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Characterization of Heat Shock Protein (HSP 70) Sequence in *Leishmania donovani*

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ABSTRACT

Heat shock protein HSP70 is the highly conserved protein along the kinetoplastid phylogenetic spectrum. A full length *hsp70* gene was amplified, cloned and sequenced. The protein sequence was aligned and phylogentic tree was constructed. The *Leishmania donovani* HSP70 protein sequence (653 amino acids) is slightly larger than the Human HSP70 protein sequence (641 amino acids) with 72% identity. The putative protein sequence is 98%, 97%, 96% and 92% identical to *L. major, L. infantum*, *L. tarentolae* and *L. braziliensis* respectively. Evolutionary relationship showed that kinetoplastid parasites are closely related to the human compared to prokaryotes, *E. coli*.

Keywords: *Leishmania donovani,* HSP 70, cloning, sequencing, cytoplasmic protein

INTRODUCTION

Leishmaniasis is a disease caused by protozoan parasite of genus *Leishmania.* At least 20 *Leishmania* species infect humans, and the spectrum of diseases that they cause ranges from self healing cutaneous leishmaniasis (CL) to mucocutaneous leishmaniasis (MCL) and often fatal visceral leishmaniasis (VL) (Murray *et al.* 2005, Herwaldt 1999). Visceral form of the disease is caused by members of the *L. donovani* complex (*L. donovani* and *L. infantum* in the Old World, and *L. chagasi* in the New World), which is a major health problem throughout the tropics with 500,000 new cases every year (Srividya 2007).

Like many other protozoan parasites, *L. donovani* must have the mechanisms for survival and multiplication under various environmental conditions since its complex life cycle involves surviving in two different hosts, a flagellated extracellular promastigote in the insect vector, and non-flagellated intracellular amastigote living inside macrophages of mammalian host. Thus, a drastic change in temperature from 26^0C to 37^0C is naturally included in its biological cycle. Some of these conditions may change the pattern of expression of particular genes and increase the synthesis of a set of proteins, highly conserved along the phylogenetic spectrum, commonly known as the heat shock proteins; HSPs (Schlessinger *et al.* 1982). The parasite is phagocytosed by macrophages and, inside the acidic phagolysosomes, promastigotes differentiate to amastigotes. The mechanisms implicated in this transformation are not completely understood, although it is known that an increase in the temperature and a decrease in the pH encountered in the phagolysosome are the key parameters to transform promastigotes into amastigotes (Garlapati *et al.* 1999, Zilberstein & Shapira 1994). The heat shock response is considered to be of vital importance for the stage-specific differentiation of *Leishmania* parasites and help to promote cell survival after a large variety of environmental stresses (Soti *et al.* 2005).

Among HSPs, HSP70 is the highly conserved protein which under non stressful condition they assist a wide range of housekeeping functions such as folding and translocation of new synthesized proteins' activation of specific regulatory proteins, replication proteins and kinases; refolding of misfolded and aggregated proteins, and protein degradation (Hartl & Hayer-Hartl 2002, Helmbrecht *et al*. 2000, Jolly & Morimoto 2000). The cellular-stress response can mediate cellular protection through expression of heat shock protein (HSP70) which can interfere with the process of cellular death (Beere *et al.* 2000) and a wide variety of stressful stimuli (Wu *et al.* 1996, 2001) but the functions of HSP70 in *Leishmania* have not been well established although it is expected that they will be analogous to similar proteins in other organisms.

MATERIALS AND METHODS

Parasite and culture condition

Leishmania donovani AG83-S (MHOM/IN/1983/AG83) promastigotes were cultured at 22^0C in modified M199 medium supplemented with 100 units/ml penicillin,

100 µg/ml streptomycin (Sigma, USA) and 10% heat inactivated fetal bovine serum (FBS) Gibco/BRL, Life Technologies Scotland, UK.

