

## Expression of *Leishmania tropica pdI-2* gene in both promastigote and amastigote phases

Dina Ali<sup>1</sup>, Chadi Soukkarieh<sup>1\*</sup>, and Mahmoud Kweider<sup>1</sup>

<sup>\*1</sup>Department of Animal Biology, Faculty of Sciences, Damascus University,  
P.O. Box 30621 Syria

**Abstract:** Parasites from the *Leishmania* genus are protozoans responsible for a group of diseases with a broad range of clinical manifestations collectively known as leishmaniasis. *Leishmania tropica* parasites are one of the causative agents of cutaneous leishmaniasis (CL). Protein disulfide isomerase (PDI) is an essential enzyme that catalyze thiol-disulfide interchange that has been reported to have role in *Leishmania* virulence and survival. There are several reports on the expression of *pdI-2* gene through different life-cycle stages of genus *Leishmania*, but none of them have focused on *L. tropica*. The present work described for the first time the expression of *pdI-2* through the life-cycle of *L. tropica*. Initially, a local isolate of *leishmania* parasite was typed using PCR technique. Then, the expression of *pdI-2* gene in logarithmic and stationary growth phase promastigotes, as well as in amastigotes, was evaluated by Reverse Transcription-PCR (RT-PCR) technique. The typing result confirmed that our local isolate belongs to *L. tropica*. Furthermore, the expression of *pdI-2* gene in both promastigotes and amastigotes forms was confirmed. The results of the present study will increase our knowledge about expression of the *L. tropica pdI-2* gene, and this may be used as an effective target for controlling CL.

**Key words:** Protein disulfide isomerase, PCR amplification, *Leishmania tropica*.

### Introduction

Leishmaniasis, malaria, schistosomiasis, filariasis, trypanosomiasis and tuberculosis, are considered by the World Health Organization (WHO) to be six of the most important tropical diseases.<sup>30</sup> Leishmaniasis currently threatens 350 million people in 88 countries around the world. Two million new cases are considered to occur yearly, with an estimated 12 million people presently infected.<sup>8,29</sup>

Leishmaniasis is caused by 20 different species which are pathogenic to humans, and belong to the genus *Leishmania*, an intracellular parasite transmitted by the bite of the phlebotomine sandfly.<sup>23</sup> During their life cycle, *Leishmania* parasites alternate between motile flagellate promastigotes in the midgut of the sandfly vector and non-motile amastigotes inside the macrophages of mammalian hosts.<sup>12</sup> Leishmaniasis can be classified into three general types of disease: cutaneous leishmaniasis (CL), mucosal leishmaniasis (ML), and visceral leishmaniasis (VL), based on the clinical manifestations of the disease.<sup>7</sup> Cutaneous leishmaniasis (CL) caused by *L. major*, *L. aethiops* and *L. tropica* in the Old World.<sup>5</sup>

To date, there is no vaccine against *Leishmania* in routine use anywhere in the world. Several different approaches to anti-leishmanial vaccine have been tested.<sup>11,13</sup> Efforts to introduce a new protein for vaccine production are currently being considered. Previous studies strongly suggest that molecules involved in facilitating folding and secretion of many of the newly synthesized virulence proteins could constitute key targets for drug development against protozoan pathogens.<sup>14,15</sup>

Protein Disulfide Isomerase (PDI), a member of the thioredoxin superfamily, is localized in the ER and responsible for introducing disulfide bonds into proteins.<sup>20</sup> During disulfide formation, two cysteines must be correctly aligned and oxidized. PDI, is a multidomain protein with four thioredoxin domains linked in tandem with a C-terminal anionic tail.<sup>9</sup>

Recently, several studies indicated a role of PDI in virulence for several pathogens.<sup>4,22,24</sup> Moreover, It has been reported to have role in *Leishmania* virulence and survival.<sup>1</sup> The characterization of these virulence factors obviously has important implications for the design of new drugs or vaccines against *Leishmania* parasites. In the work reported here, we describe for the expression of the putative *pdl-2* gene of *L. tropica* in logarithmic and stationary growth phase promastigotes, as well as in amastigotes.

## Material and Methods

### Parasites Sampling, and culture conditions

Parasites isolated from skin lesions of patients were cultured in semisolid culture medium (Agar, NaCl) containing penicillin/streptomycin 100 U/ml (Cytogen, Germany), And then were grown in RPMI-1640 medium (Sigma, USA) supplemented with L-glutamine (Sigma, Germany), penicillin/streptomycin 100 U/ml, and 10 % heat-inactivated fetal bovine serum (FBS, Cytogen, Germany) with less than five passages. This culture was then incubated at 26° C. For obtain axenic amastigotes, the parasites in stationary phase were then transferred to 37 °C and pH 5.8 with 5 % CO<sub>2</sub> and incubation was continued. Within 48 h, the complete differentiation from promastigotes to amastigote forms were occurred.

