

Stage-Specific Differential Gene Expression of Glutathione Peroxidase in *Leishmania Major* and *Leishmania Tropica*

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Abstract

Background: *Leishmania (L) major* and *L. tropica* are the etiological agents of cutaneous leishmaniasis. *Leishmania* species cause a board spectrum of phenotypes. A small number of genes are differentially expressed between them that have likely an important role in the disease phenotype. Procyclic and metacyclic are two morphological promastigote forms of *Leishmania* that express different genes. The glutathione peroxidase is an important antioxidant enzyme that essential in parasite protection against oxidative stress and parasite survival. This study aimed to compare glutathione peroxidase (TDPX) gene expression in procyclic and metacyclic and also interspecies in Iranian isolates of *L. major* and *L. tropica*.

Methods: The samples were cultured in Novy-Nicolle-Mc Neal medium to obtain the promastigotes and identified using PCR-RFLP technique. They were then grown in RPMI1640 media for mass cultivation. The expression level of TDPX gene was compared by Real-time PCR.

Results: By comparison of expression level, up-regulation of TDPX gene was observed (5.37 and 2.29 folds) in *L. major* and *L. tropica* metacyclic compared to their procyclic, respectively. Moreover, there was no significant difference between procyclic forms of isolates, while 3.05 folds up-regulation in metacyclic was detected in *L. major* compared *L. tropica*.

Conclusions: Our data provide a foundation for identifying infectivity and high survival related factors in the *Leishmania* spp. In addition, the results improve our understanding of the molecular basis of metacyclogenesis and development of new potential targets to control or treatment and also, to the identification of species-specific factors contributing to virulence and pathogenicity in the host cells.

Keywords: Glutathione peroxidase, *Leishmania*, *L. major*, *L. tropica*, Quantitative Real-time PCR.

Introduction

Leishmaniasis is a global parasitic disease caused by various species of *Leishmania (L)* that refer to the variety of diseases including self-healing cutaneous leishmaniasis (CL) to fatal visceral leishmaniasis (VL) (1). CL is the most common form of leishmaniasis and highly regarded as a significant public health problem in many tropical and subtropical regions (2, 3). In the old world including Iran, anthroponotic (ACL) and zoonotic

cutaneous leishmaniasis (ZCL) are two main forms of CL which are caused by *L. tropica*, *L. major*, respectively (2).

The emergence of leishmaniasis is not only associated with host immunity condition but also the type of parasite species is effective (4). The life cycle of *Leishmania* genus replacement between extracellular promastigotes and intracellular amastigotes. Promastigote forms also

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including two developmental stages including procyclic promastigotes and metacyclic promastigotes. Switch from non-infective procyclic (log phase) into the infective metacyclic (stationary phase) is accompanied by several morphological and genome expression variation (5). The alteration of the gene expression levels of the metacyclic leads to the emergence of parasitic infectious potency as well as adaptation to the mammalian host cell environment (6). Comparative analysis of gene expression level between both *L. tropica*, *L. major* and their metacyclogenesis process may serve to improve our understanding of molecular differences between these two species and also their metacyclogenesis stages in distinct species (6). Recent studies have also suggested the involvement of reactive oxygen species (ROS) in the pathogenesis of various parasitic diseases (7, 8). The ROS overproduction can lead to an imbalance between oxidants and antioxidants at cellular or systemic levels, causing the oxidative stress. Increased antioxidant activity may involve in pathogenicity and survival rate inside host cells. The ROS as endogenous species are produced in the mitochondrial respiratory chain during the normal metabolic process (7, 8). Antioxidants have

the central role to neutralize ROS including the expression level of genes encoding glutathione peroxidase (TDPX) and trypanothione peroxidase (TRYP1) (6, 7). Once there is the involvement of oxidative stress in physiological and pathological processes, it is necessary to know the role of antioxidant enzymes such as TDPX between different stages and species of *Leishmania*. Glutathione peroxidase comprises an enzyme group that acts as a vital antioxidant by reducing hydro-peroxides. Furthermore, one of the vital processes for the survival of the *Leishmania* parasite in different environments is the detoxification of peroxides. This is the first study that describes the expression level of a gene encoding glutathione peroxidase protein (Uniprot ID: Q4Q9B4, Gene ID: 5652808) during the metacyclogenesis process of Iranian isolates of *L. tropica* and *L. major*. Figure 1 shows a schematic picture of this study workflow. The results of this work could inform about a potential drug target for leishmaniasis control or its treatment. Thus, the aim of this study was to evaluate the TDPX gene expression level in procyclic and metacyclic promastigotes and also interspecies in Iranian isolates *L. major* and *L. tropica* by quantitative Real-time PCR technique.

