ISSN 1735-1383

Iran. J. Immunol. June 2009, 6 (2), 75-86

Manoochehr Rasouli, Ahmad Zavaran Hoseini, Bahram Kazemi, Abdolvahab Alborzi, Simin Kiany

Expression of Recombinant Heat-Shock Protein 70 of MCAN/IR/96/LON-49, a Tool for Diagnosis and Future Vaccine Research

Article Type: RESEARCH ARTICLE

The *Iranian Journal of Immunology* is a quarterly Peer-Reviewed Journal Published by Shiraz Institute for Cancer Research and the Iranian Society of Immunology & Allergy, Indexed by Several World Indexing Systems Including:
Index Medicus and Pubmed

For information on author guidelines and submission visit:

www.iji.ir

For assistance or queries, email:

iji@sums.ac.ir

Expression of Recombinant Heat-Shock Protein 70 of MCAN/IR/96/LON-49, a Tool for Diagnosis and Future Vaccine Research

Manoochehr Rasouli[1], Ahmad Zavaran Hoseini[1]*, Bahram Kazemi[2], Abdolvahab Alborzi[3], Simin Kiany[3]

[1]Department of Immunology, Faculty of Medical Sciences, Tarbiat Modares University, [2] Department of Parasitology, Shahid Beheshti University, Tehran [3] Division of Immunology, Prof. Alborzi Clinical Microbiology Research Center, Shiraz University of Medical Sciences, Shiraz, Iran

ABSTRACT

Background: Heat shock protein 70 (HSP70) is present in all organisms studied so far, and is a major immunogen in infections caused by pathogens including *Leishmania* spp. **Objective:** The aim of this study was to clone and express HSP70 from *L. infantum* strain MCAN/IR/96/LON-49 and evaluate antibody response against HSP70 in visceral leishmaniasis (VL). **Methods:** The *L. infantum* HSP70 gene segment was amplified by specific primers. It was cloned into pTZ57R vector and subcloned into pET32a (+) expression vector. The new construct was transformed in the *E.coli* Rosetta strain, and HSP70 protein was expressed in the presence of 1 mM IPTG and purified using a HiTrap chelating column. Antibody responses against HSP70 were determined by ELISA in 37 patients with visceral leishmaniasis and 63 healthy controls. **Results:** Expression of HSP70 protein was confirmed using SDS-PAGE electrophoresis and dot blot with an anti-His tag antibody. There was no difference between the sequence of nucleotides of the HSP70 gene in the present study and other reported sequences. The ELISA results indicated that the sera of 81.1% (30/37) of the patients and 6.3% (5/63) of controls reacted to *L. infantum* HSP70. **Conclusion:** The conservative nature of the HSP70 molecule is an advantage in vaccine studies, because of minor differences (6%) between the nucleotide sequences and consequently the similarity in amino acid sequences in various strains of *L. infantum.* It could therefore be used in vaccine research against leishmaniasis and also as a tool for serodiagnosis.

Keywords: *Leishmania infantum*, Heat shock protein 70, Molecular cloning

INTRODUCTION

Parasites of the genus *Leishmania* are among the most diverse human pathogens in terms of both distribution and the variety of clinical syndromes they caused. The para

*Corresponding author: Dr. Ahmad Zavaran Hoseini, Department of Immunology, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran. Tel: (+) 98 21 82883090, Fax: (+) 98 21 88220144, email: zavarana@modares.ac.ir

sites cause several forms of human diseases, including cutaneous leishmaniasis, muco sal leishmaniasis, and visceral leishmaniasis (VL) (1). It is estimated that 350 million people are at risk of acquiring infection with *Leishmania* parasites and 12 million people suffer from leishmaniasis. Furthermore, it is estimated that 2 million new cases appear annually (2).

Researchers have made serious attempts to find suitable vaccine candidates for leishmaniasis. Most of them have concentrated on antigens such as glycoprotein 63, cystein protease A and B, Lac, glycoprotein 64, M2, PSA2, LmST11, P2, A2, Leif, P8, Histone H1 and ribosome-like proteins (3-7). To achieve the desired response for the prevention of disease progression, antigens with immune-enhancing properties or adjuvants must be identified. Adjuvants provide immunostimulatory signals which can be either pathogen-derived substances or compounds related to the host itself (8). Heat-shock proteins (HSP) are prototypes of immune-enhancing structures which can act as the second signal, stimulating antigen-presenting cells and promoting Th1 responses (9).

Among HSP, HSP70 in particular is involved in the activation of antigen-presenting cells inducing cytokine secretion and the upregulation of molecules involved in antigen presentation (MHC class I, MHC class II and costimulatory markers), and in adhesion (10-13). In vivo, HSP70-peptide complexes or peptide-HSP70 fusion proteins act as a chaperon of peptides and activate T- or B-mediated adaptive immunity (10, 11, 14-16).

Several reports have shown that HSP70 family members from Trypanosomatidea can be recognized by sera from patients with VL (17-19). Since antibody response occurs against HSP70 molecule of *Leishmania* parasites, it can be considered a protein potentially useful for serodiagnosis of *Leishmania* diseases, especially the visceral form. The aim of this study was to clone the HSP70 gene of *L. infantum* strain MCAN/IR/96/LON-49 into pTZ57R, subclone it into expression vector pET23a (+) and express and purify the corresponding protein. The evaluation of rHSP70 as a diagnostic tool for VL was another objective.

