ORIGINAL RESEARCH ARTICLE

Heat Shock Protein 70 (*HSP70*) Expression in Antimony Susceptible/Resistant Clinical Isolates of *Leishmania donovani*

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Abstract

Pentavalent antimonials have long been the first line of defence against leishmaniasis, but resistance has been reported in different parts of the world. Pentavalent antimony is reduced into trivalent form in the cells and is a potential inducer of HSP70 in *L. donovani*. Expression profile of HSP70 in antimony susceptible and resistant *L. donovani* isolates were characterized by Southern blot, Northern blot and western blot analysis. *HSP70* gene copy number, gene expression and HSP70 protein expression was found uniform in both antimony sensitive and resistant clinical isolates. In laboratory condition, *Leishmania* cells respond to antimonial drug stress by three fold over expression of the HSP70 protein. The observed results indicated that HSP70 play important role in stress tolerance against antimonial drug without differential expression in antimony sensitive and resistance clinical isolates of *L. donovani*.

Keywords: Pentavalent antimony; HSP70; visceral leishmaniasis; drug resistance; PFGE; hybridization

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Introduction

Visceral leishmaniasis is a protozoan parasitic disease caused by *Leishmania donovani*. Leishmaniasis constitutes a major public health problem with increasing pattern of disease burden [1,2]. It is a neglected tropical disease (NTD) which affects mainly the poor population groups, primarily in rural areas. Unfortunately, there is still lack of effective, affordable and easy-to-use drugs for leishmaniasis treatment. Since vaccine against leishmaniasis is still under development, the control lies solely on chemotherapy [3]. However, emergence of drug resistance in parasitic protozoa is becoming a major public health problem.

Heat shock proteins (HSPs) are highly conserved proteins found in both prokaryotic and eukaryotic cells. HSP70 is a molecular chaperone which plays an important role in protein folding and assembly of polypeptides within the cell. When cells are exposed to the stressed condition, the proportion of misfolded proteins (MFPs) suddenly increases and the cell reacts by synthesizing HSPs to assist those proteins in refolding. The stress response is controlled primarily at the transcription level by a heat shock factor (HSF) [4].

It has been shown that transcript level of *HSP70* increased in *L. major* and in *L. infantum*, in response to elevated temperature conditions [5, 6, 7]. Besides elevated temperature, metals are also known to be important stress inducers in the cells. Antimonial compounds; Pentostam and Glucantime are still the drug of choice in the treatment against all forms of *Leishmania* infections [8, 9]. Since, antimonial drug resistance is becoming a common problem in many leishmaniasis endemic regions, extensive studies have been carried out to investigate the mechanism of drug resistance in the laboratory mutants. One of the mechanisms of resistance is postulated to be HSP70. *L. tarentolae* cells transfected with HSP70 gene showed resistance to SbIII or arsenite [10]. This suggests that HSP70 has a role in antimony resistance mechanism. In the present study, we have characterized the expression of Heat Shock Protein 70 in antimony sensitive and resistant clinical isolates of *L. donovani*.

Materials and Methods

Parasite and culture condition

Promastigotes of Indian *Leishmania donovani* clones AG83-S (MHOM/IN/1983/AG83), GE1 (MHOM/IN /80/ GE1F8R**)** and three untyped strains 2001, NS2, and 41 were isolated from patients with visceral leishmaniasis (VL) and were routinely cultured at 22°C in M-199 medium (Sigma, USA) supplemented with 10% heat inactivated fetal bovine serum (FBS, Gibco/BRL, Life Technologies, Scotland, UK) and 13 mg/ml penicillin and streptomycin (Sigma, USA) [11].

Clinical isolates obtained from VL patients who responded to SAG chemotherapy were designated as SAG-S (SAG sensitive) whereas VL patients that did not respond to SAG were designated as SAG-R (SAG resistant). Accordingly, SAG sensitive isolate used in this study is 2001-S whereas the four SAG-R isolates are 41-R, NS2-R, and GE1-R. The SAG-resistant isolates were maintained in the absence of drug pressure in vitro. The isolates have been passage through hamsters or BALB/c mice to retain their virulence and importantly their chemo-sensitivity profiles have remained unchanged.