Cloning of *(hsp70)* gene from *L. donovani*

A 1962 bp DNA fragment was amplified from genomic DNA using a sense primer with a flanking BamHI site, 5'-CGCGGATCCATGACATT CGACGGCGCCATC-3', at position 1-21, and the antisense primer with a flanking HindIII site, 5'–CCAAGCTTTTAGTCGACCTCCTCGACCTTGG –3', including the stop codon, at position 1939-1962. Polymerase Chain Reaction (PCR) was performed in 50 µl reaction volume containing 100 ng of genomic DNA, 25 pmol each of gene-specific forward and reverse primers, 200 µmol of each dNTP, 2mM $MgCl_2$, 5U Taq DNA polymerase (MBI Fermentas) and 5% DMSO. The condition of PCR was as follows: 94^0C for 10 min, 94^0 C for 4 sec, 62^0C for 30 seconds, 72^0C for 2 min and 35 cycles. Final extension was carried for 10 min at 72^0. A single 1.962 kb PCR Product was obtained and cloned into the Bam HI- Hind III site of pET-30a vector (Novagen). The recombinant construct was transformed into BL21 (DE3) strain of *Escherichia coli* subjected to the automated sequencing using Applied Biosystems 3730 DNA sequencer, University of Delhi (South campus). Multiple alignments of amino acid sequences were performed using Bio edit online software programme and the phylogenetic tree was constructed using PHYLIP style treefile produced by CLUSTALW.

RESULTS

Heat shock protein sequence and phylogenetic tree analysis

The gene encoding the 70kDa heat shock protein, nucleotide sequence was retreived from EMBL sequence data bank under the accession no. X52314. In order to clone the full length gene encoding hsp70, Polymerase Chain Reaction (PCR) was performed using specific oligonucleotides, The sense primer was 5'-CGCGGATCC ATGACATTCG ACGGCGCCATC-3', at position 1-21, and the antisense primer with a flanking HindIII site, '-CCCAAGCTTTTAGTCGACCTCCTCG ACCTTGG-3', including the stop codon, at position 1939-1962**.** Genomic DNA from *L. donovani* AG83 (MHOM/IN/1983/AG83) promastigotes was used as a template. A single full length 1962 bp PCR product was obtained. The gel was purified, double digested with BamH1 and HindIII restriction enzymes. The digested product was ligated with pET30a cloning vector at 16^0C overnight. The ligated product was transformed into DH5a competent cells. The positive cloned were confirmed by colony PCR, Plasmid DNA was isolated using glass milk method and sequencing. A single open reading frame consisting of correct 1962-bp was isolated. No variation in the coding sequence was found from the sequence described earlier.

The predicted HSP70 protein sequence is shown aligned with other *Leishmania* species and human HSP70 sequence (Fig. 1). The open reading frame encoded for putative polypeptide of 653 amino acids, with a predicted molecular mass of 70 kDa, which is highly similar to *L. infantum* (653 amino acids) but slightly smaller than *L. tarentolae* (657 amino acids) and *L. major* (662 amino acids), while slightly larger than the Human (641 amino acids). There was 72% identity between human and *L. donovani* HSP70 (Swiss-Prot accession no. P08107**)**. The *L. donovani* HSP70 protein sequence was found to be only 98% identical to *L. major* (Gene Bank™ accession number XP_001684564), 97% identical to *L. infantum* (Gene Bank™ accession number CAA59793) and 96% identity to *L. taentoae* (Gene Bank™ accession number AAR04339), Similarly 92% with *L. braziliensis* (Gene Bank™ accession AAG01344), 86% with *T. brucei* (Swiss-Prot accession no. P08107P11145) and 46% homology with E. coli DNAK (Swiss-Prot accession no. P04475). The carboxy-terminal region is the least conserved in the protein, as in the rest of the HSP70 from other trypanosomatids and mammals. This region contains two repetitions of GGMP terrapeptides in *L. donovani* and *L. infantum*, three repetitions in *L. major* and *T. cruzi* and variable numbers in most HSP70s from parasites sequenced so far and in the HSC70 from Human and rat (Young *et al.* 1990). While it is absent in *L. amazonensis* HSP70 protein (Bock *et al.* 1993). The significance of this sequence is not clear yet. As in other eukaryotes EEVD sequence is conserved at the C- terminus of *L. donovani* suggesting that it encodes a cytoplasmic protein and not endoplasmic reticular or mitochondrial form of the protein (Gething & Sambrook 1992). The N-terminal domain, including the ATP-binding domain, is much conserved with other HSP70 proteins.

