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Comparison of Cysteine Protease B Gene Expression between Clinical Isolates of *Leishmania tropica*, *Leishmania major* and *Leishmania infantum*

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ABSTRACT

Introduction: *Leishmania* cysteine protease B (CPB) is a parasite virulence factor that plays a vital role in host-parasitic interactions. Regarding the importance of the CPB gene, we used a quantitative real-time RT-PCR to investigate the expression of CPB in the Iranian isolates of different *Leishmania* species. **Methods:** In this study, 36 clinical samples were collected, out of which 29 belonged to cutaneous leishmaniasis (CL), 3 to viscerotropic leishmaniasis (VTL), and 5 to visceral leishmaniasis (VL) patients. Among CL isolates, 8 were *Leishmania major*, and 21 were *Leishmania tropica*, including 3 isolates from the lupoid type. After RNA extraction and cDNA synthesis, gene expression analysis was performed by qPCR. **Results:** Our results showed the highest expression of CPB in isolates of *Leishmania infantum*, followed by *L. major* and *L. tropica*. Among *L. tropica* isolates, in the lupoid forms, the mean expression of CPB was ≈6.4 times higher than that of non-lupoid isolates. In *L. major* isolates, a significant difference was observed between the level of gene expression and the duration of the infection. The expression level of CPB was associated with the severity of the infection in *L. infantum* isolates. **Conclusion:** The CPB gene expressed in all *Leishmania* species. The highest expression was in *L. infantum* species and the least expression in *L. tropica*. The transcript level of CPB increased in *L. major* isolates derived from patients with a higher number and duration of ulcers; however, further studies on more clinical samples are needed to explore our findings.

INTRODUCTION

Leishmania is a flagellate intracellular parasite that causes a group of diseases called leishmaniasis. The disease is prevalent in tropical and Mediterranean countries and affects between 12 and 15 million people around the world annually [1]. Depending on the causative species and immunological status of the host, it can manifest as self-healing cutaneous leishmaniasis (CL), diffuse cutaneous leishmaniasis (DCL), mucocutaneous leishmaniasis (MCL) and visceral leishmaniasis (VL) [2]. In the old world, including Iran, CL is mainly caused by *Leishmania tropica*, *Leishmania major*, and the species *Leishmania infantum* is the agent for visceral leishmaniasis [3]. The outcome of *Leishmania* infection depends on both host and pathogen factors that are implicated in the establishment of infection. Over the last decades, several components have been considered as virulence factors that contribute to the pathogenesis of parasite, enabling it for tissue *invasion* and survival in *macrophages* [1]. These antigenic compounds include glycoprotein 63 (GP63), lipophosphoglycan (LPG), glycosylphosphatidylinositol (GPI), Cysteine Proteases, metalloproteases, viscerotropic gene, and protein A2 [4]. Cysteine proteases (CPs) are considered as therapeutic targets due to their involvement in the biological processes during various stages of the parasite life cycle [5]. These enzymes are peptidyl hydrolyzes and classified according to their function and catalytic site [6]. Parasite cysteine proteases have been implicated in several processes such as differentiation, nutrition, host cell infection, and evasion of the host immune response [7, 8]. Three classes of CPs, including type I (CPB), II (CPA), and III (CPC) have been well described in *Leishmania* spp [9]. The CPC and CPA have single genes while CPB genes are multicopy arranged in tandem arrays and characterized by the presence of a long C-terminal extension (CTE) rich in proline, serine and/or

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threonine residues [5, 9, 10].

