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Comparison of the Lipophosphoglycan 3 Gene of the Lizard and Mammalian *Leishmania*: A Homology Modeling

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Abstract

Background: Lipophosphoglycan 3 (LPG3) is required for the LPG assembly, a well known virulent molecule. In this study, the LPG3 gene of the lizard and mammalian *Leishmania* species were cloned and sequenced. A three-dimensional structure (3D) for the target sequence was also predicted by comparative (homology) modeling.

Materials and Methods: An optimization PCR amplification was performed on genomic DNA extracted from two species of *Leishmania*. The desired PCR products were then cloned and sequenced. In addition, a homology modeling was carried out in order to create three-dimensional structure of the *Leishmania* LPG3 using SWISS-MODEL server.

Results: The GC-rich LPG3 gene of two species of *Leishmania* was successfully amplified using optimized PCR reaction consisting of betaine and 2-mercaptoethanol with bovine serum albumin and then cloned. Sequence alignment of LPG3 showed 95% identity between *Leishmania* infantum and lizard *Leishmania*. With regard to the three-dimensional structure prediction of the modeled sequence of *Leishmania* LPG3, the similarity was found in the molecular structure. Comparative analysis of functional motifs in the target sequence indicated three conserved domains and a putative C-terminal ER retention signal, as well as several post-translational modification sites. From phylogram, *L. infantum* was also found to be clustered with lizard *Leishmania* in the phylogenetic tree.

Conclusion: It is suggested that the lizard *Leishmania* may be evolutionarily more close to *L. infantum*.

Keywords: LPG3; *Leishmania infantum*; PCR additives; Homology modeling

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Introduction

Protozoan parasites of the genus *Leish*mania are responsible for a spectrum of human diseases termed *leishmaniasis*. Lack of an effective vaccine has led to attempts to identify new targets for treatment and vaccination (1). Lipophosphoglycan 3 (LPG3) encodes the *Leishmania* endoplasmic reticulum (ER) resident molecular chaperone belonging to the HSP90 family. It has been reported that LPG3 is involved in assembly of the LPG through Gal activity responsible for β-galactose addition to the mannose residue within the PG repeating unit (2-4). In all *Leishmania* species, LPG contains a repeating phosphorylated disaccharide [Gal-1, 4Man-1-PO4] repeating units (substituted with other sugars in some species), anchored to the surface of parasite by a glycophosphatidylinositol (GPI) anchor (5, 6). LPG as the predominant glycoconjugate on the surface of *Leishmania* promastigotes play critical roles in parasite survival and virulence through phagolysosomal fusion inhibition, resistance to oxidative attack, deactivation of signaling pathways in macrophages, resistance to complement lytic activity, and ultimately allowing the establishment of macrophage infection (5, 7, 8). However, LPG3 expression is not accompanied by LPG synthesis in the lizard non-pathogenic to humans *Leishmania*

species. This may be attributed to different protein sequences and/or gene regulation, resulting in changes in the parasite virulence compared to pathogenic mammal-infecting *Leishmania* species (9, 10).

It has been found previously that the addition of certain PCR additives, such as dimethyl sulfoxide (DMSO), betaine (N,N,N-trimethylglycine), poly-ethylene glycol, glycerol, 2-mercaptoethanol (2-ME), dithiothreitol (DTT) and formamide to the reaction mixture can successfully improve the yield and the specificity of GC-rich DNA sequences amplification (11-13). Although some cheap and commercially available chemical additives are included in high GC PCR kits to achieve the best results, but they are often failed to promote PCR efficiency (11). Therefore, the preparation of unusual chemicals or expensive enzymes may be occasionally needed to perform some protocols.

In this study, the efficacy of concentration-dependent combinations of different PCR additives, including DMSO, betaine, 2-ME, and BSA (bovine serum albumin) was tested for efficient PCR amplification of the LPG3 gene from mammalian L. infantum and the lizard non-pathogenic to humans *Leishmania* parasites that had no PCR-amplified products in the absence of such additives. Next, the resultant PCR products were cloned and sequenced. Since no structural information is available for the *Leishmania* LPG3, a homology modeling is needed to understand the structural and functional features of the target sequence. The homology modeling of LPG3 was performed using bioinformatics tools to predict aspects of secondary and tertiary structures, which offer the opportunity for drug targeting and/or designing vaccine candidate against *leishmaniasis*.

Materials and Methods

Leishmania strains and media

Leishmania infantum strain MCAN/IR/96/LON-49 and lizard *Leishmania* were kindly provided by Dr. Mohebali (Tehran University of Medical Sciences) and Dr. Kazemi (Shahid Beheshti University of Medical Sciences), respectively. *Leishmania* Parasites were grown in RPMI 1640 supplemented with 2 mM L-glutamine, penicillin (100 U/ml), streptomycin (100 µg/ml), and 10% heat inactivated fetal bovine serum (FBS) (Gibco-BRL, UK) at 26 °C.

