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Cloning and Expression of Recombinant Plasmid Containing LACK Gene of Leishmania Major (MHRO/IR/75/ER) in CHO Cells

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Abstract

Leishmaniasis is caused by parasitic protozoa of the genus Leishmania which, in the infected host are obligate intracellular parasite. LACK conserved among related Leishmania species. LACK is the immuno-dominant antigen of L.major which is considered as the most promising molecule for a recombinant or DNA vaccine against leishmaniasis.

MRHO/IR/75//ER (an Iranian strain) of L.major and LACK gene using PCR method was used for amplifying of LACK gene. Genomic DNA of LACK protein was extracted and amplificated as a template. Then the PCR product was cloned into PTZ57R/T cloning vector. The PT-LACK recombinant plasmid was extracted from white colonies of E.coli bacteria (TG1 strain) and sequenced. PT-LACK plasmids were digested by Hind III and EcoRI enzymes. Then, the purified digestion products were ligated to pcDNA3 vector; finally, the pc-LACK recombinant plasmid was purified from transformed E.coli (TG1 strain) and tranfection of pcTSA recombinant plasmid into the CHO cells then analysis by methods of SDS-PAGE and Western blot.

LACK gene has been cloned into pcDNA3 cloning vector. Sequence analysis of cloned LACK gene into PTZ57R/T vector high homology 89% with LmjF28.2740 (LACK gene). The recombinant plasmid containing LACK gene was expressed in CHO cells. The expression of pc-LACK recombinant plasmid was demonstrated by SDS-PAGE and Western-blot.

We cloned LACK gene of L.major in pcDNA3 vectors successfully. Recombinant plasmid was confirmed. Results indicated successful expression of pc-LACK plasmids in eukaryotic cells.

Keywords: *Cloning, Expression, Leishmania major, LACK, CHO cells.*

1. Introduction

The leishmaniasis is zoonotic parasitizes of Public health concern, caused by several species of the genus *Leishmania* (1). *Leishmania* major is wide spread throughout the world (2). The disease is prevalent in many parts of the world, with about 12 million infected cases. There are 1.5-2 millions of new cases of cutaneous leishmaniasis and 500,000 cases of visceral leishmaniasis that appear annually (3). Infection with HIV/AIDS can increase the risk of developing mainly in visceral leishmaniasis by 100- to 1000- fold (4).

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 (5). Development of either new anti-*Leishmania* drugs or a vaccine is an attractive alternative. Immunity against reinfection with *Leishmania* spp.,

suggesting that prophylactic immunization is feasible (6).

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(7), CPB(8,9), TSA(10), GP64(11), LiPO(Acidic Protein Ribosomal) (12), PSA2[13], LmSTI1(10), ORFF(14), LeIf (15)and P8, p4 (16).

A recent study comparing different DNA vaccine candidates demonstrated that the most promising gene is LACK (17). The LACK antigen, the *Leishmania* homologue for receptors for activated C kinase, is a 36 KDa protein highly conserved among related *Leishmania* species expressed in both promastigote and amastigote forms of the parasite (18). LACK is a preferential target for the early anti-parasite immune response, driving the expansion of IL-4 secreting, disease-promoting T cells (19). LACK vaccination trials using protein or DNA vectors show protection against cutaneous L.major infections by redirecting the early IL-4 responses to a protective Th1 response (18, 20).

The aim of this study was clone *L.major* LACK gene into appropriate vectors for production of recombinant plasmid containing LACK gene and the expression of pc-LACK recombinant plasmid in eukaryotic cells.

2. Materials and Methods

Parasite strains

Promastigotes of L.major (MRHO/IR/75/ER is an Iranian strain) were cultured at 24 ºC in RPMI 1640 ,s medium (bahar afshan®), supplemented with 10% inactivated fetal calf serum (FCS, Gibco®, BRL) and 100µg/ml streptomycin, 100U/ml penicillin antibiotics (Sigma®).

DNA extraction

About $2x10^6$ /ml of L.major promastigotes were concentrated by centrifugation, washed phosphate buffered saline (PBS),then DNA isolated by DNA extraction kit (fermentas®) from L.major promastigotes. The DNA stored at -20 ºC until use. The DNA extraction products were detected in 0.8% agarose gel and photographed (21, 18)

PCR amplification and gel electrophoresis

The DNA extraction products were used as a template that amplifies the LACK gene by PCR.