MATERIALS AND METHODS

Leishmania infantum. *Leishmania infantum* strain MCAN/IR/96/LON-49 promastigotes were grown at 22 °C in RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum (Gibco-BRL, USA), and 100 µg/ml gentamycin (Sigma, USA). Stationary phase promastigotes were incubated at 37 °C for 30 min to induce HSP gene transcription, and then the cells were harvested at a concentration of 1×10^7 cells/ml.

RNA Extraction and cDNA Synthesis. Total RNA was extracted from 1×10^7 promastigotes in 1 ml RPMI 1640 medium using a RNX-Plus^{TM} (Cinnagen,Iran) kit and protocol. The cDNA was synthesized with a RevertAid^{TM} H Minus First Strand cDNA Synthesis kit (Fermentas, Lithuania).

Polymerase chain reaction (PCR) and Insert Preparation. PCR amplification of the HSP70 gene was performed in a 50 µL volume using 1x PCR buffer (Cinnagen), 250 ng cDNA, 2.5 units of *Pfu* DNA polymerase (Cinnagen), 0.5 µM of each primer (*Hind*III HSP70F: 5'-**AAGCTT**ATGACATTCGAAGGC GCCAT-3' and *Xho*I HSP70R: 5'-**CTCGAG**GTCGACCTCCTCGACCTTGG-3'), 5.5 mM $MgSO_4$, and 200 µM of each dNTP under the following conditions: a denaturation step for 5 min at 95 °C followed by 35 cycles of PCR consisting of 1 min at 94 °C, annealing for 1 min at 55 °C, exten-

sion for 2 min at 72 °C and a final extension for 5 min at 72 °C. To add the 3' A tail, 2.5 units of *Taq* DNA polymerase was added to the PCR cocktail and incubated at 37 ºC for 30 min. The PCR products were analyzed on 1% agarose LE gel after electrophoresis and staining with ethidium bromide. The1989-bp band was considered as the desired DNA segment. It was cut and purified from the gel with a QIAquick Gel Extraction kit (Qiagen,USA) following the manufacturer's recommendations.

HSP70 Gene Cloning. The purified PCR products were cloned into the pTZ57R/T cloning vector and transformed in *Escherichia coli* strain DH5-α with an InsTAclone™ PCR Cloning kit (Fermentas) according to the manufacturer's protocol. Then the transformed cells were plated on Luria-Bertani (LB) agar containing 50 µg/ml ampicillin, 200 µg/ml IPTG and 20 µg/ml X-gal to screen for blue and white colonies (nonrecombinant and recombinant plasmids, respectively), and incubated at 37 ºC overnight. Ten white colonies were randomly selected and inoculated on LB agar containing ampicillin (50 µg/ml).

Miniprep Plasmid Extraction and Clone Confirmation. Four pure colonies were picked up and suspended in 90 µl of TE buffer (10 mM Tris-HCL, pH 7.4; 1 mM EDTA, pH 9.0) in 1.5 ml capped microtubes. Then 180 µl of SDS-NaOH solution (1% SDS, 0.2 M NaOH) was added and mixed gently by inversion. After 5 min of incubation at room temperature, 135 µl of 3M sodium acetate (pH 5.2) and 390 µl chloroform were added to each microtube, mixed, and centrifuged at 12000 RCF for 5 min. The upper phase of each microtube was transferred into new tubes and 390 µl isopropanol was added, and the tubes were inverted several times. The tubes were then centrifuged (12000 RCF, 10 min) and the supernatants were removed. The pellets were washed with 70% ethanol, air dried, and dissolved in 30 µl distilled water. The clones were confirmed with restriction endonucleases *Hind*III and *Xho*I analysis and electrophoresis on 1% agarose gel. The confirmed plasmid construct was sequenced by the Gastroenterology Research Center affiliated with Shahid Beheshti University of Medical Sciences, Tehran, Iran.

Subcloning of HSP70 Gene into pET32a (+). The confirmed plasmid containing the HSP70 gene and pET32a (+) expression vector (purchased from the Pasteur Institute of Iran, Tehran) was digested by *Hind*III and *Xho*I endonucleases. After electrophoresis, the desired 1989-bp and 5900-bp bands [HSP70 gene and pET32a (+), respectively] were purified from agarose gel. The HSP70 gene was subcloned into pET32a (+) and confirmed as previously mentioned, except that IPTG and X-gal were not used and only the white colonies appeared on LB agar plates. The new construct was named pET32/hsp70.

Expression and Purification of Recombinant HSP70 (rHSP70). *Escheriochia coli* Rosetta strain was transformed with pET32/hsp70 and grown at 37 ºC in LB broth containing ampicillin (50 µg/ml). To produce the recombinant HSP70, the culture was grown to an optical density (OD) of 1 at 600 nm. One milliliter of the culture was chosen as a noninduced control, and protein expression was induced with 1 mM IPTG for 4 h at 37 ºC. rHSP70 was purified by FPLC chromatography using a HiTrap chelating HP column (Amersham Biosciences,Sweden) under denaturing conditions according to the manufacturer's protocol. Immunoblots with anti-His antibody were performed to check the identity of the protein. The purity of the protein was assessed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie blue staining.

