroporation process, we plated the cells into six-well culture plates containing fresh complete RPMI medium supplemented with 5% FCS. The transfection efficiency of the NTC-EGFP was assessed using fluorescence microscopy 24 hours after transfection, illustrating the effectiveness of the electroporation approach.

Protein expression confirmation using Western blotting

The physicochemical properties of the PsSP42 protein were analyzed using the ProtParam tool (https://web.expasy.org/protparam), with a focus on key parameters, including molecular weight, pI, and instability index. We conducted Western blot analysis to confirm the expression of the PsSP42 protein in the supernatant of the transfected COS-7 cells. The previously evaluated PsSP42 protein from VR1020-PsSP42-transfected COS-7 cells[18], was used as a positive control. This protein includes a C-terminal His-tag, which facilitates subsequent enrichment processes. Employing the Ni-NTA beads, the supernatants collected from the transfected COS-7 cell culture were further enriched for Western blot analysis. Supernatants from COS-7 cells transfected with both VR1020-PsSP42 and NTC-PsSP42 constructs were harvested 48 hours post-transfection. Ni-NTA beads (50 µL) with pH

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adjusted to 8.0 to optimize His-tag/Ni-NTA interaction, were added to the supernatants and incubated on a rocker for three hours to allow for binding. Following incubation, the PsSP42-bound Ni-NTA beads were centrifuged at 250 ×g for 5 minutes. The resulting pellet was then mixed with 6× SDS-PAGE sample buffer (4.5 mM of Tris–HCl, pH 6.8; 10% v/v glycerol; 2% w/v SDS; 5% v/v 2-mercaptoethanol; 0.05% w/v bromophenol blue) and boiled for 5 minutes. After a quick centrifugation, the supernatant was subjected to SDS-PAGE using a 12.5% (w/v) polyacrylamide gel (SDS gel apparatus; Bio-Rad). The proteins were then transferred to a PVDF membrane (Thermo Scientific), which was blocked using Tris-buffered saline (1 mM of Tris/HCl and 15 mM of NaCl) containing 2.5% BSA and 0.05% Tween 20. The membrane was immersed in a mouse anti-His-tag antibody solution (1/2000 dilution; Qiagen) at room temperature for 2 hours. After washing, the membrane was treated with horseradish peroxidase-conjugated goat anti-mouse IgG antibody (1:5000 dilution; Sigma), and the chromogenic reaction was developed using DAB substrate (Merck, Germany).

RESULTS

Preparation and validation of VR1020-PsSP42 and NTC-PsSP42

The plasmid VR1020-PsSP42, provided as a gift, was used as a template for cloning the *PsSP42* into the NTC vector (Fig. 1). Specific primers (Table 1) were designed to amplify a 1100-bp fragment from the VR1020-PsSP42 vector, as depicted in the cloning pathway in Figure 1. After digestion with *Sal*I and *Not*I, the 1100-bp fragment was subcloned into the NTC9385R vector to generate NTC-PsSP42. The recombinant plasmids were verified using the above-mentioned primers. As shown in Figure 2, lanes 1 and 2 demonstrate the successful amplification of the 1100-bp fragment from both NTC-PsSP42 and VR1020-PsSP42, respectively. Digestion of NTC-PsSP42 with *Sal*I and *Not*I is demonstrated in Figure 1, with two fragments: a 1685-bp vector backbone and a 1089-bp fragment containing *PsSP42* (Fig. 2, lane 3). Furthermore, digestion of VR1020-PsSP42 with *Kpn*I yielded a 4803-bp vector backbone and a 1233-bp fragment containing *PsSP42* (Fig. 2, lane 4). Additionally, Sanger sequencing results confirmed that the amplified and cloned *TPA-PsSP42-6×His-tag* fragment showed the correct and complete sequence.

Fluorescence microscopy of COS-7 cells transfected with NTC-EGFP

In this study, the vector of interest (NTC-PsSP42) lacked fluorescent markers for microscopy. Therefore, to assess the effectiveness of the pre-set PEI and electroporation protocols for delivering the NTC plasmid, we re-evaluated transfection efficiency using the NTC-EGFP construct before conducting expression analysis. The results showed that both PEI, as a cationic transfection reagent, and electroporation successfully produced green fluorescent emissions from COS-7 cells. Our findings confirmed successful expression of EGFP in the COS-7 cells transfected with NTC-EGFP. Notably, the cells transfected with PEI exhibited greater cellular integrity compared to those subjected to electroporation (Fig. 3A and 3B).

