The production of molecular clones of *Toxoplasma gondii-derived* heat shock protein 70 kDa

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Abstract

Toxoplasmosis is a common and widespread infection in humans and many other species of warm–blooded animals. *Toxoplasma gondii*-derived heat shock protein 70 (Hsp70) may play an important role in the virulence of *Toxoplasma gondii (T. gondii)*. In the present study, *T. gondii* Hsp70 was amplified by polymerase chain reaction (PCR) from the DNA of the *T. gondii* tachyzoite RH strain through the use of specific primers with Xho1 and Xba1 restriction sites. The purified DNA fragment of the *T. gondii* Hsp70 gene was subcloned into the Xho1 and Xba1-digested eukaryotic expression vector, pcDNA3, and subsequently transformed into TOP10 chemically competent cells. A 2004 base pair (bp) band of PCR product was observed on the 0.8% agarose gel. The cDNA was inserted into the pTZ57R/T vector and then subcloned successfully into the pcDNA3 eukaryotic expression plasmid vector. The sequence of this amplified gene showed up to 100% homology with the target gene according to the Genbank database (Accession no. U82281).

Introduction

Toxoplasma gondii (*T. gondii*) is an obligate intracellular parasite that produces opportunistic infections in many different species of mammals and birds. In intermediate hosts, *T. gondii* exists in two forms: the proliferative tachyzoite phase, which is responsible for the dissemination of infection in the acute phase of disease, and the bradyzoite phase, which forms latent tissue cysts. The unique ability of *T. gondii* bradyzoites and tachyzoites to interconvert is clinically important because it is considered to be the underlying cause of toxoplasmic inflammation. This inflammation in immunocompromised hosts causes diseases such as encephalitis, chorioretinitis, and lymphadenitis (Ahmed *et al.,* 2004).

Heat shock proteins (HSPs) have been identified as a set of highly conserved polypeptides that are expressed in cells exposed to stress conditions. HSPs are grouped into families in accordance to their size in kilodaltons, which include Hsp90, Hsp70 and Hsp30 (Lyons *et al.,* 1988; Sambrook *et al.,* 2001). The Hsp70 family is well-characterized in a number of protozoa, including *T. gondii*, and its members are suspected to bind partially-folded proteins and assist in the translocation of proteins across membranes (Tsuji *et al.,* 1994). *T. gondii*-derived heat shock protein 70 (*T. gondii* Hsp70) messenger RNA was shown to be useful in the analysis of the conversion of *T. gondii* between the bradyzoite and tachyzoite phases. *T. gondii* Hsp70 inhibited the induction of nitric oxide release by peritoneal macrophages of mice that were infected with *T. gondii*. These results identify that *T. gondii* Hsp70 is a danger signal in lethal, acute infections (Mun *et al.,* 2000). It has been reported that the 70 kDa parasite stress protein may be part of an important survival strategy, by which virulent strains downregulate host parasiticidal mechanisms (Dobbin *et al.,* 2002). In the present study, we studied the clones and subclones of tachyzoite *T. gondii* cDNA that encode the C-terminal region of the Hsp70 protein family.

Material and Methods

Mice and parasites

Infection with the RH strain of *T. gondii* was maintained by intraperitoneal injection in Balb/c mice. After three days, 106 tachyzoites in the peritoneal fluid were collected and the cavity was washed with 4 mL of phosphate buffered saline (PBS).

DNA extraction

Tachyzoites were centrifuged at 900 g/min for 10 min, then lyzed in lysis buffer (100 mM Tris-HCl (pH 8.0), 1% sodium dodecyl sulphate (SDS), 100 mM NaCl, 10 mM EDTA, and proteinase K (100 µg/mL; Fermentas #EO0491) at 5ºC for 2 h). The genomic DNA was extracted by the phenol/chloroform method

followed by ethanol precipitation. Subsequently, the pellet was dissolved in TE buffer (10 mM Tris-HCl [pH 8.0] and 1 mM EDTA) and underwent polymerase chain reaction (PCR) amplification (Liu *et al.,* 2006).

