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Original Article

Cloning and Sequencing of *Leishmania major* Thiol-Specific-Antioxidant Antigen (TSA) Gene

*F Tabatabaie.[1], *F Ghaffari far [1], A Dalimi [1], Z Sharifi [2], A Zavaran Hoseini [3]

[1]*Dept. Parasitology, Faculty of Medical Sciences, Tarbiat Modarres University, Tehran, Iran*
[2]*Research Center of Iranian Blood Transfusion Organizations, Tehran, Iran*
[3]*Dept. Immunology, Faculty of Medical Sciences, Tarbiat Modarres University, Tehran, Iran*

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Abstract

Background: Leishmaniasis is caused by parasitic protozoa of the genus *Leishmania* which, in the infected host are obligate intracellular parasite. TSA is the immuno-dominant antigen of *Leishmania major* which is considered as the most promising molecule for a recombinant or DNA vaccine against leishmaniasis.

Methods: Genomic DNA of TSA protein was extracted and amplificated as a template. Then the PCR product was cloned into pTZ57R/T vector. Finally, the recombinant plasmid was extracted from transformed *Escherichia coli* (TG1 strain) and sequenced.

Results: MRHO/IR/75/ER (an Iranian strain) of *L. major* and TSA gene (Accession number LmjF15.1080) were used. Sequence analysis of cloned TSA gene into pTZ57R/T vector showed high homology of 90% with LmjF15.1080 (TSA gene) and strain "LV39" (Accession no. AF069386) and strain "Friedlin" (Accession no.AF044679).

Conclusion: We cloned TSA gene of *L. major* successfully. Recombinant plasmid was confirmed. It is ready to express recombinant protein for further studies.

Keywords: *Cloning, Sequencing*, Leishmania major, *TSA*.

Introduction

Leishmaniasis, caused by an intracellular protozoan parasite, *Leishmania major*, is widespread throughout the world (1). Leishmaniasis is a parasitic disease caused by several species of the genus *Leishmania*. The disease is prevalent in many parts of the world, with about 12 million infected cases. There are 1.5-2 million of new cases of cutaneous leishmaniasis and 500,000 cases of visceral leishmaniasis that appear annually. Infection with HIV/AIDS can increase the risk of developing mainly in visceral leishmaniasis by 100 to 1000 fold (2, 3).

Treatment of leishmaniasis is complex due to toxic and side effects and resistance against available drugs. Resistant variants in cases of cutaneous and visceral leishmaniasis have become more common and reinfection occurs rapidly (4). Development of either new anti-*Leishmania* drugs or a vaccine is an attractive alternative. Immunity against reinfection is acquired following cutaneous infection with *Leishmania* spp., suggesting that prophylactic immunization is feasible (5).

In recent years, significant progress has been made in the identification of vaccine candidates which can induce a protective response. Most of the works have focused on antigens GP63, CP (A, B), Lack, GP64, M2, PSA2, LmSTI1, P20, A2, Leif, P8, Histon H1, Ribosomal like protein (6-10).

Among the vaccine candidates, TSA (thiol-specific antioxidant protein) has been introduced as one of the predominant vaccine candidates (6). TSA is *L.major* recombinant protein homo-

***Correspondence author:** P.O. Box: 14115-331, Tehran, Iran Tel: +98-21-99011001 Fax: +98-21-88013030 E-mail: ghaffarifar@yahoo.com

logue to eukaryotic thiol-specific-antioxidant protein with molecular weight of 22.1 kDa. It is composed of 200 amino acids and placed in the chromosome of 15. TSA gene is abundant and homogeneously distributes on the surface of both extracellular and intracellular promastigote and amastigote. There are multiple copies of the TSA gene in all species of *Leishmania* analyzed. The results suggest that there are at least three copies of TSA in the *L. major* genome. TSA is chosen as vaccine candidates because it elicits a Th1 response in *L. major* infected BALB/c mice comparing to the other selected antigens. TSA DNA–vaccinated mice show excellent and stronger protection than the mice vaccinated with the other antigens of DNA-vaccine. TSA DNA–vaccinated stimulates high titers of specific IgG2 antibody, a phenotypic marker of Th1 response (1, 3, 5).

The aim of the present work was to clone *L. major* thiol-specific-antioxidant antigen (TSA) gene into appropriate vector for production of recombinant plasmid containing TSA gene.

Materials and Methods

Leishmania major

The MRHO/IR/75/ER (an Iranian strain to be isolated by Nadim et al. in 1964)) of *L. major* was provided by Pasteur Institute of Iran. Promastigotes were grown at 26 °C in RPMI1640 medium (Sigma®) supplemented with 10% heat inactivated fetal calf serum (Gibco®, BRL), and 100µg/ml gentamicine (Sigma®). Stationary phase of the promastigotes was harvested at a density of $2x10^6$/ml.