Cloning of heat shock protein 70 (HSP70) gene from *L. donovani*.

A 1962-bp DNA fragment was amplified from the genomic DNA using a sense primer with a flanking *Bam*HI site, 5′-CGCGGATCCATGACATTCGACGGCGCCATC-3′, at position 1-21, and the antisense primer with a flanking *Hind*III site, 5′ – CCCAAGCTTTTAGTCGACCTCCTCGACCTTGG – 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, 2 mM $MgCl_2$, 5U Taq DNA polymerase (MBI Fermentas) and 5% DMSO. The PCR conditions were as follows: 94^0C for 10 min, 94^0 C for 45 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 1.9 kb PCR product was obtained and cloned into the *Bam*HI - *Hind*III site of pET-30a (**Figure 1**) vector (Novagen). The recombinant construct was transformed into BL21 (DE3) strain (**Figure 2**) of *Escherichia coli* and subjected to automated sequencing..

Expression and purification of HSP70 protein.

Expression of HSP70 protein from the construct pET30a - LdHSP70 was induced at OD of 0.7 with 50 µM, 100 µM and 500 µM isopropylthiogalactoside (IPTG) (Sigma) at 37°C for different time periods. All the steps of purification were carried out at 4°C as mentioned in general materials and methods.

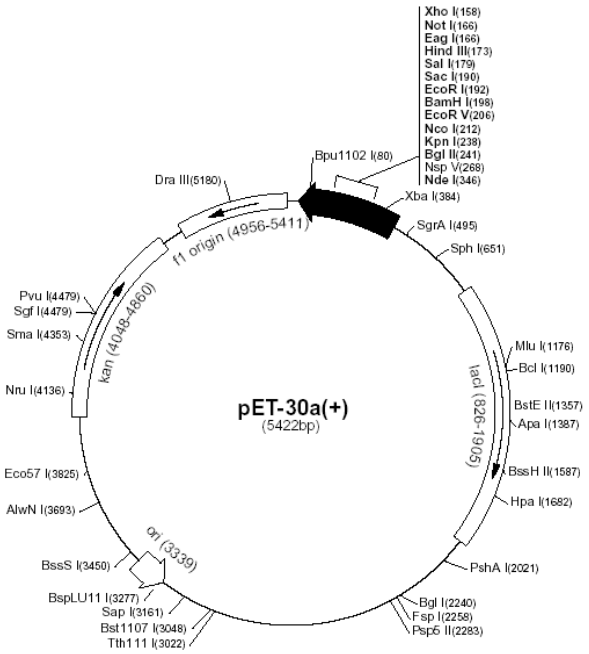

Figure 1: Restriction map of vector pET-30a(+).The *HSP70* PCR product (1.9 kb) was cloned into the *Bam*HI and *Hind*III restriction site of pET-30a vector.

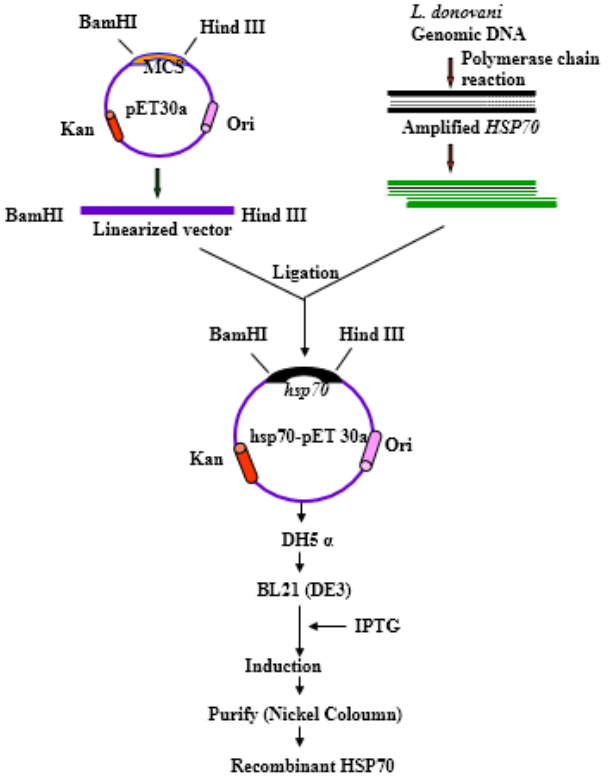

Figure 2 : Cloning strategy of heat shock protein (HSP70) in pET-30a expression vector

