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Original Article

Sequencing and Gene Expression Analysis of *Leishmania tropica LACK* Gene

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Abstract

Background: *Leishmania* Homologue of receptors for Activated C Kinase (LACK) antigen is a 36-kDa protein, which provokes a very early immune response against *Leishmania* infection. There are several reports on the expression of *LACK* through different life-cycle stages of genus *Leishmania*, but only a few of them have focused on *L.tropica*.

Methods: The present study provides details of the cloning, DNA sequencing and gene expression of *LACK* in this parasite species. First, several local isolates of *Leishmania* parasites were typed in our laboratory using PCR technique to verify of *Leishmania* parasite species. After that, *LACK* gene was amplified and cloned into a vector for sequencing. Finally, the expression of this molecule in logarithmic and stationary growth phase promastigotes, as well as in amastigotes, was evaluated by Reverse Transcription-PCR (RT-PCR) technique.

Results: The typing result confirmed that all our local isolates belong to *L.tropica*. *LACK* gene sequence was determined and high similarity was observed with the sequences of other *Leishmania* species. Furthermore, the expression of *LACK* gene in both promastigotes and amastigotes forms was confirmed.

Conclusion: Overall, the data set the stage for future studies of the properties and immune role of *LACK* gene products.

Introduction

Leishmaniasis, a vector-borne disease caused by obligate intra-macrophage protozoa, which is endemic in large areas of the tropics, subtropics and the Mediterranean basin, and has been identified as a category 1 disease by the World Health Organization (WHO) (1). *Leishmania* spp. cause a wide variety of diseases that range in severity from self-healing cutaneous leishm-aniasis (CL) to fatal visceral leishmaniasis (2). It is caused by more than 20 leishmanial species and is transmitted to humans by ~30 different species of phlebotomine sandflies (3, 4). CL is caused by many species of *Leishmania*; *L.major*, *L.aethiopica* and *L.tropica*, in the Old World (5). Nearly 90% of CL cases occur in Afghanistan, Algeria, Brazil, the Islamic Republic of Iran, Peru, Saudi Arabia and Sudan in addition to Syria (1). Two distinct developmental stages of *Leishmania* are recognized. Promastigotes are found within the sandfly. Promastigotes can be further classified as procyclic promastigotes, which multiply in the gut of the sandfly, or as the infective metacyclic promastigotes (Infective stage), found in the mouthparts and anterior gut and do not divide. These differentiate into round or oval amastigotes (Diagnostic stage), which *LACK* flagella, once in the host (6).

Leishmania homologue of receptors for activated C kinase (LACK) antigen is a 36-kDa protein expressed in promastigotes and amastigotes forms of different *Leishmania* species (7), which is a member of the family of WD40 repeat proteins. These macromolecules are evolutionarily conserved tryptophan-aspartate motif proteins and have diverse but critical functions in eukaryotes, including signal transduction, RNA processing, and cell cycle control (8). *LACK* is essential for the parasite viability and the parasite establishment in the host. In addition to its cytoplasmic localization, *LACK* was recently found to also be actively secreted, and this secretion occurring via exosomes (9). The function of *LACK* in *Leishmania* is not clear, although the immunological response to this molecule has been well studied and used for experimental vaccine studies in the mouse model (9). Since the response to *LACK* is a very early event in a *Leishmania* infection, it could also include innate elements of immunity (9). *LACK* antigen has been used as a tool to investigate various immunity-related mechanisms. It has been tested in several immunization experiments, providing heterogeneous results. The immunization of mice with *L.donovani LACK* protein protects against cutaneous *L.major* challenged (7, 10, 11). In human patients, as in mice, response to *LACK* depends strongly on the infecting *Leishmania* species (12).

In consideration of CL distribution in Syria, which is mostly caused by *L.tropica*, and the prominent protective role of *LACK* protein as a vaccine candidate against leishmaniasis, we aimed to determine the sequence of *LACK* gene of local isolate of *L.tropica* and to study the expression of *LACK* gene in both promastigotes and amastigotes forms.

Materials and Methods

Parasites Sampling

Samples were collected from patients visiting the Dermatology Hospital in Damascus, with lesions suspected to CL. Positive samples were collected directly from skin scars of the patients on slides. In addition, aspirated samples were taken from lesions for culturing in a semi-solid culture medium (13).