Genomic DNA extraction

About 2×10^6 of *L. major* promastigotes were concentrated by centrifugation, washed with phosphate buffered saline (PBS), then lysed in lysing buffer (0.1M Tris-HCl pH 8.0 containing 1% sodium dodecyl sulphate, 0.1M NaCl and l0mM EDTA) and treated with proteinase K (100 µg/ml) at 55 ^oC for 2 h.

The lysate was added to an equal volume of phenol /chloroform (25:25) to remove proteins. This mixture was centrifuged at 13000 rpm for 15 min and an equal volume of chloroform was added to the supernatant which was then re-centrifuged. The supernatant was mixed with 1/10 volume of 3 M sodium acetate and two volumes of 100% ethanol to precipitate DNA by centrifugation at 13000 rpm for 10 min. The DNA pellet was washed with 70% ethanol, dissolved in sterile distilled water and stored at -20 ^oC until use. The DNA extraction products were detected in 0.8% agarose gel and photographed (8, 10, 11).The results showed that concentration of the extracted DNA was high.

PCR amplification and gel electrophoresis

Genomic DNA isolated from promastigotes was used as a template to amplify the TSA gene by PCR. The reaction was performed in 25µl of the solution containing: 3µl of template DNA, 0.5µl of dNTP (with concentration 10mM and final concentration of 200 µM), 0.5µl of Taq DNA polymerase (with concentration 5 Unit/ml), 2.5µl of 10X PCR buffer, 0.75µl of MgCl2 (50 mM), 15.75µl of distilled water and 1µl of each of primers (10Pmol/µl).

We designed a pair of primer based on TSA gene sequence (Accession number LmjF15.1080).

Forward primer**,** 56nt: introduced Hind III recognition site, underlined:

5' - CAA TTA AA GCT TAT ATG **CAT CAC CAT CAC CAT CAC** ATG TCC TGC GGT AAC GCC AAG- 3' (1-23 nt)

For confirmation of gene expression, six-histidine (6-His tag) sequence was designed in the forward primer for diagnostic by His-tag monoclonal antibody in western- blotting.

Reverse primer, 31nt: introduced EcoRI recognition site, underlined:

5'- CAT GGA ATT CTT ACT GCT TGC TGA AGT ATCC-3' (579-600 nt), under the following conditions:

After an initial five minute denaturation at 95 °C, each cycle consisted of 60s at 95 °C, 30s at 60 °C and 45s at 72 ^oC. At the end of the 30 cycles of amplification, a final extension was continued for five minute at 72 ^oC. These primers contain restriction enzymes that are designed for gene

expression in the eukaryotic expression vector pcDNA3 down stream T 7 promotor to the CMV promoter.

The upstream primer for the TSA gene contains a HindIII site and the ATG start codon, while the downstream primer contains an EcoRI site and the TAA stop codon.

The PCR products were analyzed by electrophoresis on a 1% agarose gel and photographed. Gel was stained by ethidium bromide and DNA band visualized under UV transilluminator. The size marker used to estimate PCR products was the 1 kbp DNA ladder (Fermentas®).

The PCR products were purified using a DNA Extraction Kit from agarose gel (Ferments®) following the manufacturer's recommendations (12-14). The gel slice containing 600bp fragment band (TSA gene) was excised.

Ligation of TSA gene into pTZ57R/T cloning vector

The purified PCR products were ligated to pTZ57R/T cloning vector (InsT/Aclone ™ PCR product cloning kit, Fermentas®), following the manufacturer's protocol.

Transformation and Screening

Preparation of competent cells from *Escherichia coli* TG1 strain was performed by calcium chloride method (11).

For transformation, 10µl of ligation reaction product was added to 150µl competent cells, after vortex and spin the mixture was incubated at 42 ^{o}C for 90s, and immediately was placed on ice for 2-3 min. The transformed cells were allowed to recover in 300µl of Luria-Bertani (LB) broth medium free antibiotic by incubated at 37 ^{o}C for 1-2 h with shaking. These recovered cells were plated onto LB agar plates containing ampicillin, IPTG (Fermentas®) 200mg/ml and X-Gal (Fermentas®) 20 mg /ml to screening of blue and white colonies (no recombinant and recombinant plasmid) and incubated at 37 ^{o}C for 18 h. Twelve white and 5 blue colonies were randomly selected from each agar plate and inoculated in a LB medium containing ampicillin (100 mg/ml) and incubated at 37 ^{o}C for 18 h.

Cloning of TSA gene into pTZ57R/T vector

The plasmid was purified from white and blue colonies of bacteria by Accuprep Plasmid Extraction Kit (BioNEER®), according to the manufacturer's protocol.

Electrophoresis of extracted plasmids

Three µl of each plasmid extracted from white (pT-TSA) and blue (pTZ57R/T) colonies of bacteria were loaded on a 0.8% agorose gel and electrophoresis then photographed.

