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Original Article

Cloning and Expression of Recombinant Plasmid Containing P36/*LACK* Gene of *Leishmania infantum* Iranian Strain

Saloomeh SHIRALI [1,2], *Hamidreza HADDADZADEH [2], Mehdi MOHEBALI [3, 4], Bahram KAZEMI [5,6], Narges AMINI [2]

1. *Dept. of Biology, Faculty of Basic science, Ahvaz branch, Islamic Azad University, Ahvaz, Iran*
2. *Dept. of Parasitology, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran*
3. *Dept. of Medical Parasitology and Mycology, School of Public health, Tehran University of Medical Sciences, Iran*
4. *Center for Research of Endemic Parasites of Iran (CREPI), Tehran University of Medical Sciences, Tehran, Iran*
5. *Cellular and Molecular Biology Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran*
6. *Dept. of Biotechnology, School of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran*
7.

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***Correspondence**
Email:
hhadadad@ut.ac.ir

Abstract

Background: There are several methods, such as vaccination, to control visceral leishmaniasis. Although there is no efficient vaccine, it seem DNA vaccination with stimulates both cellular and humoral immunity apparently is the best way. The aim of this study was cloning and expression of *LACK* gene, a 36kD protein, as a candidate protein for vaccination against Iranian *L. infantum*.

Methods: Iranian strain of *L. infantum* [MCAN/IR/07/Moheb-gh] was used as a template for PCR to amplify *LACK* gene. The *LACK* gene was cloned in pTZ57R/T vector and after confirmation it was digested by restriction enzymes (BamH1) and cloned in pcDNA3.1 expression vector. Recombinant plasmid was extracted and analyzed by sequencing, restriction digestion analysis and PCR reaction. The pc- *LACK* recombinant plasmid was purified from transformed *E.coli* (DH5α) and its expression was analyzed by SDS-PAGE and Western blot.

Results: The results of sequencing, restriction digestion analysis and PCR reaction revealed that *LACK* gene was cloned correctly in pcDNA3.1 vector and the results of SDS PAGE and Western blot emphasized that *LACK* protein of Iranian *L. infantum* is a well-expressed protein.

Conclusion: We amplified, cloned and expressed Iranian *L. infantum LACK* gene successfully.

Introduction

Canine visceral leishmaniasis (CVL) is an infectious disease transmitted by sand flies and caused by *Leishmania infantum* (1). It is a zoonotic endemic disease in most of the Mediterranean area, Asia, and Latin America (2, 3). In Iran CVL is common and caused by *L. infantum* Iranian strain. The prevalence of CVL in various parts of Iran is different and it is correlated by weather condition and humidity (4). It is endemic in Ardebil, East Azerbaijan, Fars and Bushehr provinces (5).

Domestic dogs (*Canis familiaris)* are the main reservoir hosts of human visceral leishmaniasis in Iran (6) and the prevalence of VL in human, in distinct area, is associated with amount of CVL in dogs (4). In foci of canine leishmaniasis, symptomatic disease is low and due to the systemic nature of the disease, Clinical manifestations are variable (6- 8). So control of the disease in human correlated by control in dogs. It seemed among different control strategies of the disease vaccination, if an efficient vaccine being accessible, is the best possible way for eradication of this disease in dogs (6).

Immunization with naked DNA is the latest method, which promote both $CD4^+$- and $CD8^+$-mediated responses (9, 10). In the past few years, studies focused on some antigens such as *GP63, CP, TSA, GP64, LmSTI1, LeIf* and *P8, p4* and *LACK* (11).

Among of these antigens, *LACK* gene is one of the best candidates in other strains of this pathogen (12) *LACK* (*Leishmania* homologue of receptors for activated C kinase) is a 36 kDa protein localized in cytosol and external surface of the membrane (13). It is expressed in both promastigote and amastigote forms of the parasite (14). The protective effect of the *LACK* vaccine was mediated by IL-12-dependent IFN-g production (15).

The objective of this study was to investigate of the quality of *LACK* protein expression of Iran strain of *L. infantum*.

