Original Article

Cloning of Dense Granular 7 (GRA7) Gene of *Toxoplasma gondii* into pTZ57RT Vectors

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Abstract

Background: Serological assay based on dense granular (GRA) proteins of *Toxoplasma gondii* (*T. gondii*) is actually the most popular laboratory diagnostic tool to detection of toxoplasmosis. We aimed to construct a recombinant GRA7-pTZ57RT plasmid vectors that it is suitable for sub-cloning and GRA7 protein production.
Materials and Methods: Souris mice were used for maintaining of *T. gondii* tachyzoites by serial intraperitoneal passage. The tachyzoites' DNA was extracted, and the GRA7 gene was amplified by PCR. The purified DNA was inserted into pTZ57RT cloning vectors, and then transformed into TOP10 competent cells. Finally, cloning and transformation were confirmed by restriction enzymatic digestion and gene sequencing.
Results: Agarose gel electrophoresis analysis on PCR products of genomic DNA, revealed 726 bp bands that were equal to the GRA7 gene. Both white (recombinant) and blue (non-recombinant) colonies appeared on ampicillin-LB agar. Results of enzymatic digestion and gene sequencing confirmed successful cloning and transformation procedures.
Conclusion: The GRA7 gene of *T. gondii* was cloned into pTZ57RT plasmid, which is suggested to be further used as DNA vaccine or sub-cloned for production of recombinant GRA7 protein.
Keywords: Cloning, Dense Granular Protein, Toxoplasmosis

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Introduction

Toxoplasmosis is a type of infectious disease caused by an obligate, intracellular parasite called *Toxoplasma gondii* (*T. gondii*) that if not treated, can lead to extremely serious complications[1]. *T. gondii* can infect almost all warm-blooded animals and humans beings, as well[2,3]. It is estimated that nearly one third of the human populations, worldwide, might harbor *T. gondii* chronic infection, though significantly varied rate of the infection have been observed among different countries[4,5].
T. gondii can usually be transmitted through, fetus placenta (congenital toxoplasmosis), ingestion of raw or partly cooked meats, unwashed fruits and vegetables and water contaminated with animal feces containing Toxoplasma cysts[6-8]. Although toxoplasmosis in immunocompetent individuals has no symptoms, in immunocompromised people (e.g. pregnant women, organ-transplant recipients and HIV/AIDS) can cause clinical problems such as fetus abortion, systematic organ failure and even death[9,10].
Laboratory diagnosis of toxoplasmosis is possible through several approaches including biological, histological, serological methods, and more recently by molecular tools[11-14]. Various serological assays such as latex agglutination test (LAT), indirect fluorescent antibody assay (IFA) and enzyme-linked

immunosorbent assay (ELISA) are used for detection of antibodies produced against *T. gondii* in suspected individuals[12,15,16].

Setting serological assays rely mostly on antigens obtained from tachyzoites (one of three infectious forms of *T. gondii*) and conventional production of these antigens is usually expensive, time consuming, and hazardous. However, recombinant DNA technology has brought up the opportunity for production of *T. gondii* recombinant antigens in larger scales, safer manner, and with lower costs[17]. Among many various tachyzoites antigens, dense granule (GRA) proteins of *T. gondii* present higher immunogenicity, are in abundance, and are widely expressed in infected hosts for a long duration of time[18]. It is suggested that the GRA proteins, in particular GRA7, have higher potential to be used as efficient tools for laboratory diagnosis, and also as therapeutic vaccine candidate for management of toxoplasmosis[19,20].

Many researchers have attempted to produce recombinant GRA proteins for diagnosis and therapeutic management of *T. gondii* infections[17,21]. Recently, Ching and colleagues, for instance, evaluated the specificity and sensitivity of GRA2 and GRA5 recombinant proteins for serodiagnosis of toxoplasmosis, using western blot, and found that this method is capable of distinguishing between present and past infections of *T. gondii*[17,22].