The plasmid bands on agarose gel were compared.

PCR amplification of TSA gene with plasmid

The extracted plasmid from white colonies bacteria (pT-TSA) was used as a template to amplify the TSA gene by PCR performed in 25µl of solution under condition previously description in part 3. The PCR product was analyzed by electrophoresis on a 1% agarose gel and photographed. The size marker used to estimate PCR products was 1 kbp DNA ladder (Fermentas®).

Enzyme digestion of plasmid

With regard to designed HindIII and EcoRI restriction enzymes sites, respectively on forward and reverse primers and present them in extracted recombinant plasmids of white colonies bacteria (pT-TSA), these plasmids were digested by HindIII and EcoR1 enzymes. For this propose an enzyme digestion reaction was performed in 20µl of solution containing 10µl of plasmid (1-3µg), 1µl of restriction enzyme (10Unit), 2µl of 10x buffer and 7µl of DW, after spin and vortex, this mixture was incubated in 37 ^{o}C for overnight.

Because of being different of restriction enzyme buffers, each enzyme digestion was performed separately. All the digestion products were loaded on a 1% agarose gel and were extracted from agarose gel by DNA extraction kit from agarose gel (Fermentas®) (11,15-18). The band resulting from digestion by two enzymes was analyzed by electrophoresis and 1% agarose gel and photographed.

Sequencing

The extracted plasmids from the white colonies bacteria (pT-TSA) were sequenced by Takapozist Company, Iran.

Results

Fig.1 shows that genomic DNA has been extracted by phenol/chloroform method and concentration of the extracted DNA is high.

Fig. 2, shows that the size of PCR of *L. major* TSA gene is about 600 bp and similar to the *L. major* TSA gene size and no any genes is amplified exception with TSA gene. Thus, these designed primers are specific for amplifying of TSA gene.

Electrophoresis of extracted plasmids shows that pT-TSA bands place above of pTZ57R/T bands on agarose gel (Fig. 3).

Comparison the bands of the extracted plasmids of white and blue colonies shows that bands of the extracted plasmids of white colonies are heavier than the extracted plasmids of blue colonies then the TSA gene has been cloned into PTZ57R/T (Fig. 3).

When the extracted plasmids of white colonies (contain recombinant plasmids) were digested by EcoRI and HindIII restriction enzymes and the digestion product was electrophoresed, two bands were observed (Fig. 4, 5).

Results from enzyme digestion, revealed that the extracted plasmids from the white colonies bacteria (PT-TSA) were digested by EcoRI and HindIII, a 600 bp band was cut and separated that this was TSA gene, and thus the TSAgene has been cloned into PTZ57R/T (Fig. 4, 5).

Results from electrophoresis of PCR products with extracted plasmids (pT-TSA) showed a 600 bp fragment of TSA gene means. This gene was successfully cloned into the pTZ57R plasmid (Fig.6).

The extracted plasmids from the white colonies bacteria (pT-TSA) were sequenced and submitted to GeneBank under accession number: EU194915.

We used MRHO/IR/75/ER (an Iranian strain) of *L. major* and TSA gene (Accession number LmjF15.1080). Sequence analysis of cloned TSA gene into pTZ57R/T vector showed high homology of 90% with LmjF15.1080 (TSA gene) and strain "LV39" (Accession no. AF069386 isolated by Levick *et al*. in 1998) and strain "Friedlin" (Accession no.AF044679 isolated by Webb *et al*.) (7).

Fig. 7.1 and 7.2 show the results of Nucleotide Blast (blast software in Gene Bank data base and NCBI) TSA gene under accession number: EU194915 with TSA gene under accession number LmjF15.1080 and strain "LV39" (Accession no. AF069386) and strain "Friedlin" (Accession no.AF044679).

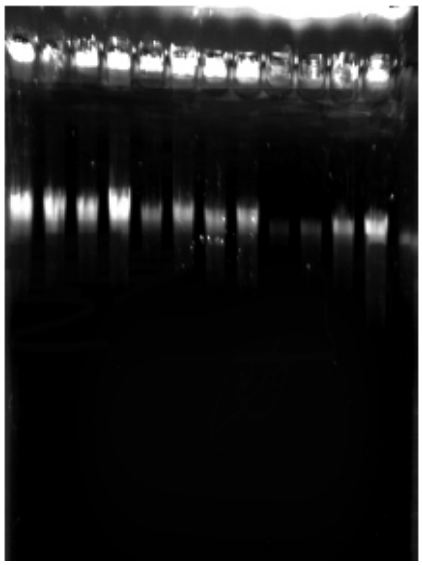

Fig. 1: Genomic DNA extraction from *Leishmania major* was performed by phenol: chloroform method and electrophoresed in 0.8% agarose gel