### DNA extraction

The total DNA of several local isolates was extracted from promastigotes (~16×10<sup>6</sup> cell/ml), washed and suspended in phosphate-buffered saline (PBS), pelleted by centrifugation at 4000 rpm for 10 min. DNA genome was, then extracted using Wizard genomic DNA purification kit (Promega, USA) according to the manufacturer's instructions.

### Assessments of DNA concentration and Purity

The DNA concentration was determined by measuring the absorbance at 260 nm. Its purity was defined as the ratio of absorbance at 260 to 280 nm wavelengths by using a Spectrophotometer (Jenway, England).

### Identification of local isolates

The species determination was performed by Polymerase Chain Reaction (PCR) using CSB2 5'-CGAGTAGCAGAACTCCCGTTCA-3' and CSB1 5'-ATTTTTTCGCGATTTTCGCAGAACG-3' primers, which are specific to the conserved region of *Leishmania* minicircle kinetoplast DNA (kDNA).<sup>26,32</sup> The PCR reaction mixture was contained 200 ng of DNA template, 0.5 μM of each primer, 0.2 mM dNTP, 2 mM MgSO<sub>4</sub>, 1X reaction buffer and 1.25 U of Taq DNA polymerase (Vivantis, USA) in a final volume of 50 μl. The PCR cycling was performed under the following conditions: 2 min at 94 °C, 40 cycles (30 sec at 94 °C; 30 sec at 54 °C of appropriate primers; 60 sec at 72 °C) and 10 min at 72 °C. Then, the PCR product was verified by electrophoresis on a 1.5% agarose gel.

### RNA Extraction (RT-PCR) Procedure

For *pdl-2* gene transcription confirmation at the level of mRNA, a RT-PCR kit was used. Initially, Total RNA was extracted from *Leishmania* promastigotes; stationary phase (~16×10<sup>6</sup> cell/ml) and logarithmic phase (~12×10<sup>6</sup> cell/ml), as well as from axenic amastigotes (~16×10<sup>6</sup> cell/ml) by Invisorb Spin Tissue RNA Mini Kit (Invitek, Germany) according to the manufacturer's. the results were then electrophoresis on 1 % agarose gel to assure the purity of our RNA of any genomic DNA contaminants. RNA was quantitated at 260 nm wave length using a spectrophotometer (Jenway, England). After that, 0.5 μg of total total RNA was used for the synthesise single-stranded cDNA using a RT-PCR kit (Fermentas) according to the manufacturer's instructions. To identify putative *pdl-2* gene, this cDNA was used as a template in a PCR with the specific primers. PDI-F 5'-TACACCGGCGCTACACCAAT-3' and PDI-R 5'-CGCCGTCAATGTAGGTTAGC-3' primers, which amplify approximately 218 bp of *pdl-2* gene. The PCR was performed in a 50 μl reaction mixture containing 2 μl of single-stranded cDNA, 0.2 mM dNTP, 0.5 μM of each primer, 2 mM MgSO<sub>4</sub>, 1 X reaction buffer and 1.25 U of Taq DNA polymerase (Vivantis, Malaysia). The PCR was conducted under the following conditions: 2 min at 94 °C, 35 times (30 sec at 94 °C; 30 sec at 58 °C of appropriate primers; 30 sec at 72 °C) and 10 min at

72 °C. Furthermore, Lmr18s-F 5'-GTGTATAGGCGGTGCGTTCT-3' and Lmr18s-R 5'-CCGATGATTACACCCCAAAA-3' primers were used to amplify about 155 bp of the conserved gene encoding, 18S ribosomal protein (Genebank Accession No. KM052753.1) as a positive control for cDNA synthesis, as well as, the total RNA was used as a negative template.

## Results

### Typing of local isolates

The total DNA of three local isolates was extracted from promastigotes. Then, the extracted DNA was electrophoresis on a 0.8 % agarose gel to assure the quality of extraction, as well as, the safety of DNA from degradation (Fig. 1). The parasite species were then confirmed by PCR technique using species-specific primers CSB2/CSB1. The PCR products were considered positive when a single band of correct size about 750 bp was observed, which corresponds to theoretical length of *L. tropica* kDNA minicircle (Fig. 2).