PCR amplification and gel electrophoresis

We designed a pair of primers based on the *Toxoplasma* sequence from GenBank (Accession No. U85648) with Xho I and Xba I restriction sites at the 5` end of the forward and reverse primer respectively. These primers (5`- CTC GAG CAT GGC GGA CTC TCC TGC T -3` and 5`- TCT AGA TTA ATC AAC TTC CTC CAC GGT -3`) amplified a 2,003 base pair (bp) heat shock protein 70 C-terminal fragment. The PCR reaction contained 1x PCR buffer, 200 µM per dNTP 40 pmol per primer, 1.5 mM $MgCl_2$, 1.25 units Taq DNA polymerase (CinnaGen, Iran) and 100 ng genomic DNA in a final volume of 100 µL. The PCR reaction consisted of 30 cycles of denaturation at 940ºC for 1 min, annealing at 590ºC for 1 min and extention for 2 min at 72°C, with an initial denaturation for 5 min and the final extension for 5 min. The amplification products were analyzed by electrophoresis on a 0.8% agarose gel stained with ethidium bromide and visualized under a UV Transilluminator (syngene).

Production of clones of the Hsp70 gene in *Escherichia coli* (*E. coli*)

The PCR product (*T. gondii* Hsp70 gene) was purified by a DNA extraction kit (Fermentas #K0513) and cloned into pTZ57R/T cloning vector through the use of T4 DNA ligase (Fermentas#EL0335). The ligation mixture was transformed into *E. coli* TOP10 chemically competent cells (Invitrogen) and distributed on lysogeny broth (LB) agar that contained 100 µg/ml ampicillin, 20 mM IPTG and 20 mg/mL X-gal. White colonies were selected.

Plasmid extraction

The bacterial colonies that contained recombinant plasmid were mass-cultured in LB medium and the plasmid was extracted through the use of the alkaline method (Sambrook, et al., 2001). Extracted plasmid was electrophoresed on 0.8% agarose gel and visualized under the UV Transilluminator.

The plasmid that was produced was named pT-TgHSP70. The pT-TgHSP70 was digested by Xho1 and Xba1 enzymes. The product was analyzed by electrophoresis on a 0.8% agarose gel and was purified through the use of a DNA Extraction kit (Fermentas).

The production of subclones of Tg-HSP70 in expression vector

The purified DNA fragment (Hsp70 gene) was subcloned into the XhoI and XbaI digested eukaryotic expression vector pcDNA3 (Novagen) and transformed into TOP10 chemically competent cells (Invitrogen). Recombinant plasmid was extracted and confirmed by restriction analysis through the use of Xho1 and Xba1 restriction enzymes. Recombinant plasmid was purified from agarose gel through the use of the DNA Extraction kit and sequenced by dideoxy chain-termination method (MWG-Biotec AG).

Results

Results in Panel A indicates a 2003bp DNA fragment as a PCR product of *T. gondii* Hsp70 gene amplified from genomic DNA of the virulent RH strain (Figure 1). The PCR product was cloned into PTZ57R/T plasmid and transformed in to *E. coli* TOP10 strain successfully.

The integrity of the pcDNA3 and Hsp70 was checked by agarose gel electrophoresis after digestion with Xho1 and Xba1 restriction enzymes and PCR (Figure 2). The observation of a 2,003-bp band on 0.8% agarose gel indicated the correct direction of insertion into eukaryotic expression plasmid vector pcDNA3.

The sequence analysis results showed that there was a homology of 100% with *T. gondii* strain RH Hsp70 gene (complete cds. Accession Number U85648) and *T. gondii* Hsp70 mRNA (complete cds. Accession Number U82281). There was 99.8% homology with T. gondii Hsp70 mRNA (complete cds. AF045559), 99.3% with *T. gondii* hsp70 mRNA (complete cds. AB019539), 98.7% with *T. gondii* strain ME49 Hsp70 gene (complete cds. U85649), and 99.8% with Toxoplasma gondii mRNA for Hsp70, partial cds. AB031071 (Figure 3).