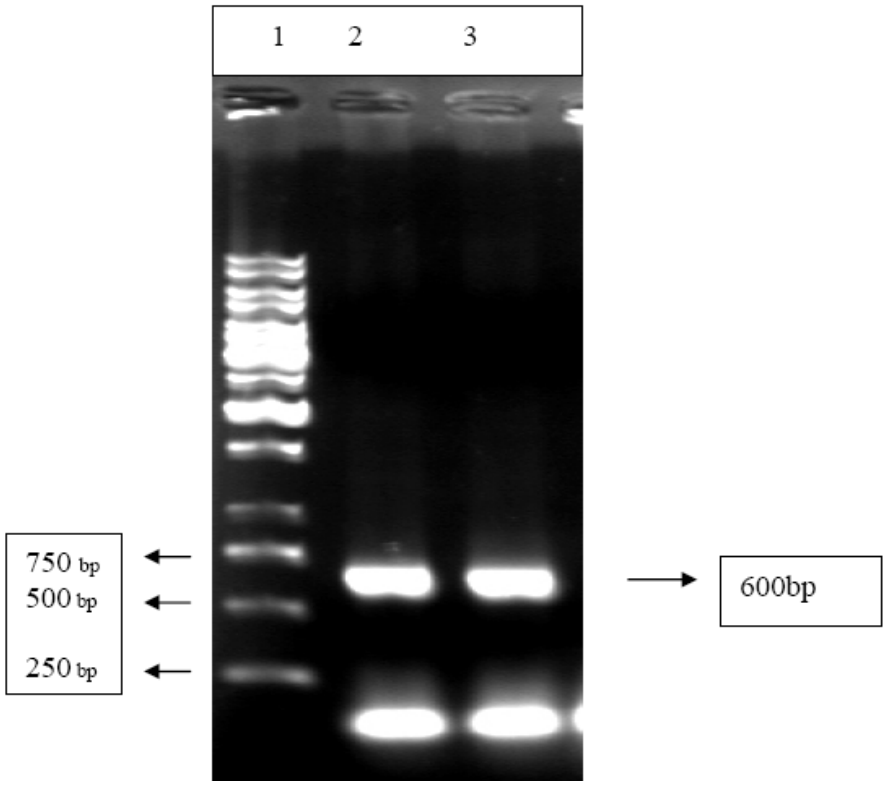

Fig .2: Electrophoresis of PCR product for *Leishmania major* thiol-specific-antioxidant (TSA) gene amplification. From left to right Line 1: 1Kbp DNA ladder, Lines 2, 3: TSA gene (600 bp)

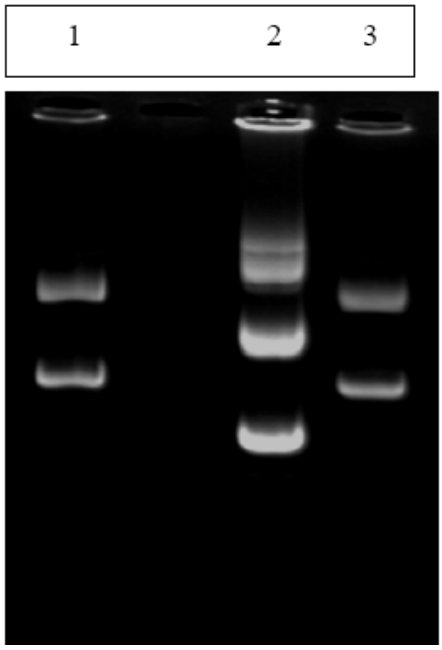

Fig.3: Plasmid extracted from white (pT-TSA) and blue (pTZ57R/T) colonies bacteria were loaded on a 0.8% agarose gel. The band of pTZ57R/T (line2) and pT-TSA (line31, 3)