Genomic DNA extraction

PCR was performed using genomic DNA extracted from *L. infantum* and lizard *Leishmania promastigotes*. Briefly, harvested *Leishmania promastigotes* were washed three times with phosphate-buffered saline (PBS) and resuspended in lysis buffer (10 mM Tris base (pH 8.5), 5 mM EDTA, 200 mM NaCl, 0.2% SDS) followed by incubation at 60 °C for 5 minutes. After adding 2.5 µl of Proteinase K and 5 µl of RNase A, the mixture was incubated for another 1 hour at 60 °C. The protein was then precipitated by adding 250 µl of 5 M NaCl to samples and centrifuging at 10,000 × g for 15 minute. Next, the DNA precipitation of the supernatant transferred to a new microtube was carried out using isopropanol and ethanol.

PCR amplification

The LPG3 gene was amplified using forward primer, F-LPG3 (5´-AGATCTATGGCGAACTCGAGCTTG C-3´) and reverse primer, R-LPG3 (5´-GCTAGC CAGATCGTCCTCGCCGACTG-3´) with BglII and NheI restriction sites underlined in each 5' end, respectively. Reaction was performed in a final volume of 50 µl containing 5 µl of 10× PCR buffer consisted of 500 mM KCl and Tris-HCl (pH 8.4), 200 ng genomic DNA, 0.25 unit of Taq DNA polymerase, 0.4 µM of each forward and reverse primers, 1.5 mM MgCl2, and 0.2 mM each dNTP under following conditions: 5 minutes at 94 °C and 35 cycles of 1 minute at 94 °C, 1 minute at 58 °C, and 2 minutes at 72 °C, with 10 minutes at 72 °C for final extension. PCR optimization was performed in the presence of various concentrations of DMSO, betaine and 2-ME with BSA. The same PCR condition was also used for the amplification of the LPG3 gene from lizard *Leishmania*, but the annealing was carried out at 61.5 °C for 1 min. The PCR products, analyzed by 1% agarose gel electrophoresis, were then cleaned up from the gel using QIAquick Gel Extraction Kit (Qiagen, USA) following the manufacturer's instructions.

Cloning and sequencing

The eluted PCR product was cloned into the pTZ57R/T vector using InsT/AcloneTM PCR Product Cloning kit (Fermentas, Lithuania) following the manufacturer's procedures and transformed into competent *E. coli* DH5α strain. The presence of recombinant plasmid in the *E. coli* clones, selected by blue/white screening, was confirmed by gene-specific PCR and digestion with *BglII* and *NheI* restriction enzymes. Isolated positive clones were sequenced using dideoxynucleotide chain termination method (MWG Operon's Sequencing Service, Germany). The obtained sequences were then submitted to the GenBank.

Structural modeling

The amino acid sequences of LPG3 of two *Leishmania* species were downloaded for structural modeling from NCBI. Sequence alignment and phylogenetic tree construction were then performed

Workbench V.7.5.1. This aligned file was put on server and the program was run for homology modeling of the target sequence. Tertiary structures of the *Leishmania* LPG3 were modeled on the basis of different template structures using SWISS-MODEL server.

Results

The LPG3 gene was successfully amplified from *L. infantum* and lizard *Leishmania* genomic DNA under the optimized PCR condition. LPG3 belongs to GRP94/HSP90 family which plays an essential role in LPG synthesis pathway by adding β-galactose to Man (a1-PO4)-glycan core-PI (Figure 1).

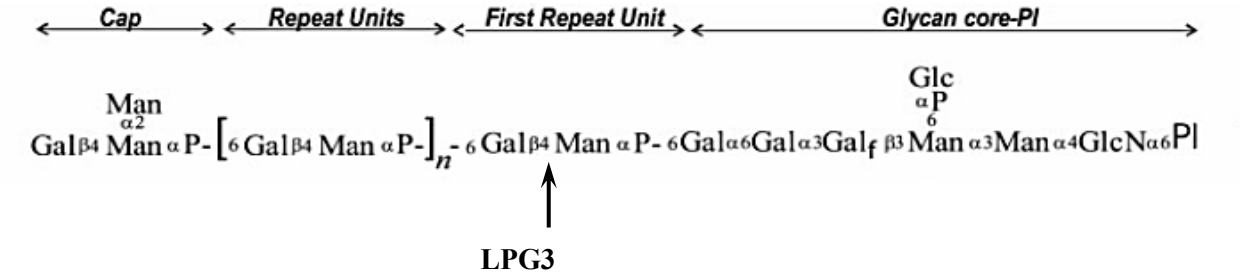

Figure 1. Structure of *Leishmania* lipophosoglycan (LPG) and the GalT activity of LPG3 in β-galactose addition to Man (α1-PO4)-glycan core PI.

We obtained 2316 bp and 2313 bp products (with 60.8% and 59.1% GC content, respectively) that had previously failed to amplify with the addition of inexpensive PCR enhancing additives, including DMSO, betaine and 2-ME with BSA to the reaction mixture.