Expression of PsSP42 protein in the supernatant of transfected COS-7 cells

The coding sequence for *PsSP42* was integrated into two vectors, each containing an N-terminal signal peptide and a C-terminal hexa-His-tag. To confirm the expression of the PsSP42 protein, we conducted a Western blot analysis on the supernatants of the transfected COS-7 cells using an anti-His antibody. We further assessed the physicochemical properties of the PsSP42 protein using the ProtParam tool. The signal-peptide-free protein, consisting of 317 amino acids, had an approximate molecular weight of 35.9 kDa, a pI of 8.94, and an instability index of 16.53, indicating acceptable stability. According to the metric scores, values below 40 are considered stable. The COS-7 cell line was transiently transfected with either VR1020-PsSP42 or NTC-PsSP42 plasmids, employing either PEI or electroporation methods. Following 48 hours of incubation, the supernatants were harvested, enriched with the Ni-NTA beads, and the proteins were then electrophoresed on a 12.5% SDS-PAGE gel. As observed in Figure 4, a 39.6 kDa band, corresponding to the PsSP42 protein containing a TPA signal peptide and 6×His tag, was detected. This observation confirmed the secretion of PsSP42 into the supernatants of both VR1020-PsSP42 and NTC-PsSP42 transfected COS-7 cells, regardless of the transfection method, as indicated by arrows in Figure 4.

DISCUSSION

During a sand fly bite, various salivary compounds containing bioactive molecules are transmitted to the host alongside the parasite. These compounds play a crucial role in modulating the host's immune response, either enhancing protection or exacerbating the progression of leishmaniasis[21]. Research has shown the efficacy of salivary gland homogenates[23] and specific proteins, such as apyrase and maxadilan, derived from *Phlebotomus kandelakii*[24] and *Phlebotomus papatasi*[25] sand flies. In 2015, Katebi et al. developed

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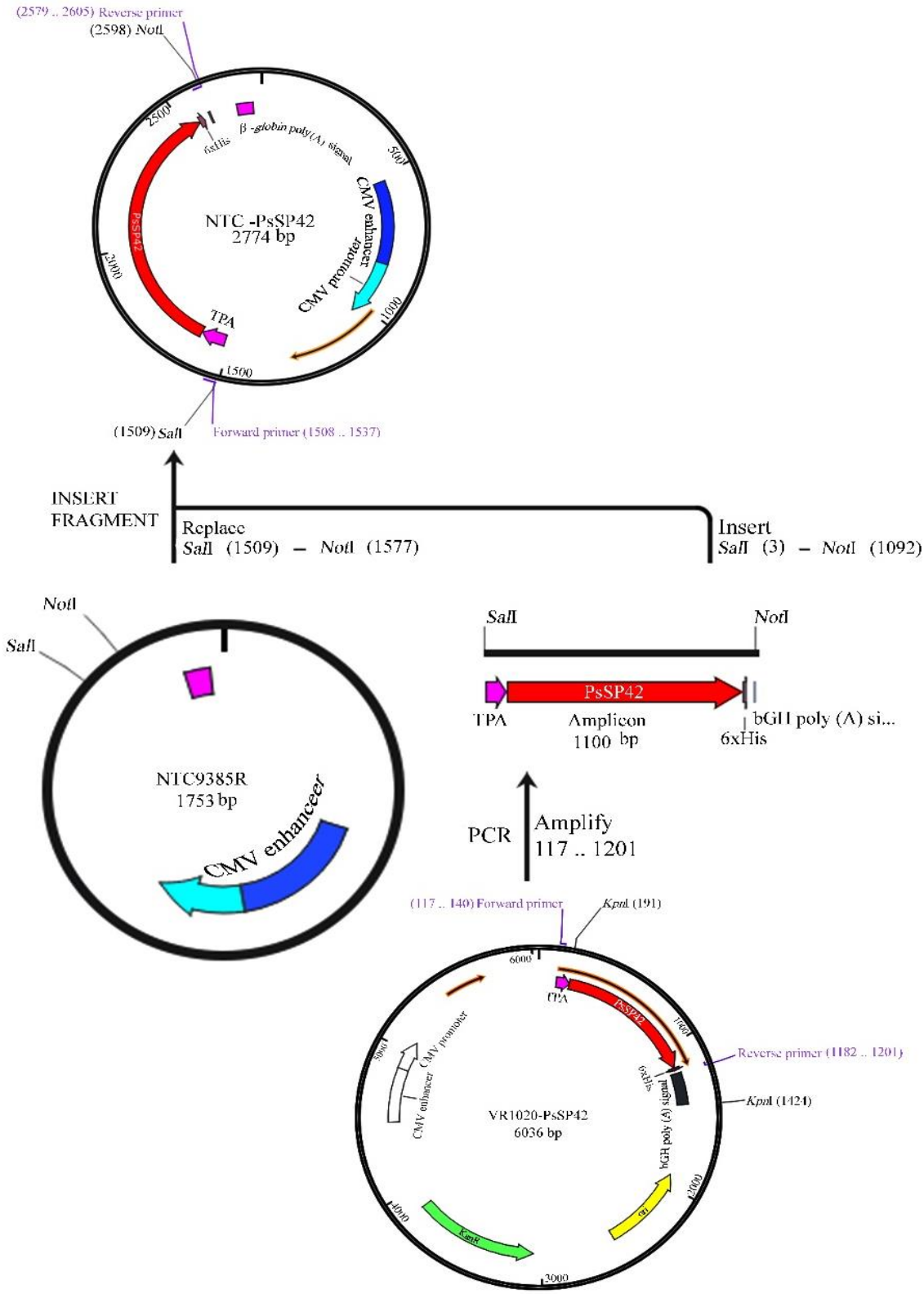

