

## Cloning, expression and immunoreactivity of recombinant *Toxoplasma gondii* GRA5 protein

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### ABSTRACT

**Background and Objectives:** *Toxoplasma gondii* is an obligatory intracellular parasite which causes severe diseases in the fetus of pregnant women and immunocompromised patients. Serological tests based on recombinant protein are one of the main diagnosis methods for the detection of specific antibodies in serum samples. Dense granule antigenic proteins derived from *T. gondii* (TgGRAs) are potential antigens for the development of diagnostic tools.

**Materials and Methods:** DNA was extracted from *T. gondii* (RH-strain) tachyzoites and PCR reaction was done using corresponding primers for GRA5 antigen. The PCR product was purified and ligated into pTG19-t vector and then subcloned into *Xho*I and *Bam*HI digested pGEX6p-1 expression vector. Recombinant plasmid was transformed into *E. coli* (BL21 DE3) and induced by 1mM IPTG and analyzed by 15% SDS-PAGE. Expressed protein was confirmed by western blot analysis.

**Results:** There was no difference among the sequences of *T. gondii* GRA5 gene from different isolates. The recombinant plasmid pGEX-6p-1/GRA5 induced by IPTG was expressed in *E. coli*. It was a GST fusion protein and could react with human positive sera analyzed by western blot.

**Conclusion:** The GRA5 gene of *T. gondii* isolates is highly conservative. This antigen as a recombinant protein was successfully expressed in *E. coli*, which showed high immunoreactivity.

**Keywords:** *Toxoplasma gondii*, Dense granule antigen; GRA5, Immunoreactivity

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## INTRODUCTION

*Toxoplasma gondii* is an obligate intracellular parasite which belongs to phylum Apicomplexa (1). This protozoan parasite in addition to the human infects all warm-blooded animals. One-third of the world's populations are infected with this organism (2). *T. gondii* has three infectious stages including tachyzoite, bradyzoite, and sporozoite (3). The parasite is transmitted by ingestion of contaminated foods with oocyst, raw or poorly cooked meat of livestock and birds with tissue cysts or via transplacental transmission (4). Sulfonamide and pyrimethamine is the effective therapy for toxoplasmosis. In acute toxoplasmosis, the asexual stage is the appropriate stage for treatment while, bradyzoites or tissue cyst cannot be eradicated in chronic toxoplasmosis. As a result, prevention and treatment of toxoplasmosis are important to conduct (5).

Early diagnosis of the disease is of great importance in the prevention of the disease, and planning for effective treatments, in high risk people like immunocompromised patients and pregnant women. One of the main routine diagnosis approaches is the detection of specific antibodies using the serology tests (6). The routine diagnostic methods for *T. gondii* infection are not satisfactory. Due to the probable long time presence of IgM antibody in sera, the precise detection between acute and chronic toxoplasmosis becomes difficult (7). The use of recombinant antigens representative of acute or chronic infection might further enhance their application for discriminating acute from chronic infection (8). Various specific antigens of *T. gondii* including rhoptry, microneme (9), dense granule and surface proteins have been reported as seromarkers in previous studies for detection of *T. gondii* specific serum antibodies in both acute and chronic *Toxoplasma* infection (10, 11). Dense granule (GRA) proteins are high immunogenic proteins which are found in both tachyzoites and bradyzoites stages of the parasite (10). The *T. gondii*'s dense granule antigens, an important fraction of antigens that circulate in the bloodstream during the first days following infection are secreted in abundance and therefore have higher potential to develop tools for laboratory diagnosis of toxoplasmosis (12). During the host cell invasion, GRA5 is released into the parasitophorous vacuole (PV). Recombinant *T. gondii* antigens which produced in bacterial or yeast expression systems have several advantages com-

pared to whole parasite extracts such as lower cost of production and purification and precisely known antigen composition of the test which it allows better standardization procedures for serologic tests (13).

We previously produced the recombinant GRA7 antigen and evaluated its efficiency for detection of *Toxoplasma* infection (10, 14). This study aims to cloning and expression of the recombinant gene encoding GRA5 protein (rGRA5) of *T. gondii* in bacteria which can be used as vaccine or diagnostic tool for toxoplasmosis.

## MATERIALS AND METHODS

**Parasite preparation.** Mice were purchased from the laboratory animal center of Shahid Beheshti University of Medical Sciences and the *T. gondii* tachyzoites (RH strain) were maintained by serial intraperitoneal passage in mice and were harvested from peritoneal fluid of the infected mice after 3 to 4 days of infection. The fluid was centrifuged and tachyzoites were washed and subsequently resuspended in sterile phosphate buffered saline (PBS) prior to usage.