Discussion

HSPs are considered to be highly conserved molecules of the biosphere with a wide distribution in nature (Tsuji *et al.,* 1994). They are known to play an important role in the folding, unfolding or translocation of proteins, as well as in the assembly and disassembly of protein complexes. Due to their mentioned helper functions, HSPs have been termed molecular chaperones (Zügel *et al.,* 1999). Tachyzoites of *T. gondii* have been reported to express a specific type of HSPs that is known as *T. gondii* Hsp70 (*T.g.*Hsp70) (Lyons *et al.,* 1995). It has been reported that one of its major properties is as a down-regulator of host immune responses in *T. gondii* -infected mice. Expression of *T.g.*Hsp70 was increased in the *T. gondii*-infected mice just before death and therefore indicated the molecule to be a danger signal in lethal, acute *T. gondii* infection (Mun *et al.,* 2000; Piao *et al.,* 2004).

In the present study, we studied the expression of Hsp70 in a virulent RH strain of *T. gondii*. The gene was extracted and cloned into the eukaryotic expression vector pcDNA3. Sequence analysis indicated complete homology of our virulent *T. gondii* Hsp70 mRNA with

Figure 1: Panel A: Electrophoresis of PCR product for Hsp70 fragment of *T. gondii* amplification. Line 1: 1 kb ladder. Line 2: Hsp70 gene (2,003-bp). Panel B: Comparison of pTZ57R/T and pT-Tg Hsp70 plasmids by electrophoresis. Line 1: pTZ57R/T after digestion with two enzymes. Line 2: Plasmid extracted from blue (pTZ57R/T) colony (super coil). Line 3: Plasmid extracted from white (pT- Tg Hsp70) colony. Line 4: pT-Tg Hsp70 after enzyme digestion with two enzymes (Hsp70 2,003 bp, pTZ57R/T 2885 bp). Line 5: ladder 1 kb.

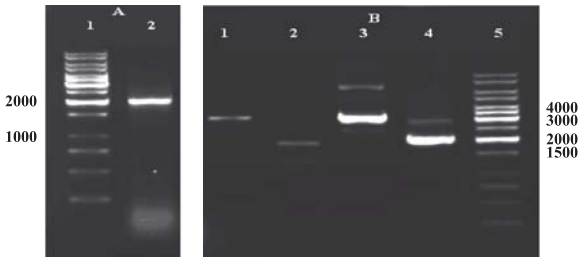
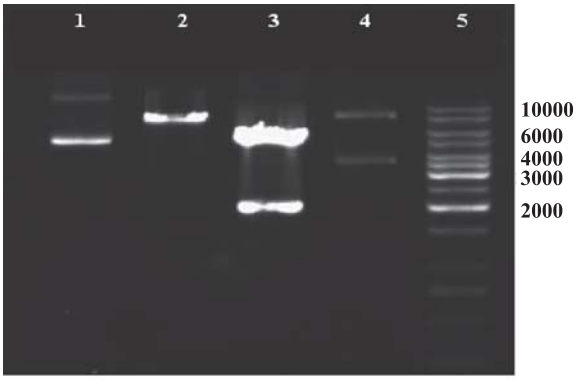


Figure 2: Comparison of pcDNA3 and pcDNA3 and Hsp70 plasmids and digestion of extracted pcDNA3 and Hsp70 after transformation. Line 1: Plasmid extracted from pcDNA3 & Hsp70 colony. Line 2: pcDNA3 and Hsp70 after enzyme digestion with Xho1 enzyme. Lines 3: pcDNA3 and Hsp70 after enzyme digestion with two enzymes (Hsp70, 2,003-bp, pcDNA3, 5,400-bp). Line 4: pcDNA3. Line5: ladder 1 kb.