Microscopic examination

Smears on glass slides, were air dried, fixed with methanol for a few seconds and stained with Giemsa. Then, the stained smears were examined using light microscope 100×(Olympus, Japan)(3, 14).

In vitro cultivation of Leishmania

Cultures were obtained by needle aspiration from the border of the lesions. The aspirated fluid was discharged into the culture tubes contain a semisolid medium; agar and RPMI-1640 (Sigma, Germany) supplemented with 10% heat-inactivated fetal bovine serum (FBS),100U/ml L-Glutamine and 100U/ml penicillin-streptomycin (Cytogen, Germany). The cultures were incubated at 26°C. After a few days, one drop of media was examined using inverted microscope 40× (Optika, Italy). Once promastigotes were formed, the cultures were transferred to RPMI-1640 supplemented with FBS, L-Glutamine and penicillin-streptomycin as described above. To obtain axenic amastigotes, promastigotes in stationary phase in 25cm^2 ventilated flask were incubated at 37°C and pH 5.8 with 5% CO_2. for about 48hour (15-18).

DNA extraction

DNA was extracted from 5ml of *Leishmania* in vitro culture (~16×10^{6}cell/ml). Promastigotes were harvested and washed with (1×) phosphate-buffered saline (PBS) pH7.4, pelleted by centrifugation at 3000rpm for 15minutes at room temperature. Then, genomic DNA was isolated using Wizard Genomic DNA Purification Kit (Promega, USA) according to the manufacturer's instructions.

PCR amplification

Polymerase Chain Reaction (PCR) was performed using CSB2 5'-CGAGTAGCAGAAAC TCCCGTTCA-3' and CSB1 5'-ATTTTTCGCG ATTTT CGCAGAACG-3' primers for species determination, which are specific to the conserved region of *Leishmania* minicircle kinetoplast DNA (kDNA)(19, 20). Leishmanial DNA isolated from reference strains *L.major* (MHOM/SY/91/LEM2397) and *L.tropica* (MHOM/SY/90/LEM2066) was used as a positive control (Reference strains are a gift from French National Reference Centre for-*Leishmania*(Montpellier, France). The total volume of PCR reaction is 30µl including (1×) Go Taq Buffer, dNTPmix (~0.2mM each dNTP), GoTaq DNA polymerase (1u) (Promega, UAS), and DNA template 0.25µg. DNA amplification was performed in the PeQlab thermocycler (PEQLAP, Germany) with 45 cycles, using an initial heating 94°C for 2min. Each cycle was divided into three stages: denaturation (94°C–30sec), annealing (54°C–1min), and elongation (72°C–1min). After the reaction, the material was kept at 72°C for 10min. PCR products were separated on 1% agarose gel. Fragment sizes were determined with bands of a DNA length standard (1kb DNA Ladder, Promega, USA). The length of the amplified PCR products is about 750bp.

Cloning and sequencing of LACK gene

LACK gene was amplified with the use of GoTaq DNA polymerase and LACK-FL-F 5'-ATGAACTACGAGGGTCACCT-3' and LACK-FL-R 5'-CTCGGCGTCGGAGATGGACC-3' specific primers. Primers were designed according to *LACK* gene sequence of *L.major* (GenBank accession number AF363975.1). PCR products were purified using Invisorb Fragment CleanUp kit (Stratec molecular, Germany). Then, the purified fragments were cloned into the open plasmid pDrive cloning vector (QIAGEN, Germany), and sequenced by Genetic Analyzer system ABI-310 using universal specific primers for the plasmid; FP (CGCCAGGGTTTTCCCAGTCACGAC) and RP (TCACACAGGAAACAGCTATGAC) (Applied Biosystems, USA).

Comparison of LACK sequences between *Leishmania* spp.

