CLONING AND EXPRESSION OF *TOXOPLASMA GONDII* DENSE GRANULE ANTIGEN 2 (*GRA2*) GENE BY *PICHIA PASTORIS*

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Abstract. Detection of *Toxoplasma gondii* infection is essential in pregnant women and immunosuppressed patients. Numerous studies have shown that the recombinant production of several *Toxoplasma* antigens, including dense granule antigens (GRAs) has high potential as diagnostic reagents. In the present study, we produced GRA2 using *Pichia pastoris* system. RNA of *T. gondii* RH strain tachyzoite was used as a template to produce cDNA clones of full-length *GRA2* via reverse transcriptase PCR. Amplicons were inserted into pPICZα A and the recombinant plasmid transformed into *P. pastoris*, X-33 strain. The expressed recombinant protein was identified by SDS-PAGE and Western blotting. A recombinant protein of ~28 kDa was produced, which could be detected by toxoplasmosis positive human sera indicating that the recombinant protein retained its antigenicity. The present study indicates that *P. pastoris*-expressed *GRA2* should be useful for detection of *Toxoplasma* infection.

Keywords: *Toxoplasma gondii*, *GRA2*, expression, Western blot

INTRODUCTION

Toxoplasma gondii is an apicomplexan parasite of worldwide distribution, which can infect most warm blooded vertebrates, including man, causing the disease toxoplasmosis. Members of the cat family serve as the definitive hosts, and a variety of birds and mammals serve as intermediate hosts in the parasite's life cycle (Dubey *et al*, 1998). Although *T. gondii* infects a large proportion of the world's human populations, it is an uncommon cause of disease. Certain individuals are at high risk including congenitally infected fetuses and newborns and immunologically impaired individuals (Rorman *et al*, 2006). Toxoplasmosis most often occurs in persons with defects in T cell-mediated immunity, such as patients receiving corticosteroids or cytotoxic drugs and patients with hematologic malignancies, organ transplants or acquired immunodeficiency syndrome (HIV-AIDS). On the other hand, primary or chronic (latent) infection with *T. gondii* is asymptomatic in immunocompetent individuals.

Most cases of *T. gondii* infection elude diagnosis because they either are asymptomatic or mimic common viral infection in which they reside in the host tissues (Patrick, 2002). Due to this, toxoplasmosis must be considered carefully in the differ-

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ential diagnosis of a large variety of clinical presentations. The correct diagnostic tests must be performed and interpreted appropriately with the patient's clinical presentation. The use of serologic tests to detect specific antibodies to *T. gondii* is the primary method of diagnosis (Montoya, 2002). Most commercial serological test kits are expensive and can be less specific as they use total antigens originating from *T. gondii* tachyzoites grown on host cells or in the peritoneal cavity of mice. Use of recombinant antigens could overcome these drawbacks. In past few years, many attempts have been made to clone and express *T. gondii* genes or gene fragments using difference systems.

Dense granule antigen 2 (GRA2) is a member of the GRA antigenic protein family located in the dense granules of *T. gondii* tachyzoite. They are secreted into the tachyzoite parasitophorous vacuole after invasion and also are present at the cyst wall of the slower-growing bradyzoite (Kumolosasi *et al*, 1994). GRA2 (28 kDa) has an essential survival role in the encystations stage of the parasite and in the maintenance of the cyst wall. Immunization with this antigen mediates a higher humoral and cell mediated response in chronic infection condition (Brinkmann *et al*, 1993; Pistoia *et al*, 1996). For these reasons, we have cloned and expressed *T. gondii GRA2* gene in *Pichia pastoris* in order to develop an improved diagnosis test and to produce vaccine in the future.

MATERIALS AND METHODS

Parasite

Tachyzoites of the *T. gondii* RH strain were obtained from the Institute for Medical Research Kuala Lumpur, Malaysia. Tachyzoites were grown in MBDK cell monolayer in RPMI medium supplemented with 10% fetal calf serum at 37ºC in 5% CO_2 atmosphere.