**Insert DNA preparation.** 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. The parasite genomic DNA was extracted from the *T. gondii* tachyzoites by Genimic DNA Extraction Kit, DNG-plus (CinnaGene, Iran, DN8118C) using the kit manufacture's guidelines. Continuously, the genomic DNA quantity and quality was controlled by electrophoresis on 1% agarose gel and a biophotometer (Eppendorf, UK) at 260 and 280 nm. Nucleotide sequence of the gene encoding GRA5 of *T. gondii* was taken from NCBI. Primer was designed on the basis of the sequence of GRA5 gene (Genbank: L06091, GI:2270893). The following primers were used in PCR reaction;

GRA5 F: 5'-GGA TCC ATG GCG TCT GTA AAA CGC GT- 3'

GRA5 R: 5'-CTC GAG TTA CTC TTC CTC GGC AAC T- 3'

These primers had *Bam*HI and *Xho*I restriction sites to facilitate the cloning process. PCR was performed in a PCR tube containing: 200 ng (1.5µl) extracted DNA as template, 20 pmol (0.25µl) of each

primer, and 7.5  $\mu$ l of PCR master mix 2 X (Bie & Berntsen A/S). The target gene was amplified by an initial denaturation at 96 °C for 5 min and then cycled 30 times with denaturation at 96 °C for 30s, followed by annealing at 60 °C for 30s and finally an extension step at 72 °C for 1 min followed by 20 min final extension at 72 °C. Subsequently, the PCR product was run on 2% agarose gel against a standard DNA Ladder (Fermentas, Germany). The PCR product was cut and purified using a DNA purification kit (Fermentas, Germany) prior to cloning. The purified DNA was ligated into pTG19-t vector by using T/A PCR product cloning kit (Vivantis, Malaysia).

**Ligated vector transformation.** Before transformation, Luria-Bertani (LB) agar plate contained 100 $\mu$ l/ml of ampicillin, were prepared and spreaded with 5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactoside (X-Gal) and isopropylthio- $\beta$ -D-galactocide (IPTG). For each ligation reaction, 50 $\mu$ l aliquoted of frozen competent cells (Top10) was thawed on ice and added 2  $\mu$ l of the ligation reaction to the competent cells and mixed gently by stirring with the pipette tip. The cells were incubated on ice for 30 min then heats shocked for 30 s in the 42 °C water bath, and then immediately were placed on ice for 2 min. The transformed cells were spreaded on each labeled LB-ampicillin plate with X-Gal and IPTG. The plates were inverted and placed at 37 °C incubator for overnight. The positive colonies which had white color were identified either by restriction enzyme and screening by PCR method after transformation.

**Sub-cloning and gene expression.** The GRA5 gene was sub-cloned into *Xho*I and *Bam*HI digested PGEX6p-1 expression vector and transformed into *E. coli* (BL21). Recombinant plasmids were selected and transformed into bacterial cell (BL21 DE3). Bacterial colony containing recombinant plasmid was picked and mass-cultured in Luria-Bertani (LB) medium supplemented with 100  $\mu$ l/ml ampicillin. The culture was induced by 1mM IPTG when OD<sub>600</sub>=0.5 to 0.7 and sampling were done after induction for various incubation periods and analyzed by 15% SDS-PAGE.

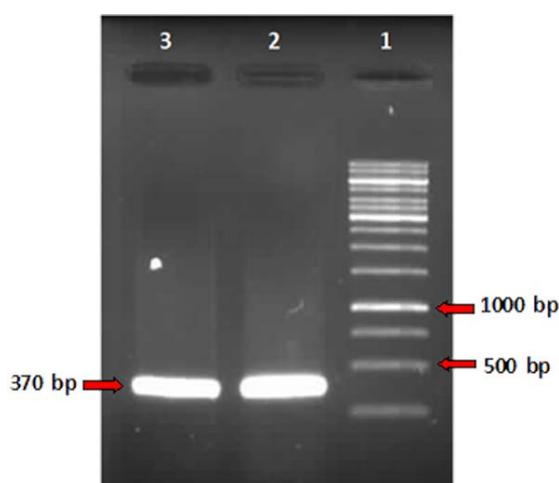
**Western-blot analysis.** For western blot analysis following SDS-PAGE, the gels' protein content was transformed on nitrocellulose papers, and stabilized by a UV Cross Linker. The nitrocellulose papers were