We designed a pair of primer based on LACK gene sequence. Forward primer, 35nt: introduced Hind III recognition site, underlined:

5'ATT AAG CTT ATG AAC TAC GAG GGT CAC CTG AAG GG 3'

Reverse primer, 27nt introduced EcoRI recognition site, underlined:

5' TTA GAA TTC TTA CTC GGC GTC GGA GAT 3'

The DNA Extraction Kit from agarose gel (Fermentas®) was used for PCR products purification according to the manufacturer recommendations.

The ligation of LACK gene into PTZ57R/T vector, transformation of ligation PTZ57R/T-LACK and screening, PT-LACK plasmid extraction and detection of the PT-LACK recombinant product described previously (22).

Ligation of LACK gene into pcDNA3 cloning vector

The purified digestion products were ligated to pcDNA3 cloning vector by T4 DNA ligase. The reaction was performed in 20 µl of the solution containing: 10 µl of the purified digestion products (1-3 µg), 2 µl of 10x buffer, 1 µl of T4 DNA ligase (10 Unit), 7 µl of D.W(distilled-water), after vortex and spin, this mixture was incubated in 37 ºC for overnight.

Transformation of ligation pcDNA3-LACK

For transformation, 10-15 µl of ligation reaction product was added to 150 µl competent cells, after vortex and spin, the mixture was incubated at 42 ºC for 90s, and immediately was placed on ice for 2-3 min. The transformed cells were allowed to recover in 300 µl of Luria-Bertani (LB) broth medium free antibiotic by incubated at 37 ºC for 1-2 h with shaking. These recovered cells was plated onto LB agar plates containing ampicillin (100 mg/ml) and incubated at 37 ºC for 18 h.

White colonies were randomly selected from each agar plate and inoculated in a LB medium containing ampicillin (100 mg/ml) and incubated at 37 ºC foe 16-18 h.

PcDNA3-LACK plasmid purification

The plasmid was purified from colonies of bacteria by Accuprep Plasmid Extraction Kit (BioNEER®), according to the manufacture; s protocol.

The pc-LACK recombinant plasmid was detected by 3 methods:

1-Electrophoresis of plasmid purification: 3 µl of each plasmid extraction from pc-LACK and pcDNA3 colonies of bacteria were loaded on a 0.8% agarose gel and electrophoresis then photographed. The plasmid bands on agarose gel were compared.

2-PCR amplification of LACK gene with pc-LACK plasmid: the pc-LACK was used as a template to amplify LACK gene by PCR under condition previously description in part 3. The PCR product was analyzed by electrophoresis on 1% agarose gel and photographed.

3-Enzyme digestion of pc-LACK plasmid: with regard to designed Hind III and EcoRI restriction enzyme sites, in pc-LACK recombinant plasmid, and existence them sites on forward and reverse primers, pc-LACK plasmids were digested by Hind III and EcoRI enzymes. For this purpose was performed in 20 µl of solution containing 10 µl of plasmid (1-3 µg), 1 µl of each restriction enzyme (10 Unite), 2 µl of 10x buffer and 6 µl of D.W, after vortex and spin, this mixture was incubated in 37 ºC for overnight.

Tranfection of recombinant pc-LACK into the CHO cells

For confirmation in vitro transfection of CHO (Chine's Hamester Ovary) cells, they were grown to 50-80% confluence at 37°C and 5% CO2 in 35 mm wells in Dulbecco's modified Eagle's medium (DMEM, Gibco) each containing 100 U ml^{-1}penicillin and streptomycin and 10 % fetal calf serum (FCS). The cells were washed in a serum free medium. The transfection was performed with a transfection Kit (Genejuice Transfection Kit, Novagene, USA) according to the instructions of the manufacturer.

SDS-PAGE and Western blot analysis

The transfected and non-transfected as control cells were harvested for 48 h or 72 h following the transfection and lysed in sample buffer. After the cells were concentrated by centrifugation and their protein profile was resolved in 10% reducing SDS-PAGE according to the method of Laemmli. The recombinant *L.major* LACK protein was expressed and separated by SDS-PAGE and stained with Coomassie blue and for western blot transferred into nitrocellulose membrane.