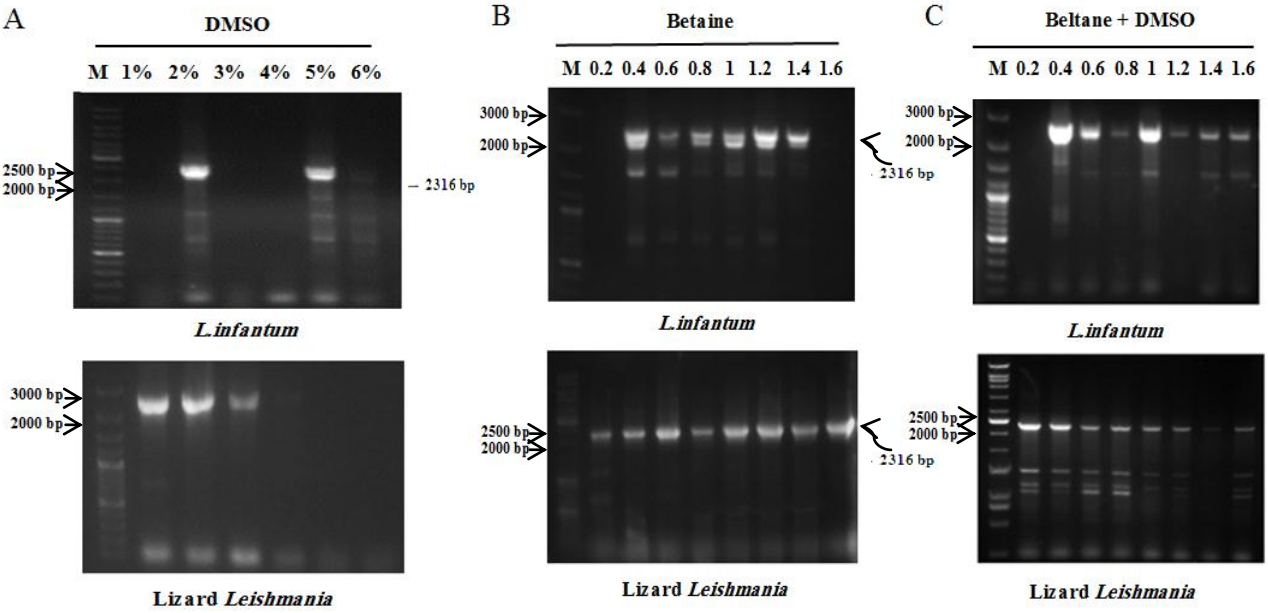

Figure 2. Effects of various concentration of (a) DMSO and (b) betaine either alone or (c) the combination of two additives with 50 µg/µl of BSA on the PCR amplification of the LPG3 gene of *L. infantum* and lizard *Leishmania*. The PCR products were electrophoresed on 1 % agarose gel. Lane M, DNA size marker.

PCR was conducted with concentration ranges between 1-6% (v/v) DMSO, 0.2-1.6 M betaine and 0.2-1.6 mM 2-ME with 50 µg bovine serum albumin (BSA) /µl, or a combination of either two (DMSO and betaine) or three additives (DMSO, betaine and concentrations ranging from 0.4M to 1.4M concentration although non-specific amplification products were produced in PCR of the LPG3 gene from *L. infantum*. These non-specific amplification

products were disappeared at similar concentration in the case of lizard *Leishmania* (Figure 2b). When DMSO (2% v/v) was added as a co-additive in the reaction mixture consisted of various concentrations of betaine with BSA (50µg/µl), both specificity and yield could be improved in L. infantum despite the observed reduction in lizard *Leishmania* (Figure 2c). The additive effect of 2-ME combined with BSA in enhancing PCR yield and specificity was greater in *L. infantum* than that of lizard *Leishmania*.