The goal of the present study was to construct a recombinant GRA7-pTZ57RT plasmid vectors for the purpose of sub-cloning of the GRA7 gene in prokaryotic and eukaryotic plasmids, in future.

Methods

Proliferation of *T. gondii* tachyzoites: Souris mice were purchased from the laboratory animal center of Shahid Beheshti University of Medical Sciences (Tehran, Iran), and were used for maintaining of *T. gondii* tachyzoites by serial intraperitoneal passage. Briefly, 0.5 ml of mouse peritoneal fluid containing 2×10^4 of fresh and active RH strain of *T. gondii* tachyzoites were injected intraperitoneally into mice. After lapse of 4-5 days following injection, the infected-mice were sacrificed, and the multiplied *T. gondii* tachyzoites were harvested from the peritoneal fluids, were centrifuged at 13000 rpm for 1 min. and re-suspended in sterile phosphate buffered saline (PBS) with pH 7.2 containing streptomycin-penicillin.

Tachyzoites DNA Extraction: The harvested *T. gondii* tachyzoites were washed twice with 10 mM PBS, followed by centrifugation at 13000 rpm for 1 min. (Eppendorf Micro-centrifuge 5417R) and re-suspension. DNA extraction was then performed using DNG™-Plus kit (SinnaGen Inc, Tehran, Iran), according to the manufacturer's instruction. In details, 100 µl of *T. gondii* tachyzoites suspension was mixed with 400 µl of DNG™-Plus solution in a l.5 ml microtube, and was vortexed for 15-20 seconds. After wards, 300 µl of Isopropanol was added to the mixture, followed by incubation at -20°C for 20 minutes, and then centrifugation at 12,000 rpm for 10 minutes. After that, the supernatant was removed, and 1 ml of 75% Ethanol was added to the pellet, was vortexed gently for 3-5 seconds, and centrifuged at 12,000 rpm for 5 minutes. This step was repeated once again. The Ethanol was then poured off completely, and the pellet was dried at 65°C for 5 minutes, up to dry. Finally, DNA pellet was dissolved in 50 µl of sterile distilled water by gentle shaking and placing at 65°C for 5 minutes, and were preserved at -20°C.

Amplification of GRA7 gene: The GRA7 gene of *T. gondii* was amplified by polymerase chain reaction (PCR) with a pair of primers, as follows;

GRA7 F: 5`- gga tcc atg gcc cga cac gca att- 3`

GRA7 R: 5`- gaa ttc cta ctg gcg ggc atc ctc- 3`

These primers had *BamHI* restriction sites to facilitate cloning.

PCR assay was carried out in a 15 µl reaction mixture containing 7.5 µl master mix (Bie & Berntsen A/S), 1 µl of equally-mixed primers, 1.5 µl DNA and 5 µl ddH_2O. The following thermal cycling condition was used for the PCR reaction: a 5-min hold at 96°C, followed then by 30 three-step cycles of; denaturation at 95°C for 30 seconds, annealing at 62°C for 30 seconds, and extension at 72°C for 45 seconds. The reaction was ended with a final 10 min extension at 72°C.

The amplified DNA was run onto 1% agarose gel containing Ethidium Bromide, and was then visualized under Ultraviolet transilluminator. Molecular weight of the PCR product was determined and verified using a 1000 bp standard ladder (Fermentas, Berlin, Germany). Finally the PCR product was cut and purified using a DNA purification kit (Fermentas, Berlin, Germany) prior

to cloning.

Construction of Recombinant Vectors: The purified DNA was inserted into pTZ57RT cloning vectors using T/A PCR product cloning kit (Fermentas, Berlin, Germany) according to the manufacturer's protocol. In summary, 3 µl of purified PCR product was mixed with 1.5 µl of pTZ57R plasmid vector (200 ng), 3 µl of 10X ligation buffer, 1 µL of T_4 DNA Ligase (5u), and 18.5 µl of ddH_2O in 1.5 mL microtube (a total of 30 µl reaction mixture) and was incubated overnight at 22°C to let the ligation reaction take place. Then, the ligation product was stored at -20°C.