Patient and Control Sera. Sera were obtained from 37 infants with VL hospitalized in Namazi Hospital, Shiraz, Iran. Their diagnosis was confirmed based on the observation

of *Leishmania* amastigotes in stained bone marrow smears. Sera were also obtained from 63 healthy infants living in areas endemic for VL but without any history of the disease, and were used as negative controls. Having obtained informed consent from the parents of both the control and the patients, we collected blood samples from them.

Enzyme-Linked Immunosorbent Assay (ELISA). To perform ELISA, 96-well plates were coated with 100 µl of rHSP70 at a concentration of 2.5 µg/mL in 0.05 M sodium carbonate buffer, pH 9.6, and incubated at 4 ^{o}C overnight. Afterwards the plates were washed 6 times with 300 µl/well of phosphate buffered saline (PBS), pH 7.4, containing 0.05% Tween-20 (PBS-T) and blocked with 250 µl/well of 1% bovine serum albumin (BSA) in PBS for 1 h at 37 ^{o}C. After 6 washes with PBS-T, 100 µl of diluted sera (1:50 in PBS-T containing 0.5% BSA) was added to each well and the plates were incubated at 37 ^{o}C for 2 h. The plates were washed, and 100 µl of horseradish peroxidase-conjugated anti-human IgG (Sigma) was added to the wells and incubated for 1 h at 37 ^{o}C. The plates were washed as before, and 100 µl of 3,3′,5,5′-tetramethylbenzidine substrate (Cell Signalling Technology) was added to each well; then the plates were incubated at room temperature in the dark for 15 min. The reaction was stopped by adding 50 µl of 0.5 M sulfuric acid (Merck, Germany), and absorbances of the wells was read at 450 nm.

Statistical Analysis. The cut-off value for positivity was defined as the median plus two standard deviations of the absorbance of the negative control group (18). Statistical significance of the differences in mean absorbances were determined by Student's *t*-test, with the level of significance set at <0.05.

RESULTS

Induction of HSP70 transcripts in *Leishmania infantum.* After growing the promastigotes of *L. infantum* strain MCAN/IR/96/LON-49 at 22 °C, the stationary phase of the promastigotes was incubated at 37 °C for 30 min to induce HSP70 gene transcription. This process resulted in an increased HSP70 mRNA level. The PCR reaction with HSP70 cDNA of heat-shocked promastigotes showed stronger bands compared to the unshocked counterparts in the electrophoretic analysis (data not shown).

Cloning and Subcloning of HSP70 Gene Fragment. The 1989-bp DNA fragment corresponding to the HSP70 gene was amplified successfully using DMSO as a chemical denaturant in the PCR cocktail (Figure 1). The PCR-amplified product was cloned successfully into pTZ57R/T after being extracted from agarose gel. The target DNA was obtained by *Hind*III/*Xho*I digestion and verified by agarose gel electrophoresis (Figure 2). This construct was sequenced and the result (Figure 3) was compared with the data available in GenBank (accession numbers X85798 and XM_001470287). Our results showed 94% identity with the data available in GenBank (data not shown). Figure 4 shows the subcloning of HSP70 gene into pET32 a (+) after digestion with *Hind*III/*Xho*I.

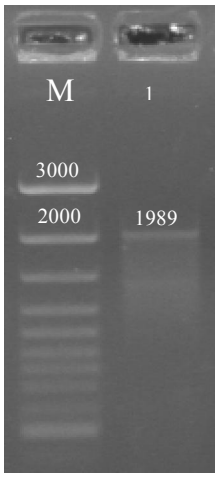

Figure 1. Electrophoretic analysis of HSP70 PCR product. Lane 1, 1989-bp band corresponds to HSP70 gene. Lane M shows a 100-bp DNA ladder.

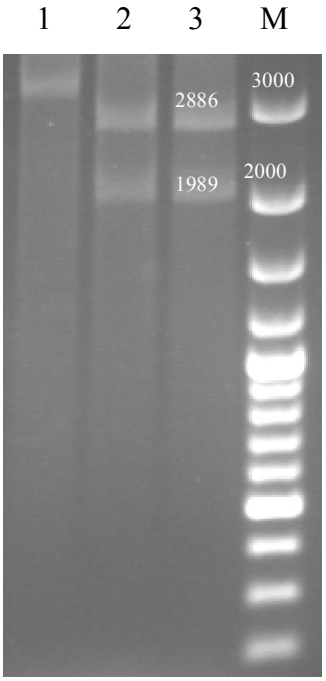

Figure 2. Electrophoretic analysis of pTZ57R/hsp70 construct. Lane 1, uncut plasmid. Lanes 2 and 3 plasmid digested with *Hind*III/*Xho*I restriction endonucleases. Lane M, 100-bp DNA ladder

Expression and Purification of Recombinant HSP70 in E. coli. The prokaryotic expression vector pET32/hsp70 was transformed in the *E. coli* Rosetta expression host and HSP70-expressing clones were screened using PCR and confirmed with *Hind*III/*Xho*I digestion and electrophoretic analysis (Figure 5). *Escherichia coli* Rosseta/pET32a (+)/hsp70 was induced by 1 mM IPTG for 4 h. The expression of HSP70 was detected as the appearance of a unique band with a molecular mass of more than 66 kDa in SDS-PAGE (Figure 6). The results of dot blot tests with the anti-His tag confirmed the presence of rHSP70 (Figure 7). With the HiTrap chelating HP column, rHSP70 showed good purity by SDS-PAGE analysis (Figure 8).