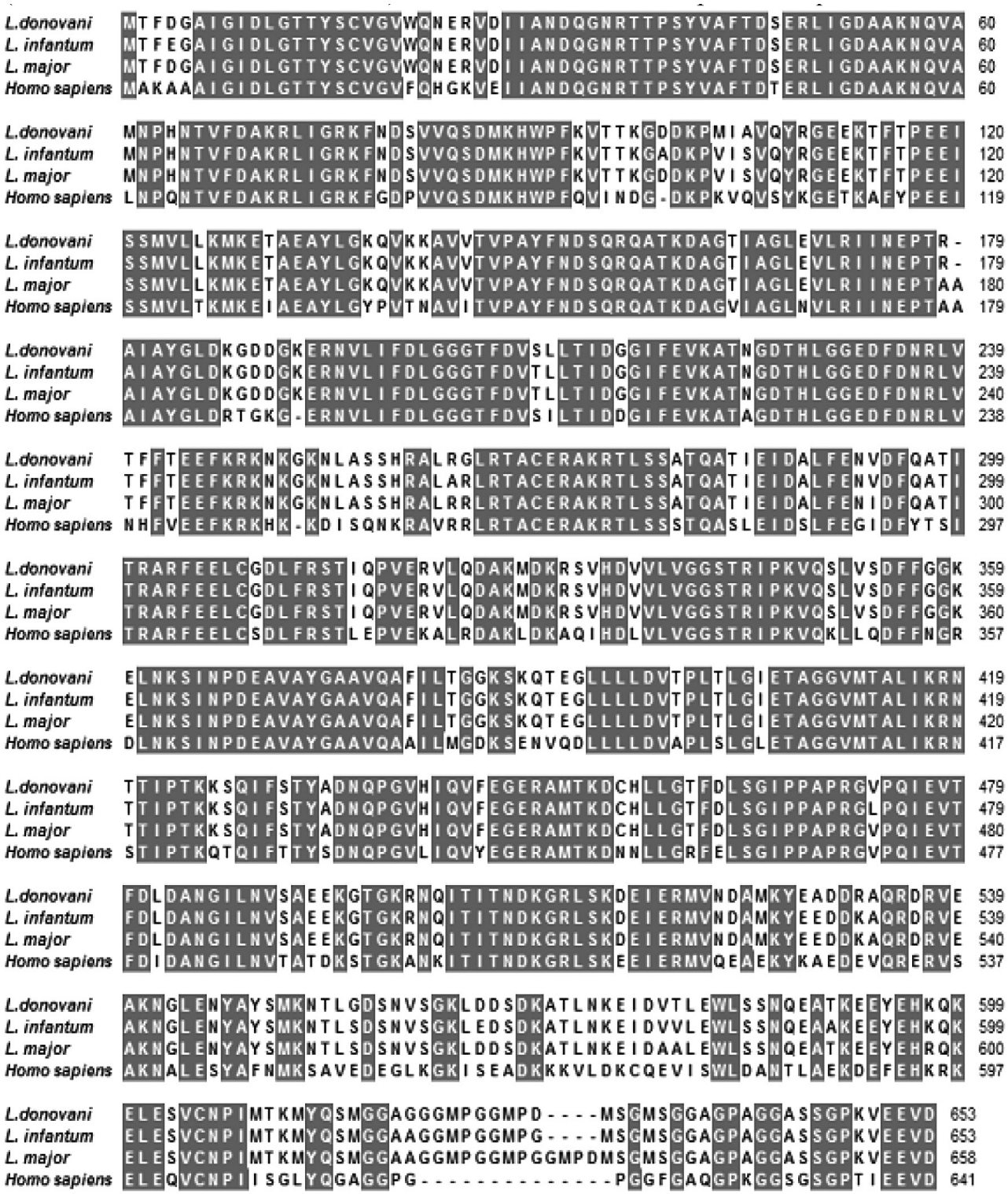

Fig. 1. Multiple sequence alignment of HSP70 sequences from L. donovani (CAA36551), L. major (XP_001684564), L. infantum (CAA59793) and Human (P08107) using Bio edit online multiple sequence alignment software. The amino acids are numbered to the left of the respective sequences. Residues that are identical or similar with other HSP70 are indicated in black showing complete identity.