The membrane strips were blocked with 1% BSA-PBST20 overnight and sequentially probed with *Leishmania* antibody-positive mice sera and an anti-mouse IgG horseradish-peroxidase (Sigma) diluted in 1% BSA -PBST20 (1/10000), specific binding was revealed with diaminobenzidine (DAB) (DAKO, Denmark) (23, 24). The Western-blotting analysis was recognized by specific polyclonal and monoclonal antibodies.

3. Results

We used MRHO/IR/75//ER (an Iranian strain) of *L.major* and LACK.

Genomic DNA has been extracted by DNA Extraction Kit (Fermentas®) and concentration of the DNA extraction is high.

The purified plasmids from the white colonies bacteria (PT-LACK) were sequenced.

The Sequence analysis of PTZ57R/T-LACK recombinant plasmids showed high homology of 89% with LmjF28.2740 (LACK gene) [22].

Results, detection methods the pc-LACK recombinant plasmid product:

Electrophoresis of extracted plasmids shows that pc-LACK bands place above of pcDNA3 bands on agarose gel (***Figure 1***).

Comparison: the bands show that the pc-LACK bands heavier than the pcDNA3 bands. Therefore, LACK gene has been cloned into pcDNA3 (***Figure 1***).

Figure 1. *Electrophoresis of pc-LACK recombinant plasmid and pcDNA3 plasmid were loaded on a 0.8% agarose gel. The band of pcDNA3 (line 1) and pc-LACK (line 2)*

The pc-LACK recombinant plasmids were digested by EcoRI and Hind III restriction enzymes.

Electrophoresis of the digestion product showed two bands on agarose gel.

Results from enzyme digestion, revealed that the pc-LACK recombinant plasmids were digested by EcoRI and Hind III, a 939 bp band was separated, thus the LACK gene has been cloned into pcDNA3 (***Figure 2***).

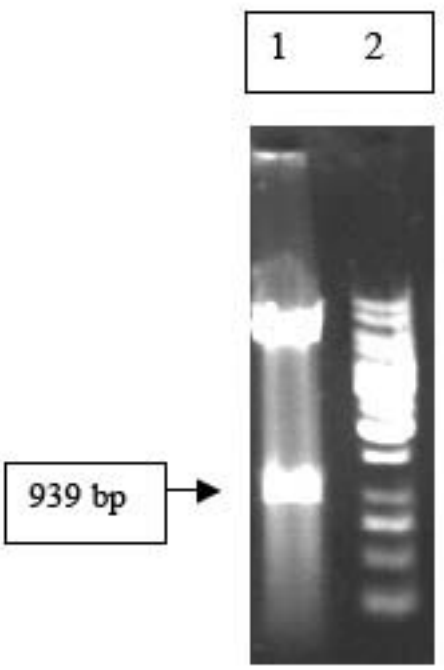


Figure 2. *Electrophoresis of extracted pc-LACK following the transformation after digestion by enzymes.*
Column 2: 1 kbp DNA ladder, column 4: pc-LACK and column 1: pcLACK digested by EcoRI and Hind II

Results from electrophoresis of PCR products with the pc-LACK plasmid showed a 939 bp fragment of LACK gene. Then gene LACK was successfully cloned into the pcDNA3 coloning vector (***Figure 3***).

A band at about 36 KDa was recognized by *Leishmania* antibody-positive mice sera in protein extracts of the cells transfected with pc-LACK. LACK protein was not detected in the non-transfected control cells (***Figures 4***, ***5***).