ATGACATTCGAAGGCGCCATCGGCATCGACCTGGGCACGACGTACTCGTG
CGCGGGTGTGTGGCAGAACGAACGCGTGGACATCATCGCGAACGACCAGG
GTAACCGCACGACACCGTCGTACGTTGCGTTCACGGACTCGGAGCGGCTGA
TCGGCGATGCCGCAAAGAACCAGGTGGCAATGAACCCGCACAACACGGTGT
TCGACGCGAAGCGCCTGATTGGCCGCAAGTTCAACGACTCGGTTGTGCAGT
CGGACATGAAGCACTGGCCGTTCAAGGTGACGACGAAGGGCGCCGACAAG
CCCGTGATTTCGGTGCAGTACCGCGGCGAGGAGAAGACCTTCACGCCGGAG
GAGATCAGCTCGATGGTGCTGCTGAAGATGAAGGAGACGGCGGAGGCGTA
CCTGGGCAAGCAGGTGAAGAAGGCCGTGGTGACGGTGCCGGCGTACTTCAA
CGACTCGCAGCGCCAGGCAACGAAGGACGCCGGCACGATTGCTGGCCTGGA
GGTGCTGCGCATCATCAACGAGCCGACGGCGGCGGCCATCGCGTACGGCCT
GGACAAGGGCGACGACGGCAAGGAGCGCAACGTGCTGATCTTCGACCTTGG
CGGCGGCACGTTCGATGTGACGCTGCTGACGATCGACGGCGGCATCTTCGA
GGTGAAGGCGACGAACGGCGACACACACCTTGGCGGCGAGGACTTCGACA
ACCGCCTCGTCACGTTCTTCACCGAGGAGTTCAAGCGCAAGAACAAGGGTA
AGAACCTGGCGTCGAGCCACCGCGCGCTGCGCCGTCTGCGCACGGCGTGCG
AGCGCGCGAAGCGCACGCTGTCGTCCGCGACGCAGGCGACGATCGAGATCG
ACGCGCTGTTCGAGAACGTTGACTTCCAGGCCACCATCACGCGCGCGCGCTT
CGAGGAGCTGTGCGGCGACCTGTTCCGCAGCACGATCCAGCCGGTGGAGCG
CGTGCTGCAGGACGCGAAGATGGACAAGCGCTCCGTGCACGACGTCGTGCT
GGTGGGCGGGTCCACGCGCATCCCGAAGGTGCAGAGCCTGGTGTCCGACTT
CTTCGGCGGCAAGGAGCTGAACAAGAGCATCAACCCCGACGAGGCTGTGGC
GTACGGCGCGGCGGTGCAGGCGTTCATTCTGACGGGCGGAAAGAGCAAGCA
GACGGAGGGCCTGCTGCTGCTGGACGTGACGCCGCTGACGCTGGGCATTGA
GACGGCCGGTGGCGTGATGACGGCGCTGATCAAGCGCAACACGACGATCCC
CACCAAGAAGAGCCAGATCTTCTCGACGTACGCGGACAACCAGCCCGGCGT
GCACATCCAGGTGTACGAGGGCGAGCGCGCGATGACGAAGGACTGCCACTC
GCTGGGCACGTTCGACCTGTCCGGCATCCCGCCGGCGCCGCGCGGTGTGCC
GCAGATCGAGGTGACCTTCGACCTGGACGCGAACGGCATCCTGAACGTGTC
CGCGGAGGAGAAGGGCACGGGCAAGCGCAACCAGATCACCATCACGAACG
ACAAGGGCCGTCTGAGCAAGGACGACATCGAGCGCATGGTGAACGACGCG
TCCAAGTACGAGCAGGCGGACAAGGAGCAGCGCGAGCGCGTGGACGCGAA
GAACGGGCTGGAGAACTACGCCTACTCGATGAAGAACACGATCAGCGACCC
CAACGTTGCCGGCAAGCTGGACGAGAGCGACAAGGAGGCGCTGAACAAGG
CGATCGAGGCGGCGCTGAGCTGGCTGAACAGCAACCAGGAGGCGTCGAAG
GAGGAGTACGAGCACCAGCAGAAGGAGCTGGAGAACACGTGCAACCCGAT
CATGACGAAGATGTACCAGAGCATGGGCGGCGCTGGCGGTGCTCCGGGCGG
CATGCCTGGCGGTATGCCAGACATGTCCGGCATGGGAGGCATGGGCGGCAT
GGGCGGTGCCTCGGCCCCCGACGCCGGCGCGTCCTCCGGCCCCAA**GG-**
TCGAGGAGGTCGAC

Figure 3. Nucleotide sequence of the HSP70 gene segment inserted into pTZ57R cloning vector. The bold nucleotides show the sequences used as primers.