The nucleotide sequences of *LACK* gene in a local isolate of *L.tropica* (GenBank accession number: KM042903) was compared with reported *LACK* sequences in *L.donovani, L.major, L.amazonensis, L.braziliensis, L.chagasi, L. infantum and, L. mexicana* and from three recently reported *L.tropica* Iranian isolates (GenBank accession numbers: AF363974.1, AF363975.1,

AF363977.1, AF363978.1, U27569.1, U49695.1, AF363976.1, JX305923, JX305924, and KC763809, respectively). Sequence alignment and dendrogram prediction were performed using Geneious v4.8 software, available from www.geneious.com.

Total RNA Extraction and cDNA Synthesis

Total RNA was extracted from 5ml of *Leishmania* promastigotes; stationary phase (~16×10^6cell/ml) and logarithmic phase (~12×10^6cell/ml), as well as from axenic amastigotes (~16×10^6cell/ml). The extraction was carried out using SV Total RNA Isolation System (Promega, USA) according to the manufacturer's instructions. Total RNA concentration was measured at 260nm wavelength using spectrophotometer (Jenway, England), and 500ng was loaded into 1% agarose gel electrophoresis to inspect its integrity and quality after extraction. Furthermore, 100ng of total RNA was applied as a negative template in a PCR reaction using *LACK* gene specific primers; LACK-F 5'-GCAGCTGTTCAAGATCAACG-3' and LACK-R 5'- GGATCAGGTTGTCCTTGTGG-3'. Then, PCR products were examined on 1% agarose gel to assure the purity of our RNA of any genomic DNA contaminants. Hereinafter, total RNA (0.5µg) was reverse transcribed into single-stranded cDNA using First Strand cDNA Synthesis Kit (Fermentas, Canada) according to the manufacturer's instructions, and the resulted cDNA (60ng) was used as PCR template.

In PCR reaction, two couples of specific primers were used. LACK-F/R primers, which amplify 230bp of *LACK* gene, and Lmr60s-F 5'-ATGCCGCCGAAGTTCGACCC-3' and Lmr60s-R 5'-CGTCGCCACACGGTTCTTGA-3' primers, which amplify 200bp of the encoding gene for 60s ribosomal protein L12 (GenBank accession number: XM_001683687) as a positive control for cDNA synthesis. Three independent experiments were undertaken for the selected genes in all described samples.

Results

Typing of local isolates

Typing was accomplished using PCR technique and species-specific primers CSB2/CSB1. PCR result was considered positive when a single band of correct size ~750bp was observed, which corresponds to *L.tropica* kDNA minicircle, while the band with 560bp corresponds to *L.major*. This result confirmed the identity of the analyzed parasites in comparison with the reference strains (Fig. 1).

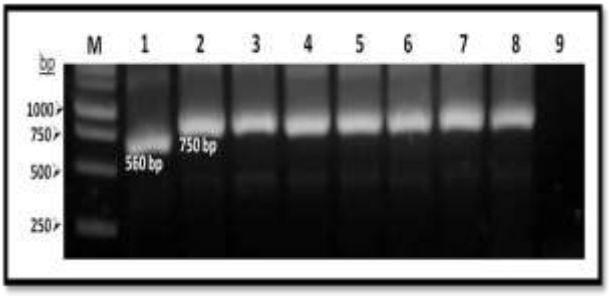

Fig. 1: PCR result from tested leishmania isolates and from two reference strains using species-specific kDNA minicircle CSB2/CSB1 primers. Lane M: marker (1kb DNA ladder); Lanes 1and 2 reference strains *L. major* and *L. tropica*, respectively. Lanes 3-8: local isolates of *L.tropica*. Lane 9: negative control

Cloning and sequencing of LACK gene

Full-length *LACK* gene was amplified from *L.tropica* genomic DNA using two specific primers LACK-FL-F/R. GoTaq DNA polymerase, used in PCR reaction, adds overhang A to each end of the target sequence, which allows the gene insertion into linerized pDrive cloning vector. After bacterial transformation, blue/white colony screening was performed to select positive colonies, confirmed by colony PCR using two plasmid specific primers (FP/RP) resulting in a band of bp (data not shown). Plasmid constructs from positive colonies were prepared and sent for sequencing. Sequence alignment analysis revealed the presence of similar *LACK* sequence form all tested *L.tropica* local isolates, and the resulted sequence

(Fig.2) was sent for registration in NCBI data- base (GenBank accession number: KM042903).

