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Gene Cloning of *Toxoplasma gondii* Heat Shock Protein 90

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Abstract: In the present study we have isolated *Toxoplasma* tachyzoites from infected mice, RNA was extracted and RT-PCR reaction was conducted to obtain the *Toxoplasma gondii* heat shock protein 90 cDNA and gene was amplified using PCR. A 2126 bp fragment as PCR product was cloned in pGEMEX-1 expression vector and confirmed by restriction analysis.

Key words: *Toxoplasma gondii*, heat shock protein 90, cloning

INTRODUCTION

Toxoplasma gondii is a ubiquitous obligate intracellular protozoan parasite, which distributes via host circulation and causes some cell defects. A hallmark of the asexual cycle is an interconversion between the rapidly dividing tachyzoite and the more slowly dividing bradyzoite, which is essential for disease propagation and causation. Tachyzoites are responsible for acute illness and congenital neurological disease, while bradyzoites can remain latent within the tissues for many years, representing a threat to immunocompromised patients (Markel *et al*., 1996).

Acquired toxoplasmosis is a benign disease which usually doses not any symptoms, while congenital toxoplasmosis is a malignant disease (Markel *et al*., 1996). Intra uterine infection varies based on pregnancy trimester. The risk of congenital disease is low (10 to 25%) when maternal infection occurs during the first pregnancy trimester and goes high (60 to 90%) during the third pregnancy trimester. However, congenital disease is more fatal when infection is acquired in the first trimester (Jones *et al*., 2003).

The induction of bradyzoite development *in vitro* has been associated with changes in temperature, pH and other stress inducers known also to activate the expression of heat shock proteins (Tomavo *et al*., 1991). *Toxoplasma* tachyzoites are release from host cell at acute phase of infection and invade to uninfected cells. The tachyzoite excrete an 82 kDa protein named heat shock protein 90 (Hsp90) to facilitating their invasion (Ahn *et al*., 2003). It is a potential drug target and inhibition of this protein may protect of infection (Echeverria *et al*., 2005).

Since there are not enough informations about the Hsp90, in this study we decided to clone the gene for further application e.g., prevention of disease, diagnosis of infection and using as chaperonic molecule in other investigations.

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MATERIALS AND METHODS

Parasite

Mice were infected via intraperitoneum by *Toxoplasma* tachyzoites RH strain. *Toxoplasma* tachyzoites were isolated one week after infection through mice peritoneum punction and rinsed by PBS buffer many times until ready for RNA extraction.

RNA Extraction

Toxoplasma total RNA was extracted from tachyzoites under RNase free condition described previously (Uckert *et al*., 1998) with brief modification. Briefly, were mixed tachyzoites with 200 μL RNX^{plus} buffer (CinnaGen Iran) containing phenol and guanidine. The mixture was incubated for 5 min at room temperature and then 50 μL of chloroform was added, mixed and centrifuged at 12000 rpm for 15 min at 4°C. Supernatant (containing RNA) was transferred to a new RNase free micro tube. Total RNA was precipitated by ethanol and finally solved in 10 μL of diethyl pyrocarbonate treated water (Uckert *et al*., 1998).

cDNA Synthesis

Reverse Transcription (RT) was performed under RNase free condition by using 5 μg of template RNA which was incubated in a 20 μL reaction mixture containing 40 pico mol of *Toxoplasma* Hsp90 specific antisense primer (HspR 5`-GAA TTC TTA GTC GAC CTC CTC CAT CT-3`), 100 unit of reverse transcriptase enzyme (MMULV, Fermentas, Lithuania), 1x RT buffer, 20 unit RNasine (Fermentas Lithuania) and 0.2 mM dNTP for 1 h at 42°C (Pfeffer, 1998).

PCR Reaction

PCR reaction was prepared in 50 μL volume containing 1 μg of synthesized cDNA, 40 pico mol concentration of each forward and reverse *Toxoplasma* Hsp90 specific primers (HspF 5`-GAG CTC ATA TGG CGG ACA CCG AGA CCT T- 3` and HspR 5`-GAA TTC TTA GTC GAC CTC CTC CAT CT-3), 1.5 mM $MgCl_2$, 0.1 mM dNTP, 1X PCR buffer, 1.5 unit of Taq DNA polymerase (CinnaGen, Iran). PCR process was carried out with 30 cycles in thermocycler machine (Eppendrof, personal model) under following conditions: denaturation at 94°C for 40 sec, annealing at 65°C for 60 sec and extension at 72°C for 60 sec. Before PCR cycle's initiation, reaction was incubated at 94°C for 5 min and after PCR cycle's termination, reaction was incubated at 72°C for 5 min (Pherson and Moller, 2000).