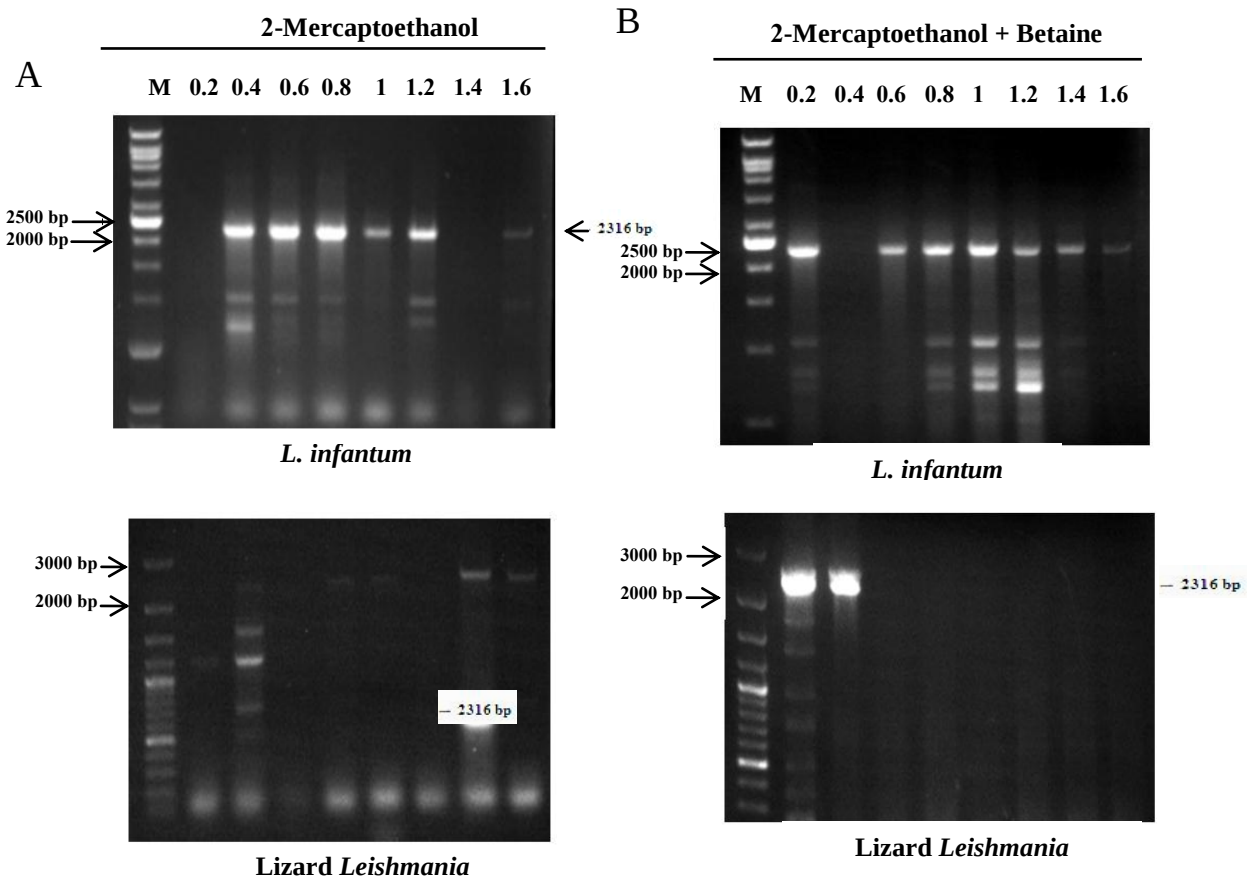

Figure 3. Effects of different concentrations of (a) 2-mercaptoethanol (2-ME) either alone or (b) in combination with betaine and 50 µg/µl of BSA on the PCR amplification of the LPG3 gene of *L. infantum* and lizard *Leishmania*. The PCR products were electrophoresed on 1% agarose gel. Lane M, DNA size marker.

The ffective concentration of 2-ME was found to be at 0.4 to 0.8 mM associated with rather non-specific amplification products while the addition of 2-ME was failed to promote target amplification of lizard *Leishmania* (Figure 3a).

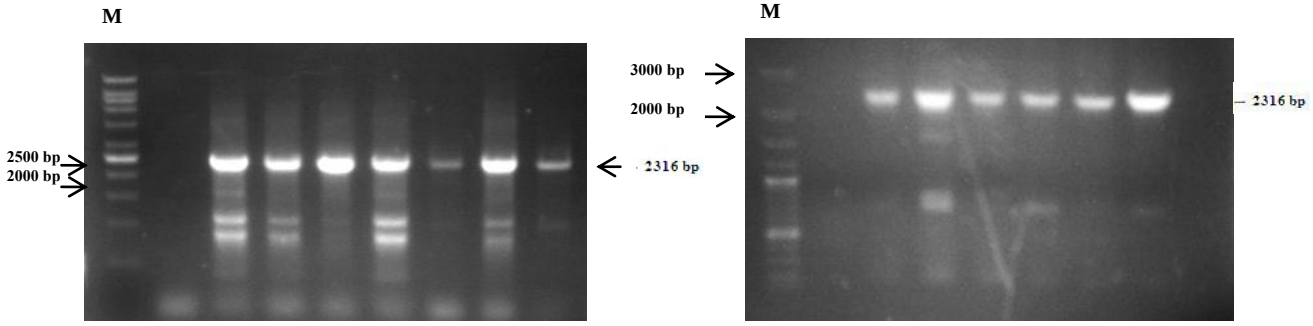

Figure 4. Gel electrophoresis of PCR product of the *L. infantum* and lizard *Leishmania* LPG3 gene in different reaction mixtures. Lane M, DNA size marker.