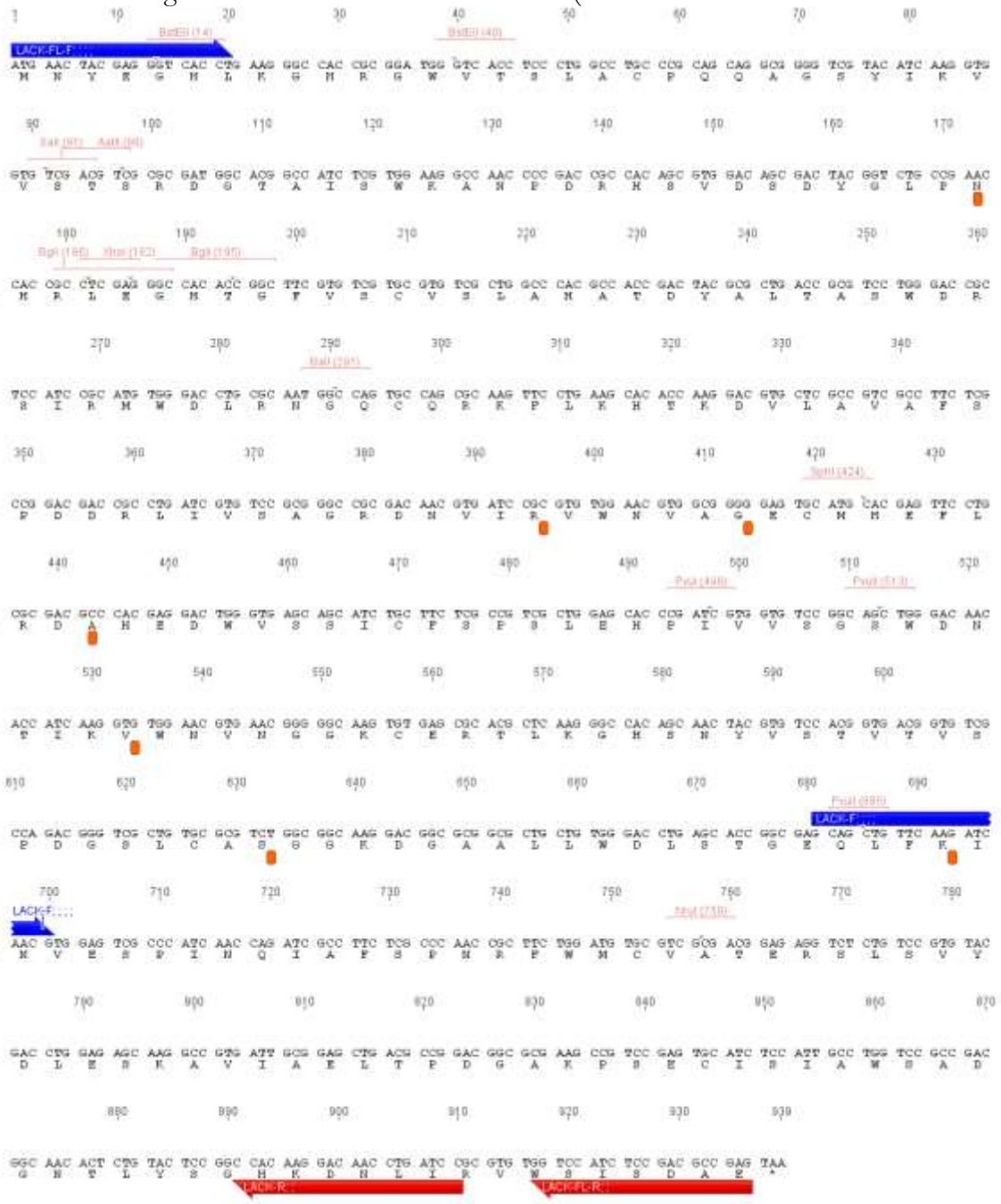

Fig. 2: Nucleotide and deduced amino acid sequences of *L.tropica LACK* gene. The nucleotide sequence is shown in the 5'-3' direction, numbered from the first base of start codon as the position 1. Deduced amino acid sequences are located below the nucleotide sequence. Positions of *LACK* gene specific primers and of several cutting restriction enzymes from the most commonly used ones are shown. Dotes below the sequence indicate the nucleotides that differ between *L.tropica* and other species