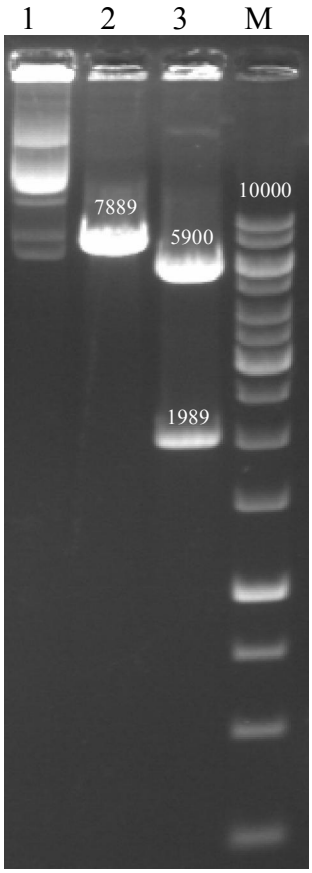

Figure 4. Electrophoretic analysis of pET32a (+)/hsp70 construct. Lane 1, uncut plasmid. Lane 2, pET32a(+)/hsp70 digested with *Hind*III. Lane 3, pET32a(+)/hsp70 digested with *Hind*III/*Xho*I. Lane M, 1-Kb DNA ladder.

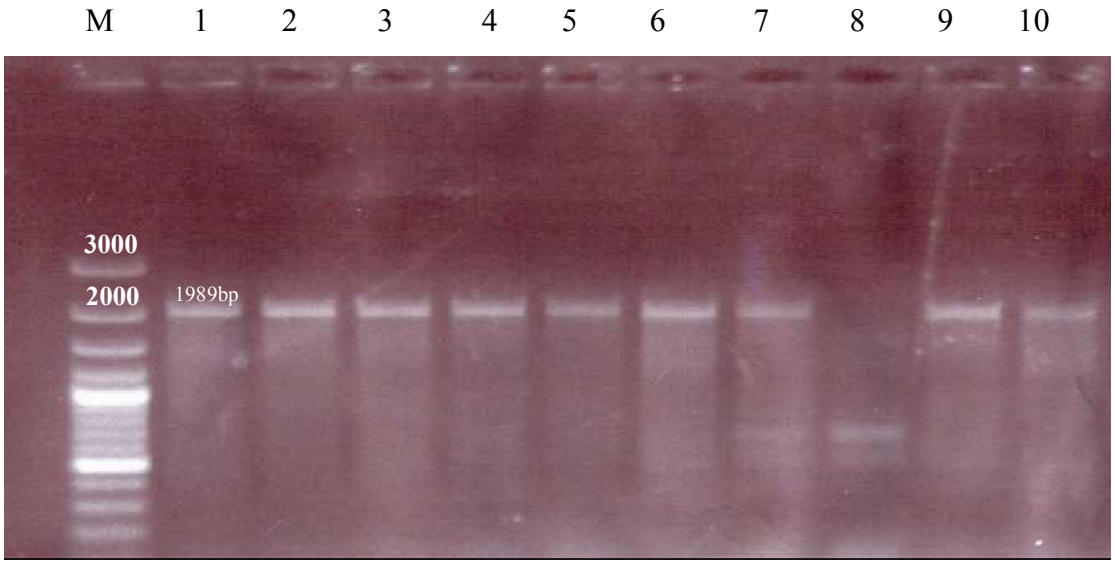

Figure 5. Screening of *E.coli* Rosetta clones expressing HSP70 using PCR. Lanes 1-7 and 9-10 show clones expressing HSP70, and Lane 8 shows a nonexpressing clone.

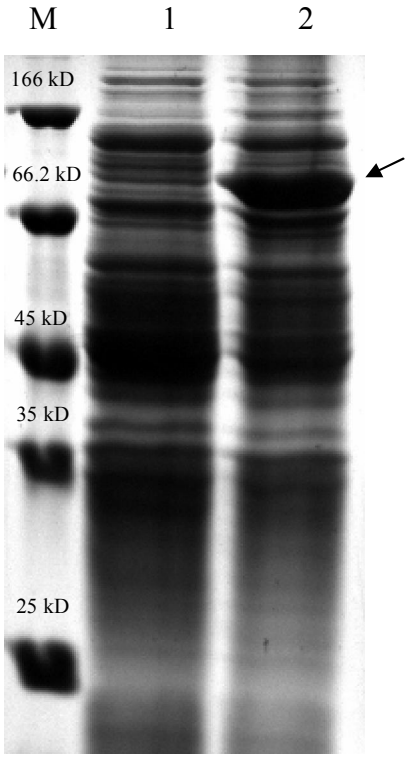

Figure 6. SDS-PAGE analysis of *E.coli* Rosetta transformed with pET32a(+)/hsp70 extract. Lane 1, extract of transformed bacteria not induced by IPTG. Lane 2, extract of transformed bacteria induced with 1 mM IPTG for 4 h at 37 ^{o}C (the arrow shows a band corresponding to the rHSP70 protein). Lane M, protein size marker.