The improved PCR efficiency was also observed in the combination of betaine and 2-ME with BSA, but some products were nonspecifically formed along with the desired product (Figure 3b). When these

PCR additives were combined together, the amplification of the target DNA was not promoted in the lizard *Leishmania*. In the case of *L. infantum*, a low yield of PCR product was observed (Figure 4).
Next, the resultant PCR products were cloned into the TA cloning vector (pTZ57R/T) and the presence of insert in the recombinant plasmids, termed pT-LPG3, was detected by digestion using *BglII* and *NheI* restriction enzymes and PCR (Figure 5).

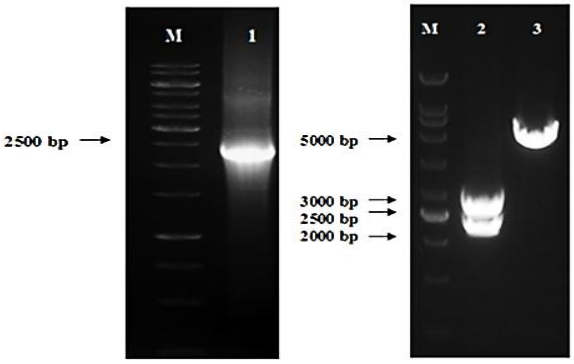

Figure 5. Confirmation of pT-LPG3 vector by PCR and restriction enzyme digestion. Lane 1, PCR product; Lane 2, the products of digestion with BglII and NheI; Lane 3, undigested vector. Lane M, DNA size marker. The products were electrophoresed on 1% agarose gel.

These products were then sequenced via the dideoxy chain termination method on an automated sequencer. The obtained LPG3 sequences of both *Leishmania* species were registered to the GenBank database under accession no. HQ400675.1 and JN87191301, respectively.
The crystal structure of LPG3 has not yet been recorded, therefore, alignment and homology modeling of the target sequence were performed. Sequence comparison indicated highly conserved amino acid residues in two species of *Leishmania*. The deduced amino acids alignment showed 95% identity between *L. infantum* and lizard *Leishmania*. In addition, the LPG3 gene was compared with LPG3/GRP94 genes from other Leishmania species, which had 81-98% identity with some sequences recorded in GenBank, including XP001566424.1, XP003872486, XP001466598.1, XP003722150, and AAM0039001 (Table 1).
It is thought that a significant similarity in the nucleotide and/or amino acid sequences is almost always manifested in three-dimensional structure, indicating molecular homology. Therefore, comparative (homology) protein structure modeling built a three-dimensional model for the target sequence based on multiple template structures using the SWISS-MODEL server (Figure 6). The best model of LPG3 was found to be monomer. About 49.94% α helix (Hh), 14.53% extended strand (Ee), 3.76% β turn (Tt) and 37.78% random coil (Cc) have been predicted for LPG3 protein of *L. infantum*, whereas LPG3 protein of lizard *Leishmania* contains 49.68% α helix (Hh), 13.88% extended strand (Ee), 4.8%β turn (Tt) and 31.65% random coil (Cc).

Table 1. The pairwise sequence alignment was performed using CLC Main Workbench 7.5.1 software which illustrates the LPG3 identity among different *Leishmani*a species. Each square represents one pairwise alignment.

Identity Species (%)	*L. infantum*	*L. major*	*L. infantum JPCM*	*L. donovani*	*L. mexicana*	*lizard Leishmania*	*L. panamensis*	*L. braziliensis*
L. infantum		97.67	97.80	97.67	97.15	95.49	81.62	82.00
L. major	97.67		97.02	96.89	95.98	93.30	81.88	82.26
L. infantum JPCM	97.80	97.02		99.87	97.41	93.30	82.38	82.51
L. donovani	97.67	96.89	99.87		97.28	93.17	82.38	82.51
L. Mexicana	97.15	95.98	97.41	97.28		92.65	82.00	82.26
lizard Leishmania	95.49	93.30	93.30	93.17	93.65		78.09	78.46
L. panamensis	81.62	81.88	82.38	82.38	82.00	78.02		98.48
L. braziliensis	82.00	82.26	82.51	82.51	82.26	78.46	98.48	

Further structural analysis of the modeled sequence presented several distinct motifs which are hallmarks of the GRP94/HSP90 protein family, including Histidine Kinase-like ATPase domain (box I; residues 52-217), nucleotide binding domain (box II; residues 154-159), and a conserved dimerization domain (box III; residues 673-716) in LPG3. Notably, they have an EDDL sequence (box IV), which is different from the KDEL ER retrieval signal of GRP94 in eukaryotes, at its C-terminus to achieve

ER localization that was similar to the related *L. infantum* GRP94 (2).