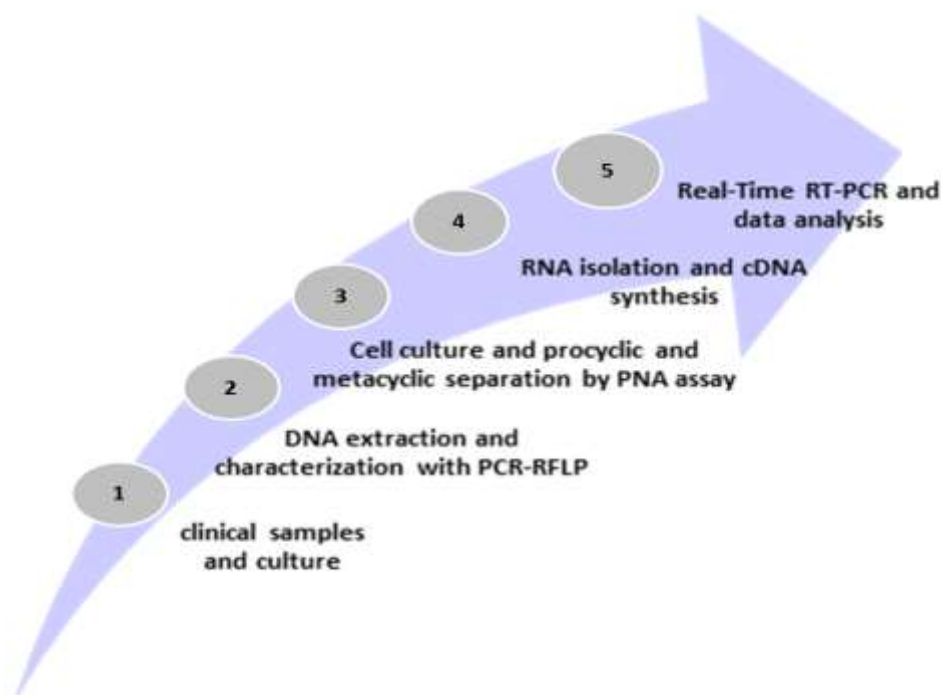


Fig. 1. Experimental workflow of quantitative Real-time PCR analysis of glutathione gene expression level in *L. major* and *L. tropica* isolates.

Materials and methods

Samples culture and characterization

In this study, we used the *Leishmania* parasite isolates obtained from the cutaneous lesions of Iranian patients infected with *L. major* and *L. tropica* from an endemic place for each species. Written informed consent was obtained from each patient prior to participation. All the subjects were new cases, without taking medication. The primary isolates initially were grown on N.N.N (Novy-Nicolle-Mc Neal) medium and for mass production, parasites were cultured in RPMI-1640 medium (Gibco, Germany) supplemented with 10–15% fetal bovine serum (Gibco, Germany) and 100 U/ml penicillin and 100 µg/ml streptomycin and incubated at 24–25 °C. In order to sample characterization, parasites DNA was extracted from promastigotes using the DNG-PLUS commercial kit (SinaClon-Iran) according to the manufacturer's instructions. We performed a polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) methods with internal transcribed spacer1 (ITS1) according to the method described previously (9). Since in this study procyclic and metacyclic promastigotes were evaluated for glutathione peroxidase expression level, peanut agglutinin (PNA; Sigma) assay and morphometric analysis were used to separate of purified procyclic and metacyclic stages as described previously (5, 9).