Protein determination

Protein concentration was determined by Bradford's method using bovine serum albumin (BSA) as standard [12].

Antibody production

The purified recombinant HSP70 protein (20 µg) was subcutaneously injected in mice using Freund's complete

adjuvant followed by two booster doses of recombinant HSP70 protein (15 µg) with incomplete adjuvant at two-week intervals to produce the polyclonal antibody against the recombinant HSP70 protein. The mice were bled two weeks after the second booster dose and sera was collected for Western blot analysis.

Isolation of genomic DNA and total RNA.

Genomic DNA was isolated from ~ 2 x 10^9 cells from 10-15 ml culture (mid log phase promastigotes) as described in general materials and methods. 5 µg of genomic DNA was digested overnight with *Hind*III and subjected to electrophoresis in 0.8% agarose gels. The gel was run at a constant voltage of 30V overnight. Total RNA was isolated from 2 × 10^8 promastigotes using TRI reagent™ (Sigma) according to manufacturer's instructions. The isolated RNA was stored in diethyl pyrocarbonate (DEPC) treated water in small aliquots at –80°C. For Northern blot analysis, 15 µg of total RNA was fractionated by denaturing agarose gel electrophoresis and transferred to nylon membrane following the standard procedure.

Pulse Field Gel Electrophoresis (PFGE)

Leishmania chromosomes were separated by PFGE. Low melting agarose blocks, containing embedded cells (10^8 log phase promastigotes/ml) were electrophoresed in a contour clamped homogenous electric field apparatus (CHEF DRIII, Bio-Rad) in 0.5 × TBE with buffer circulation at a constant temperature of 14°C. *Saccharomyces cerevisiae* chromosomes were used as chromosomal size markers. Pulse field gel electrophoresis (PFGE) running conditions were as follows: initial switch time, 60 s; final switch time, 120 s; run time, 24 h; current 6V/cm; including angle 120°. The transfer of PFGE separated chromosomes from agarose gels to nylon membrane was achieved by capillary method as described by [13].

Hybridization of Northern, Southern and PFGE blot

Pre-hybridization was done at 65°C for 4 hours in a buffer containing 0.5 M sodium phosphate; 7% SDS; 1mM EDTA (pH 8.0) and 100 µg/ml sheared denatured salmon sperm DNA. The blots were hybridised with denatured α-[P^{32}]-dCTP-labelled DNA probe (PCR probe described for the *L. donovani HSP70* coding region) at 10^6cpm/ml. The probe was labeled by random priming (NEB Blot®Kit, New England Biolabs, Inc.). Membranes were washed sequentially as follows: 2× SSC, 0.1% SDS; 1× SSC, 0.1% SDS; 0.5× SSC, 0.1% SDS; 0.2× SSC, 0.1% SDS for 10 min each at 65°C until the non-specific counts had substantially reduced. Membranes were air-dried and exposed to imaging plate. The image was developed by PhosphorImager (Fuji film FLA-5000, Japan) using Image Quant software.

Western blot analysis

Late log phase promastigotes (1 × 10^8) were harvested and the resultant cell pellet was lysed by sonication and cell supernatants were prepared by centrifugation at 20,000 x g. 50 µg of protein from each cell line were fractionated by SDS-polyacrylamide gel electrophoresis and blotted on nitrocellulose membrane using electrophoretic transfer cell (BIO-RAD). Western Blot analysis was done using the ECL kit (Amersham Pharmacia Biotech) according to the manufacturer's protocol. Polyclonal antibody to purified recombinant *L. donovani* HSP70 generated in mice was used for the Western blot analysis. Autoradiograms were analyzed by using model FLA 5000 imaging densitometer (Fuji, Japan). The results shown are from a single experiment typical of at least three giving identical results.