Comparison of LACK sequences between Leishmania spp

The nucleotide sequence of *L.tropica LACK* from the local isolates (KM042903) was aligned with other reported *LACK* sequences from different *Leishmania* spp. as well as with three previously published *L.tropica LACK* gene sequences (JX305923, JX305924, and KC763809). Sequence alignment of *LACK* genes from these records revealed that the local isolates (KM042903) manifest three *L.tropica* hallmark nucleotide substitutions; an A instead of G (A/G) at position 173 (resulting in an asparagine instead of a serine), a G/C at 414 (glycine) and a T/C at 633 (serine). However, in the fourth position at 396 C/A (Arginine), the *LACK* gene from the local isolates differs from all other *L.tropica* isolates and resembles the other *Leishmania* species *LACK* genes by possessing an A instead of C. Interestingly, the *L.tropica* isolate (KC763809), which found to be very close to our local isolates in the dendrogram of *LACK* genes, was found to share with the local isolates a unique substitution; a G/A (valine) at position 534 comparing with other strains including those from *L.tropica*. Another unique substitution for our local isolate is a C/G (alanine/glycine) at position 443, and this change seems to be tolerated by *L.tropica* at this site since an S (G or C) was found in this location of the isolate (KC763809) from the same species. One confusing notice was regarding the G (lysine) at position 693, which characterizes all *Leishmania* species including *L.tropica* local isolates and the isolate (JX305924) but not the isolate (JX305923), which has an A (lysine) substitution.

However, it seems that both possibilities are also acceptable for this site in *L.tropica* since an R (G or A) was found in this location in the isolate (KC763809) from the same species (Fig.2).

The result of the alignment of the *LACK* gene sequences from all records was presented in a dendrogram (Fig.3).

Remarkably, the sequence of *LACK* gene has almost perfect matching among *L.tropica* isolates. The cluster of similarity in this species could be easily distinguished from two other clusters; the first which is closer in similarity and contains *L.donovani, L.chagasi,* and *L.infantum,* and a second distant cluster gathering *L.amazonensis, L.major, L.braziliensis, and L.mexicana*. However, *LACK* gene seems to be a highly conserved gene in *Leishmania* genius since a relatively great degree of similarity (reaching 98.1%) was observed between the farthest two sequences in the dendrogram; the local isolates of *L.tropica* and *L.mexicana*(Fig.3).

Total RNA Extraction and cDNA Synthesis

Total RNA was extracted successfully from cultured promastigotes (from stationary and logarithmic phases) and axenic amastigotes. Furthermore, total RNA electrophoresis illustrated smear of mRNA and three bands of rRNA, showing an acceptable integrity and purity of the prepared RNA (Fig.4A, B, and C). Moreover, the purity of the RNA from genomic DNA contaminants was confirmed by PCR reaction, using *LACK* gene specific primers where a negative amplification is expected (Fig.4D).

Then, total RNA was reverse transcribed, and the synthesized cDNA was used as template to amplify a small domain from *LACK* full-length gene using LACK-F/LACK-R primers.

This resulted in a single band of 230bp in all tested conditions, confirming the presence of *LACK* gene transcripts in both amastigotes and promastigotes; stationary and logarithmic phases, as well as a single band about 200bp refers to positive control transcripts (Fig.5).