In different species of *Leishmania*, the number and sequence of the CPB gene are different. In *Leishmania mexicana*, CPB is located in one locus with 19 copies arranged in a tandem repeat. However, in the *Leishmania donovani* complex, there are five copies of it [11, 12]. CPB is a cathepsin L-like protein that plays a vital role in host-parasitic interactions and immune invasion [5, 10]. The generation of CP-deficient mutants showed that CPB contributes to parasite infection of macrophages and induce lesions in BALB/c mice [11]. In Iran, CPB and CPA from *L. major* and *L. infantum* were cloned and characterized, and have been confirmed to elicit a protective response in the form of recombinant proteins and in a DNA vaccine [13, 14]. It has been reported that a cocktail vaccine containing CPA and CPB encoding plasmids induces long-lasting protection against the *L. major* in the mouse model [15]. On the other hand, reaction of recombinant form of CPA and CPB with the sera of active and recovered VL cases from humans and dogs can suggest these antigens as diagnostic markers [16]. In particular, CPB and its long C-terminal extension (CTE) showed a higher level of IgG reactivity in asymptomatic dogs than in dogs with VL proposing their importance in serodiagnostic of asymptomatic form [17]. CPB is mostly expressed in the amastigote stage and to lesser extent in infective metacyclic promastigote [18]. However, there is not any study regarding the RNA transcript expression of CPB in promastigote form. According to the critical role of CPB in the pathogenesis and immune invasion [4], in the current study we evaluated the expression of cysteine protease B gene (CSB) in different isolates of *L. tropica*, *L. major* and *L. infantum*, exhibiting different clinical symptoms, in Iran using quantitative Real-Time PCR method. This study aimed to investigate the association between different *Leishmania* species and the expression level of CPB gene.

MATERIAL AND METHODS

In this study, 36 clinical samples were collected, out of which 29 belonged to cutaneous leishmaniasis, 5 belonged to visceral leishmaniasis, and 3 belonged to viscerotropic leishmaniasis. Among CL isolates, 21 were *L. tropica*, including three lupoid forms, and 8 were *L. major*. These samples were collected from patients who referred to the leishmaniasis Lab of School of Public Health, Tehran University of Medical Sciences (TUMS). The lupoid samples were isolated from patients who were diagnosed with a positive serum using DAT for more than two years. Visceral specimens included 5 *L. infantum* and 3 viscerotropic *L. tropica* isolates, which were cryopreserved in the laboratory and previously diagnosed with a positive serum using DAT (Table 1).

Table 1. Number of isolates from different *Leishmania* species

Diseases	Number of samples (Total no. = 36)	Kind of species
Cutaneous leishmaniasis	25	*L. major* (n=8) *L. tropica* (n=17)
Cutaneous leishmaniasis (Lupoid form)	3	*L. tropica*
Visceral leishmaniasis	5	*L. infantum*
Viscerotropic leishmaniasis (VTL)	3	*L. tropica*

Sampling, examination, and culture. Samples were prepared from ulcer exudates of patients with CL, and visceral samples recovered from cryopreserved conditions (Nitrogen tank -196°C). All the samples were cultured in RPMI-1640 medium (Gibco, Germany) supplemented with 10-15% fetal bovine serum (Gibco, Germany), penicillin (100 U/ml) and streptomycin (100 µg/ml) and then incubated at 24-25ºC [3].

DNA extraction and PCR-RFLP. DNA was extracted from promastigotes using the High Pure PCR Template Preparation Kit (Roche-Germany) according to the manufacturer's instructions. For identification of the species, the internal transcribed spacer1 (ITS1) sequence was amplified followed by the RFLP method as described previously [19].

RNA extraction and cDNA synthesis. Total RNA was extracted from the promastigotes during the stationary-phase by homogenization in a 1ml tripure reagent according to the manufacturer's instruction (Roche, Mannheim, Germany). For the elimination of DNA contamination, DNase1 (1 unit/µL) (Frementas, Ontario, Canada) was used for 45 min at 37°C. cDNA synthesis was performed using the cDNA Synthesis Kit (Roche, Germany) and the integrity of cDNA evaluated with alpha-tubulin primers (F: CAGGTGGTG-TCGTCTCTGAC) (R: TAGCTCGTCAGCACGAAGTG) as housekeeping gene [20].