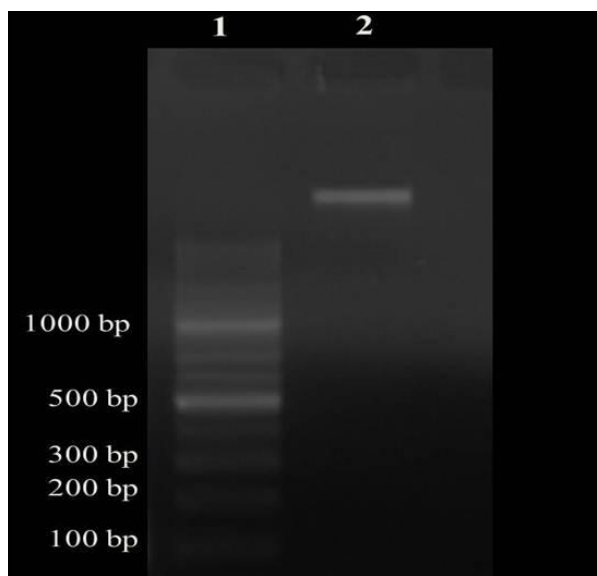


Fig. 1. Electrophoresis of extracted DNA. Lane: 1, DNA ladder molecular marker; Lanes: 2, total DNA.

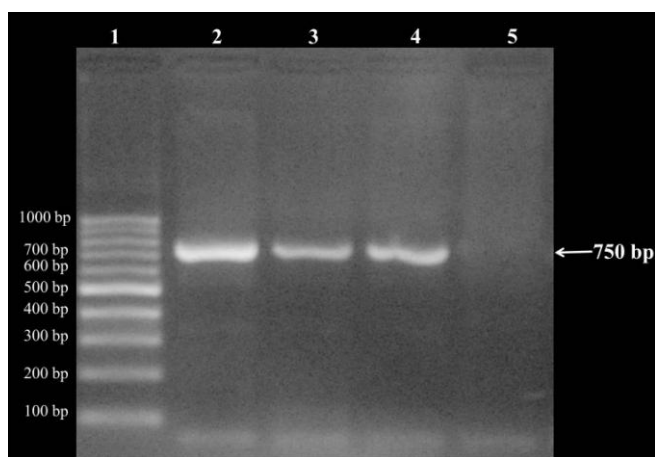
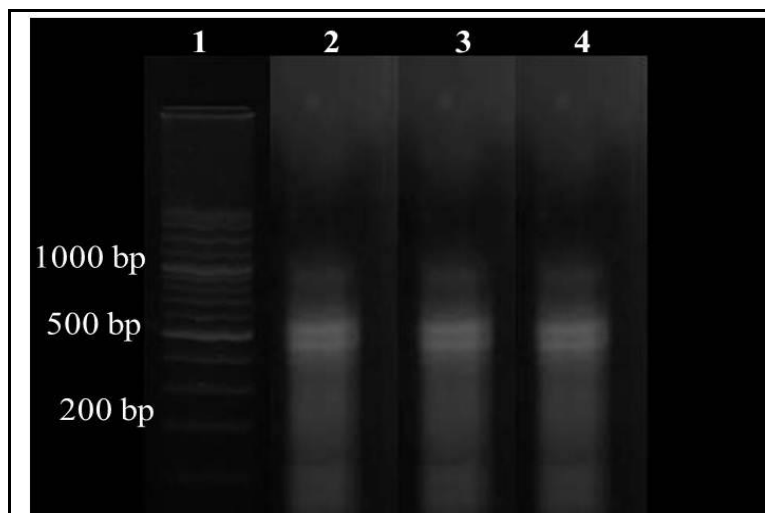


Fig. 2. PCR results from tested *leishmania* isolates using species-specific kDNA minicircle CSB2/CSB1 primers. Lane: 1, DNA ladder molecular marker 100 bp; Lanes: 2, 3 ,and 4, local isolates of *L. tropica*. Lane: 5, negative control.

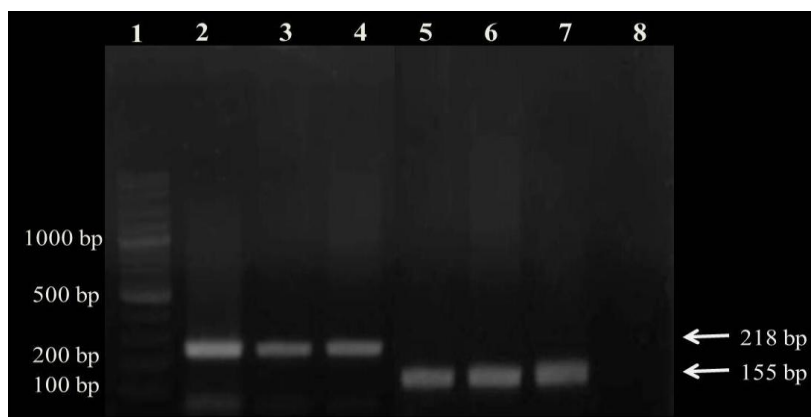
### Total RNA Extraction and cDNA Synthesis