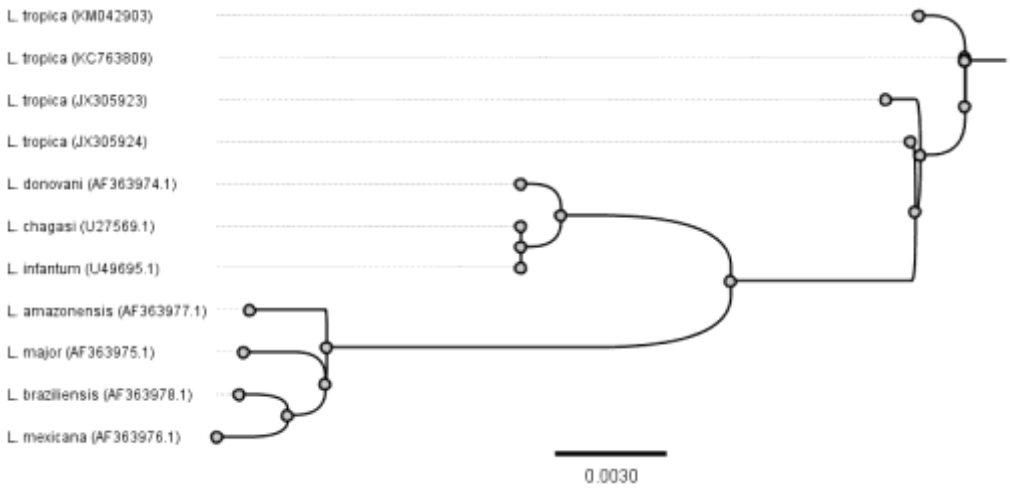

Fig. 3: A dendrogram of *LACK* gene sequences from different *Leishmania* species that were aligned with the local *L.tropica LACK* gene (KM042903)

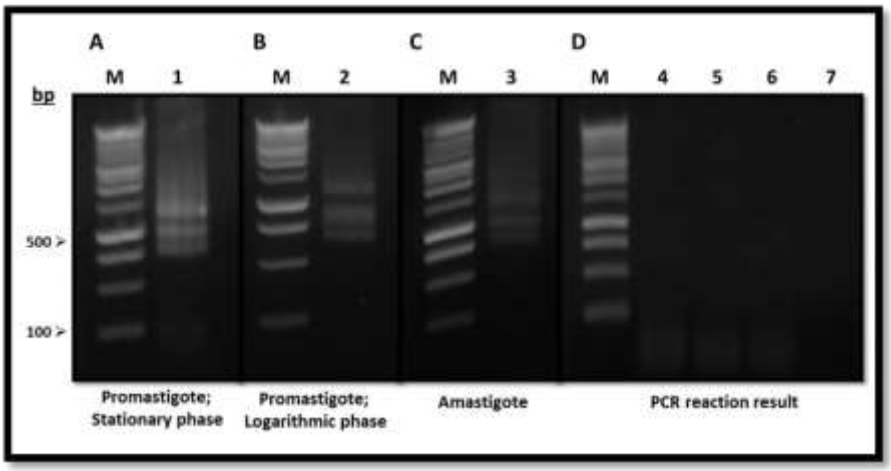

Fig. 4: Evaluation of the quality and purity of the extracted total RNA.**A, B** and **C**: depict smear of mRNA and three bands of rRNA reflecting the excellent extraction of total RNA in promastigotes; stationary and logarithmic phases, and axenic amastigotes (Lanes 1, 2 and 3), respectively. **D**: Result of PCR reactions confirming the purity of extracted total RNA from genomic DNA contaminants in promastigotes; stationary and logarithmic phases, and axenic amastigotes (Lanes 4, 5 and 6), respectively. Lane 7: negative control for PCR reaction

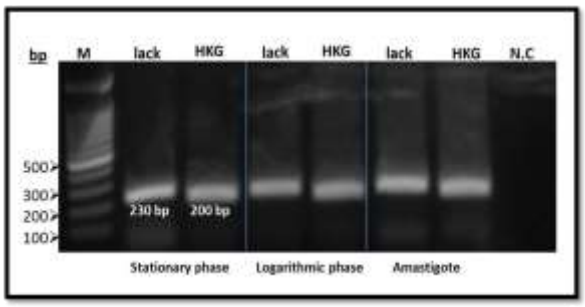

Fig. 5: PCR result of synthesized cDNA. PCR reaction was applied on cDNA of *LACK* gene of promastigotes; stationary and logarithmic phases, and amastigotes, respectively, and cDNA of 60s ribosomal protein L12 gene (a positive control) of promastigotes; stationary and logarithmic phases, and amastigotes, respectively. The PCR product size is approximately 230bp of the target gene (*LACK*), and 200bp of the housekeeping gene (HKG). Lane M: marker (BenchTop 100bp DNA ladder); and lane 7: Negative control