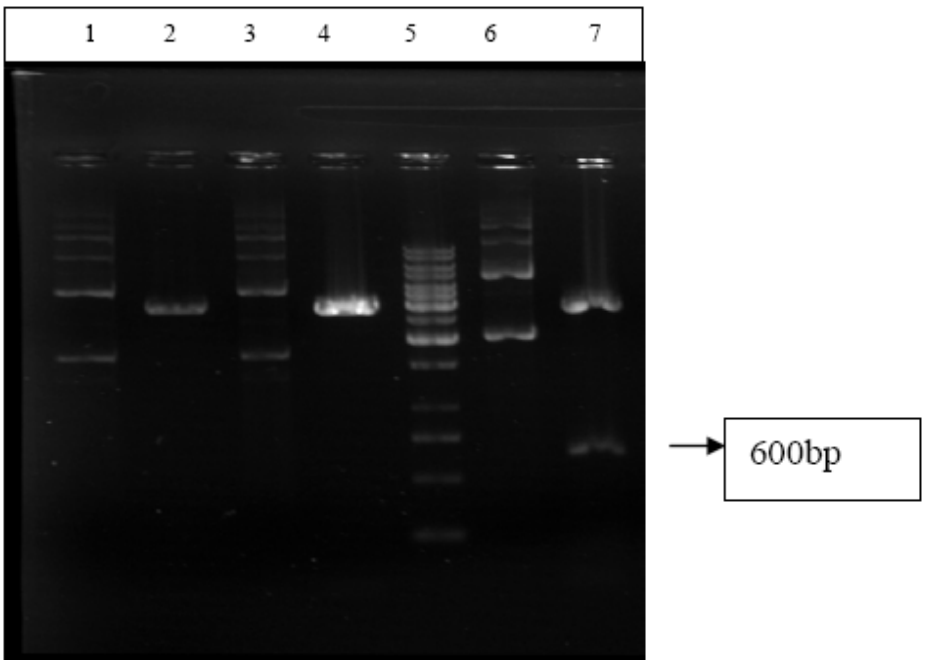

Fig. 4: Electrophoresis of pTZ57R/T and extracted pT-TSA following the transformation before and after digestion by enzymes:
Columns 1 & 3: pTZ57R/T (uncut), Columns 2 & 4: pTZ57R/T (cut), Line 5: DNA ladder(1Kbp DNA ladder), Line 6: pT-TSA and Line 7: pT-TSA digested by *EcoRI*, *HindIII*

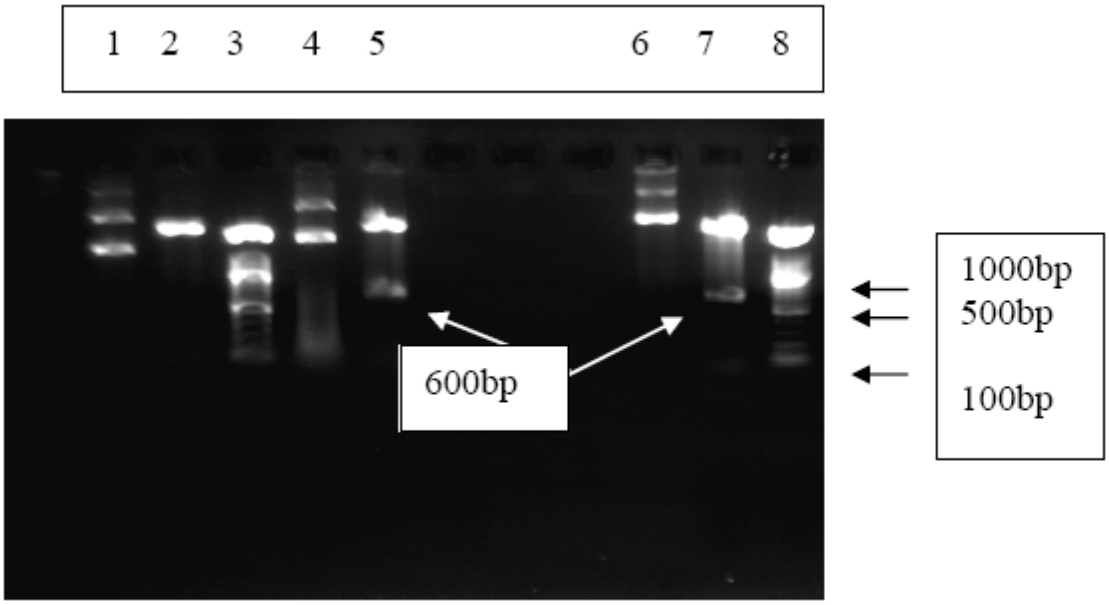

Fig.5: Electrophoresis of pTZ57R/T and extracted pT-TSA following transformation before and after digestion by enzymes:
Line 1: pTZ57R/T (uncut), Line 3: pTZ57R/T (cut), Lines 3 and 8: DNA ladder (100 bp DNA ladder), Lines 4 and 6: pT-TSA and Lines 5 and 7: pT-TSA digested by *EcoRI*, *HindIII*