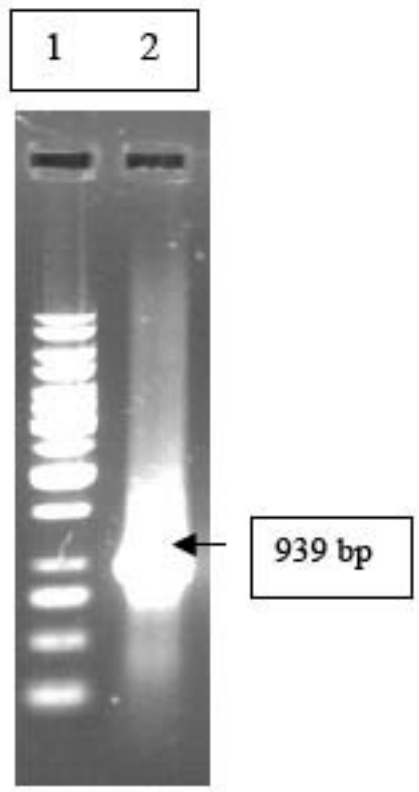
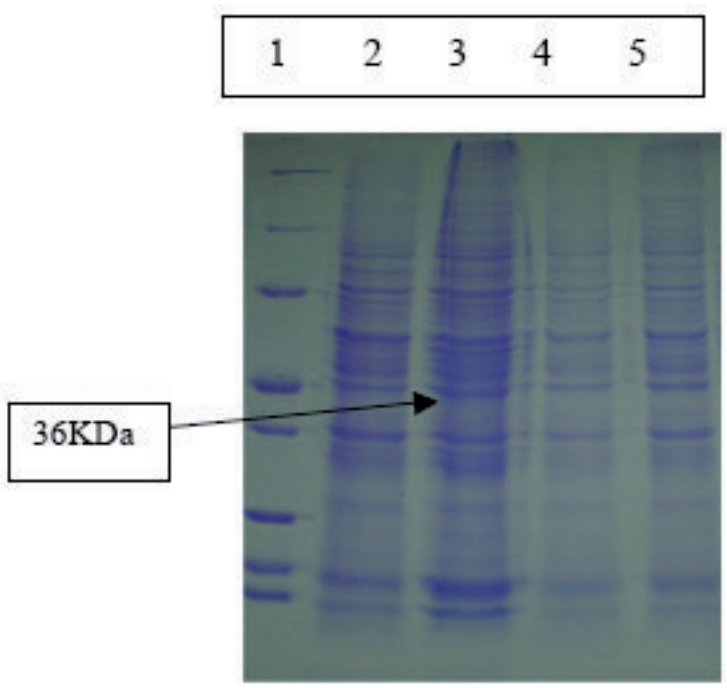
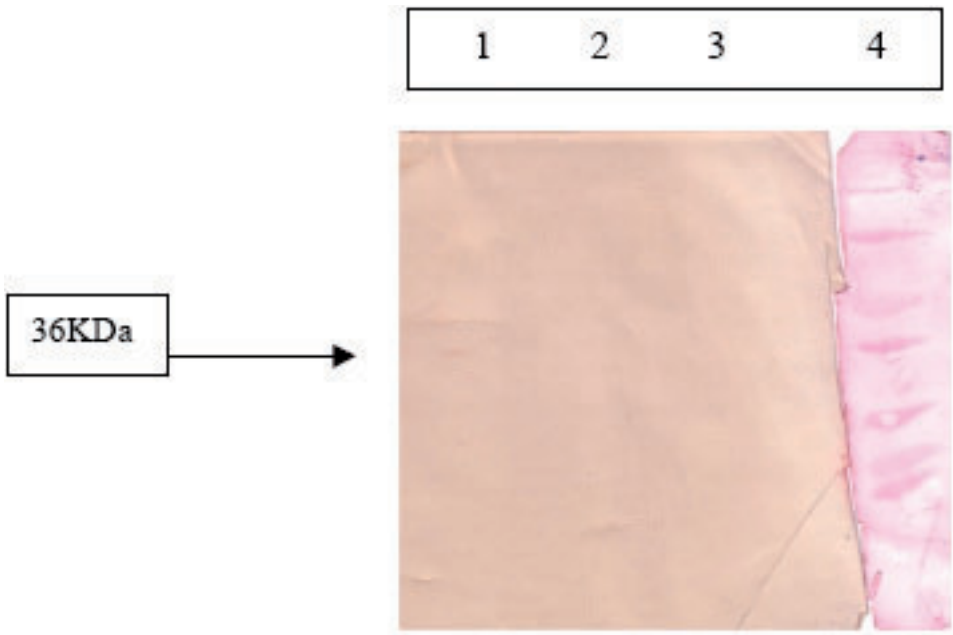


Figure 3. *Electrophoresis of PCR, amplification of LACK gene with pc-LACK.*
Line 1: 1 kbp DNA ladder, line 2: PCR amplification of LACK gene with pc-LACK