Transformation into competent hosts: Prior to transformation, Luria-Bertani (LB) agar culture medium (Xgal, Sigma) containing Ampicillin (100 µg/ml), Isopropylthio-β-D-galactoside (IPTG), and 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) was prepared.

For transformation, 2 µl of the ligation product (pTZ57RT-GRA7 recombinant plasmids) was added into 50 µl of *E. coli* strain TOP10 competent cells thawed on ice, and then were mixed gently by stirring with the pipette tip. The mixtures was incubated on ice for 30 minutes, heat shocked for 1 min in water bath of 42°C, and were then immediately placed on ice and incubated for 2 minutes. The transformed cells were cultivated on LB medium followed by an overnight incubation at 37°C. The pTZ57RT-GRA7-positive bacteria appeared as white-color colonies and pTZ57RT-GRA7-negative bacteria as blue-color colonies.

Cloning verification and gene sequencing: The white-color and blue-color colonies were selected, and the presence of pTZ57RT-GRA7 recombinant plasmids was verified and compared by restriction enzyme analysis using *BamHI* and PCR assay, followed by gel electrophoresis. In addition, special DNA bands identified as GRA7 were extracted from the gel using DNA purification kit, and then, they were sent to commercial laboratory (Bioneer Inc.) for gene sequencing. Finally, the similarities and differences of the cloned gene sequence with respect to GRA7 gene of *T. gondii* was evaluated on the website of www.ncbi.nlm.nih.gov/blast.

Results

Amplification of GRA7 gene of *T. gondii*: A pair of primers specific for the GRA7 gene of *T. gondii* was recruited for amplifying the gene by PCR assay, which also introduced BamHI restriction sites to make cloning possible. Results of agarose gel electrophoresis analysis on PCR products of genomic DNA extracted from the tachyzoites revealed 726 bp products that regarding the size were equal to the GRA7 gene of *T. gondii* (Fig. 1). This indicated that the reaction conditions were optimized and the designed primers were specific for amplification of the GRA7 gene.

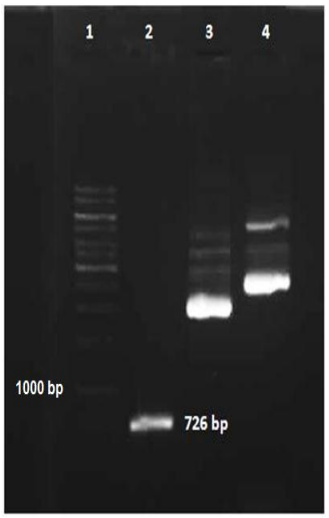

Figure 1. Gel electrophoresis analysis on PCR product of GRA7 and the plasmids used for cloning: Lane 1; DNA ladder (1000bp), lane 2; GRA7 gene, lane 3; non-recombinant supercoil plasmids, and lane 4; linear plasmids

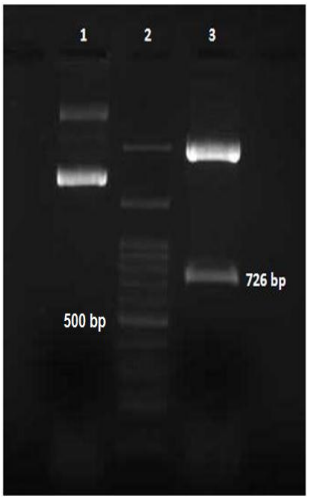

Figure 2. Gel electrophoresis analysis on cloning products following restriction enzymatic digestion: Lane 1; non-recombinant plasmids, lane 2; DNA ladder (100bp), and lane 3; the GRA7 and linear plasmids