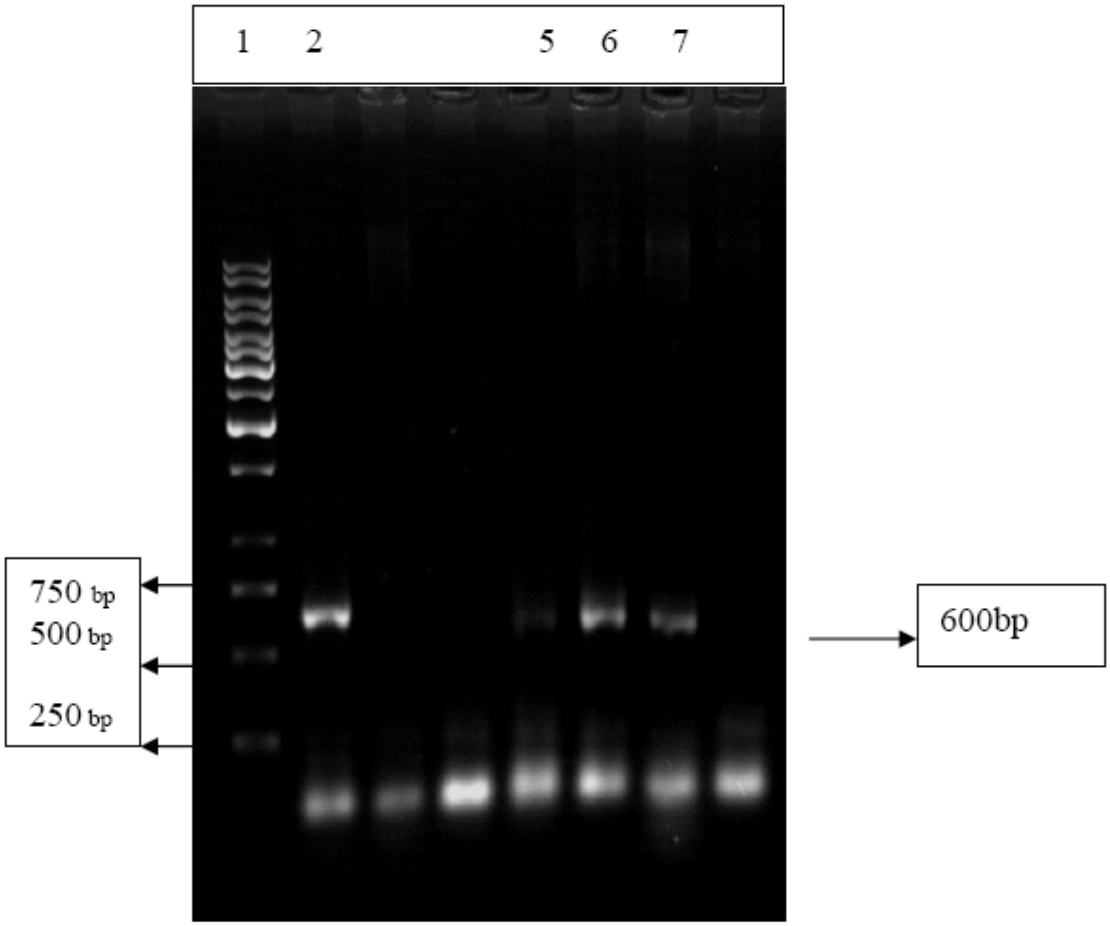

Fig.6: Electrophoresis of PCR amplification of TSA gene with pT-TSA.
Line 1: 1Kbp DNA ladder, Lines 2, 5, 6, 7: PCR amplification of TSA gene with pT-TSA

Discussion

Leishmaniasis has not shown any tendency towards subsidence in recent years. Although measures may be taken against vectors and reservoirs, and the identification of new drugs is a very desirable goal, particularly in view of the emerging drug resistances, the development of safe and efficient vaccines remains the best hope of achieving definitive control of the disease. *Leishmania* TSA protein is known antigenic in both murine and human systems and is constitutively expressed in both promastigote and amastigote life stages. The molecular weight of TSA protein is 22.1 kDa, its size is 600bp, consists of 200 amino acids, and placed in chromosome15 (1, 3, 6). Currently, the most successful vaccine attempts in humans and animals have been achieved with live-attenuated, whole-killed *Leishmania* promastigotes (with or without IL-12) and autoclaved *Leishmania* with BCG also Radio attenuated promasitigotes and biochemically altered leishmanial parasites. A single synthetic T-cell epitope from gp63 administered (synthetic peptide) with Th1 stimulating poloxamer 407 conferred protection against *L. major* in BALB/c mice.