Results

Heat shock protein 70 (HSP70) sequence analysis

The gene encoding the heat shock protein 70 kDa (HSP70) nucleotide sequence was retrieved 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'-CGCGGATCCATG ACATTCGACGGCGCCATC-3' at position 1-21 and the antisense primer with a flanking *Hind*III site, 5'-CCCAAGCTTTTAGTCGACCTCCTCGACCTTGG- 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 PCR fragment was purified from the gel and double digested with *Bam*H1 and *Hind*III restriction enzymes. The digested product was ligated with pET30a cloning vector at 16°C overnight. The ligated product was transformed into DH5α competent cells. The positive clones were confirmed by colony PCR. Further, the cloned *HSP70* PCR fragment was confirmed by sequencing. A single open reading frame consisting of 1962-bp was isolated. No variation in the coding sequence was found from the sequence described earlier.

Over-expression and purification of full-length *L. donovani* HSP70 in *E. coli*

In order to overexpress and purify recombinant protein, the encoding *L. donovani* HSP70 sequence was cloned inframe in pET-30a expression vector. The resultant pET-30a -LdHSP70 construct was transformed into *E. coli* and protein over expression was induced using IPTG (**Figure 3A and 3B**) as described in general materials and method. A protein with a molecular weight that matched the estimated 70 kDa according to amino acid composition of *L. donovani* HSP70 with His_6 tag and S-tag present at its N-terminal end was also induced. The recombinant protein was purified by Ni^{2+}-NTA affinity chromatography column Purification of His-tagged *L. donovani* HSP70 by metal affinity chromatography (**Figure 3C**) yielded ~200 µg of purified protein from 1-litre bacterial culture.

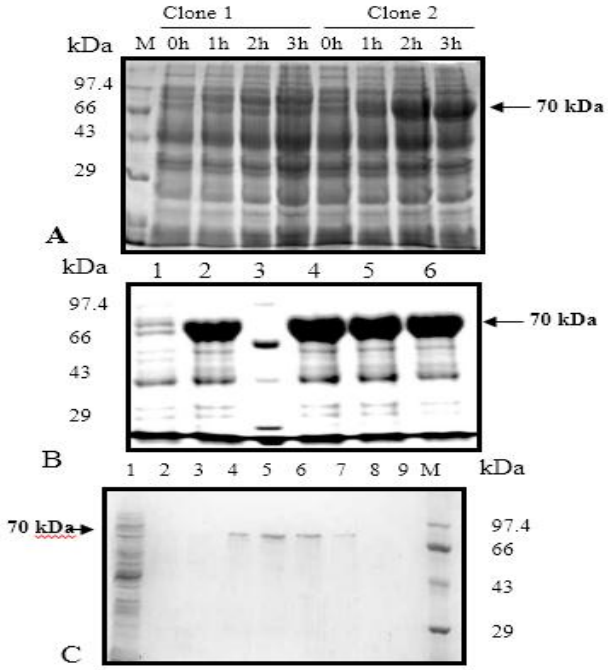

Figure 3: Overexpression and purification of L. donovani HSP70 protein. (A) Coomassie blue staining of SDS–PAGE showing overexpression of full-length *L. donovani* HSP70 protein in *E. coli*. The pET-30a bacterial extract before induction (lane 2 and 6) and after induction (lanes 3-5 and 7-9) of two positive clones at 1, 2, and 3 h, respectively with 1 mM IPTG. Arrow shows the induced recombinant HSP70 protein. Broad range protein MW marker (MBI-Fermentas) was used to identify the size of the recombinant protein (Lane 1). (B) The pET30a bacterial extract before induction (lane 1), molecular marker (lane 3) and three hour after induction (lane 2, 4, 5 and 6) with 50 µM, 100 µM, 200 µM and 500 µM IPTG respectively. (C) Purification profile of soluble form of HSP70 protein by Ni- NTA affinity bead affinity chromatography resin. Lane 1 flow through, lanes 2 and 3, washes with 20 mM and 60 mM immidazole, Lane 4-7 elutes using 100 mM, 150 mM, 200 mM, and 250 mM immidazole, Lane 8 Ni- beads, lane 9- blank. All the purification fractions were run along with the molecular weight protein marker (lane M).