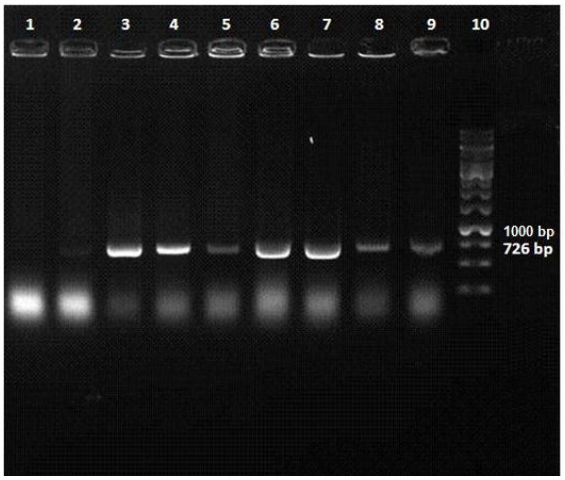

Figure 3. Gel electrophoresis analysis on PCR product of blue colonies (lane 1), white colonies (lanes 2-9) obtained from the present study. Lane 10 presents DNA ladder (1000 bp). Results showed that the 726-bp GRA7 gene was successfully cloned (lanes 2-9)

Confirmation of pTZ57RT-GRA7 Cloning: Results of agarose gel electrophoresis analysis on constructed pTZ57RT-GRA7 recombinant plasmids following restriction enzymatic digestion revealed two different bands, the first band, which was heavier, indicating linear plasmids and the second, a lighter 726 bp band, indicating the GRA7 gene (Fig. 2). These findings confirmed that the GRA7 gene was successfully cloned into pTZ57RT plasmids.

Verification of pTZ57RT-GRA7 Transformation: Both white and blue bacterial colonies appeared on the ampicillin-LB agar medium following an overnight cultivation of TOP10 competent cells in exposure to the constructed pTZ57RT-GRA7 recombinant plasmids. However, more than 90% of the colonies were white, which indicated successful transformation of pTZ57RT-GRA7 recombinant plasmids into the TOP10 hosts. In addition, agarose gel electrophoresis analysis on plasmids extracted from blue and white colonies exhibited 726 bp bands of GRA7 gene, which indicated that white colonies had received pTZ57RT-GRA7 recombinant plasmids through successful transformation (Fig. 3).

Results of Gene Sequencing: Results of gene sequencing of cloning products are given in fig. 4. Evaluation and comparison of gene sequencing results revealed only 24 changes in cloned GRA7 gene compared to the wild type (consensus) of the gene, which confirmed that the GRA7 gene of *T. gondii* had been successfully cloned into pTZ57RT recombinant plasmids.

Discussion

GRA proteins of *T. gondii* were first described as secreted antigens, since they were secreted following incubation of the parasites with serum; it was found that these substances, as regulated secretory proteins, are exported through the Golgi apparatus in response to specific stimulation[23]. Widely detected and continuously expressed in hosts infected with *T. gondii*, this has made them as appropriate candidates for tracking the infection and pertinent events18. Serological assay is actually the most popular laboratory diagnostic tool for toxoplasmosis designed based on detection of *T. gondii* GRA proteins.