PCR amplification of *GRA2* gene

Total RNA of *T. gondii* RH strain tachyzoite was extracted using TRIZOL Reagent (Sigma, St Louis, MO). Reverse-transcription PCR was performed in order to construct cDNA using SuperScript® II RT kit (Invitrogen, Carlsbad, CA). In short, 1 µl of total RNA was heated at 75ºC for 5 minutes and then rapidly chilled on ice. The denatured RNA was added with random hexamer and reverse transcriptase and incubated at 37ºC for 1 hour. PCR then was conducted using *GRA2* specific primers: GRA2 forward (5′ GAA TTC ACC ATG TTC GCC GTA AAACA 3′) and GRA2 reverse (5′ GAA TTC CAT GTC AAT AAT TCG TCT GCC ACT 3′) containing'*Eco*RI restriction site (underlined). PCR was carried out in a 25 µl reaction mixture containing 1 U *Taq* DNA polymerase (Fermentas Life Sciences, Burlington, Canada) using the following thermal cycling conditions: 95ºC for 10 minutes, 30 cycles of 94ºC for 30 seconds, 50ºC for 1 minute, and 72ºC for 2 minutes and a final heating at 72ºC for 10 minutes.

Recombinant plasmid construction and transformation

The PCR amplicon of the full length *GRA2* generated was purified and subsequently ligated into pPICZα A (Invitrogen, Carlsbad, CA) by overnight incubation at 16ºC. The recombinant vectors then were transformed into *E. coli* TOP10F′, which were grown on Luria Bertani agar, supplemented with zeocin (50 µg/ml, Invitrogen, Carlsbad, CA). Positive recombinant clones were selected, grown in zeocin-supplemented Luria Bertani broth and their plasmids were extracted, which were sent to a commercial laboratory

(Bioneer Corporation, Daejeon, South Korea) for sequencing to confirm the orientation and integrity of the *GRA2* gene. Transformation of *P. pastoris* with the recombinant pPICZα A was performed according to the manufacturer's protocol of the EasySelect™ *Pichia* Expression kit (Invitrogen, Carlsbad, CA). Positive recombinant *P. pastoris* clones were selected for expression study.

Expression of recombinant *GRA2* in *P. pastoris*

A single recombinant *P. pastoris* colony was inoculated into 25 ml of buffered complex medium containing 1% (v/v) glycerol. The culture was grown at 28ºC for 24 hours. Cells were harvested and resuspended in 100 ml of buffered complex medium containing 0.5% (v/v) methanol. Culture was allowed to grow for 5 days. Methanol was added every 24 hours to a final concentration of 0.5% to induce expression. *P. pastoris* cell/supernatant (1 ml) was collected every 24 hours after methanol induction for protein extraction and analysis. Non-recombinant *P. pastoris* host cells (X-33 strain) and X-33 transformed with parent vector (without insert) were similarly treated and analyzed as negative controls. The growth conditions in shake flasks such as methanol concentration (0.5, 0.75 and 1%) and inducing time were optimized.

Protein extraction and analysis

Culture supernatants were collected by pelleting at 3,000*g*. An equal volume of 20% (v/v) trichloroacetic acid was added to the supernatant and the mixture was stored overnight at -20ºC. The mixture then was centrifuged at 12,000*g* for 30 minutes and the pellet washed with acetone, and centrifuged at 12,000*g* for 5 minutes, followed by air drying at room temperature for 10 minutes. The protein was resuspended in phosphate-buffered saline (PBS).

The recombinant proteins were evaluated by Western blot assays using five sera of patients diagnosed with toxoplasmosis and five healthy donors. The serological status of these samples was pre-determined using Captia™ *T. gondii* IgG and *T. gondii* IgM Kits (Trinity Biotech, Co Wicklow, Ireland). These samples were obtained from the Diagnostic Laboratory at the Department of Parasitology, University of Malaya. Isolated proteins were separated by 12% SDS-PAGE and transferred by electroblotting to polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA) (Sambrook and Russell, 2001). The proteins were incubated with human immune or non-immune serum (at 1:200 dilution). Bound antibodies were detected with alkaline phosphatase-conjugated goat anti-human IgM/IgG (diluted 1:2,500) (Bio-Rad, Hercules, CA). Protein bands were visualized using 5-bromo-4-chloro-3-indolyl phosphate/nitro-blue tetrazolium chloride (BCIP/NBT) as a chromogenic substrate.