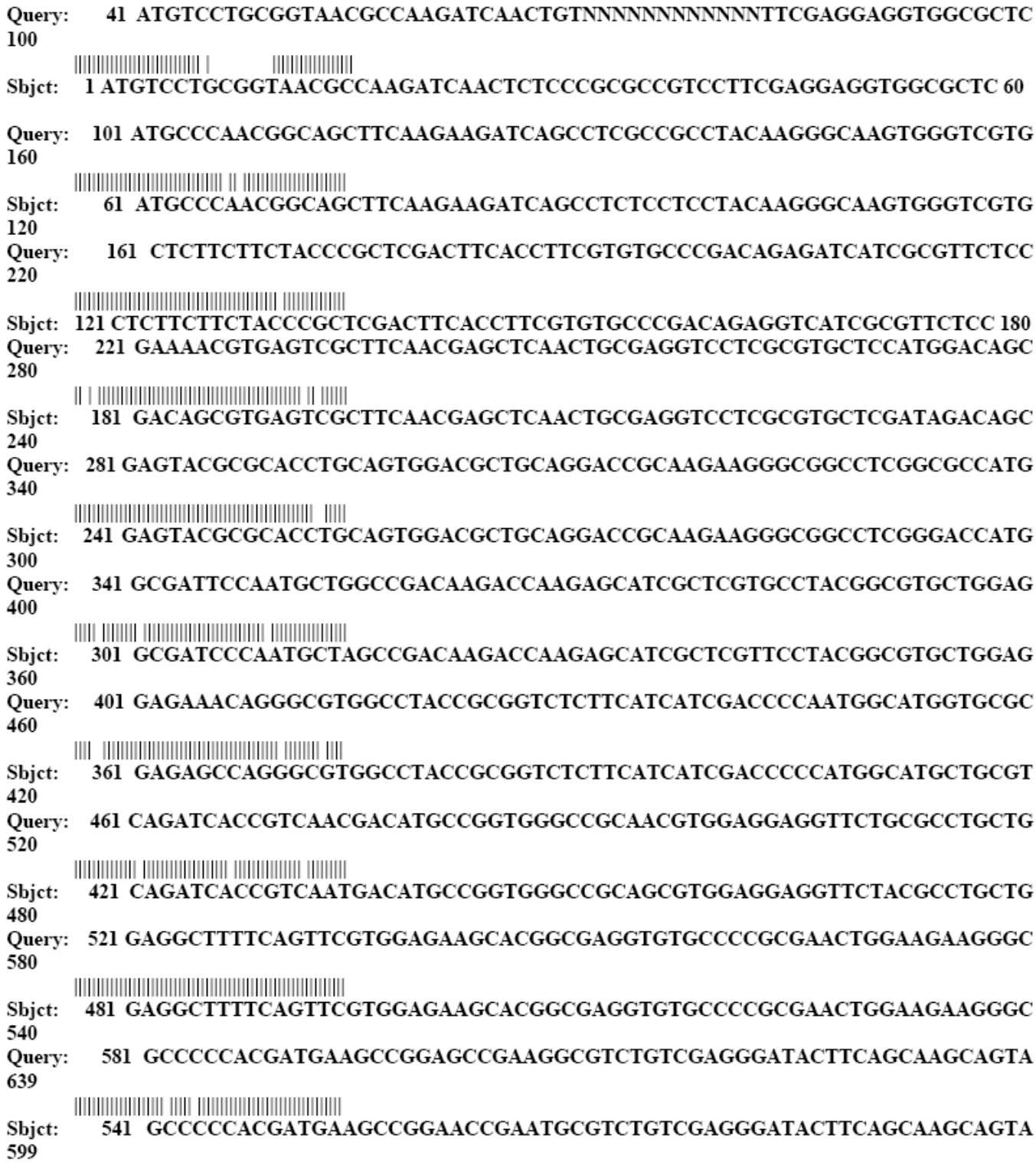

Fig.7.1: The Blast results of TSA gene under accession number EU194915 in comparison with TSA gene under accession number LmjF15.1080

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28148   27  TTGCCCCCA-CC-GCCATGTCCTGCGGTAACGCCAAGATCAACTGTCCCGCGCCGCCCTT  84
AF069386 121   ...A...G..A.....................C.........T....  178
AF044679 11   .....A...G..A.....................C.........T....  70

28148   85  CGAGGAGGTGGCGCTCATGCCCAACGGCAGCTTCAAGAAGATCAGCCTCGCCGCCTACAA  144
AF069386 179  .....T..T.....  238
AF044679 71   .....T..T.....  130

28148   145 GGGCAAGTGGGTCGTGCTCTTCTTCTACCCGCTCGACTTCACCTTCGTGTGCCCGACAGA  204
AF069386 239  .....  298
AF044679 131  .....T.G.....  190

28148   205 GATCATCGCGTTCTCCGAAAACGTGAGTCGCTTCAACGAGCTCAACTGCGAGGTCCTCGC  264
AF069386 299  .G.....C.G.....  358
AF044679 191  .G.....C.G.....  250

28148   265 GTGCTCCATGGACAGCGAGTACGCGCACCTGCAGTGGACGCTGCAGGACCGCAAGAAGGG  324
AF069386 359  .....G..A.....  418
AF044679 251  .....G..A.....  310

28148   325 CGGCCTCGGCG-CCATGGCGATTCCAATGCTGGCCGACAAGACCAAGAGCATCGCTCGTG  383
AF069386 419  .....-.A.....C.....A.....T  477
AF044679 311  .....-.A.....C.....A.....T  369

28148   384 CCTACGGCGTGCTGGAGGAGAAACAGGGCGTGGCCTACCGCGGTCTCTTCATCATCGACC  443
AF069386 478  .....GC.G.....  537
AF044679 370  .....GC.....  429