then cut into vertical strips and blocked using bovine serum albumin (BSA), as blocking solution, for 2 hours at room temperature by constantly shaking. After washing, the nitrocellulose strips were incubated with human positive serum (1:200) for 1 hours, washed and treated with the Horse Radish Peroxidase (HRP)-conjugated rabbit anti serum antibodies IgG, at room temperature for 1 hours. Afterwards, the strips were washed and treated with solution containing Tris, H<sub>2</sub>O, H<sub>2</sub>O<sub>2</sub>, and DAB (3, 3'-diaminobenzidine) followed by incubation at room temperature for 15 min. Finally, the reaction was stopped by washing in H<sub>2</sub>O and analyzed brown bands.

## RESULTS

**PCR results.** After designing the specific primers for the gene encoding GRA5, the PCR reaction was done and the 370bp appropriated bands were detected on 2% agarose gel (Fig. 1).

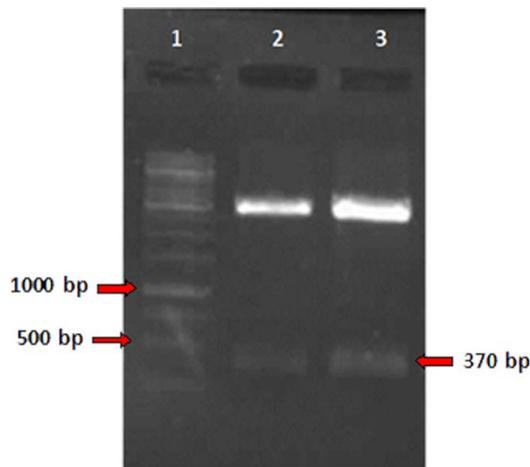
**Ligated vector transformation.** The purified PCR product was successfully cloned into pTG19-t vector. The recombinant plasmids were then successfully transformed into *E. coli*. After incubation at 37 °C during overnight, colony screening were done successfully using alpha-complementation test. The recombinant plasmid was extracted from white colonies and digested by *Bam*HI and *Xho*I (Fig. 2) and released fragment (GRA5 gene) was sub-cloned



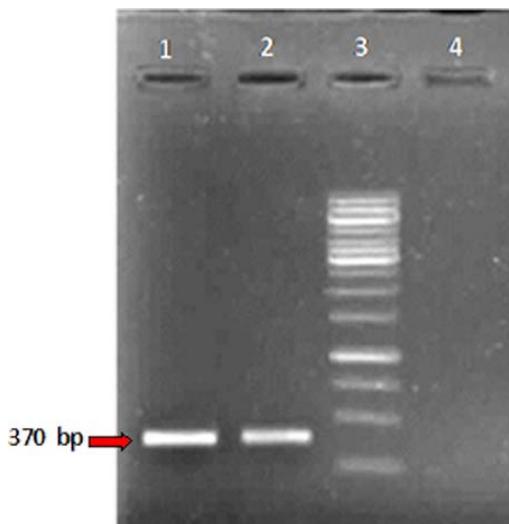
**Fig. 1.** The 2% agarose gel electrophoresis of PCR product. Lane 1: 1000 bp DNA ladder marker; Lane 2 and 3: PCR products of GRA5 gene.

into *Bam*HI and *Xho*I digested pGEX6p-1 expression vector. Fig. 3 shows the confirmed recombinant plasmids by restriction analysis and PCR (Fig. 3).

**Protein Expression.** After transforming the recombinant plasmid into *E. coli* BL21 strain, the plasmid promoter was induced by IPTG. Bacterial samples were lysed and electrophoresed on 15% SDS-PAGE. Gel was stained by coomassie brilliant blue. As shown in Fig. 4, the protein band was ap-

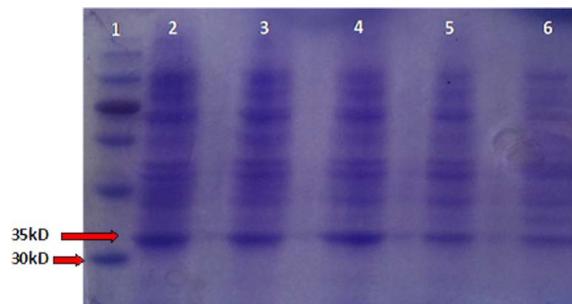


**Fig. 2.** The 1.5% agarose gel electrophoresis of digested recombinant pTG19-t. Lane 1: 1000 bp DNA ladder marker; lane 2 and 3: *Bam*HI and *Xho*I digested recombinant pTG19-t.