SDS-PAGE analysis of expressed gene product

Figure 4. *Line 3: Transfected with LACK after 48hr, Line 1: Untransfected CHO cells after 48hr, Lines 4, 5: Transfected with pcDNA3 after 48hr, Line 2: Low molecular weight markers (LMW 14, 18, 25,35,45,66,116 KDa).*



Western blot analysis of expressed gene product

Figure 5. *Line 1: Transfected with LACK, Line 2: Untransfected CHO cells,Transfected with pcDNA3, Line 4: LMW(14,18,25,35,45,66,116 KDa)*

4. 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 (2, 25). The LACK antigen, the leishmania homologue for receptors for activated C kinase, is a 36 KDa protein highly conserved among related *leishmania* species expressed in both promastigote and amastigote forms of the parasite (26). The molecular size of LACK protein is 939bp, consists of 313 amino acids, and placed in chromosome 28.

DNA vaccine has some unable features in comparison with traditional vaccine. 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 (27, 28).

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 $CD8^+$ Tcells(18, 24)

LACK vaccination trials using protein or DNA vectors show protection against cutaneous *L.major* infections by redirecting the early IL-4 responses to a protective Th1 response (18, 20). This protective effect is mediated by IL-12 dependent INF-γ production (26).

Despite such promising results, there are no vaccines in active clinical and veterinary medical use (26, 29).

In such situation, the PSA2 seems to be not very effective. On the other hand, with the Gp63, used as a DNA vaccine candidate in our experiments, the beneficial effect seems to be transitory. In a previous report, 30% of the immunized BALB/c mice with a DNA vaccine encoding to the Gp63 overcame leishmaniasis infection (30). The third candidate is represented by a construct which encodes to the LACK antigen. This construct gave the most promising results and especially when it is used in its truncated form (LACKp24). Nevertheless, and despite the promise that it has raised, the LACK DNA vaccine candidate is unable to generate a full protection against *L.major* in BALB/c mice. Previous reports using the LACKp24 as a DNA vaccine candidate were able to show almost a full protection (31-32).

Experiments indicate that the non-specific immune responses triggered by the ISS sequences are not strong enough alone to induce a significant protective effect. However, if associated with a selected gene, for instance the gene encoding to LACKp24 as in pCMV3ISS-LACKp24, the ISS sequences exert a potent adjuvant effect which boosts the immune responses to the selected gene when compared to what was raised with the plasmid pCMV-LACKp24 exempt of the tandem repeats of three ISS motifs. Nevertheless, one should know that the plasmid pCMV-LACKp24 contain the ampicilin resistance gene which include two motifs of ISS sequences. Therefore, we can presume that the presence of ISS sequences is not enough by itself to promote the level of immune responses, but their number and may be their positions could be as important.(17)

In the study, the groups of mice that received DNA IL-12 or DNA IL-18 showed higher levels of IFN- γ than the group primed with DNAp36 alone. The higher levels of IFN-γ were obtained when DNA IL-12 was administered together with DNAp36, results significantly different than the amount produced when mice were primed with DNA IL-18+ DNAp36/LACK.(33)

In the present study, the LACK protein gene open reading frame was modified by PCR amplification. The resulting PCR product was digested with Hind III and EcoRI digested PTZ57R/T system. The insert was excised using Hind III and was ligated into expression vector pcDNA3 downstream to the CMV promoter. Cloning of gene LACK into PTZ57R/T and pcDNA3 was confirmed by restriction enzymes (EcoRI and Hind III) and PCR amplification of LACK.

The extracted recombinant plasmids (PT-LACK) were sequenced.

In this research, we used MHRO/IR/75/ER (an Iranian strain) of *L.major* and LACK. Sequence analysis of cloned LACK gene into pTZ57R/T vector showed high homology of 89% with LmjF28.2740 (LACK gene).

Southern blot hybridization analysis indicated that there are multiple copies of the LACK gene in all species of Leishmania analyzed. Northern blot analyses demonstrated that the LACK gene is constitutively expressed in *L.major* promastigotes and amastigotes (2, 6).

Conclusion

In conclusion, expression of the LACK protein could be a preliminary step for further research to design effective vaccine against leishmaniasis (17, 34 and 35).

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