RNA extraction and cDNA synthesis

Total RNA was extracted from 10⁷ promastigotes (procyclic and metacyclic) of *L. major* and *L. tropica* samples during the log-phase using RiboEx reagent (GeneAll Biotech, Korea) according to the manufacturer's protocol. The quality and quantity of the RNA were evaluated by electrophoresis and Nanodrop 1000, respectively. To prevention of any gene contamination, total RNA was subjected to DNase I enzyme (Fermentas, Burlington, Canada). Three independent RNA samples were used for each real-time PCR experiment. Complementary DNA (cDNA) was synthesized using 3 µg RNA, 1λ oligo-dT (Thermofisher,

USA) using a RevertAid™ First Strand cDNA Synthesis Kit (Thermo Fisher Scientific). The gene expression analysis was assessed by qRT-PCR using SYBR Green 2x Master Mix Green (Ampliqon). RT-qPCR was performed by a StepOnePlus™ Real-Time PCR System (Applied Biosystems, Foster City, CA).

Quantitative Real-time PCR method

Real-time PCR was conducted to evaluate the differences in the RNA expression level of TDPX gene among log (procyclic) and stationary (metacyclic) phases of *L. major* and *L. tropica* samples. Specific primers were designed by Gene Runner software version 6.5.50 (www.generunner.net). The sequence of the forward (F) and reverse (R) primers used in RT-qPCR are the following; F: TTCATGGTACTGGCGTTCCC and R: ACCCTTGACGTTCTCTCA. PCR was performed in 20 µl reactions 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 for target genes and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene as a housekeeping gene using a StepOne™ Real-time PCR System (Applied Biosystems, Life Technologies, USA). The PCR condition was as follows: activation at 95 °C for 3 min, amplification at 95 °C for 10 s, 60 °C for 1 min for 40 cycles. The data were normalized against the GAPDH transcript level and 2^{-ΔΔct} method was used to calculate the relative expression.

Statistical Analysis

The data are reported as the mean ± standard deviations (SDs). The significance of differences for expression levels in procyclic and metacyclic stages of *L. major* and *L. tropica* isolates was determined by Student's *t*-test and the level of significance acceptable was 95% (p < 0.05). Statistical analyses were performed with GraphPad Prism 5.0 (GraphPad Software, Inc., San Diego, CA).

Results

The identities of the isolates were performed by using PCR-RFLP technique (6). Real-time RT-PCR was used to evaluate the relative expression level of TDPX gene in the developmentally procyclic and metacyclic stages of *L. major* and *L. tropica* Iranian isolates and to compare it with each other. Fig. 2a shows a significant upregulation of the TDPX gene in the metacyclic stage of *L. major* isolates (5.37 folds; $p < 0.001$) compared to its procyclic stage. Also, this gene in the metacyclic stage of *L. tropica* isolates significantly upregulated (2.29 folds; $p < 0.01$)

compared with the procyclic stage (Fig.2b). In addition, the relative expression level of the TDPX gene between each specific stage of the *L. major* and *L. tropica* isolates was investigated, and no significant differences were detected between procyclic stages of two studied species (Fig. 3a), while TDPX gene expression level difference was significant between their metacyclic stages. According to Fig.3b TDPX gene was expressed 3.05 folds ($p < 0.01$) in *L. major* metacyclic compared to *L. tropica* metacyclic.