Electrophoresis

PCR products were submitted to electrophoresis using 1% agarose gel. Gel was stained by ethidium bromide and DNA band visualized under ultraviolet light (UV transilluminator) (Boffey, 1984).

Gene Cloning

EcoRV blunt digested pBluescript and PCR product were electrophoresed in 1% Low Melting Point (LMP) agarose gel and expected DNA bands were sliced under long wave UV. DNAs were recovered by DNA purification kit (Fermentas Cat. No. k0513). Recovered PCR product and EcoRV blunt digested pBluescript were 3` tailed using dATP and dTTP respectively by terminal deoxy nucleotidyl transferase (Eun, 1996; Gaastra and Klemm, 1984a). PCR product was ligated to pBluescript (Gaastra and Hansen, 1984b) via T/A cloning method, transformed into *E. coli* XLI-blue strain competent cells (Hanaham, 1983) and dispensed on LB agar plate containing 50 μg mL^{-1} ampicillin. Colonies were screened by X-gal and IPTG to discriminate between recombinant (white)

and no recombinant (blue) plasmids (Bothwell *et al*., 1990). SacI and EcoRI restriction sites were established on 5` end of forward and reverse primers respectively. Recombinant plasmid was digested by SacI and EcoRI enzymes and a 2126 bp DNA band fragment encoded protein of Hsp90 was released. Digested plasmid was electrophoresed in 1% low melting point agarose gel and released DNA fragment (Hsp90 gene) was sliced under long wave UV then recovered by DNA purification kit (Fermentas Cat. No. k0513). The purified DNA fragment was subcloned in SacI and EcoRI digested PGEMEX-I expression vector. The reaction was transformed in *E. coli* XL1- blue competent cells and the positive colonies containing plasmid was mass cultured in LB medium. Recombinant plasmid was extracted (Feliciello and Chinal, 1993) and confirmed by restriction analysis.

RESULTS

Toxoplasma tachyzoites were isolated from peritoneum of infected mice and rinsed by PBS buffer. Total *Toxoplasma* RNA was extracted and cDNA was synthesized by specific *Toxoplasma* HSP90 antisense primer. PCR reaction was carried out by specific *Toxoplasma* sense and antisense primers.

The 2126 bp as expected PCR product of *Toxoplasma* tachyzoite Hsp90 gene is shown in Fig. 1.

PCR product was cloned in Bluescript plasmid and then subcloned in PGEMEX-I expression vector. Figure 2 shows recombinant compare with no recombinant plasmid.

Figure 3 shows electrophoresis of digested plasmid on LMP agarose gel. Gel was sliced and recovered by DNA purification kit, recovered DNA was 3` T tailed by terminal transferase and used for T/A cloning method.

PstI enzyme has one restriction site on HSP90 sequence but PGEMEX-I haven't restriction site for this enzyme. Therefore, recombinant plasmid was digested by PstI, but PGEMEX-I didn't. Figure 4 shows recombinant plasmid digested with PstI enzyme and confirmation of recombinant plasmid.

The PCR product of *Toxoplasma gondii* heat shock protein 90 was sequenced and deposited to GenBank under accession number.