Quantitative Real-Time PCR. The cysteine protease B (CPB) and alpha-tubulin genes were amplified by a Real-Time PCR assay. The CPB primers, forward (5-ACAGCTCCT-CTTTCATGGAC) and reverse (5-AATGTGTGAGGA-CAGGTACG) were designed using the Primer 3 software version 0.4.0 http://frodo.wi.mit.edu). The 20 µl reactions contained 1 µl cDNA target, 100 nM forward and reverse primers and 1x SYBR® Premix Ex *Taq*TM II (Takara, Tokyo, Japan). The amplification was carried out in an Applied Biosystem step one (ABI company), programmed for 10 min at 94°C, 40 cycles of 5 s at 94°C and 20 s at 60°C followed by a melt curve analysis using temperature increments of 0.2°C every 30 s to ascertain amplification of the expected product. The relative expression of the CPB gene was calculated by comparing the cycle thresholds (CTs) of the target gene with that of the

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housekeeping gene (Alpha-tubulin) using the relative expression software tool (REST, http://rest.genequantification.info) as previously described [21].

Data Analysis. The expression ratio results of the genes of interest were calculated for significance by a Pairwise Fixed Reallocation Randomization Test and calculated by REST. Samples with a probability value of <0.05 were considered significantly different among the groups. Statistical analysis between groups was performed using one-way analysis of variance (ANOVA). All experiments were conducted at least three times and the results were expressed as the mean ± standard deviations (SDs.) [21].

RESULTS

Expression of CPB gene in different *Leishmania* species (interspecies). Real-time RT-PCR was used to determine the relative expression of CPB gene in the *L. tropica*, *L. major* and *L. infantum*. The mean expression of CPB gene in the isolates of *L. tropica*, *L. major* and *L. infantum* was 1.493, 2.179, and 3.022, respectively (*P*-value <0.05). The highest level of RNA expression was observed in *L. infantum* isolates and the lowest in *L. tropica* isolates (Figure 1).

Expression of CPB in *L. tropica* isolates (Intraspecies). The average expression of the CPB gene was the highest in *L. tropica* isolates derived from lupoid forms (*P*-value <0.05). As shown in Fig. 2, the average expression of CPB was 23.78 in the isolates from lupoid forms (n=3), which was 6.4 fold higher than that of non-lupoid *L. tropica* isolates (n=17, 3.68). There was no significant relationship between the CPB RNA expression among the *L. tropica* isolates, according to the number of ulcers, duration, and location of the infection, sex, and age (data not shown). Also, the average transcript expression of the CPB gene in *L. tropica* isolates derived from viscerotropic leishmaniasis cases (n=3) was 3.03 which was not statistically significant compared to non-lupoid forms.

Expression of CPB in *L. major* isolates (Intraspecies). In *L. major*, as shown in Fig. 3, the highest expression was observed in the isolates obtained from patients who had 3, 2, and 1 lesion, respectively. The RNA level of CPB was elevated in line with the increase of the ulcer's duration; in this line, the highest expression was exhibited in the isolates derived from ulcers occurred 3-5 months before the sampling. Additionally, CPB RNA transcript expression of *L. major* isolates did not differ by sex and age (data not shown).

Expression of CPB in *L. infantum* isolates (Intraspecies). The expression of the CPB gene was associated with the severity of the infection in 5 isolates of *L. infantum*. The highest expression was observed in an isolate obtained from a dog with an acute form of visceral leishmaniasis and a high titer of anti-leishmanial antibody (1: 5120) revealed by Direct Agglutination Test (DAT) while the lowest expression rate was observed in an isolate from a cat spleen (Fig. 4).

