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Original papers

Comparison of gene expression of pyruvate kinase and tryparedoxin peroxidase in metacyclic promastigote forms of *Leishmania* (*L.*) *tropica* and *L. major* by real-time PCR

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ABSTRACT. Two predominant forms of cutaneous leishmaniosis are anthroponotic CL (ACL) and zoonotic CL (ZCL) caused by *Leishmania* (*L.*) *tropica* and *L. major* in Iran and many countries, respectively. Since differential gene expression play an important role in outcome of the infection, we compared relative gene expression value of pyruvate kinase (PyrK) and tryparedoxin peroxidase (TryP) in metacyclic forms of Iranian isolates of *L. major* and *L. tropica*. Clinical isolates of CL patients were sampled in endemic foci of Iran and identified by PCR-RFLP. Then, we employed real-time PCR to evaluation of the expression level of PyrK and Tryp genes in *L. major* and *L. tropica*. By this comparison, up-regulation of PyrK and Tryp genes was observed in metacyclic stage. Moreover, the average mRNA expression of PyrK and Tryp genes in *L. major* was 1.69 and 3.72 folds of its expression in *L. tropica* isolates. The results of this study could open the new window for further investigations of the correspondence between parasite gene expression level and disease pathology. Species-specific parasite factors contributing to virulence and pathogenicity in the host may be mostly due to the some of the differential regulation of conserved genes between species.

Keywords: quantitative real-time PCR, *Leishmania*, *L. major*, *L. tropica*, metacyclic promastigote, pyruvate kinase (PyrK), tryparedoxin peroxidase (TryP)

Introduction

Leishmaniosis is one of the world's most neglected vector-borne infectious diseases with various clinical manifestations which affecting 12 million people and 350 million at risk of infection in 98 countries. So far, no vaccine are available against leishmaniosis and currently used drugs have high side effects [1,2]. Two epidemiologically important forms of cutaneous leishmaniosis (CL) have been reported in Iran and other Middle-East countries; zoonotic CL (ZCL) and anthroponotic CL (ACL) caused by *L. major* and *L. tropica*, respectively [3]. Numerous factors influence disease severity, but the most important determinant of the form of leishmaniosis is the species of *Leishmania* involved. The life cycle of the parasite is digenetic and dimorphic including two extracellular and intracellular stages. The procyclic promastigotes differentiate to metacyclic promastigotes inside the gut of female sandflies and amastigote forms residue in the infected vertebrate host macrophage cells [4]. Metacyclic promastigotes change their gene expression levels to achieve infectivity and adaptation in the vertebrate host cells such as human macrophages. The species-specific gene regulation in infective stage of *Leishmania* has provided valuable data about interspecific gene expression profile differences and also providing an indication of genes involved in biology, pathogenesis and host-pathogen interaction mechanisms in the two different species of CL causing agents [5,6]. Increased mitochondrial activity, response to stress and energy metabolism may involve in high infectivity and maintaining the parasite inside macrophages [7]. Among the energy metabolism involved enzymes, pyruvate kinase (PyrK) is the final enzyme that contributed in glycolysis pathway.

Leishmania parasite uses cytosolic peroxidase such as tryparedoxin peroxidase (TryP) and glutathione peroxidase for detoxification of peroxides, in which is a pivotal event for the survival of digenetic parasites living in two disparate biological environments [8,9]. Real-time PCR is advantageous over conventional PCR because it is faster, less labour-intensive and reduces risk of contamination [10]. Comparative assessment have shown important differences in expression level between both species. This analysis may serve to improve our understanding of parasite difference between these two closely related species that could be linked to their different disease phenotypes and to provide further windows into their molecular mechanism of pathogenicity as distinct species. This is the first study that describes the expression level of genes encoding PyrK and TryP in metacyclic stage of *L. major* compared to *L. tropica* by quantitative molecular method. The results of this comparison can provide information about pathogenicity, biology and virulence differences that could be led to potential targets for drug discovery and/or vaccine development effort. Hence, we have conducted a comparative analysis of gene expression value of PyrK and TryP between metacyclic forms of Iranian isolates of *L. major* and *L. tropica* by real-time PCR technique.