Materials and Methods

Leishmania infantum

Leishmania infantum Iran strain [MCAN/IR/07/Moheb-gh] was provided by the school of Public Health Tehran University of Medical Sciences. Promastigotes were grown in RPMI 1640 medium (Gibco®, Germany) supplemented with 5% fetal calf serum and 200IU /ml penicillin G [crystalizeh].

Genomic DNA extraction

1ml of RPMI medium was centrifuged at 12000g for 10 minute. The pellet was washed with PBS [pH=7.2] 3 times. DNA extracted by DNA extraction kit-MBST-IRAN and electrophoresed on 1% agarose gel.

PCR amplification and cloning in pTZ57R/T vector

The extracted DNA was used as a template to amplify the *LACK* gene by PCR. The reaction was performed in 100µl of the solution containing: 1µl of template DNA, 2µl of dNTP (100 µmol), 0.5µl of Taq DNA polymerase (5u/µl), 10µl of 10X PCR buffer, 2 µl of $MgCl_2$ (50 mmol), 79.5µl of distilled water and 2µl of each of primers (10 Pmol/µl).We designed a pair of primer based on *LACK* gene sequence (Accession number U49695) with BamHI and HindII restriction enzymes on 5` forward and reverse primer *LACK* F 5-ggatcc A TGA ACT ACG AG-G GTC ACC -3 Reverse primer: introduced HindIII. recognition site: *LACK* R 5` aagcttTTA CTC GG-C GTC GGA GAT GGA-3. PCR product was purified (Purification kit MBST-IRAN), cloned into pTZ57R/T vector (fermentase® T/A vector cat. No #K1213) and transformed in competent cell (*E.coli* DH5α). Recombinant plasmid was confirmed by PCR, restriction analysis and sequencing methods.

Ligation of LACK gene into pcDNA3.1 cloning vector

Recombinant pTZ57R/T-*LACK* was digeted by BamHI and released *LACK* gene purified and sub cloned into pcDNA3.1 shuttel vector. Briefly, the reaction was performed in 10 µl of the solution containing: 5 µl of the purified *LACK* gene, 2 µl of 5x buffer, 0.5 µl of T4 DNA ligase (5u/µl) (fermentase®), 1.5 µl of D.W (distilled-water) in 4°C for overnight and transformed in to competent cell as previously described (16).

Detection of pc-LACK recombinant plasmid

Analysis of recombinant colony was done by three methods: colony PCR reaction, Restriction analysis and Sequencing. The universal primers are used for detection are T7Promoter, TAA TAC GAC TCA CTA TAG GC and BGH-rev, CTA GAA GGC ACA GTC GAG GC.

SDS-PAGE and Western blot analysis

A single colony of pc-*LACK* was cultured, when its OD reach to 0.6 was induced by 0.1 molar IPTG. Samples were given at 0,1,2,3 and 5 hours after induction. The expression was checked by SDS-PAGE technique.

Electrophoresed proteins were transferred to nitrocellulose membrane and western blot analysis was done and specific protein was detected by *Leishmania* antibody-positive dog serum and anti-dog conjugate (anti-IgG dog Sigma ®). Specific binding was revealed with diaminobenzidine (DAB) (DAKO, Denmark).

For dilution of serum in Western blot, the best antigen-Antibody reaction was found by Dot blot.

Results

Gene Cloning in pcDNA3.1

Leishmania infantum Genomic DNA was extracted and *LACK* gene amplified by PCR reaction. Then gene was cloned into PTZ57R/T vector and after confirmation plasmid by colony PCR and restriction analysis, recombinant plasmid was digested by BamHI and sub cloned into pcDNA3.1 expressing vector (Fig.1). Recombinant plasmid was confirmed by colony PCR (Fig.2) and Restriction analysis (Fig.3).