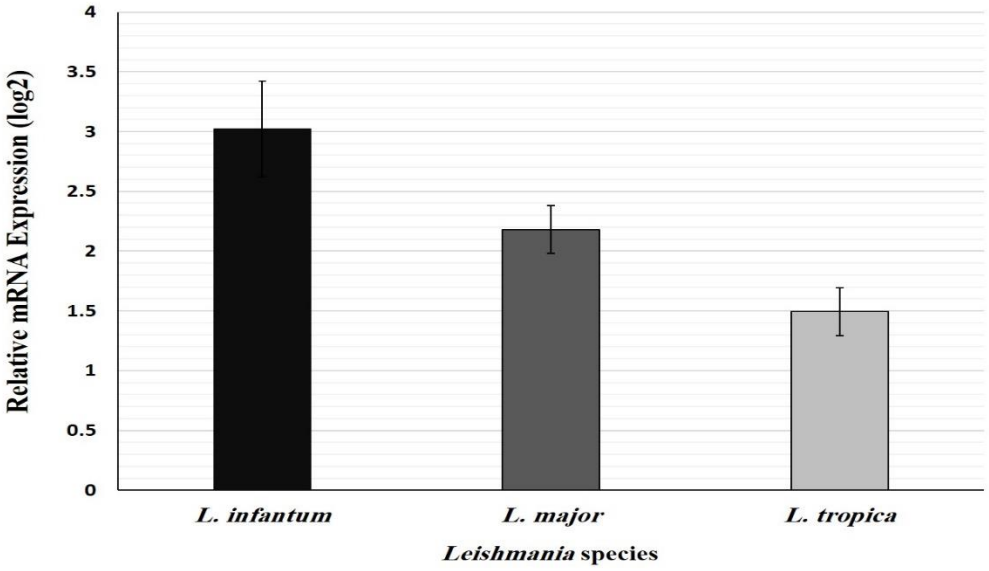


Fig. 1. Comparison of the mean of cysteine protease B gene (CPB) expression in isolates of different *Leishmania* species. The values are the mean ± SD of two independent experiments. The mean expression of the CPB gene between *Leishmania* species is statistically significant (*P*<0.05).

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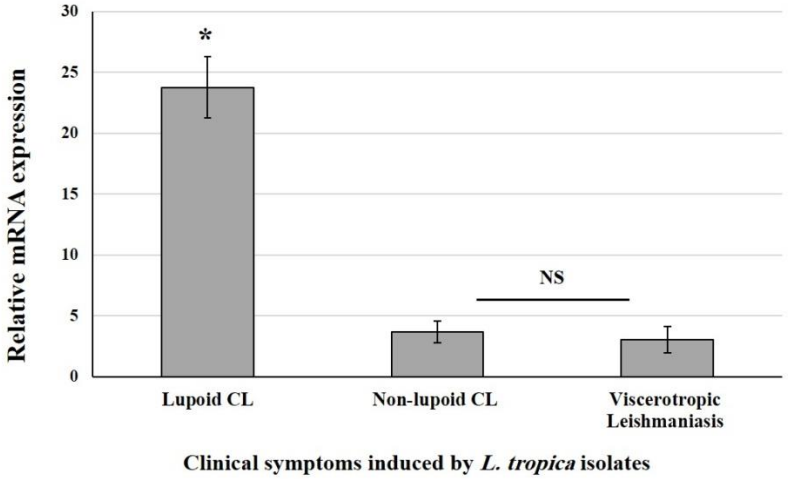


Fig. 2. Relative RNA expression of the CPB gene based on the clinical symptoms. The expression of alpha-tubulin was used to normalize the data. The values are the mean ± SD of two independent experiments. *; Significantly different (P<0.05); NS, non-significant. Lupoid CL cases (n-3); Nonlupoid (n=17) and viscerotropic *Leishmania* (n=3).