Materials and Methods

***Leishmania* samples and culture.** Clinical isolates of CL patients were collected in endemic foci of ACL (Bam city in Kerman Province) and ZCL (Gonbad city in Golestan Province) of Iran. All primary isolates initially were grown on NNN (Novy-Nicolle-Mc Neal) medium and for mass production, parasites were transferred to RPMI1640 medium (Gibco, Germany) supplemented with 10% FBS (Gibco, Germany), 100 U/ml penicillin, and 100 µg/ml streptomycin (Gibco, Germany) at 25°C. Promastigotes were cultured with repeated medium for 6–10 days for achievement the metacyclic promastigotes phase. During this time, the numbers of parasites were counted with light microscope. To ensure the collection of purified metacyclic promastigotes, we also performed agglutination assay by peanut agglutinin (PNA; Sigma) and morphometric analysis [11,12]. Briefly, we added PNA to PBS-washed metacyclic promastigotes to a final concentration of 30 µg/ml for 1×10^7 cells/ml. The next stage was involved separation of PNA- cells (metacyclic promastigotes) from PNA^+ cells (procyclic promastigotes) by centrifugation at 100 g for 10 min [11,12]. Finally, the last supernatant was centrifuged at 3500 g for 20 min to recover PNA^- promastigotes. The cell body size and flagellum lengths for 300 parasites in each day were measured by light microscopy, and promastigotes whose flagellum/body length ratio was ≥2 were considered metacyclic form as described [11,12]. Then, the parasites (10^7 cells/ml) were centrifuged at 3500 rpm for 20 min at 4°C and then were washed 3 times with sterile phosphate-buffered saline (PBS; pH: 7.4) and collected in two aliquots in -70°C for DNA and RNA extraction.

DNA extraction and species identification. Cultured metacyclic promastigotes from the stationary phase were washed with PBS (pH 7.4) before subjected to DNA extraction. Using DNG-PLUS commercial kit (Sinaclon-Iran) according to the manufacturer's instructions. For the evaluation of quality, the total extracted DNA were electrophoresed on 1.5% agarose gel. We used the primers L1TSR (5′-CTGGATCATTTTCCGATG-3′) and L 5.8S (5′-TGATACCACTTATCGCACTT-3′) to amplify the internal transcribed-spacer-1 (ITS1) region of the parasites' ribosomal-RNA gene, followed by *HaeIII* digestion of the resulting amplicons as described previously [12]. After using the restriction enzyme, banding patterns of the isolates were obtained in comparison with the molecular profiles of the WHO reference strains of *L. tropica* (MHOM/IR/99/YAZ1), and *L. major* (MRHO/IR/75/ER).

RNA extraction and cDNA synthesis. Total RNA was extracted from 10^7 metacyclic promastigotes using RiboEx reagent (GeneAll Biotech, Korea) as described by the manufacturer. The quality (based on the appearance of the spectra) and quantity of RNA were assessed using Nano Drop (ND-1000, Thermo Scientific Fisher, US). Three independent RNA samples were used for each real-time PCR experiment. Complementary DNA (cDNA) was synthesized from 3 µg of total RNA using RevertAid™ First Strand cDNA Synthesis Kit (Thermo Fisher Scientific Inc) following the manufacturer's instructions.

Real-time PCR. Primers for targeted genes were designed using Gene Runner software version 6.5.50 (wwwgenerunner.net). The sequence of the primers has shown in Table 1. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene was included for normalization purposes, referred to as

Table 1. Oligonucleotide sequences of genes amplified in this study

Genes	Primer sequence (5'→3')	Primer sequence (3'→5')
Pyruvate Kinase (PyrK)	CTAACGCGCACACGATCTCT	AAGATCATGTCCACGCCCTG
Tryparedoxin Peroxidase (TryP)	AGTCGCTTCAACGAGCT	CTTGTCGGCTAGCATTG
GAPDH	GAAGTACACGGTGGAGGCTG	CGCTGATCACGACCTTCTTC

GAPDH: Glyceraldehydes-3-phosphate dehydrogenase

internal control. Equal amounts of cDNA were run in triplicate and amplified in 20 ml reaction containing 1 µl cDNA target, 100 nM forward and reverse primers and 1x SYBR Green RealQ Plus Master Mix (Ampliqon, DK-5230 Odense M, Denmark). Experiments were carried out in duplicate using a StepOne TM Real-time PCR System (Applied Biosystems, Life Technologies, USA). The PCR condition was as follows: activation at 95°C for 10 min., amplification at 95°C for 15 s, 60°C for 1 min. for 40 cycles. The relative expression value of each gene was determined based on the threshold cycle (Ct) value of the target genes, normalized to that of reference genes (GAPDH) using the $2^{-\Delta\Delta Ct}$ method.