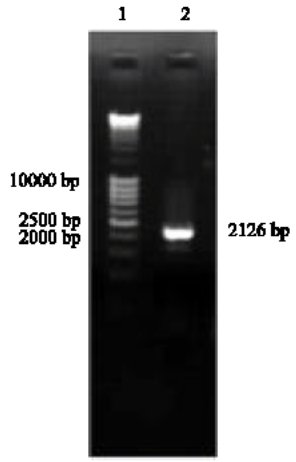

Fig. 1: Electrophoresis on 1% agarose gel, Lane 1: 100 bp DNA ladder marker, Lane 2: PCR product of *Toxoplasma* HSP90 gene (2126 bp)

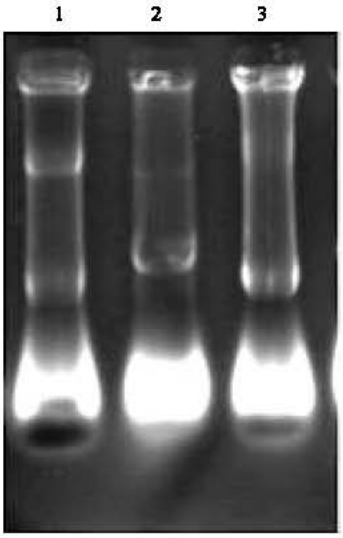

Fig. 2: Electrophoresis on 0.8% agaroe gel, Lanes 1 and 3: No. recombinant plasmid, Lane 2: Recombinant plasmid

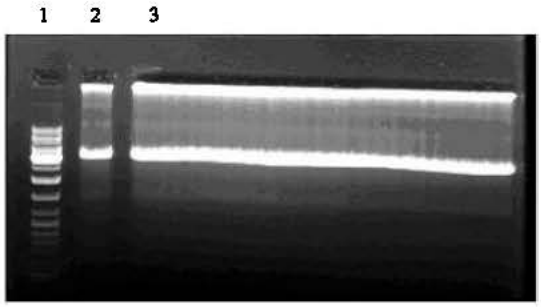

Fig. 3: Electrophoresis on 1% LMP agaroe gel, Lane 1: 100 bp DNA ladder marker, Lanes 2 and 3: Digested plasmid

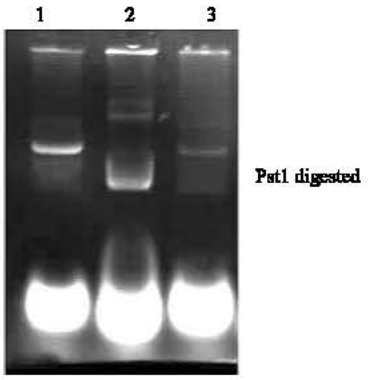

Fig. 4: Electrophoresis on 0.8% agaroe gel, Lanes 1 and 3: Plasmid, digested with PstI (recombinant) Lane 2: Plasmid, undigested with PstI (nonrecombinant)

DISCUSSION

Heat shock protein 90 (HSP90) excrete from extracellular tachyzoites of *Toxoplasma gondii* (Ahn *et al*., 2003). Although there are not enough informations about *Toxoplasma gondii* HSP90 molecule, but it must be important in toxoplasmosis. The 82 kDa heat shock protein (HSP90) is facilitating the entrance of tachyzoite into the host cells and its monoclonal antibody will protect this phenomena (Ahn *et al*., 2003).

Hsp90 is a chaperonic molecule which involves in different cell process like differentiation, apoptosis and cell proliferation (Heike *et al*., 1996; Sreedhar *et al*., 2004; Cox and Miller, 2003). This chaperonic molecule is very stable and has an important role in protein folding and some cell stresses (Zügelm and Kaufmannm, 1999). Heat shock proteins are used in vaccination in infection diseases (Zügelm and Kaymannm, 1999). It was used a DNA vaccine against *Candida albicans* in mouse (Raska *et al*., 2005) and also is a potential anticancer vaccine (Blachere *et al*., 1993). The *Toxoplasma* Hsp90 have cloned by Ahn *et al*. (2003) and they realized that Geldanamycin, a benzoquinone ansamycin antibiotic, known to interfere with HSP90 function, did not affect the secretion of *Toxoplasma* Hsp90 from extracellular tachyzoites. However, prevention of its expression will inhibit entrance of tachyzoite into the host cell as well as the intracellular proliferation (Ahn *et al*., 2003). Further studies are requisite about its function in living the parasite in host cell and its function as chaperonic molecule.

We like others (Ahn *et al*., 2003; Rojas *et al*., 2000) have cloned the heat shock protein gene of *Toxoplasma* tachyzoite named Hsp90 in expression vector and it is ready for further application e.g., prevention of disease, diagnosis of infection and using as chaperonic molecule in other investigations.

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