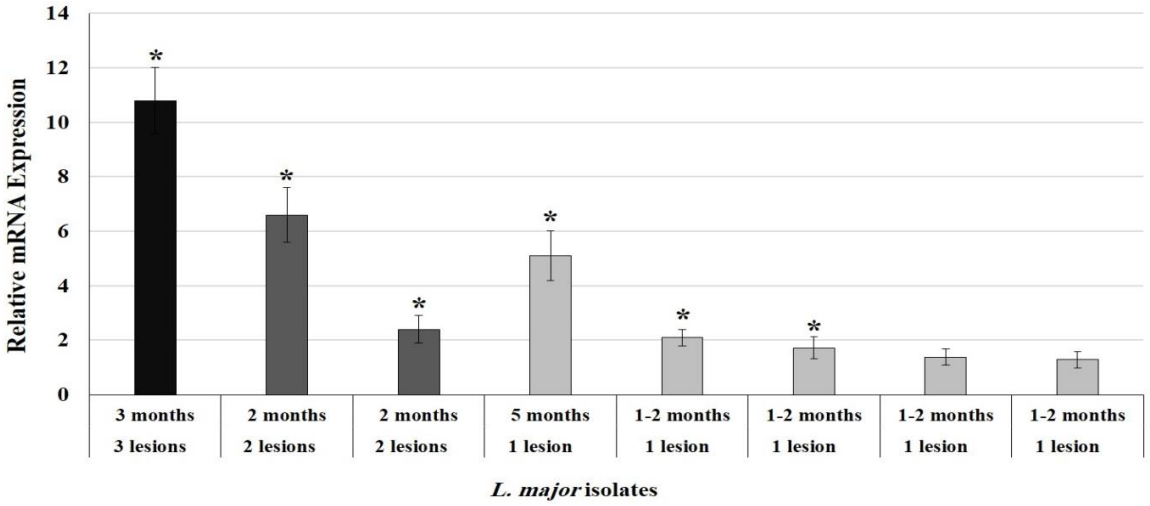


Fig. 3. Relative mRNA expression of the CPB gene in 8 *L. major* isolates derived from CL patients according to the number and duration of ulcers (each column represents one patient). The expression of alpha-tubulin was used to normalize the data. The values are the mean ± SD of two independent experiments. *Significantly different (P<0.05).

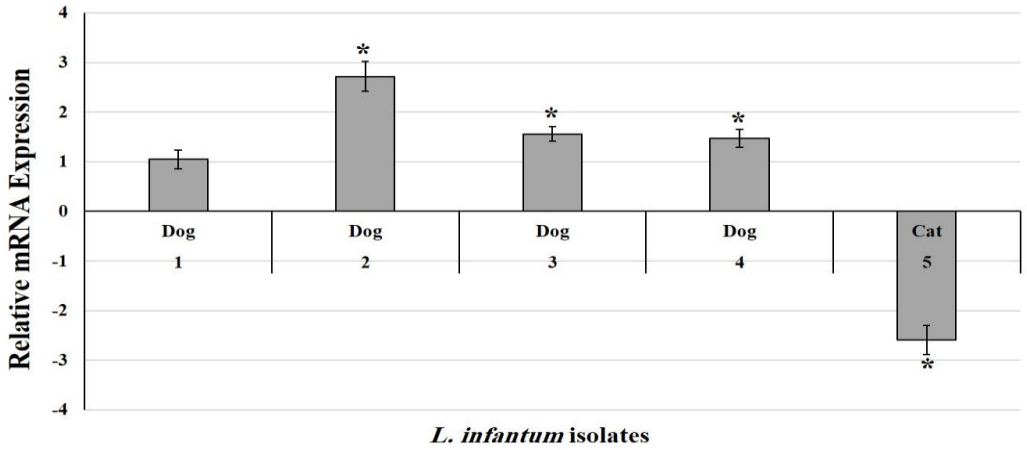


Fig.4. The level of RNA expression of the CPB gene in 5 *L. infantum* isolates. The highest expression in the isolate (No. 2) obtained from a dog with acute VL and the lowest expression was in the isolate (No. 5) derived from a cat without clinical symptoms. The values are the mean ± SD of two independent experiments. *Significantly different (P<0.05)