Polyclonal antibody production and Western blotting

Western blot using size-fractionated parasite protein, antiserum could detect a band of anticipated *L. donovani* HSP70 size ~70 kDa in promastigote extracts. In antimony resistant *L. tarentolae* mutants, HSP70 was reported to increase more than 4-fold compared to the wild-type cells [10]. The overexpression of HSP70 remained stable in *L. tarentolae* mutants after several hundreds of passages without drug [10]. To test the link between HSP70 expression and resistant phenotype in antimony resistant *L. donovani* field isolates, we performed Western blot analysis using polyclonal antibody. A Western blot using equal amount of parasite protein (50 µg each) extract from antimony sensitive (AG83-S and 2001-S) and resistant (GE1-R, 41-R and NS2-R) field isolate did not show any differential expression of HSP70 protein (**Figure 4**).

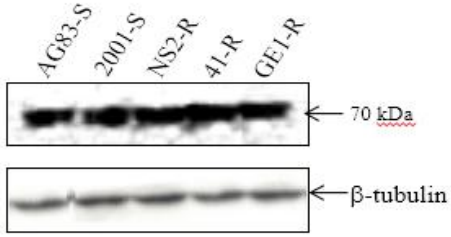

Figure 4. Western blotting using anti-His-HSP70 antibody raised against LdHSP70 in BALB/C mice. HSP70 protein in fractionated extracts obtained from SAG-S and SAG-R cultures harvested after 48 h of growth. The same blots were reprobed with antibody against *β*-tubulin protein to normalize the loading on to each lane of the gel.

Southern blot hybridization of HSP70 gene

The copy number of *HSP70* gene varies considerably among various *Leishmania* species [6, 10, 14]. To determine *HSP70* gene copy number in *L. donovani* field isolates, Southern blot analysis was performed using 1.9 Kb HSP70 PCR product as a probe as described under general materials and methods. *Hind*III which digests outside the gene producing a 10 kb restriction fragment (expected size) was used to check the copy number of the gene. The probe hybridized to a single fragment of 10 kb in all the field isolates (**Figure 5**) indicating that *HSP70* exists as a single copy gene in these strains. Further, we checked the chromosomal localization of *HSP70* gene by Southern blot hybridization of PFGE blot. The *HSP70* probe hybridized to a single chromosomal band of 1.2 Mb region that corresponds to the chromosome number 28 of the *L. infantum* genome database (**Figure 6**).

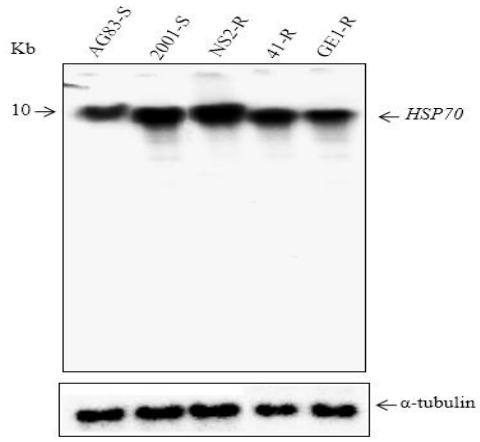

Figure 5. Southern blot analysis of *HSP70* gene in SAG–S and SAG–R *Leishmania donovani* field isolates. Total genomic DNA of *Leishmania* strains were isolated and digested with *Hind*III restriction enzyme. The blot was probed with 1.9 Kb full length *HSP70* gene. The blot was reprobed with *a-tubulin* to monitor the amount of digested DNA layered on the gel.