Discussion

In this study, we showed by the molecular typing with PCR technique that all *Leishmania* local isolates were belonging to *L.tropica*. Such method is widely used for distinguishing the different species of *Leishmania*, simply by amplifying highly conserved sequence blocks of kDNA minicircles (19, 20). Recently, PCR is used for visceral and cutaneous leishmaniasis diagnosis (21). In compared studies of PCR assays, PCR based on kDNA was the most sensitive diagnostic assay for CL and recommended for routine diagnosis (22). The genomic DNA of one isolate belonging to *L.tropica* was extracted and *LACK* gene was amplified, cloned and sequenced. *Ltropica LACK* nucleotide sequences from the local isolates were identical and manifest a great similarity with *L.tropica LACK* gene from published records. This provides further confirmation of the identity of our local isolates based on the analysis of *LACK* gene. Furthermore, *LACK* gene from local isolates showed significant homology to *LACK* sequence from several other species including *L.donovani, L.major, L.amazonensis, L.braziliensis, L.chagasi, L.infantum and L.mexicana*, implying that *LACK* protein may have an important functions for the parasite life cycle. This high similarity of *LACK* nucleotide sequences may refer to identical amino acid sequence among the different *Leishmania* spp. Melby et al. (2001) had shown that *L.donovani LACK* deduced amino acid sequence was identical to the published *L.chagasi* and *L.infantum* sequences (GenBank accession numbers U27569 and U49695, respectively) and differed at only 1 or 2 amino acids from the *L.major, L.mexicana, L.amazonensis,* and *L.braziliensis* sequences they obtained (10). In this work, *LACK* protein from our local strains was found to differ in two amino acids from other *Leishmania* spp. because of nucleotides substitution at position 173 and 443.

We demonstrated that *LACK* gene was expressed in logarithmic and stationary phases present procyclic and metacyclic promastigotes respectively, as well as in amastigotes in *L.tropica*. *LACK* protein is believed to be expressed by all *Leishmania* spp. in both promastigote and amastigote stages (12, 23). Recently, Hajjaran et al. (2012) study approved that *LACK* protein is one of the over expressed proteins in Iranian isolate of *L.tropica* promastigotes (24). The continuous expression and production of *LACK* protein reflects its important role in various stages of the parasite life cycle, and his major role was confirmed by several studies; *LACK* is required for vertebrate parasitization, plasminogen activation, and can function as an aggravating factor in animal host causing early phase infection. In addition, *LACK* can induce both proliferative and cytokine responses in peripheral blood mononuclear cells (8, 9, 11, 25). To date, there have been numerous attempts at developing a successful vaccine against leishmaniasis (2). The protective role of *LACK* as a vaccine in humans is not straightforward (25). On the other hand, *LACK* administered with IL-12 is able to direct the T cells toward a Th1 response in mice (26). However, driving of the immunodominant *LACK* response toward a Th1 phenotype could be the key for preventing the ill effects of Th2 responses (25). Immunization with recombinant antigens or plasmid DNA encoding *Leishmania* antigens represents a promising approach to vaccinate against leishmaniasis. These DNA vaccines can induce both humoral and cell-mediated immune responses and results in long lasting immunity (27).Both models, *LACK* DNA and recombinant *LACK* protein can induce protection in mice that were immunized and challenged with *L.major* promastigotes (27). These results, which demonstrated a successful application of *LACK* DNA/Protein vaccine in the infectious disease models, open up an avenue for the development of vaccines against Old World cutaneous leishmaniasis caused by *L.tropica*.

This report is limited to study the sequence and the expression of *LACK* gene at mRNA level in different stages of *L.tropica* parasite life cycle. However, it will obviously be essential to validate these findings at protein level when the antibody is available, as post-transcriptional mechanisms have been shown to regulate protein levels in *Leishmania* (28).

Conclusion

Our study confers a base for producing the recombinant *LACK* protein since it is of particular interest as a vaccine candidate against CL. It focuses on determining *LACK* gene sequence in *L.tropica* (local isolates), comparing its sequence with those reported in database for other *Leishmania* spp. Finally, verifying of *LACK* gene expression at mRNA level in two different phases of the parasite life cycle; logarithmic and stationary phase of promastigotes, as well amastigotes.

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