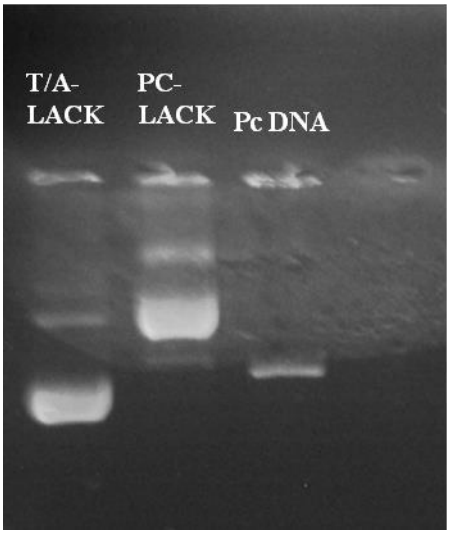

Fig. 1: Electrophoresis of T/A-*LACK* and pc- *LACK* recombinant plasmids and pcDNA3 plasmid were loaded on a 1% agarose gel./ Column1: T/A-*LACK* recombinant plasmid/Column2: pc- *LACK* recombinant plasmid/Column3: The band of pcDNA3

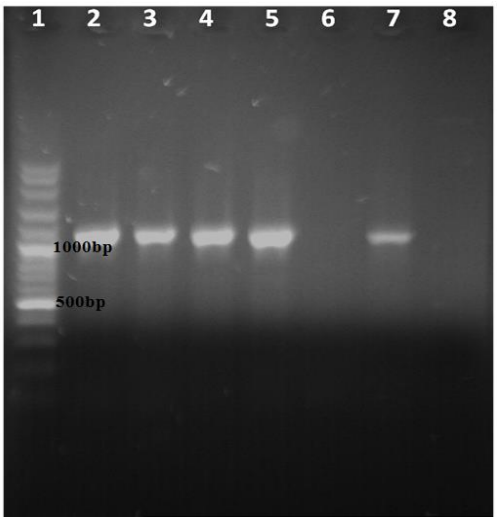

Fig.2: Electrophoresis of PCR, amplification of *LACK* gene/with pc-*LACK*./ column 1: DNA ladder/column 2-7: PCR amplification of *LACK* gene with pc-*LACK*/column 8: control negative

Recombinant pcDNA3.1 plasmid was purified and sequenced by dideoxy chain termination method and Compared with *LACK* gene (Accession no U49695.1) by www.ncbi.nlm.nih.gov/blast-showed high homology of 98 % (Fig. 4).

SDS PAGE and Western blot analysis

A 36 KD band was recognized by *Leishmania* antibody-positive [polyclonal] dog sera in protein extracts of the cells transfected with pc-*LACK*. In Western blot *LACK* protein was not detected in the non-transfected control cells. (Fig. 5, 6)

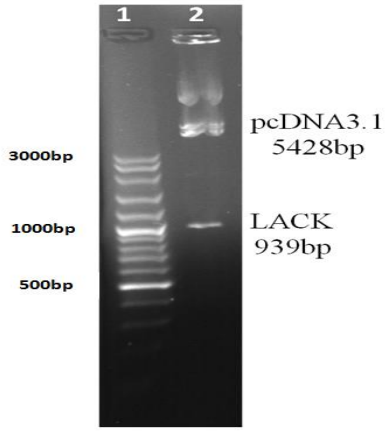

Fig. 3: Electrophoresis of extracted pc-*LACK* after digestion by enzyme/ Column 1: DNA ladder/column 2: pc*LACK* digested by BamH1

Fig. 4: Sequencing of *LACK* gene with universal primers of pc-DNA3.1

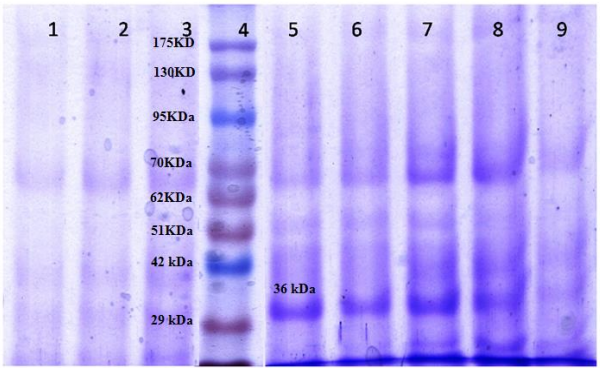

Fig. 5: SDS-PAGE analysis of expressed gene product
Column1:DH5α before expressing by IPTG (control)
Column2: pc-DNA3.1 before expressing by IPTG (control)
Column3: pc-*LACK* before expressing by IPTG (control)/ Column4: Pre stain protein ladder
Column5: pc-*LACK* 1h after expressing by IPTG
Column6: pc-*LACK* 2h after expressing by IPTG
Column7: pc-*LACK* 3h after expressing by IPTG
Column8: pc-*LACK* 4h after expressing by IPTG
Column9: pc-*LACK* 5h after expressing by IPTG