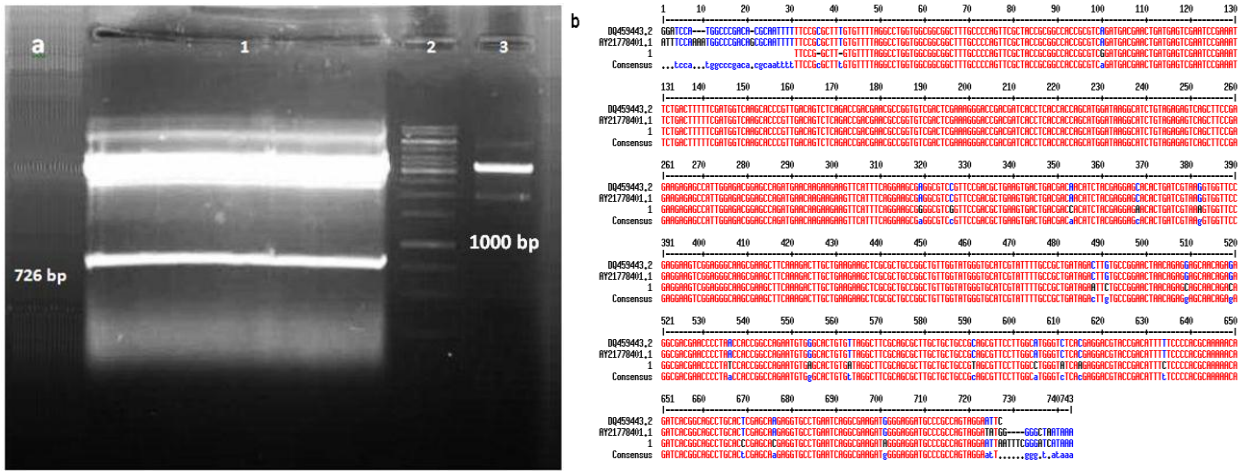

Figure 4. Gene sequencing of GRA7 cloning products: a) cloning product of GRA7 gene used for sequencing. Lane 1, digested product containing linear plasmid and GRA7 gene. Lane 2, DNA ladder (1000bp). Lane 3, recombinant plasmids. b) Sequencing results GRA7 gene revealed few differences compared to the wild type (consensus) of the gene.

In the present study, we successfully cloned a 726 bp gene fragment of GRA7 into the pTZ57RT plasmids. The results of electrophoresis assay following restriction enzymatic digestion, and also the gene sequencing evaluation of the recombinant GRA7-pTZ57RT plasmids confirmed that the cloning, transformation and amplification procedures were successfully and accurately achieved. We obtained and preserved recombinant colonies of Top10 strained of *E. coli* containing GRA7-pTZ57RT plasmids that can be used for sub-cloning and protein expression of GRA7 within suitable prokaryotic and eukaryotic hosts, and subsequently for developing serological tools for clinical use.

Although, to the best of our knowledge, we were the first in Iran to use pTZ57RT plasmid as vector for cloning of GRA7 gene, cloning and expression of GRA7 protein of *T. gondii* and its immunoreactivity have been previously investigated by few numbers of studies[24,25]. Jacobs and colleagues, for instances, for the first time assessed immunoreactivity of the newly discovered GRA7 protein by western blot immunoassay, using human sera originally used for screening, and they demonstrated that this protein reacted with a monoclonal antibody that was raised against the entire parasite[25]. Subsequently, they evaluated capacity of a recombinant GRA7 protein, expressed by GRA7-pmTNFMPH and GRA7-pIGFH111 plasmids, for detection of IgG antibodies, and introduced this protein a tool for studying the immune response to *T. gondii*[26]. More recently, Wang et al. evaluated GRA1 and GRA7 as potential diagnostic markers for *T. gondii* infection in dogs by ELISA assay[27].

In addition to diagnostic applications, DNA products of GRA7 cloning and amplification have also be used as DNA vaccine to efficiently boost a complete immune response against *T. gondii*. For example, It was demonstrated that liposome-formulated RA7-pVAX IgG plasmids induced a significant immune response including; significant increased IFNγ and IgG2 level against *T. gondii*[28]. Moreover, Jongert and colleagues found that RA7-DNA vaccine elicited a polarized Th1/Th2 immune response against *T. gondii* brain cyst formation in mice[29]. Considering the results of these studies[28,29], our constructed GRA7-pTZ57RT plasmids can be evaluated as a vaccine DNA candidate by future studies.

Conclusion

We successfully cloned GRA7 gene of *T. gondii* into pTZ57RT plasmid, which was confirmed following restriction enzymatic digestion and gene sequencing. The cloned gene can either be subsequently cloned into protein expression vectors to produce large amount of the GRA7 recombinant protein for developing serological diagnostic assays, or be used as DNA vaccine to stimulate immune responses against *T. gondii*. However, further studies needs to be carried out in order to produce recombinant GRA7 protein and to evaluate its immunoreactivity and immunogenicity, as well.

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