Fig. 1. Vector maps and cloning pathway for the VR1020-PsSP42 and NTC-PsSP42 plasmids. The VR1020-PsSP42 plasmid contains a strong CMV promoter, a transcription termination sequence from bovine growth hormone, kanamycin resistance gene, TPA coding sequence, and a C-terminal His-tag. *Kpn*I restriction enzyme sites and *PsSP42*-specific primer sites are indicated in the figure. The NTC-PsSP42 plasmid contains a strong CMV promoter, TPA coding sequence, and a C-terminal His-tag. *Sal*I and *Not*I restriction enzyme sites, as well as *PsSP42*-specific primer sites, are also depicted. The diagram illustrates the cloning path from VR1020-PsSP42 to NTC-PsSP42.

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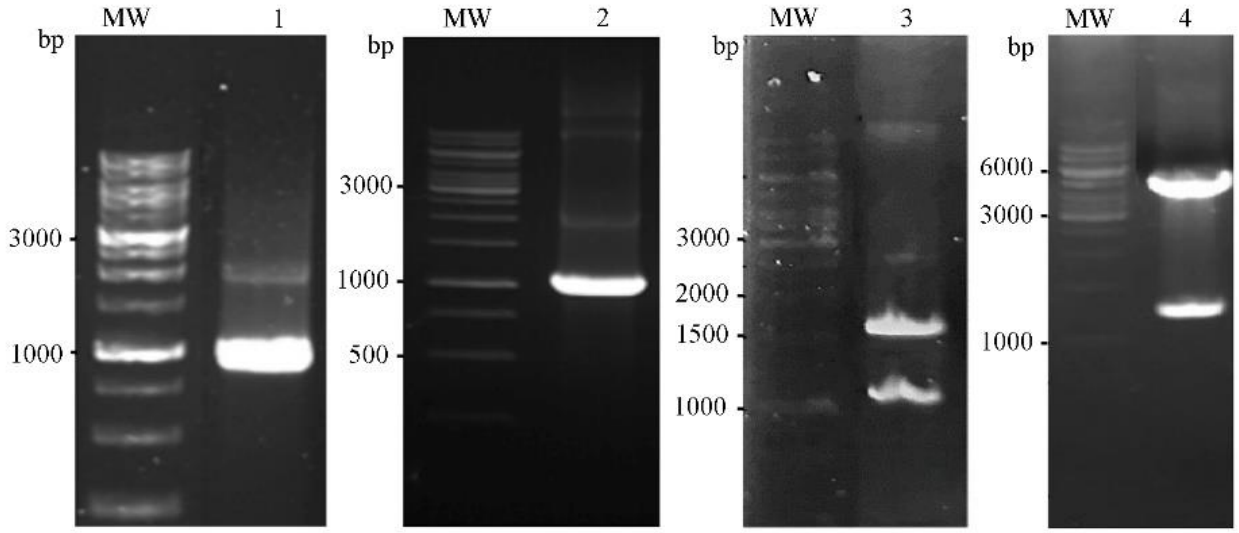