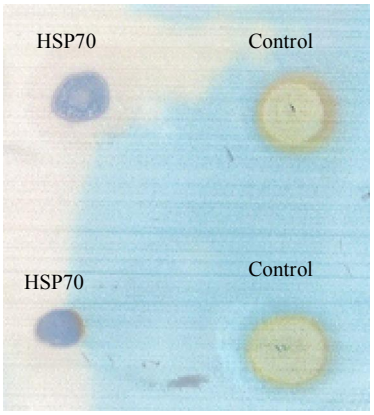

Figure 7. Dot-blot analysis using anti-His tag antibody. Blue dots show the extract of transformed bacteria (*E.coli* Rosetta/pET32a(+)/hsp70) induced with 1 mM IPTG, and yellow dots show the extract of the transformed bacteria without IPTG induction. The color version of this figure is available at: www.iji.ir.

Reactivity of Sera from Visceral Leishmaniasis Patients with rHSP70. An enzyme-linked immunosorbent assay was developed using rHSP70 as a diagnostic test for VL. The optimum rHSP70 concentration (2.5 µg/ml) and the optimum serum dilution (1:50) were determined with a checker board method (data not shown). The cut-off value for positivity, which was defined as the median plus two standard deviations in the absorbance of negative control group, was 1.425 (0.673 + 0.752).

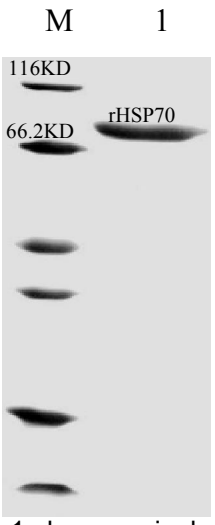

Figure 8. SDS-PAGE analysis. Lane 1 shows a single band corresponding to rHSP70 purified with a HiTrap Chelating HP column. Lane M, protein size marker.

Absorbances (mean ± S.D.) were significantly higher in patients with VL than in negative controls (1.931 ± 0.670 vs. 0.790 ± 0.376, $p<0.001$). The test showed negative results for 7 out of 37 patients with VL and positive results for 4 out of 63 negative controls (Figure 9). Therefore, the sensitivity and specificity of the test were 81.1% and 93.65%, respectively.

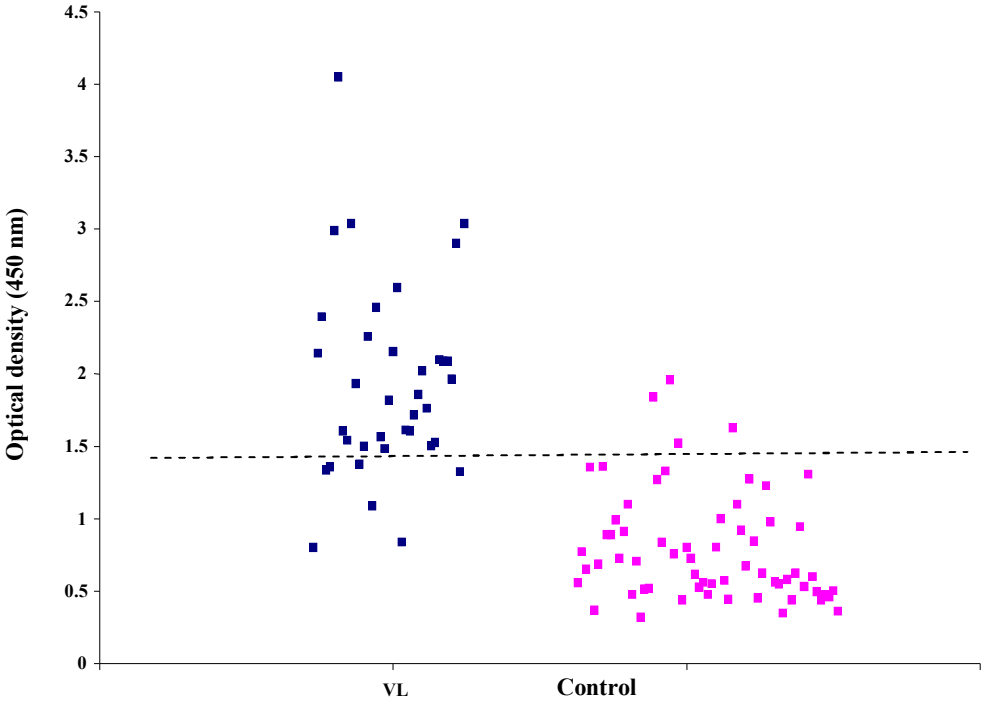

Figure 9. Comparison of antibody responses to *L. infantum* HSP70 in 37 visceral leishmaniasis patients (VL) and 63 negative controls. ELISA was used to determine HSP70-specific antibody responses. The dashed line shows the cut-off value (median +2 S.D. of the value in negative controls.