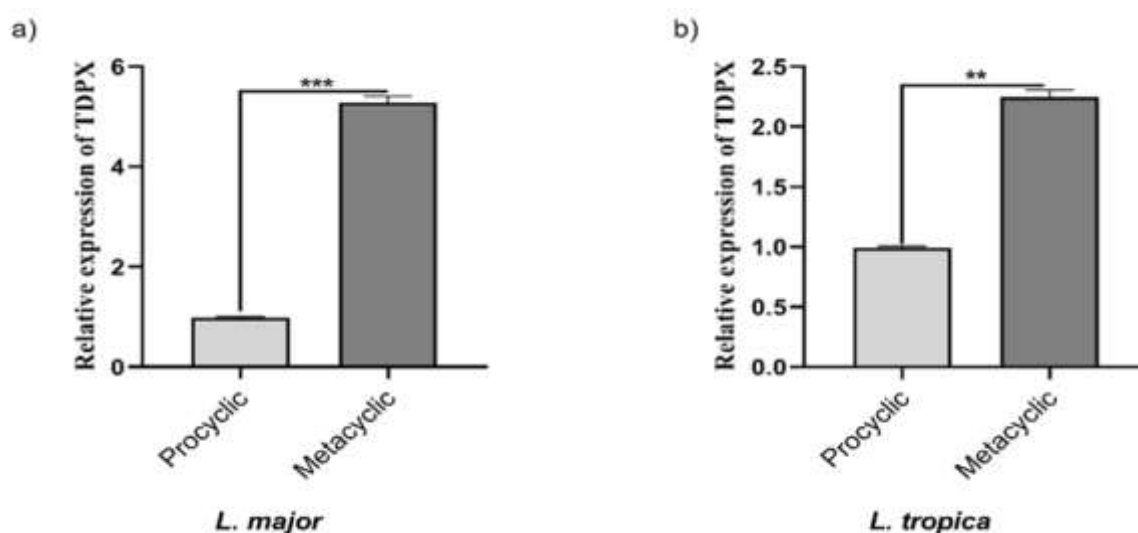


Fig. 2. Comparison of TDPX gene expression level in procyclic and metacyclic promastigotes forms of a) *L. major* and b) *L. tropica* Iranian isolates. ** $p < 0.01$; and *** $p < 0.001$.

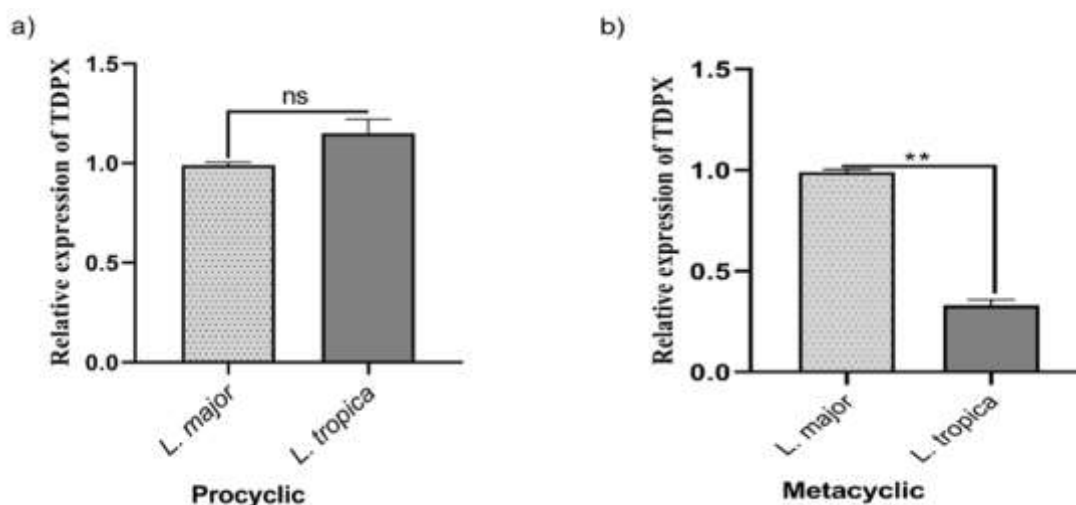


Fig. 3. Comparison of TDPX gene expression level in a) procyclic and b) metacyclic promastigotes forms of Iranian isolates of *L. major* and *L. tropica*. ** $p < 0.01$.

Discussion

Leishmania parasites pass through different developmental phases including procyclic promastigote, metacyclic promastigote and amastigote forms in mammalian host cells with an alteration in gene expression profile and morphological changes. The parasites adapt themselves to changes in the infective stage and environment condition, in order to survive inside host cells. The full molecular basis of the procyclic to metacyclic conversion is limited. Large scale methods such as microarray genome analysis enable the studies of the gene expression level differences between life stages of the parasite and several studies exist in this field (6, 10-13). Studies have also been reported by real-time PCR to investigate on parasite gene expression (6, 14). We, therefore, chose to study the alteration of TDPX gene expression in Iranian isolates of *L. major* and *L. tropica* during metacyclogenesis, and also between species, that may contribute to the metacyclogenesis process and hence may play a role in *Leishmania* infectivity, pathogenicity, survival and ulcer phenotype differences among studied isolates.