Fig. 2. Confirmation of the presence of the *PsSP42* sequence in both endotoxin-free purified NTC-PsSP42 and VR1020-PsSP42 plasmid constructs using PCR reactions and enzymatic digestion. Lane 1: PCR amplification of the *PsSP42* sequence (1100 bp) from the NTC-PsSP42 recombinant vector; lane 2: PCR amplification of the *PsSP42* sequence (1100 bp) from the VR1020-PsSP42 plasmid; lane 3: *Sal*I and *Not*I digestion of the NTC-PsSP42 led to production of fragments with 1685 bp and 1089 bp; lane 4: *Kpn*I digestion of the VR1020-PsSP42 resulted in fragments of 4803 bp and 1233 bp; Mw: 1-kb DNA ladder

a recombinant *Leishmania tarentolae*-PpSP15 and successfully assessed its immunogenicity and protective efficacy against *Leishmania major* infection in BALB/c mice. Their findings confirmed a robust cellular immune response, providing significant protection against the infection[26]. Similarly, Lajevardi et al. explored the potential of sand fly salivary proteins by evaluating a recombinant *Leishmania tarentolae* strain that co-expresses *PpSP15* and *PsSP9*. Their results demonstrated that immunized mice developed a strong protective Th1-type immune response[27]. An additional study by Gholami et al. indicated that immunization with a plasmid encoding the PsSP9 salivary protein from *P. sergenti* induced a pronounced Th1-type immune response, characterized by a strong delayed-type hypersensitivity in BALB/c mice[15]. Given the crucial role of sand fly salivary proteins in triggering immunity against leishmaniasis, *PsSP42*, a member of the apyrase family from *P. sergenti*, was selected for evaluation as a potential DNA vaccine candidate. However, thorough verification of protein expression in mammalian cells is essential before employing these constructs as DNA vaccine candidates in animal models.

The VR1020 and NTC9385R plasmids were strategically selected for evaluation in mammalian cells. Both plasmids contain a CMV promoter; however, VR1020 is larger (5064 bp) and includes a kanamycin resistance gene. In contrast, the NTC9385R-MCS plasmid, which is smaller in size (1753 bp), has recently gained significant attention due to the absence of antibiotic resistance genes. This feature not only eliminates the risk of developing antibiotic resistance in the host but also facilitates the translocation of the smaller plasmid across the cell membrane, enhancing protein expression efficiency[20,28]. The NTC9385R-MCS plasmid includes a chimeric CMV promoter and an HTLV-IR-U5 sequence containing a 5' splice acceptor site and a synthetic 3' acceptor site derived from the rabbit β-globin intron, significantly improving the efficiency and expression of the target protein[20,29-31]. Additionally, it encodes an antisense RNA (RNA-out) that suppresses the expression of SacB, which encodes levansucrase, an enzyme that is toxic in the presence of sucrose[32]. Thus, plasmid selection is effectively optimized in a sucrose-containing environment, independent of antibiotic pressure.

In a previously published study by Seyed et al., the NTC-EGFP plasmid was compared to the conventionally used pcDNA plasmid (pcDNA-EGFP) to evaluate transfection efficiency in COS-7 cells. Expression analysis using quantitative real-time PCR, which measured the copy number of EGFP mRNAs, indicated significantly higher levels of EGFP mRNA in pcDNA-transfected cells compared to NTC-transfected cells, despite equal plasmid concentrations. This finding contrasted with the intense EGFP fluorescence observed in NTC-EGFP-transfected cells. The discrepancy could be explained by the presence of an HTLV-I-related region in the NTC vector, which promotes translation efficacy even when total mRNA levels are lower. These