DISCUSSION

Heat shock proteins, which have been conserved from the earliest forms of life to the most advanced organisms, are important in protecting cells from stress, which was the first role attributed to them (20). Another role of HSP is their participation in immune responses (9). These proteins are potent inducers of innate and acquired immunity. It was demonstrated that HSP complexes bind to the professional APC via surface receptors (such as TLRs, scavenger receptors and CD19), are taken up into the APC where the peptide is processed and presented with MHC class I (21). Thus, HSP allow exogenous antigens access to the endogenous antigen-processing pathway.

Heat shock proteins may have a dual role in vaccine development against infectious diseases. Because pathogen-derived HSP might be the early targets in the immune responses against pathogens, they could be exploited as antigens for vaccine development (22). Moreover, because HSP potently stimulate innate and antigen-specific pathways, they are promising as vaccine adjuvants for a broad spectrum of pathogens (9). This role has been proposed for HSP70 derived from *Toxoplasma gondii*, which can induce dendritic cells maturation and stimulate IL-12 responses (23). In light of the importance of HSP molecules, we tried to clone and express HSP70 protein derived from *L. infantum* strain MCAN/IR/96/LON-49, a native strain in Iran, to be used as a tool for future studies of vaccines against *Leishmania* parasites and for the diagnosis of VL. The PCR product of HSP70 cDNA of heat-shocked promastigotes showed stronger bands in comparison to unshocked promastigotes. The effect of temperature on the transcription of HSP70 mRNA was consistent with the report of Requena and colleagues (24), who observed that incubation of the stationary phase of *L .infantum* promastigotes at 37 ^{o}C for 30 min increased HSP70 mRNA transcripts.

As shown in Figure 1, the appearance of a 1989-bp band in the agarose gel indicates successful amplification of the HSP70 gene. According to the data available in GenBank (http://www.ncbi.nlm.nih.gov), the *L. infantum* HSP70 gene is a GC-rich segment (more than 64%). Amplification of this kind of gene segment is hard and most of the time it is unsuccessful. This problem was solved by adding 5% DMSO to the PCR cocktail. The results of the present study suggest that DMSO could be useful for the successful amplification of GC-rich gene segments.

The HSP70 PCR product was cloned in pTZ57R/T, the newly synthesized construct was sequenced, and the results were compared with the data available in GenBank (Accession numbers X85798 and XM_001470287). The results obtained from sequencing revealed 94% identity between the nucleotide sequences reported in Genbank and ours. Similarity between the sequences of different strains of *L. infantum* is not surprising, because HSP are known as conserved proteins (20). The conservative nature of the HSP70 molecule is an advantage in vaccine studies; due to the minor differences between nucleotide sequences and consequently the similarity in amino acid sequences in various strains of *L. infantum HSP70*, it is possible to generalize the findings to other strains of the organism.

Several studies have shown that members of the HSP70 family are target antigens during the course of VL (25, 26). Hence, one of the aims of the present study was to test the usefulness of the rHSP70 as a target antigen for serodiagnosis of VL. Our data indicated that 30 out of 37 patients tested positive, leading to a sensitivity of 81.1%. Quijada and colleagues reported that 78.95% of the sera from VL patients reacted with *L. infantum* HSP70 (19), which is in agreement with our data. The percentage of positive sera shows

that *L. infantum* HSP70 is a major antigen during the course of the disease. We also found that 4 out of 63 negative controls had a positive test, although they did not have history of VL. This resulted in a specificity of 93.65%. Based on the conservative nature of HSP70 family members, a degree of cross-reactivity can be expected in serodiagnosis assays, and this was the likely cause of positive results in 6.3% (4/63) of our negative controls.

We therefore conclude that the conservative nature of the *L. infantum* HSP70 molecule is an advantage for future vaccine studies. The similarities of HSP70 nucleotides and consequently the similarity of its amino acid sequences among different strains of *L. infantum* allows researchers to expand these results to other strains of the parasite. This protein can also be used as a tool for the serodiagnosis of visceral leishmaniasis.

AKNOWLEDGEMENTS

We would like to thank Dr. Hassan Khajehei for providing editorial assistance. This study was financially supported by Tarbiat Modares University (grant No. 150/19172) and Prof. Alborzi Clinical Microbiology Research Center affiliated with Shiraz University of Medical Sciences (grant No. 85-2).