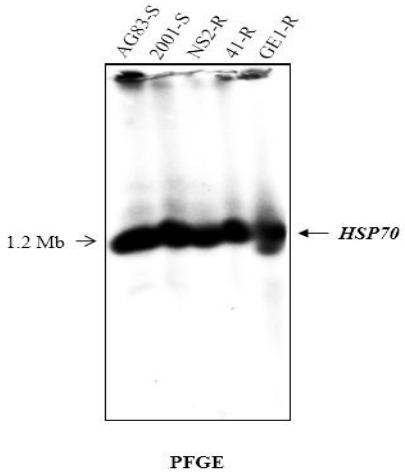

Figure 6. PFGE analysis of SAG-S and SAG-R *L. donovani* isolates indicating chromosomal localization of *HSP70* gene. Chromosomes of *L. donovani* isolates were separated by CHEF and Southern blots were hybridized with the *HSP70* probe. The size of hybridizing band was identified using chromosomes of *S. Cerevisae* as marker

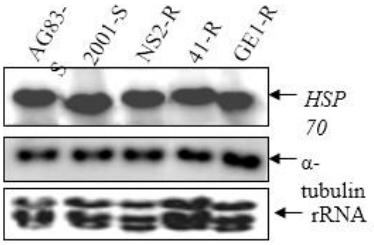

Figure 7. Expression analysis of *HSP70* gene in *L. donovani* Northern blot analysis of mRNA from SAG-S and SAG-R isolates (log phase culture). 15 µg of total RNA was loaded per lane, transferred and hybridized with *HSP70* probe. A α-tubulin probe and was used to monitor the amount of RNA layerd on the gel. The rRNA stained with ethidium bromide was used to normalize the RNA loading.

Northern blot hybridization of HSP70 gene

Since, we couldn't find any difference in HSP70 protein expression and copy number of gene in between SAG-S and SAG-R *L. donovani* field isolates, we checked transcript level of *HSP70* gene expression. Northern blotting of SAG-S and SAG-R *L. donovani* field isolates revealed a single transcript of ~ 3.5 kb in all the strains (**Figure 7**). Densitometric analysis of hybridizing bands corresponds to the internal control α-tubulin showed similar expression of *HSP70* gene in between SAG sensitive and resistant field isolates.

Induction of HSP70 by SbIII in promastigote of *L. donovani* field isolate

Leishmania cells respond to SbIII exposure by increasing HSP70 protein [10]. Induction of HSP70 was found more than four (>4) fold in *L. tarentolae* and *L. infantum* cells when incubated for 24 hours at IC_{50} concentration of the drug [10]. To test whether strain were incubated with 15 µM (IC_{50} concentration of AG83-S) SbIII for 24 hours. A time dependent increase of HSP70 protein expression in *L. donovani* field isolates was observed. Protein expression increased by more than > 1.7 fold at 12 hours and more than three (>3) fold after 24 hours compared to the cells incubated without drug (**Figure 8A and 8B**).

trivalent antimony could induce the expression of HSP70 protein in *L. donovani* field isolates, we used polyclonal antibody raised against HSP70 antigen in BALB/C mice. Promastigotes of *L. donovani* (AG83-S)

Discussion

Physiological role of the HSP70 as a molecular chaperon has been well described [15]. Induction of HSP70 can protect cells by binding to misfolded proteins in variety of adverse conditions [16]**.** Besides its physiological cytoprotective role an increased expres

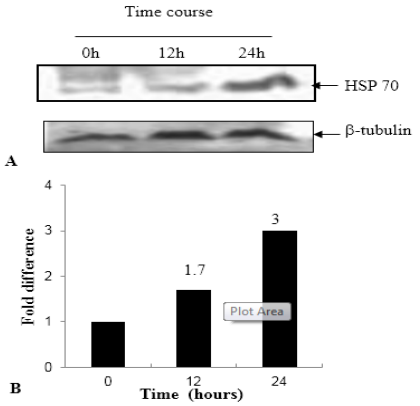

Figure 8. Effect of SbIII on HSP70 protein expression at different time points. (A) Western blot analysis of HSP70 in *L. donovani* promastigotes after treatment with 15 µM SbIII for 0 h, 12 h and 24 h. The same blot was reacted with antibody against β-tubulin protein to normalize the loading on to each lane of the gel. (B) Densitometric scanning of the western blot in A. The bands were quantified by scanning on a densitometer and fold difference in signal intensities relative to the zero control were plotted.