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DISCUSSION

Several studies have examined changes in the expression of the *Leishmania* parasite gene during different stages of the parasite. These studies have shown that less than 5% of mRNA transcripts is dis-regulated during promastigote to amastigote differentiation. Also, it has been demonstrated that changes in the level of expression of virulence factors in different species of *Leishmania* parasites affect the pathogenesis of parasites [22, 23]. Among the virulence factors, cysteine proteases are enzymes known to play a pivotal role in the establishment of infection [24]. CPB is associated with a Th2 cytokine response by increasing IL-4 production, degrading the transcription factor NF-κβ and IL-12, thereby diminishing the Th1 cytokine response [25, 26]. Regarding the importance of cysteine proteases, in this study, for the first time, the RNA expression of cysteine protease B (CPB) among different *Leishmania* species (interspecies) was investigated. Our results revealed that the level of RNA expression of CPB gene was the highest in *L. infantum* and *L. major*, respectively and the least in *L. tropica*. Among CL isolates, the average expression of CPB was significantly higher in *L. major* compared to *L. tropica* isolates. Since *L. infantum*-induced visceral form, (the most invasive leishmaniasis) and *L. major* induce a more devastating form of CL compared to *L. tropica*, the higher level of CPB expression is expected in these species. Moreover, in agreement with our findings, a study by Kariyawasam *et al.* [27] revealed that pooled virulence factors transcript was lower in *L. (V.) panamensis* isolates than that of *L.(V.) braziliensis* and *L. (V.) guyananesis* at 24 h post macrophage infection, which might reflect that a strain of *L. (V.) panamensis* originated from a patient with non-severe CL while *L. (V.) braziliensis* isolates derived from more clinically invasive lesions. These findings suggested the most expression of virulence factors could be exhibited in the species inducing more severe clinical forms [27, 28].

In the current study, the RNA expression of the CPB gene among each of the isolates (Intraspecies) was investigated. In *L. tropica* isolates (Intraspecies) the lupoid-form isolates exhibited the highest expression compared to the isolates from non-lupoid and VTL. Moreover, the average expression of CPB was the least in *L. tropica* isolates derived from VTL and was approximately similar to that of non-lupoid isolates. The expression of this gene in the lupoid forms was approximately 6.4 times higher than that of non-lupoid *L. tropica* isolates, which is statistically significant ($P<0.05$). As there is no biomarker that can predict the possibility of recurrence of the wound to prevent its occurrence, upregulation of CPB in lupoid forms could be suggested this gene as a potential biomarker to detect the possibility of recurrence and lupoid form; however, due to the limitation of lupoid samples further study on more clinical samples is needed to confirm our findings.

Among 8 *L. major* isolates, the transcript level of the CPB increased in the isolates derived from patients with a higher number and duration of ulcers. In this context, a correlation between the CPB expression and virulence has been reported in *L. amazonensis* promastigotes [29]. Also, the highest expression of CPB was reported in *L. (V.) braziliensis* isolates derived from more clinically invasive lesions in comparison with a strain of *L. (V.) panamensis* originated from a patient with non-severe CL, suggesting an association of clinical phenotype and expression level of virulence factors [27]. Likewise, our findings suggest a probable correlation between the duration of wound and CPB gene expression; however, since the progression of wound is multifactorial further analysis is needed to confirm it.

In visceral samples, *L. infantum* isolates displayed the highest CPB expression compared to the other species. Among 4 *L. infantum* isolates drived from dogs, the highest expression was observed in a case with a high titer of anti-leishmaniasis by DAT. Consistent with our results, in a former study on *L. viannia* subgenus isolates, it was revealed that RNA expression level of CPB was upregulated during post-macrophage infection. These findings may associate with disease severity through reducing the Th1-directed cytokine profile and immunologic response to infection [30, 31].

It is worth mentioning that different clinical features are reported in CL or VL in several parts of the world in different hosts; nonetheless, many manipulating factors have not yet understood [32]. Several studies revealed that the genetic heterogeneity of parasite might induce diverse clinical manifestations [33, 34]. Besides, the immune system and immunodeficiency syndrome play a vital role in the clinical outcome and severity of the infection. It has also been reported that single nucleotide polymorphisms (SNPs) occurring in CPs, which can vary according to the parasite's life stage, could be related to clinical characteristics such as a dermotropic rather than a viscerotropic status [35].

The cysteine protease B gene (CPB) is expressed in the promastigote forms of all three species of *L. tropica*, *L. major* and *L. infantum* of Iran. As the results showed, the highest expression was in *L. infantum*, and then *L. major* and the least expression in *L. tropica*. The transcript level of CPB increased in *L. major* isolates derived from patients with a higher number and duration of ulcers. Further studies on more clinical samples are needed to explore our findings.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest associated with this manuscript.

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