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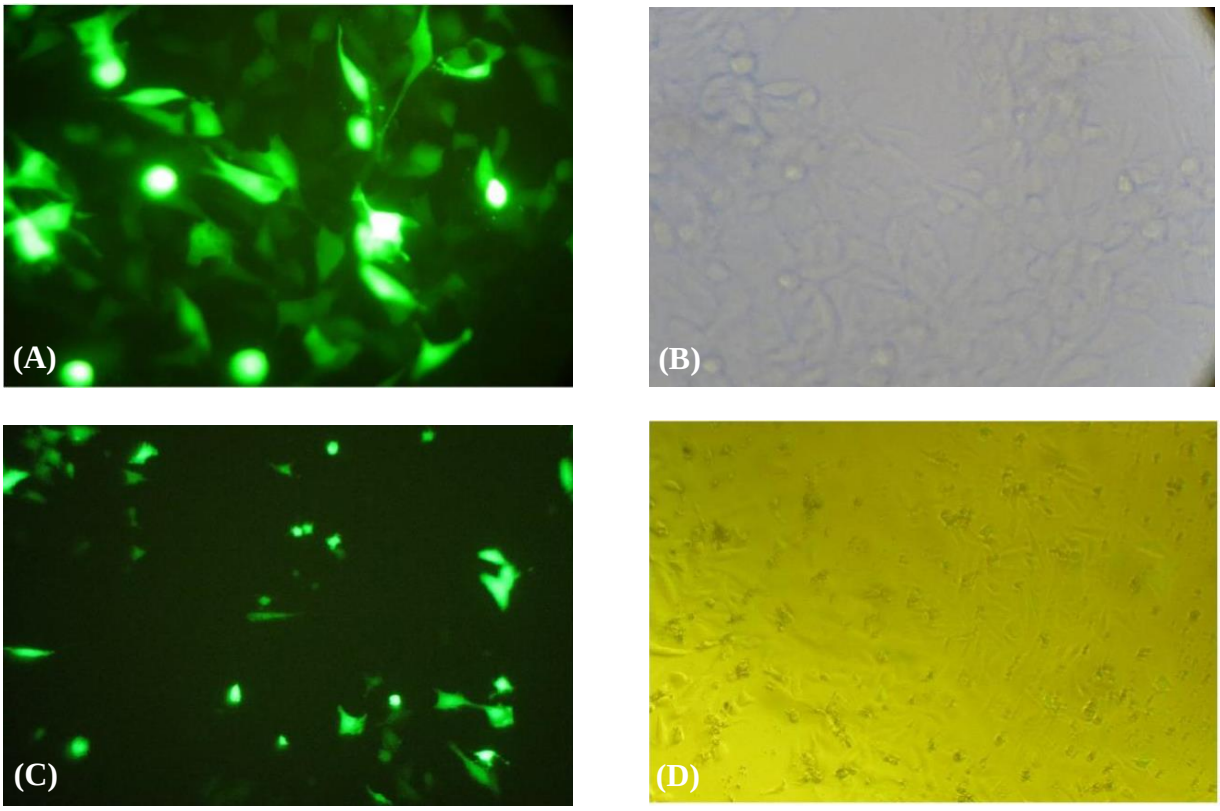

Fig. 3. In vitro evaluation of EGFP expression in COS-7 cells transfected with NTC-EGFP using fluorescence microscopy 24 h post-transfection. The NTC-EGFP-transfected COS-7 cell utilizing PEI after switching the fluorescent light on (A) and off (B). The NTC-EGFP-transfected COS-7 cell with an electroporation pulse after (C) and before (D) switching the fluorescence light.

observations suggest that the reduced mRNA levels in NTC-transfected cells are compensated by increased protein translation compared to the conventional CMV-based plasmids, such as pcDNA[20]. Based on this background, two DNA vaccine constructs encoding sand fly apyrase (PsSP42), including the conventional VR1020 plasmid and a novel NTC construct, were compared for protein expression in the transfected COS-7 cells. His-tagged PsSP42 proteins, enriched using Ni-NTA beads from both NTC-PsSP42- and VR1020-PsSP42-transfected COS-7 cells, were electrophoresed. Western blot analysis clearly demonstrated comparable expression levels of PsSP42 from both constructs following electroporation, confirming that the applied electrical pulse could effectively facilitate the delivery and expression of both plasmids (VR1020-PsSP42 and NTC-PsSP42), irrespective of their structural differences. Furthermore, Western blot results showed successful expression of PsSP42 protein in the supernatants obtained from PEI-transfected COS-7 cells for both VR1020-PsSP42 and NTC-PsSP42 constructs.