expression of HSP70 protein in antimony resistant *L. tarentolae* and *L. infantum* laboratory mutants and the increased expression of HSP70 was stable after hundreds of passages without the drug [10]. If the linkage between HSP70 overexpression and antimony resistant phenotype observed in those laboratory mutants is also true for *L. donovani* field isolates, *HSP70* gene could be one of the important biomarker for monitoring antimony resistance in the field conditions. With this hypothesis we have cloned the *HSP70* gene in bacterial expression vector, purified the recombinant protein and raised the polyclonal antibody against HSP70 antigen in BALB/C mice. We checked the HSP70 protein expression in two antimony sensitive (AG83-S and 2001-S) and three natural antimony resistant (41-R, CK2-R and NS2-R) *L. donovani* clinical isolates using Western blotting. We found constitutive expression of HSP70 protein in both SAG-S and SAG-R clinical isolates. No difference in HSP70 protein expression was observed in antimony sensitive and resistant field isolates.

Further, we characterized the *HSP70* gene in SAG-S and SAG-R *L. donovani* Indian field isolates to check the chromosomal localization and copy number of the gene. The copy number of *HSP70* gene has been reported to be considerably varied among different trypanosomatids such as single copy in *L. braziliensis*, 2 copies in *L. tarentolae*, 4 copies in *L. major*, 6 copies in *L. infantum*, 7 copies in *L. amazonensis* and *T. cruzi* [5, 6, 10, 14, 17, 18]. In *L. donovani* Indian field isolates, the copy number of HSP70 gene was found to be single copy. No difference in gene copy number was observed in between SAG-S and SAG-R field isolates. To further verify the *HSP70* gene amplification, we performed Southern blot hybridization of PFGE blot. *HSP70* probe hybridized to a highly amplified single band at the 1.2 Mb region which corresponds to the 28 chromosome of the *L. infantum* genome database.

Transcript level of the *HSP70* gene has been shown to be transiently induced by environmental stress such as exposure to heat shock or heavy metals [19]. During heat shock, *HSP70* transcription rate increases rapidly and then decline slowly [20]. Increased *HSP70* transcript level has been shown in *Leishmania* when cells were exposed to heat [6]. The transcription mechanism has shown to be controlled at the posttranscriptional level specifically by the 3'-untranslated region of the gene in contrast to most other organisms [7, 21]. Similar regulation has also been described in the related parasite *T. brucei* [5]. Increased expression of HSP70 has been reported in antimony resistant *L. tarentolae* and *L. infantum* mutants compared to wild type cells [10]. The increased expressions of HSP70 in laboratory mutant were found to be stable after hundreds of passages without the drug [10]. Earlier studies indicated that HSP70 overexpression in antimony resistant mutants was the stable phenotype. To check whether similar mechanism is operating in field condition, we performed Northern blot hybridization of *HSP70* gene in between natural antimony resistant *L. donovani* clinical isolates to that of the antimony sensitive parasites. No difference in HSP70 transcript level was found between SAG-S and SAG-R clinical isolates.

Arsenite and Sb III have been described as the known inducer of the HSP70 in *Leishmania* [10, 22, 23]. By gene transfection experiment, it has been shown that HSP70 contribute first line of defense against Sb III [10] suggesting the role of HSP70 in antimonial drug resistance. In the present study, we checked HSP70 protein expression in promastigotes of *L. donovani* (AG83-S) in presence of Sb III. Interestingly, we could find considerable over expression of HSP70 protein when cells were incubated in IC_{50} concentration of Sb III. Protein over expression was about 1.7 fold at 12 hours that increased up to 3 fold at 24 hours time period. The observed results suggest that *Leishmania* cells respond to antimonial drug stress by over expression of the HSP70 protein.

Acknowledgements

The authors thank University Grants Commission, New Delhi, India for providing JRF/SRF to Mahendra Maharjan to conduct this study.

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