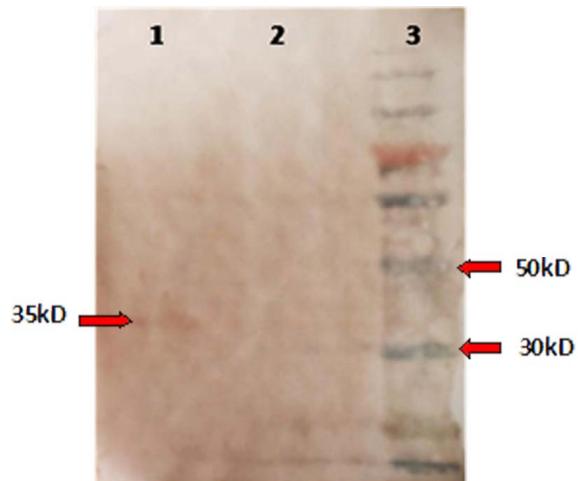


**Fig. 3.** The 1.5% agarose gel electrophoresis of recombinant pGEX-GRA5 plasmid, lane 1 and 2: PCR products using specific primers for GRA5; lane 3: 1000 bp DNA ladder; lane 4: non-recombinant pGEX-GRA5 plasmid.

proximately 35KDa (including GST fusion protein and GRA5) in induced cells compared with non-induced cells. SDS-PAGE was performed by two separate gel and the protein bands were transferred into nitrocellulose membrane using western blotting device (Fig. 4). The recombinant protein was successfully tested in western blotting assays with human serum of toxoplasmosis sample and one negative serum as control (Fig. 5) which showed the acceptable immunoreactivity.



**Fig. 4.** SDS-PAGE analysis of bacterial cells. Lane 1: Protein size marker; Lane 2: Bacterial cells containing recombinant pGEX6p-1 8 hr after induction; lane 3: Bacterial cells containing recombinant pGEX6p-1 6 hr after induction; lane 4: Bacterial cells containing recombinant pGEX6p-1 4 hr after induction; lane 5: Bacterial cells containing recombinant pGEX6p-1 2 hr after induction. Lane 6: Induced bacterial cells.



**Fig. 5.** Western blot analysis using human serum (1:200), lane 1:BL21 lysates containing recombinant pGEX6p-1 plasmid; lanes 2: BL21 lysates without GRA5 insert; lane 3: protein as marker.

## DISCUSSION

*T. gondii* is an intracellular parasite which can infect every nucleated cell. The precise diagnosis of *Toxoplasma* infection in pregnant women and immunocompromised patients has critical importance (15). Delay in diagnosis in infected women who have been infected during the first trimester of pregnancy can lead to abortion. The latter can lead to congenital toxoplasmosis (16). *T. gondii* has a complex life cycle and specific secretory organelles such as micronemes which are apical, cigar-shaped organelles involved in recognition of and adhesion to the host cell, rhoptries which located at the anterior region and probably driving the installation of the parasite inside the host cell and dense granules which are involved in the maturation of the PV. All the proteins located in these organelles specifically the dense granule antigens are highly potential for diagnostic (13).

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 (17). Widely detected and continuously expressed in hosts infected with *T. gondii*, has made them as appropriate candidates for tracking the infection and pertinent events (18). As a result, the immunogenicity and prolonged expression of GRA proteins make them one of the promising candidates for recombinant protein production. Many studies have suggested that recombinant production of several *Toxoplasma* antigens, such as dense granule antigens (GRAs) has high potential as diagnostic reagents (19). GRA antigens such as GRA2, GRA6, GRA7 and GRA8 diagnostic performance has been investigated via ELISA for discriminating acute from the chronic *Toxoplasma* infections (20). GRA5 is a 21 kDa hydrophobic protein consisting of a N-terminal hydrophobic signal peptide and a hydrophobic transmembrane domain (21). It was reported that GRA5 appears in both soluble and hydrophobic forms (22). During the host cell invasion, the GRA5 is secreted into the PV as a soluble form (23). The recombinant proteins have been previously applied for *Toxoplasma* serodiagnosis by many researches (24, 25). The antigenicity of rGRA5 has been checked by western blotting analysis (26).