TDPX protein is an antioxidant enzyme involved in response to oxidative stress that is a therapeutics opportunity (15) and the parasites need it to pre-adaptation and survive in the host intracellular environment. According to literature, antioxidant gene expression changes such as TDPX occurs in metacyclic compared with procyclic during the metacyclogenesis process. When procyclic promastigotes transition into metacyclic promastigotes forms, is accompanied by emerging of motility-related protein expression. Therefore, an increased level of proteins involved in motility is in agreement with high parasite movement and infectivity. The reactive oxygen species (ROS) as endogenous species are produced in the mitochondrial respiratory chain during the normal metabolic process (7, 9). Antioxidants have the central role to neutralize ROS including glutathione peroxidases (TDPX) and trypanothione peroxidases (TRYP1) (7). According to our data, the TDPX gene upregulated in the metacyclic stage of both *L. major* and *L. tropica* isolates. Overexpression of this protein as a part of the antioxidant enzyme class confirms the parasite answer to oxidative stress. Similarly, in our previous work on the protein

profile of *L. tropica* during the metacyclogenesis process by using proteomics method, this protein upregulated in the metacyclic stage compared with procyclic stage in proteome level, in which validates this paper results (5). The results of other studies on *L. donovani* antioxidant enzymes also revealed more expression in drug-resistant parasites (16). Other documents reported that there is a link between trypanothione peroxidase with survival, virulence and drug response in *L. donovani* (17). Furthermore, one of the vital processes for the survival of *Leishmania* parasite in the cellular environment is the detoxification of peroxides. With regard to the contents mentioned about the function of antioxidant proteins, it can be concluded that increased expression of the TDPX gene in metacyclic forms of *L. major* and *L. tropica* isolates in this study, is because promastigotes undergo a high level of replication. It also naturally in the face of increased ROS during cell division due to high energy consumption and even age rising in procyclic and metacyclic forms increased the antioxidant and cell redox homeostasis related proteins. Since the metacyclic is an infective form of *Leishmania* and able to interact with the mammalian cells, alteration in genome expression during procyclic conversion into metacyclic form could be a way for pre-adaptation to host cell environment and intracellular survival. For this, the *Leishmania* alters some genes expression level such as TDPX to compatibility with the oxidative stress conditions of the host cells. Furthermore, overexpression of parasite protective complex could indicate the significant role of this gene in host-parasite interaction and increased infectivity of parasites. Similarly, previous studies using genomic microarrays and proteomics analysis showed more abundant expression of gene and protein in metacyclic and amastigote forms (18, 19). In the current study, a comparison of TDPX gene expression level between the procyclic of studied parasites showed no significant differences between *L. major* and *L. tropica* isolates, whereas the corresponding level significantly differed between *L. major* and *L. tropica* metacyclic forms. A few numbers of species-specific genes affected pathogenesis and clinical manifestation, but the expression level of genes usually differ between

different species (20). Since ZCL phenotype is severe rather than ACL, the increased level of TDPX gene expression in *L. major* can be related to high adaptive and survival ability in host cells and emerging of intense ulcers caused by *L. major*. Therefore, this gene may be a potential proper target for metacyclogenesis and infectivity stop and improves new potential targets to control or treatment. However, further studies must be undertaken with a large number of isolates and detailed investigations to better understand the distinct gene expression pattern found between *L. major* and *L. tropica*.

It is the first study is evaluating on the TDPX gene in a number of different Iranian cutaneous Leishmania parasite species isolates (*L. major* and *L.*

tropica) including ZCL and ACL forms. The average expression of the TDPX gene was different significantly between metacyclic and procyclic stages of isolates. In addition, TDPX gene expression was higher in *L. major* compared to *L. tropica*. Our findings suggest that TDPX can be considered as potential molecular markers for metacyclogenesis and screening of cutaneous leishmania species isolates.

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The authors declare that they have no conflicts of interest with the contents of this article.

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