Data analysis. All experiments were done at three replicates and data are reported as the mean ± SD (standard deviations) and comparison between *L. major* and *L. tropica* groups were analysed by student's t-test. The level of significance acceptable was 95% and *P-value* <0.05.

Results

PCR-RFLP analysis

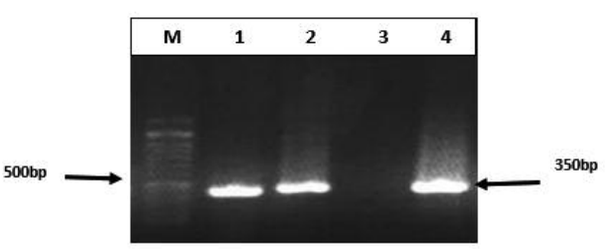

Figure1. Result of *Leishmania* genus DNA with ITS1 primers. M: marker 100bp; lanes 1-2: samples; lane 3: blank; lane 4: standard strain of *Leishmania.*

After DNA extraction, samples were characteri zed by PCR-RFLP. Quality assessment of PCR product were performed on agarose gel and 350 bp band correspond to *Leishmania* genus were confirmed (Fig. 1). After digestion of PCR product with *HeaIII* enzyme, isolates and the reference strain exhibited two bands (135 bp, 215 bp) on agarose gel which correspond to *L. major* and two bands (57 bp, 185 bp) correspond to *L. tropica* (Fig. 2).

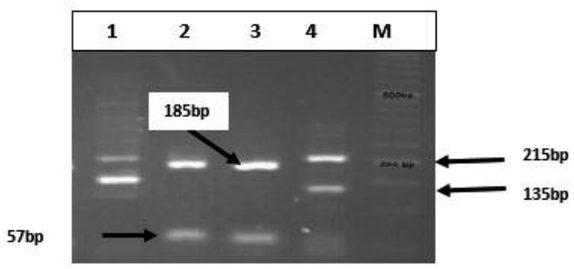

Figure 2. Result of RFLP patterns of ITS1 amplicons digestion with *HaeIII* enzyme.
Lane 1: standard strain of *L. tropica*; lane 2: standard strain of *L. major*; lane 3: *L. major* sample; lane 4: *L. tropica* sample; M: marker 50bp.

Real-time PCR analysis

Real-time-PCR was conducted to examine the relative differential expression of pyruvate kinase and tryparedoxin peroxidase genes between metacyclic stage of *L. tropica* and *L. major* isolates. Figure 3 shows up-regulation of PyrK and TryP genes in metacyclic of *L. major* isolates compared with *L. tropica*. The average mRNA expression of PyrK and TryP genes in *L. major* was 1.69 and 3.72 fold of its expression in *L. tropica*. Moreover, there was significant difference in the mentioned genes expression between procyclic and metacyclic forms of the both of *L. major* and *L. tropica* (result not shown).

Discussion

Gene expression analyses by microarray and real-time PCR in *Leishmania* species have been performed successfully for the detection of differentially expressed, stage-specific, species-specific genes and drug resistance mechanism study in this parasite [5,13–15]. A review of previous

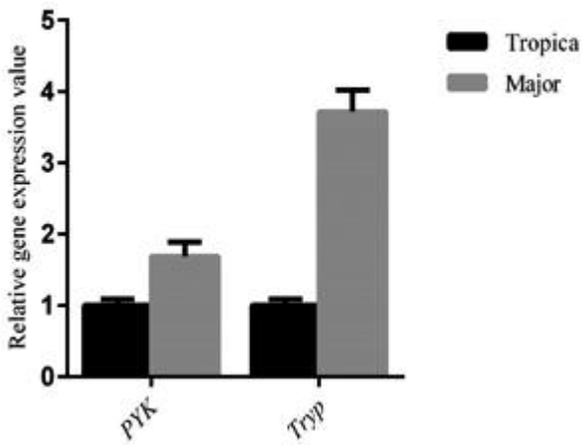

Figure 3. Relative gene expression pattern of two correspond genes of differential expressed proteins between *L. major* and *L. tropica* metacyclic stage by real-time PCR.The expression of GAPDH was used as internal control gene. PyrK: pyruvate kinase; Tryp: tryparedoxin peroxidase.