Given the structural differences between the two plasmids, it was anticipated that they would behave differently during transfection, and the NTC construct is expected to exhibit greater efficiency than the larger vector due to its smaller size. However, Western blot analysis confirmed that both constructs achieved similar protein expression levels across both transfection

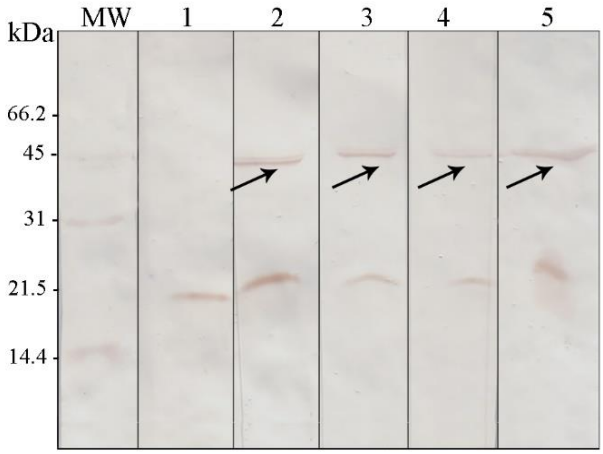

Fig. 4. Confirmation of PsSP42 protein expression in the transfected COS-7 cells using Western blot analysis. Lane 1: COS-7 cell line (as a negative control); lane 2: supernatant from COS-7 cells transfected with NTC-PsSP42 using PEI; lane 3: NTC-PsSP42 using electroporation; lane 4: VR1020-PsSP42 using PEI; lane 5: VR1020-PsSP42 using electroporation. The arrows indicate the 39.6 kDa protein.

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methods after 48 hours. These findings indicate a promising potential for successful protein expression through in vivo transfection, particularly when compared to PEI-based transfection, which cannot be ethically used in animals. However, it is important to note that in vivo electroporation protocols may not fully replicate the results observed under in vitro conditions. These issues will be addressed in the context of DNA vaccination within our experimental model (unpublished data).

CONCLUSION

DNA vaccines have received increasing attention in vaccine development due to their unique ability to induce a strong Th1-type immune response. Since effective expression of vaccine components in eukaryotic cells is crucial for triggering immune activation, it is advisable to evaluate their expression under different in vitro conditions before immunization in mouse models. This study investigated two delivery strategies, PEI and electroporation, for transfecting a conventional VR1020 plasmid and a novel NTC construct, which differ significantly in size and protein expression capacity. As expected, both plasmids demonstrated comparable protein expression levels in vitro, regardless of the transfection method used. Such evaluations are essential for obtaining a comprehensive understanding of construct performance before advancing to in vivo studies.

DECLARATIONS

Acknowledgments

The authors sincerely thank Dr. Jesus G. Valenzuela and Dr. Shaden Kamhawi (Vector Molecular Biology Section, Laboratory of Malaria and Vector Research, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Rockville, Maryland, USA) for the generous gift of some DNA vaccine constructs. We also thank Mr. Shahram Gholam-Alizadeh for his technical assistance (Department of Immunotherapy and Leishmania Vaccine Research, Pasteur Institute of Iran, Tehran, Iran). The funders had no role in the study design, data collection and analysis, decision to publish, or manuscript preparation. Grammatical revisions to some parts of the manuscript were conducted with the assistance of ChatGPT.

Ethical approval

This study was conducted by the Institutional Animal Care and Research Advisory Committee of the Pasteur Institute of Iran (ethical code: IR.PII.REC.1400.076) in complete compliance with the Particular National Ethical Framework for Biomedical Research (MOHME-2005).

Consent to participate

Not applicable

Consent for publication

All authors reviewed the results and approved the final version of the manuscript.

Authors' contributions

SHJ: conceptualization, data curation, formal analysis, investigation, methodology, and writing–original draft; HR: conceptualization, funding acquisition, and writing-review & editing; AN: funding acquisition; TT: investigation; NS: data curation, formal analysis, methodology, supervision, validation, writing–original draft, and writing– review & editing; ZE: methodology, EG: conceptualization, data curation, formal analysis, investigation, methodology, supervision, validation, writing original draft, and writing review & editing; SR: conceptualization, funding acquisition, project administration, resources, supervision, validation, visualization, and writing–review & editing.

Data availability

All relevant data can be found within the manuscript.

Competing interests

The authors declare that they have no competing interests.

Funding

This research received financial support from the Pasteur Institute of Iran (grant ID 1268) and Bu-Ali Sina University, Hamedan (grant number: 1400-1016).

Supplementary information

The online version does not contain supplementary material.

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[DOI: 10.61882/ibj.5055]

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