RESULTS

By using *T. gondii* total RNA as the primary source, cDNA of *GRA2* was constructed by reverse transcription PCR. Fig 1 shows the expected band of *GRA2* cDNA of 1,050 bp. *GRA2* cDNA was cloned into pPICZα A and transformed into *E. coli* TOP10F'. The orientation of the insert in each recombinant plasmid was determined by PCR as follows. As the position of 5' AOX and 3' AOX primer are fixed on the plasmid, amplification was dependent on the orientation of the *GRA2* specific primers, which in turns is determined by the orientation of the insert itself. Thus, the 5'AOX was used in tandem with the

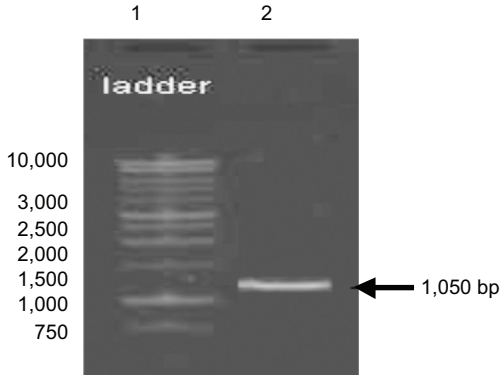

Fig 1–*GRA2* fragment generated from reverse transcription PCR. The desired band size of 1,000-1,500 bp was obtained by reverse transcription PCR using both the *GRA2* forward and reverse primers as described in Materials and Methods. Lane 1, 1 kb DNA ladder; lane 2, cDNA of *GRA2* obtained from reversed transcription PCR.

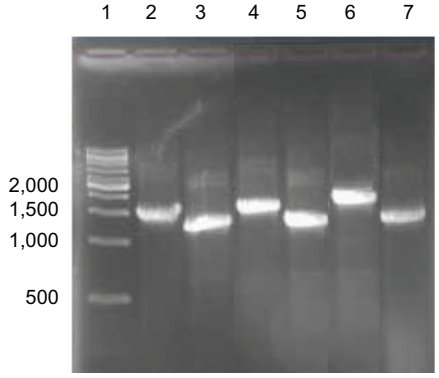

Fig 2–PCR confirmation of the orientation of *GRA2* insert in recombinant pPICZα A. The orientation of the insert in the positive clone was determined by PCR. Amplification was only possible if the primers were on opposite strands. As the position of 5′ AOX and 3′ AOX primer are fixed on the plasmid, amplification is dependent on the orientation of the GRA2 specific primers, which in turn is determined by the orientation of the insert. The 5′AOX was used in tandem with the GRA reverse primer. Lane 1, DNA ladder; lane 2, clone 5 using 5′ and 3′ AOX primers; lane 3, clone 5 using 5′ AOX and GRA2 reverse primers; lane 4, clone 12 using 5′ and 3′ AOX primers; lane 5, clone 12 using 5′ AOX and GRA2 reverse primers; lane 6, clone 13 using 5′ and 3′ AOX primers; lane 7, clone 13 using 5′ AOX and GRA2 reverse primers.

GRA reverse primer. Three clones were identified carrying the insert in the correct direction (Fig 2). Alignment of the published *T. gondii GRA2* gene retrieved from NCBI with the cloned *GRA2* was conducted using NCBI nucleotide blast program (http://blast.ncbi.nlm.nih.gov/Blast.cgi), which revealed 99% similarity compared with the published *GRA2* sequence (Accession number: HM014012.1) with only two nucleotides different (data not shown).

A time course expression study was carried out to determine the optimal time for maximum expression of the recombinant protein. Maximum expression of a protein of 28 kDa was at day 5 when induced with 1% methanol (data not shown). This protein was not detected in the control samples. This 28 kDa protein was most likely to be the putative recombinant GRA2. The identity of this antigen was confirmed in a Western blot using anti-*Toxoplasma* human immune serum (Fig 3). The recombinant protein was further tested in Western blot assays with 5 human serum of toxoplasmosis samples and 5 negative controls which showed that all toxoplasmosis positive samples reacted with recombinant GRA2 (data not shown). These results indicated that the recombinant GRA2 shared common epitopes with the native GRA2.