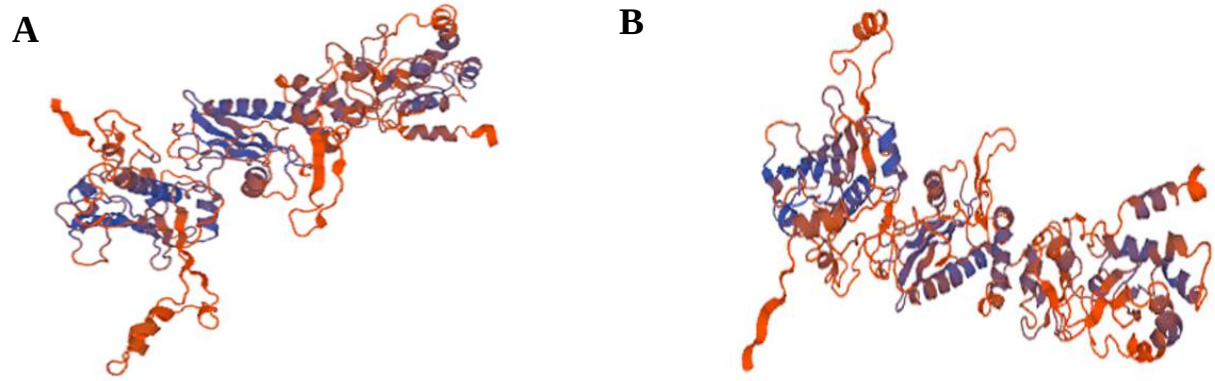

Figure 7. Ribbon presentations of the modeled LPG3 of (a) *L. infantum* and (b) lizard *Leishmania* were predicted by SWISS-MODEL server.

This may suggest the utilization of the other signal for ER localization in the *L. infantum* LPG3. Various post-translational modification sites were identified in LPG3 protein of both *Leishmania* species. N-glycosylation sites were found at Asn-3, -63, -306, and -402 in LPG3 protein of *L. infantum*, as well as at Asn-3, -63, and -402 in LPG3 protein of lizard *Leishmania*. Seven protein kinase C (PKC) hosphorylation sites were predicted in *L. infantum* and eight PKC sites were predicted in the case of lizard *Leishmania*. One to two amidation sites were also present in *L. infantum* at position 564 and in lizard *Leishmania* at position 285 and 564 of the LPG3 protein (Figure 7). It is remarkable that GRP94 is present in the parasites *Leishmania* and *Typanosoma* even though it is not found in bacteria, yeast, most fungi and other unicellular organisms (14). A phylogenetic tree was constructed using the neighbor-joining method. It indicates that *Leishmania* LPG3/GRP94 evolved separately from the GRP94/HSP90 family members in *Trypanosoma*, *E. coli*, *Yeast* and human, perhaps due to its different functions. The formation of a cluster between the *L. infantum* and lizard *Leishmania* was observed in the phylogenetic tree (Figure 8).

Discussion

In this study, we report the cloning of LPG3 gene from the lizard and mammalian *Leishmania* species. *LPG3*, a GRP94/HSP90 homolog chaperon, is one of the biosynthetic enzymes involved in assembly of the LPG as the main virulence factor in the establishment of initial macrophage infection (2). *L. infantum* LPG3 has also been shown to modulate immune responses as an immune reactive protein (3, 15, 16). Further, we found that the target sequence from two species of *Leishmania* is remarkably amplified under optimized PCR reaction conditions. The efficiency of PCR amplification has been reported to be increased by adding organic solvents (17). DMSO and other additives such as betaine and BSA not only reduce the formation of secondary structures like hairpins caused by GC-rich regions but also remove inhibitors present either in the DNA template preparation or in the reaction buffer and increase enzyme stability as well (18-22).

The addition of either DMSO or betaine with BSA in the reaction mixture showed substantial improvement in PCR amplification yield although non-specific amplification products were observed. When all of the three agents were included in the reaction mixture, both PCR yield and specificity were achieved in the amplified LPG3 gene. The modified protocols including either DMSO with betaine and/or DMSO with certain denaturants such as for mamide has been used to improve the specificity of PCR-amplified products (23). The co-additive effect of 2-ME has been less used on the enhancement of PCR efficiency. 2-ME as a reducing compound is known to stabilize Tag DNA polymerase and reduce the formation of secondary structures. It has been shown that the yield of PCR amplification may be improved by adding sorbitol or 2-ME as antioxidants (11, 13, 24). A study revealed that the combination of BSA, glycerol and DTT had the most additive effect on the amplification of VT1 and VT2 fragments of enterohemorrhagic *E. coli* (EHEC) (25). Current results showed that the addition of 2-ME with betaine and BSA to the reaction mixture produces remarkably high yield with no undesired non-specific products. The PCR products were subsequently cloned and sequenced, followed by submitting in GenBank with accession numbers.

Altogether, the findings may provide a basis for amplifying targets which cannot be amplified under standard reaction conditions due to unpredictable performance of commercially available PCR kits.