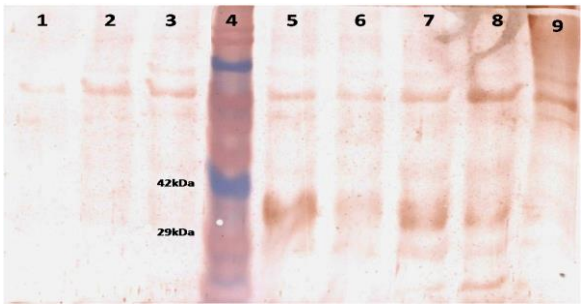

Fig. 6: Western blot analysis of expressed gene product
Column1:DH5α before expressing by IPTG (control)
Column2: pc-DNA3.1 before expressing by IPTG (control)
Column3: pc-*LACK* before expressing by IPTG (control)/ Column4: Pre stain protein ladder
Column5: pc-*LACK* 1h after expressing by IPTG
Column6: pc-*LACK* 2h after expressing by IPTG
Column7: pc-*LACK* 3h after expressing by IPTG
Column8: pc-*LACK* 4h after expressing by IPTG
Column9: pc-*LACK* 5h after expressing by IPTG

Discussion

ZVL is a zoonotic lethal protozoan disease of man and animals. Canine's family, particularly domestic dogs are considered the main reservoir of zoonotic transmission to humans (17).

Dogs usually develop the systemic form of infection, with a highly variable clinical appearance that may involve any organ, tissue, or body fluid and is manifested by non-specific clinical signs. Both symptomatic and asymptomatic *Leishmania*-infected dogs act as a source of parasites for VL transmission (17).

Although anti *Leishmania* drugs successfully used for human, VL therapy show low efficacy in canines (9, 18). Thus, recent studies have tended to CVL control instead of treating. Control methods are variable, such as reservoir control, vector control, insecticide - impregnated materials and culling. But none of them were useful (8, 9, 19).

Due to the simple nature of the parasite and this fact that recovery and resistance are the results of reinfection in leishmaniasis, vaccination against VL is feasible (13, 20).

Studies on a protective vaccine candidate have advanced in recent years and several vaccination methods and several antigens were tested. Immunization with naked DNA (DNA vaccination) is a new approach that promote both $CD4^+$ and $CD8^+$ mediated responses and helped to inducing a protective response against infection (10, 15, 21, 22). These mechanisms of action make it attractive for control of *Leishmania* (10).

The efficacy of immunogenicity of *LACK* gene was proved by in previous study. Kelly et al. found that *LACK* is required for parasite viability and it can be a potential drug target for leishmaniasis (20). *LACK* might bind and enhance plasminogen activation in vivo promoting the formation of plasmin and might contribute to the invasiveness of the parasite (13).

In some studies, the efficacy of vaccine based on *LACK* was investigated. For example, the study on the combination of DNA vectors expressing IL-12 + IL-18 and a booster with a vaccinia virus recombinant expressing *LACK* in mice revealed that this combined prime/booster immunization re-

gime is an efficient approach to protect against leishmaniasis (14). Heterologous prime-boost regime using DNA and recombinant vaccinia virus vectors expressing *LACK* was tested in some studies and relative protection was achieved (22- 25). *LACK* DNA vaccine induced a robust parasite-specific Th1 immune response (IFN-γ) but it is not protective against cutaneous or systemic *L. donovani* challenge (15).

Cloning and expression of the *LACK* gene of *L. major* Iranian strain for immunology study and production of recombinant vaccine was done before (26, 27). According to prevalence and importance of visceral leishmaniasis in Iran and Necessity of production an efficient vaccine, we did this study.

Conclusion

The results of the blast in gene bank and western blot analyze showed that the gene correctly cloned in vector and it is active in immunological aspect. Therefore, this study is a start of design a recombinant vaccine against canine visceral leishmaniasis in the future.

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