28148   444 CCAATGGCATGGTGCGCCAGATCACCGTCAACGACATGCCGGTGGGCCGCAACGTGGAGG  503
AF069386 538  ..C.....C....T.....T.....G.....  597
AF044679 430  ..C.....C....T.....T.....G.....  489

28148   504 AGGTTCTGCGCCTGCTGGAGGCTTTTCAGTTCGTGGAGAAGCACGGCGAGGTGTGCCCCG  563
AF069386 598  .....A.....  657
AF044679 490  .....A.....  549

28148   564 CGAACTGGAAGAAGGGCGCCCCCACGATGAAGCCGGAGCCGAAGGCGTCTGTCGAGGGAT  623
AF069386 658  .....A.....  717
AF044679 550  .....A....T.....A....  609

28148   624 ACTTCAGCAAGCAGTA  639
AF069386 718  .....  733
AF044679 610  .....  625
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Fig.7.2: The Blast results of TSA gene under accession number EU194915 in comparison with strain "LV39" (Accession no. AF069386) and strain "Friedlin" (Accession no.AF044679).

The produced antibodies against liposphoglycan (LPG) may prove to be effective in limiting the transmission of cutaneous leishmaniasis in its vector *Phlebotomus dubosqi*. *Leishmania* vaccine preparations have evolved from crude parasite preparations to defined molecules administered as recombinant proteins or DNA vaccines. But these vaccines have been proven not to be able to induce complete and long lasting protection. DNA vaccines have some notable features in comparison with traditional vaccines, they are easy to produce, relatively

inexpensive, homogeneous, heat stable, and believed to be safer than subunit or viral vector based vaccines. DNA vaccine can induce strong, long lasting and powerful humoral and cellular immunity. They have also the potential to increase immunogenicity through modifications of the vector or incorporation of adjuvant-like cytokine genes. DNA vaccines may be especially useful for protection against cutaneous leishmaniasis, since the development of naturally acquired immunity to a primary exposure to *L. major*, involving low dose infection in the skin has recently been shown to depend on both $CD4^+$ and $CD8^+$ T cells. Immunization of BALB/c mice with TSA DNA vaccine confers high levels of protective immunity (humoral and cellular), induces CTL activity and solid protection as well as stimulates high titers of specific IgG1, IgG2a antibody and INFγ, a phenotypic marker of Th1 response. In recent years, significant progress has been made in identification of vaccine candidates which can induce a protective response. Most of the works have focused on antigens: GP63, CP (A,B), Lack, GP64, M2, Leif, PSA2, LmSTI1, P20, A2, P8, Histon H1 and Ribosomal like protein. But they did not induce complete and long lasting protection (19-24).

The TSA protein gene open reading frame was modified by PCR amplification to contain an amino-terminal six-histidine tag with the synthetic oligonuleotides. The resulting PCR product was digested with NdeI and EcoRI and subcloned into an NdeI-and EcoRI-digested pET17b vector. Recombinant TSA protein containing an amino-terminal six-histidine tag was expressed in *Escherichia coli* with the pET17b system (7). Webb et al. suggested that the TSA protein was useful as a component of a subunit vaccine against leishmaniasis (7). In optimization of DNA vaccine against cutaneous leishmaniasis, a cDNA encoding a truncated LACK was cloned in–frame downstream to a kozak consensus sequence and an initiation codon into pECEvector. The insert was excised using HindIII and ligated into expression vector Pc DNA3 downstream to the CMV promoter. The full-length sequences of LmSTI1 and TSA were PCR amplified from *L. major* genomic DNA using sequence specific primers and subcloned into pcDNA3.1 (BamH1 and EcoRI sites). These results establish intradermal vaccination using DNA encoding multiple *Leishmania* antigens as a way to optimize of CD4+ and CD8+ T cells necessary for potent and durable protection against cutaneous leishmaniasis (7) demonstrated that the recombinant leishmanial antigens LmSTI1 and TSA induced excellent protection in both murine and nonhuman primate models of human cutaneous leishmaniasis (as a promosing candidate subunit vaccine against human leishmaniasis).

The recombinant TSA protein with IL-12 induces excellent protection in BALB/c mice. TSA DNA produces $CD8^+$ response, confirming the idea that $CD8^+$ T-cell may constitute an important component of the protective immune response. In addition, TSA DNA is a vaccine of choice because specific immunity will be generated against the increased number of parasite epitopes (1-3, 5).

Cloning of gene TSA was confirmed by restriction anzymes (EcoRI and Hind III) and PCR amplification of TSA.

The extracted plasmids from the white colonies bacteria (pT-TSA) were sequenced and submitted to GeneBank under accession number: EU194915.

In conclusion, production of TSA protein could be a preliminary step for further research in designing sophisticated diagnostic kit or effective vaccine against leishmaniasis.

Acknowledgements

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The authors declare that they have no Conflict of Interests.

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