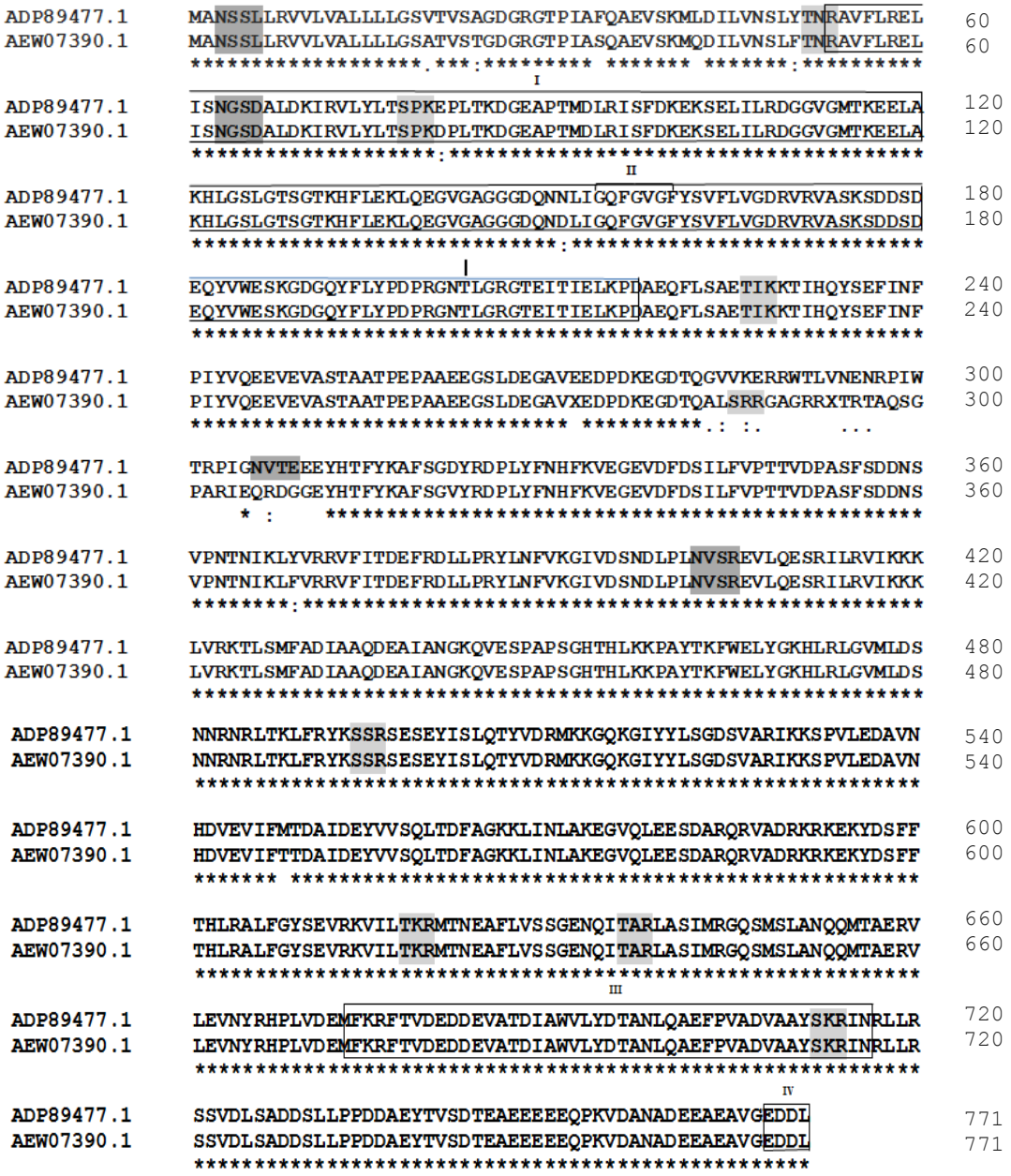

Figure 8. Deduced amino acid alignment of the LPG3 gene of *L. infantum* (ADP89477.1) with lizard *Leishmania* (AEW07390.1). The alignment was done using the ClustalW2 program. Asterisks (*) and colons (:) below the alignment represent identical and similar residues, respectively. The boxes indicate Histidine Kinase-like ATPase domain (box I), GXXGXG conserved motif in all HSP90 family proteins (boxII), dimerization domain (box III), and the C-terminal ER retention signal (box IV). Potential N-glycosylation sites are indicated by dark gray, and protein kinase C phosphorylation sites are marked by light gray. The numbers on the right indicates amino acid positions.

Notably, the lizard *Leishmania* as a new protein expression system has been previously used to express *L. infantum* LPG3 as a single band of about 97 kDa was observed using western blotting (26). Additionally, a homology model of LPG3 was built by SWISS-MODEL server which is used for automated comparative modeling of three-dimensional (3D) protein structures because of unavailable crystal structure for the target sequence. This is the first homology model for the *Leishmania* LPG3. Sequence comparison showed a high degree of identity between the lizard and mammal-infecting *Leishmania* species. Further, several distinct functional motifs and various post-translational modification sites were predicted in the modeled sequence, indicating a remarkable structural similarity. The phylogenetic analysis also

indicated that the lizard *Leishmania* is evolutionarily related to *L. infantum*. This is in agreement with biogeographic data, since both the lizard *Leishmania* and *L. infantum* are found only in the Old World.