The study of *pdl-2* gene expression was confirmed by RT-PCR method. Total RNA was successfully extracted from cultured promastigotes (from stationary and logarithmic phases) and axenic amastigotes. Then,

extracted total RNA was verified by 1 % agarose gel electrophoresis and resulted in illustrated smear of mRNA and three bands of rRNA (Fig. 3). After that, total RNA was reverse transcribed into cDNA, and the synthesized cDNA was used as template to amplify at about 218 bp from *pdl-2* gene using specific PDI-F/PDI-R primers, finally the results were verified by 1 % agarose gel electrophoresis (Fig. 4). Moreover, The gene expression analysis showed that *L. tropica pdl-2* gene is expressed in both promastigotes (from stationary and logarithmic phases) and axenic amastigotes (Fig. 4, lanes 2, 3, 4), comparing with the band about 155 bp refers to positive control transcripts (Fig. 4, lanes 5, 6, 7). In addition, the purity of the RNA from genomic DNA contaminants was also confirmed by PCR using *pdl-2* gene specific primers (Fig. 4, lane 8).



**Fig. 3.** Evaluation of the quality and purity of the extracted total RNA. Lane: 1, DNA ladder molecular marker 100 bp; Lanes: 2, 3, and 4 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, respectively.



**Fig. 4.** PCR results of synthesized cDNA. Lane: 1, DNA ladder molecular marker 100 bp; Lanes: 2, 3, and 4 the PCR product size is approximately 218 bp of the *pdl-2* gene of promastigotes; stationary and logarithmic phases, and axenic amastigotes, respectively; Lane: 5, 6, and 7, about 155 bp of 18S rRNA gene (a positive control) of promastigotes; stationary and logarithmic phases, and axenic amastigotes, respectively; lane: 8, Negative control.

## Discussion

Protein disulfide isomerase (PDI) is a multifunctional protein of the thioredoxin superfamily. PDI mediates proper protein folding by oxidation or isomerization and disrupts disulfide bonds by reduction; it also has chaperone. In this paper, we report for the first time the expression of the *L. tropica pdl-2* gene.

The study of *pdl-2* gene expression was confirmed by RT-PCR method. Moreover, The gene expression analysis showed that *L. tropica pdl-2* gene is expressed in both promastigotes (logarithmic and

stationary phases) and axenic amastigotes, finally the results were electrophoresis on 1% agarose gel (Fig. 4). These data may suggest that *L. tropica* PDI could constitute a potential drug target against *Leishmaniasis*. In a previous work, an increased expression of PDI in a highly virulent strain of *L. major* was demonstrated, thereby suggesting that PDI may have a role in *Leishmania* pathogenesis.<sup>1</sup> It is expressed and secreted at both promastigote and axenic amastigote phases.<sup>3,4,18</sup> In other study, Expression of the *L. donovani pdl* gene as determined by Northern blot analysis showed that the putative *L. donovani pdl* gene was transcribed in both life stages of the *L. donovani* parasites (promastigotes and axenic amastigotes).<sup>22</sup> More recently, Ben Khalaf et al., 2012 demonstrated that LmPDI protein of *L. major* is expressed in both metacyclic promastigotes and axenic amastigotes.<sup>2</sup> The Expression levels of native LmPDI in both phases were analyzed by Western blotting.<sup>2</sup> PDIs may also be involved in the host mucosal immune system, inducing secretory IgA.<sup>10</sup> For example, *Toxoplasma gondii* PDIs are recognized by host IgA.<sup>19</sup>

In prokaryotes, such as *Neisseria meningitidis*, two membrane PDI proteins have been found to be essential for bacterial growth and biogenesis of functional type IV pili.<sup>28</sup> In parasites such as *Chlamydia*, it has been shown that PDI on the cell surface is required for the effective attachment of the parasite.<sup>6</sup> Furthermore, PDI of *Toxoplasma gondii*, was reported as playing a role in the parasite-host cell interaction.<sup>17,26</sup> Moreover, in *Leishmania* species, Protein disulfide isomerase (PDI), is reported to be involved in its virulence and survival.<sup>1</sup> Currently, there are no effective vaccines against leishmaniasis, and treatment using pentavalent antimonial drugs is occasionally effective and often toxic for patients. Recently, It was demonstrated for the first time the immunogenicity of PDI protein of *L. donovani* both in vitro as well as in vivo, educing a dominant Th1-type cytokine profile.<sup>16</sup> These data may suggest that PDI could constitute a potential drug for the development of vaccines against Old World cutaneous leishmaniasis caused by *L. tropica*.

## Conclusion

This work describes the expression of a *L. tropica pdl-2* gene in logarithmic and stationary growth phase promastigotes, as well as in amastigotes. In the absence of studies on the role that could be played by the PDI-2 as a vaccine against the disease-causing types of cutaneous leishmaniasis as the *L. tropica*, which now causes leishmaniasis in Syria, the methodology to produce the protein in different ways makes it candidate as a potential drug for the development of vaccines against Old World cutaneous leishmaniasis.

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