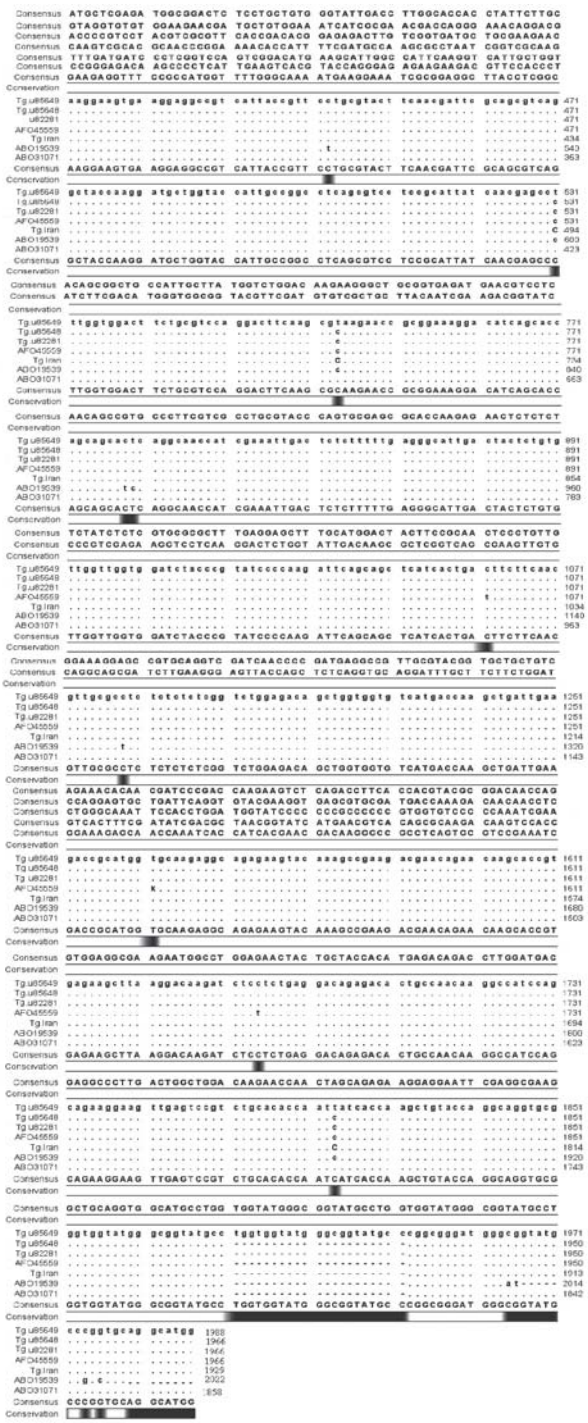
the Hsp70 gene under accession number U82281 and U85648.

Lyons & Johnson (1995) reported expression of Hsp70 only in virulent strains grown in vivo or in the immunocompetent host, while no expression was observed in virulent strains grown in vitro or in the immunocompromised host. However, molecular genetic studies have revealed the presence of a single copy of Hsp70 gene in both virulent and avirulent strains of *T. gondii* (Lyons *et al.,* 1988).

Mun *et al*., (2000) reported that destructive tachyzoites expressed low levels of *T.g.*Hsp70 whereasand virulent tachyzoites expressed high levels of T.g.Hsp70. After *T. gondii* infection, the conversion stage from encysted bradyzoites to destructive tachyzoites took more than three days, after which they converted to virulent tachyzoites (Ahmed *et al.,* 2004; Mun *et al.,* 2000).

In accordance with the sequence analysis of Lyons

Figure3: Comparison of Hsp70 gene amplified in the present study (Tghsp70, IRAN) with Hsp70 gene under accession number U82281, U85648, AF045559, AB019539, U85649, AB031071.



& Johnson (1988), the genes that encode Hsp70 in virulent and avirulent strains are identical at the amino acid level, with the exception of the number of seven residue repeat units (GGMPGGM) at the 3'-end of the

gene. In avirulent strains, five copies of this repeat are detected, while virulent parasites have only four copies (Johnson, 1999; Lyons *et al*., 1988).

Measurement of *T.g.*Hsp70 RNA expression in tissues may be of use in clinical diagnosis, in order to understand the inflammatory level caused by *T. gondii* infection (Multhoff, 2007). In addition, the role of *T.g.*Hsp70 in the functional maturation of dendritic cells suggests *T.g.*Hsp70 as a useful molecule for the development of a vaccine against *T. gondii* infection (Kang *et al.,* 2004).

Ahmed *et al.* (2004) demonstrated that vaccination with *T.g.*Hsp70 DNA vaccine strongly enhanced the induction of protective immunity in mice. Therefore, application of *T.g.*Hsp70 protein for the diagnosis of infection, prevention of disease and as chaperone molecules could be suggested as potential subjects for further studies.

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