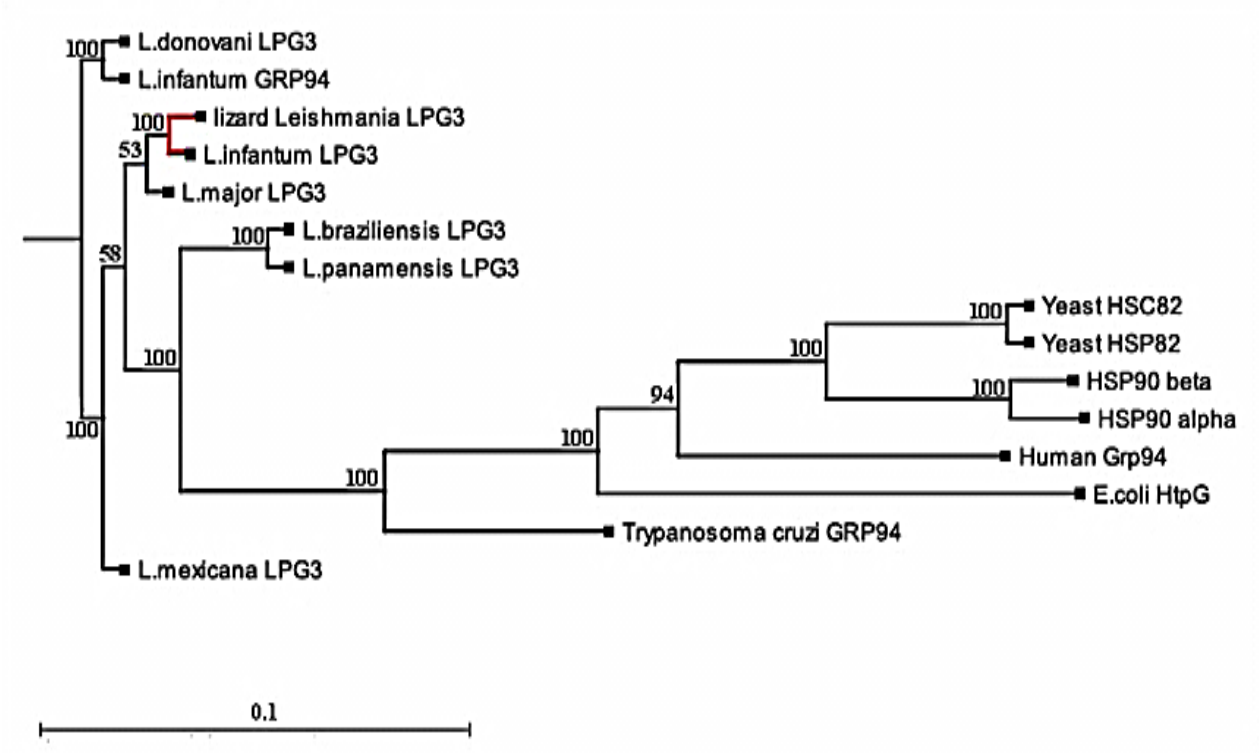

Figure 9. Analysis of phylogenetic relationships among some HSP90 family members. The sequence comparison of human cytosolic HSP90α (AAI21063.1), HSP90β (NP_031381.2), human GRP94 (AAH66656.1), Yeast HSP82 (NP_O15084.1) , Yeast HSC82 (NP_013911.1), *Leishmania braziliensis* (XP 001566424), *Leishmania* Mexicana (XP 003872486), *Leishmania infantum* JPCM5 (XP001466598), Leishmania major (XP003722150), *Leishmania donovani* (AAM0039001), *E. coli* HtpG (AP_001122.1), *Leishmania infantum* (ADP89477.1), lizard *Leishmania* (AEW07390), *Leishmania panamensis* (AIO00026), and *Trypanosoma cruzi* GRP94 was performed using CLC Main Workbench 7.5.1 software. *L. infantum* and lizard *Leishmania* LPG3 were highlighted in red. Numbers at the nodes are bootstrapping values. The scale bar indicates the number of 0.1 residue substitutions per site

In general, we have shown that the use of known inexpensive and readily available PCR additives may substantially improve the specific amplification of the target sequence to use for subsequent cloning and sequencing. Based on comparative analysis, a closer similarity was also demonstrated in two species of *Leishmania*. Furthermore, homology modeling showed that they are not only related evolutionarily but also have a structural similarity as well. Thus the results may provide some new insights into the molecular knowledge of the LPG3 in the lizard and mammalian *Leishmania* species and offer further functional research for designing drug targets or vaccine candidates against leishmaniasis.

Acknowledgments

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Conflict of Interest

There is no conflict of interests.

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