In this study, the recombinant GRA5 protein was used as an immunogenic antigen for toxoplasmosis

diagnosis. A fragment of the GRA5 gene was cloned successfully into prokaryotic expression vector. The pGEX6p-1 was used to allow high level expression of GRA5 using tac promoter. The pGEX-6p-1 expression plasmid belonging to the gene-fusion category of vectors, and expressed rGRA7 as glutathione S-transferase (GST) fusion, which facilitated the purification process (27). The antigenicity of GRA5 was investigated by Western blotting using the human positive sera for *T. gondii*. The recombinant antigen strongly reacted with *Toxoplasma*-specific IgG and IgM antibodies in the positive sera. No specific band was observed with healthy sera. Meanwhile, in our previous study, the sensitivity and specificity of recombinant GRA7 antigen have also been evaluated for the serodiagnosis of *Toxoplasma*-infected patients' sera through the western blot analyses (11).

Very few studies exist regarding cloning, expression and immuno-reactivity characterization of recombinant GRA5 protein of *T. gondii* which have revealed similar results to the findings of present study. For example, the recombinant GRA5 protein cloned and expressed for detection of human toxoplasmosis using western blot analyses by Xiao Tang Ching et al. (2013). They found that rGRA5 protein was 100% specific for analysis of toxoplasmosis-negative human sera. In addition, rGRA5 was able to detect the acute and chronic *T. gondii* infections (26). However, Ching et al. used different vector pRSET B in comparison with this study. The recombinant GRA5 protein will then be purified and used in immunization studies in mice model in order to determine its potential role in eliciting an immune response towards *T. gondii* infection. Moreover, it can be used in all in-house ELISA diagnosis kit to detect toxoplasmosis in infected patients.

In conclusion, we successfully cloned GRA5 gene of *T. gondii* into pTZ57RT plasmid, which was confirmed following restriction enzymatic digestion and gene sequencing. This study showed that rGRA5 cloned and expressed by *E. coli* using pGEX-6p-1 plasmid. Therefore, rGRA5 protein can be considered as a potential tool for serodiagnosis and detection of toxoplasmosis.