REFERENCES

1 Reed SG, Scott PA. Immunologic Mechanisms in Leishmania. In: Cunningham MW, Fujinami RS, (Editors). Effects of Microbes on the Immune system. 1st ed. Philadelphia, PA: Lippincott Williams & Wilkins; 2000. p. 537-54
2 Reithinger R, Dujardin JC, Louzir H, Pirmez C, Alexander B, Brooker S. Cutaneous leishmaniasis. Lancet Infect Dis. 2007;9:581-96.
3 Mauël J. Vaccination against Leishmania infections. Curr Drug Targets Immune Endocr Metabol Disord. 2002;2:201-26.
4 Webb JR, Kaufmann D, Campos-Neto A, Reed SG. Molecular cloning of a novel protein antigen of Leishmania major that elicits a potent immune response in experimental murine leishmaniasis. J Immunol. 1996;157:5034-41.
5 Taslimi Y, Zadeh-Vakili A, Taheri T, Doustdari F, Salmanian AH, Rafati S. Immunization with the hybrid protein vaccine, consisting of Leishmania major cystein proteinase type I (CPB) and type II (CPA), partially protects against leishmaniasis. Vaccine. 2004;22:1930-40
6 Ahmed SB, Bahloul C, Robbana C, Askari S, Dellagi K. A comparative evaluation of different DNA vaccine candidates against experimental murine leishmaniasis due to L.major. Vaccine. 2004;22:1631-9.
7 Rafati S, Salmanian A, Hashemi K, Schaff C, Belli S, Fasel N. Identification of Leishmania major cysteine proteinases as targets of immune response in humans. Mol Biochem Parasitol. 2001; 113:35-43.
8 Hilf N, Radsak M, Schild H. Host derived adjuvants. In: Kaufmann SHE (Editor). Novel vaccination strategies. 1st ed. Weinheim, Germany: Wiley-VCH; 2004. p.129-45.
9 Segal BH, Wang XY, Dennis CG, Young R, Repasky EA, Manjili MH, et al. Heat shock proteins as vaccine adjuvants in infections and cancers. Drug Discov Today. 2006;11:534-40
10 Asea A, Kraeft SK, Kurf-Jones EA, Chen LB, Finberg RW, Koo GC, et al. HSP70 stimulates cytokine production through a CD14-dependent pathway, demonstrating its dual roles as a chaperon and cytokine. Nat Med, 2000;6:435-42
11 Somersan S, Larsson M, Fonteneau JF, Basu S, Srivastava P, Bhardwaj N. Primary tissue lysate are enriched in heat shock proteins and induce the maturation of human dendritic cells. J Immunol, 2001;167:4844-52
12 Basu S, Binder RJ, Suto R, Anderson KM, Srivastava PK. Necrotic but not apoptotic cell death releases heat shock proteins, which deliver a partial maturation signal to dendritic cells and activate the NF-kappa B pathway. Int Immunol. 2000;12:1539-46.
13 Kuppner MC, Gastpar R, Gelwer S, Nössner E, Ochmann O, Scharner A, et al. The role of heat shock protein (hsp70) in dendritic cell maturation: hsp70 induces the maturation of immature dendritic cells but reduces DC differentiation from monocyte precursors. 2001;31:1602-09
14 Melcher A, Todryk S, Hardwick N, Ford M, Jacobson M, Vile RG. Tumor immunogenicity is determined by the mechanism of cell death via induction of heat shock protein expression. 1998;4:581-87
15 Wallin RP, Lundqvist A, Moré SH, von Bonin A, Kiessling R, Ljunggren HG. Heat-shock proteins as activators of the innate immune system. Trends Immunol. 2002;23:130-5.
16 Nicchitta CV. Re-evaluating the role of heat-shock protein-peptide interactions in tumour immunity. Nat Rev Immunol. 2003;3:427-32
17 De Andrade CR, Kirchhoff LV, Donelson JE, Otsu K. Recombinant Leishmania Hsp90 and Hsp70 are recognized by sera from visceral leishmaniasis patients but not chagas' disease patients. J Clin Microbiol. 1992;2:330-5
18 Krautz GM, Peterson JD, Godsel LM, Krettli AU, Engman DM. Human antibody responses to Trypanosoma cruzi 70-kD heat-shock proteins. Am J Trop Hyg. 1998;2:137-43.

19 Quijada L, Requena JM, Soto M, Alonso C. Analysis of the antigenic properties of the L.infantum HSP70: design of synthetic peptide for specific serodiagnosis of human leishmaniasis. Immunol Let. 1998;63:169-74
20 Binder RJ. Haet-shock protein-based vaccines for cancer and infectious disease. Expert Rev Vaccines. 2008;3:383-93.
21 Srivastava P. Roles of heat-shock proteins in innate and adaptive immunity. Nat Rev Immunol. 2002;2:185-94.
22 Zugel U, Kaufmann SH. Immune response against heat shock proteins in infectious diseases. Immunobiology. 1999;201:22-35
23 Kang HK, Lee HY, Lee YN, Jo EJ, Kim JI, Aosai F, et al. Toxoplasma gondii-derived heat shock protein 70 stimulates the maturation of human monocyte-derived cells. Biochem biophys Res Commun. 2004;322:899-904
24 Folgueira C, Quijada L, Soto M, Abanades DR, Alonso C, Requena JM. The translational efficiencies of the two Leishmania infantum HSP70 mRNAs, differing in their 3'-untranslated regions, are affected by shifts in the temperature of growth through different mechanisms. J Biol Chem. 2005; 280:35172-83.
25 Wallace GR, Ball AE, MacFarlane J, el Safi SH, Miles MA, Kelly JM. Mapping of a visceral leishmaniasis-specific immunodominant B-cell epitope of Leishmania donovani Hsp70. Infect Immun. 1992;60:2688-93.
26 Amorim AG, Carrington M, Miles MA, Barker DC, de Almeida ML. Identification of the C-terminal region of 70 kDa heat shock protein from Leishmania (Viannia) braziliensis as a target for the humoral immune response. Cell Stress Chaperones. 1996;1:177-87