studies searching for potential drug targets molecules involved in *Leishmania* metabolism identified several enzymes for drug targets. Pyruvate kinase (involved in glycolysis) and tryparedoxin peroxidase (as an antioxidant) are considered essential for the survival of *Leishmania* in host environment and introduced as appropriate therapeutics targets [16,11]. In addition, different structure of *Leishmania* energy metabolism involved proteins compared with *Homo sapiens* because of evolutionary distance, make those as potential drug targets [17]. The two genes evaluated in this study were selected also because of they showed differential expression value in the different stage and different species of *Leishmania* [11,18,19]. These genes may be required for survival in the amastigote stage and also may be directly responsible for the disease pathogenesis and different lesion manifestation. The gene pattern differences between the two species of *Leishmania* and within the same species causing distinct pathologies that govern the outcome of infection and pathogenesis in the mammalian host cells are unknown. Assessment of *in vivo* mRNA level highlighted substantial differences in gene expression patterns, providing an indication of the genes involved in pathogenesis in different forms of leishmaniosis [5]. Tryparedoxin peroxidase (TryP) is one of the trypanothione metabolism pathway that introduced as another potential drug target in literatures review and it is a key enzyme in the defence mechanism against oxidative stress condition [20,21]. Glycolysis occupies a central role in cellular metabolism, and is of particular importance for the catabolic production of ATP in protozoan parasites such as *Leishmania* and *Trypanosoma*. In these organisms pyruvate kinase plays a key regulatory role [22]. Since *Leishmania* is infectious in the metacyclic form and has the ability to interact with the mammalian host, it has to pre-adapt to host cell environment for survive in it. For this purpose, the parasite changes the some genes expression such as TryP to compatibility with the oxidative stress conditions of the host cells. In addition, we observed overexpression of these genes in metacyclic form compared with procyclic form in both of *L. major* and *L. tropica* isolates. In fact, these changes can indicate the importance of these genes in host-parasite interaction and increased infectivity. It is thus possible that the higher expression of genes involved in glycolysis or response to stress can generate sufficient ATP necessary and appropriate condition for the intracellular remodelling and morphological differentiation of metacyclic parasites. Similarly, previous studies using genomic microarrays and proteomics analysis showed more abundant expression of gene and protein in metacyclics and amastigotes of the parasite [11,23,24]. Also, the difference in expression of these genes in these two species can be interpreted as such that the both species of *L. major* and *L. tropica* caused different cutaneous lesion morphologically and phenotypically. A few species parasite genes implicated in pathogenesis and clinical manifestation, but the parasite gene expression levels differ highly among different species [25]. Therefore, the increased expression in the genes evaluated in this study can be related to the severity of the ulcer in Zoonotic cutaneous leishmaniosis. Regarding the shorter communicative term of the *L. major* than the *L. tropica*, the high expression of PyrK and TryP genes in the *L. major* can be the reason for the earlier appearance of clinical manifestation in cutaneous leishmaniosis patients caused by this species. Also, high expression of TryP gene in *L. major* can be attributed to its high ability of this species against oxidative stress. Therefore, these genes and their-related encoding proteins may be appropriate targets for the prevention of metacyclogenesis and host-pathogen interaction interruption. However, further investigations and detailed studied are needed for

species-specific drug and vaccine target ability of the mentioned proteins.

Analysis of comparative gene expression pattern of pyruvate kinase and tryparedoxin peroxidase between *L. major* and *L. tropica* metacyclic forms by real-time PCR demonstrated high expression of PyrK and TryP in Iranian isolates of *L. major* compared with *L. tropica*. The conservation in gene organization between *L. major* and *L. tropica* contrasts their distinct pathogenesis, suggesting that highly regulated changes in gene expression may be involved. Therefore, these enzymes may play critical roles in infectivity, virulence, pathogenesis and pre-adaptation of parasite for survival in host macrophage cells.

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