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## REFERENCES

- McConkey G A, Martin H L, Bristow G C, Webster J P. *Toxoplasma gondii* infection and behaviour–location, location, location? *J Exp Biol* 2013; 216: 113-119.
- Hill D, Dubey J. *Toxoplasma gondii* prevalence in farm animals in the United States. *Int J Parasitol* 2013; 43: 107-113.
- Dubey J, Lindsay D, Speer C. Structures of *Toxoplasma gondii* tachyzoites, bradyzoites, and sporozoites and biology and development of tissue cysts. *Clin Microbiol Rev* 1998 Apr; 11(2):267-299.
- Dubey J. Pathogenicity and infectivity of *Toxoplasma gondii* oocysts for rats. *J Parasitol* 1996; 951-956.
- Indrasanti D, Haryanto A, Artama W. Expression and isolation of recombinant microneme 3 (MIC3) protein of *Toxoplasma gondii* local isolate on *Escherichia coli* (BL21). *Anim Prod* 2011; 13: 131-137.
- Selseleh M, Keshavarz H, Mohebbi M, Shojae S, Selseleh M, Eshragian M R, et al. Production and evaluation of *Toxoplasma gondii* recombinant GRA7 for serodiagnosis of human infections. *Korean J Parasitol* 2012; 50: 233-238.
- Aubert D, Maine G, Villena I, Hunt J, Howard L, Sheu M, et al. Recombinant antigens to detect *Toxoplasma gondii*-specific immunoglobulin G and immunoglobulin M in human sera by enzyme immunoassay. *J Clin Microbiol* 2000; 38: 1144-1150.
- Golkar M, Azadmanesh K, Khalili G, Khoshkholgh-Sima B, Babaie J, Mercier C, et al. Serodiagnosis of recently acquired *Toxoplasma gondii* infection in pregnant women using enzyme-linked immunosorbent assays with a recombinant dense granule GRA6 protein. *Diagn Microbiol Infect Dis* 2008 May;61(1):31-39.
- Holec L, Gąsior A, Brillowska-Dąbrowska A, Kur J. *Toxoplasma gondii*: enzyme-linked immunosorbent assay using different fragments of recombinant microneme protein 1 (MIC1) for detection of immunoglobulin G antibodies. *Exp Parasitol* 2008; 119: 1-6.
- Arab-Mazar Z, Seyyed-Tabaei S J, Mirahmadi H. Cloning of dense granular (GRA) 7 gene of *Toxoplasma gondii* into pTZ57RT vectors for sub-cloning in prokaryotic and eukaryotic plasmids. *Novelty in Biomedicine* 2014; 2: 114-119.
- Fallahi Sh, Seyyed Tabaei SJ, Pournia Y, Zebardast N, Kazemi B. Comparison of loop-mediated isothermal amplification (LAMP) and nested-PCR assay targeting the RE and B1 gene for detection of *Toxoplasma gondii* in blood samples of children with leukaemia. *Diagn Microbiol Infect Dis* 2014 Jul; 79(3):347-354.
- Hughes H, Van Knapen F. Characterisation of a secretory antigen from *Toxoplasma gondii* and its role in circulating antigen production. *Int J Parasitol* 1982; 12: 433-437.
- Holec-Gąsior L, Kur J. *Toxoplasma gondii*: Recombinant GRA5 antigen for detection of immunoglobulin G antibodies using enzyme-linked immunosorbent assay. *Exp Parasitol* 2010; 124: 272-278.
- Arab-Mazar Z, Fallahi Sh, Koochaki A, Haghghi A, Tabaei S J S. Immunodiagnosis and molecular validation of *Toxoplasma gondii*-recombinant dense granular (GRA) 7 protein for the detection of toxoplasmosis in patients with cancer. *Microbiol Res* 2016; 183: 53-59.
- Fallahi Sh, Mazar Z A, Ghasemian M, Haghghi A. Challenging loop-mediated isothermal amplification (LAMP) technique for molecular detection of *Toxoplasma gondii*. *Asian Pac J Trop Med* 2015; 8(5):366-372.
- McAuley J B. Congenital toxoplasmosis. *J Ped Infect Dis* 2014; 3(suppl 1): S30-S35.
- Coppens I, Andries M, Liu J L, Cesbron-Delauw M-F. Intracellular trafficking of dense granule proteins in *Toxoplasma gondii* and experimental evidences for a regulated exocytosis. *Europ J Cell Biol* 1999; 78: 463-472.
- Velmurugan GV, Tewari AK, Rao JR, Baidya S, Kumar MU, Mishra AK. High-level expression of SAG1 and GRA7 gene of *Toxoplasma gondii* (Izatnagar isolate) and their application in serodiagnosis of goat toxoplasmosis. *Vet Parasitol* 2008; 154: 185-192.
- Lau Y-L, Fong M-Y, Mohd Idris M, Ching X-T. Cloning and expression of *Toxoplasma gondii* dense granule antigen 2 (GRA2) gene by *Pichia pastoris*. *Southeast Asian J Trop Med Pub Health* 2012; 43(1): 10.
- Golkar M, Rafati S, Abdel-Latif M S, Brenier-Pinchart M-P, Fricker-Hidalgo H, Sima B K, et al. The dense granule protein GRA2, a new marker for the serodiagnosis of acute *Toxoplasma* infection: comparison of sera collected in both France and Iran from pregnant women. *Diagn Microbiol Infect Dis* 2007 Aug;58(4):419-426.
- Nam H-W. GRA proteins of *Toxoplasma gondii*: maintenance of host-parasite interactions across the parasitophorous vacuolar membrane. *Korean J Parasitol* 2009; 47(Suppl): S29-S37.
- Lecordier L, Mercier C, Sibley L D, Cesbron-Delauw M-F. Transmembrane insertion of the *Toxoplasma gondii* GRA5 protein occurs after soluble secretion into the host cell. *Mol Biol Cell* 1999; 10: 1277-1287.
- Lecordier L, Mercier C, Torpier G, Tourvieille B, Dar-

- cy F, Li L J, et al. Molecular structure of a *Toxoplasma gondii* dense granule antigen (GRA 5) associated with the parasitophorous vacuole membrane. *Mol Biochem Parasitol* 1993; 59: 143-153.
24. Ching X T, Lau Y L, Fong M Y. Heterologous expression of *Toxoplasma gondii* dense granule protein 2 and 5. *Southeast Asian J Trop Med Public Health* 2015; 46: 375-387.
25. Hiszczyńska-Sawicka E, Oledzka G, Holec-Gasior L, Li H, Xu J B, Sedcole R, et al. Evaluation of immune responses in sheep induced by DNA immunization with genes encoding GRA1, GRA4, GRA6 and GRA7 antigens of *Toxoplasma gondii*. *Vet Parasitol* 2011; 177(3-4): 281-289.
26. Ching X T, Lau Y L, Fong M Y, Nissapatorn V, Andiappan H. Recombinant dense granular protein (GRA5) for detection of human toxoplasmosis by Western blot. *Biomed Res Int* 2014; 2014: 690529.