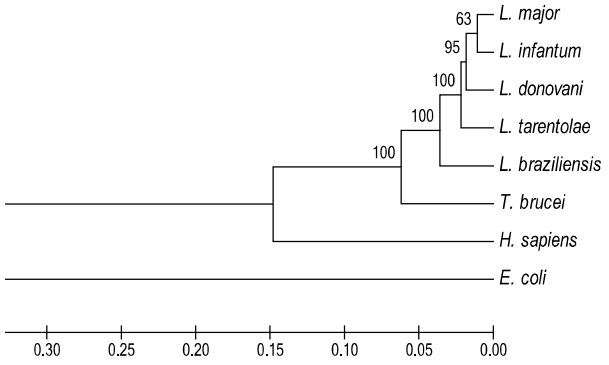

Fig. 2. Bootstrap analysis of phylogenetic tree by UPGMA method using the amino acid sequences of HSP70 from L. donovani (CAA36551), L. infantum (CAA59793), L. major (XP_001684564), L. braziliensis (XP_001566325), L. tarentolae (AAR043339.1), T. brucei (XP_829198.1), E. coli (NP_414555.1) and Human (P08107). The tree view program under the CLUSTAL W program viewed the phylogenetic trees derived from the multiple alignments.

A phylogenetic tree has been constructed using the *L. donovani* HSP70 sequence and other representative HSP70 sequences. The tree indicates close evolutionary relationship of *L. donovani* with *L. infantum* and *L. major* followed by *L tarentolae*, *L. braziliensis*, and *T. brucei* among the kinetoplastid protozoa. The kintoplastid HSP70 sequences are even much closer to human compared to prokaryotes *E. coli* (Fig. 2).

DISCUSSION AND CONCLUSION

Leishmania donovani HSP70 protein sequences are highly homologous among the trypanosomatids. The sequence identity is more than 90% among the *Leishmania* species, more than 80% with the *Trypanosoma* species and 72% identity with the human. N- terminal region of the sequences among the species is highly conserved compared to the C- terminal region. In C- terminal region most striking difference is due to presence of GGMP tetra peptide repeats (Engman *et al.* 1989) which is varied from none in *L. amazonensis* (Bock and Langer 1993) to multiple in *T. cruzi* (Engman *et al.* 1989). GGMP motif is also found in HSP70 of other parasites such as *P. falciparum* (Bianco *et al.* 1986). The presence of EEVD sequence at the C-terminus of HSP70 protein is the characteristic of cytoplasmic form of the HSP70 protein (Gething & sambrook 1992) which is present most of the trypanosomatids including *L. donovani*.

Amino acids 'D', 'E', 'R' and 'K' are the charged residues and the major heat shock protein in eukaryotes (HSP70) is distinguished by multiple charge clusters (Karlin *et al.* 1995).The charge clusters regions of HSP70 might in some extent interact ionically to help orient and position the protein to interact in a hydrophobic manner with special residues of the target protein in its unfolded state, further more misfolded proteins may also permit charge-backbone interactions of HSP70 with the target peptide (Karlin & Brocchieri 1998). Mitochondrial form of the HSP70 protein of *Leishmania* do not posses the charge cluster (Karlin & Brocchieri 1998). Multiple sequence alignment of the cytoplasmic form of HSP70 amino acid sequence of *L. donovani*, *L. infantum* and *L. major* showed at least six conserved charge cluster. Hence HSP70 protein sequence of *L. donovani* is more than 90% identical with other *Leishmania* group causing cutaneous leishmaniasis and cutaneous leishmaniasis. The parasitic HSP70 protein sequence is evolutionary more close to human than the prokaryotes.

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