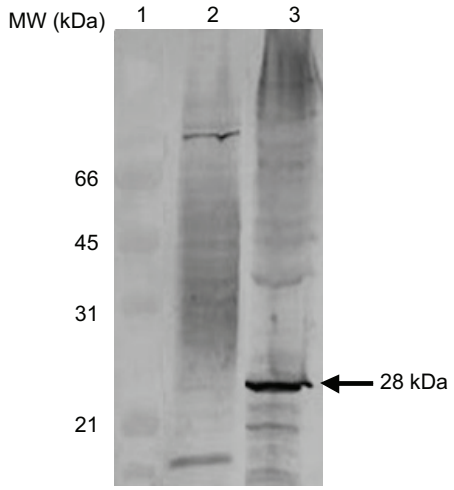

Fig 3–Western blot analysis of total protein extracted from the culture supernatant of recombinant *P. pastoris* secreted *T. gondii* GRA2. Lane 1, protein molecular weight standards; lane 2, total protein extracted from medium of non-recombinant *P. pastoris* culture; lane 3, total protein extracted from medium of a recombinant *P. pastoris* clone after 5 days of induction with 1% methanol. Human anti-*Toxoplasma* immune serum detected the 28 kDa protein in lane 3 (arrow), thus confirming the identity of the recombinant *T. gondii* GRA2.

DISCUSSION

Previous studies on dense granule of *T. gondii* have shown that GRA2 is a crucial protein for the formation of the intravacuolar membranous nanotubular network (MNN) and that one of the functions of the MNN is to organize the parasites within the parasitophorous vacuole (Mercier *et al*, 1998; Stedman and Joiner, 1999; Lebrun *et al*, 2007). Thus, it has an essential survival role in the encystations stage of the parasite and in the maintenance of the cyst wall of the bradyzoites. Studies also revealed that strong immune response involving both the humoral and cell mediated response in challenging the infection was observed in immunized mice (Brinkmann *et al*, 1993; Pistoia *et al*, 1996). Therefore, *GRA2* has been selected as a potential candidate to be heterogeneously expressed using *P. pastoris* system for development as a vaccine against the infection and for diagnosis.

In earlier studies, various bacterial heterologous expression systems have been used in cloning the *T. gondii* antigens, however most did not succeed as the recombinant proteins were either insoluble and misfolded (Lueking *et al*, 2000) or correctly folded but in low yield (Cregg, 1999). In order to have high level expression and appropriate folding, the methylotrophic yeast, *P. pastoris* was chosen as an expression system of GRA2. A number of proteins of *T. gondii* have been previously expressed in *P. pastoris*. For example, *T. gondii SAG1* has been successfully cloned and expressed using the *P. pastoris* system and immunized into mice resulting in a strong protection against the infectious stage of *T. gondii* tachyzoites (Bulow and Boothroyd 1991; Debard *et al*, 1996). Similarly, the recombinant protein of full-length and truncated SAG2 from *P. pastoris*, which has a posttranslational modification, has been postulated to be suitable as a diagnostic tool in detecting anti-*Toxoplasma* IgG and IgM antibodies (Lau *et al*, 2006, 2008; Fong *et al*, 2008). *P. pastoris* has also been identified as a robust system as it has successfully expressed a combination of antigen SAG1-GRA2, which has a potential role in the development of multiantigenic vaccines (Zhuo *et al*, 2007).

The expression system by *P. pastoris* can be either intracellular or extracellular. In this study, in order to facilitate purification, an extracellular expression vector

pPICZα A was used to allow high level expression and secretion of *GRA2* using AOX1 promoter. This expression vector contains the secretion α-factor peptide signal, which fuses with the cloned GRA2 protein and subsequently it is targeted to the medium. In addition, it has a purification polyhistidine tag at the C-terminal (Higgins and Cregg, 1998; Cregg, 1999).

Antigenicity of GRA2 was investigated by Western blotting using human toxoplasmosis positive sera. The recombinant antigen strongly reacted with IgG and IgM antibodies in the positive sera. No specific bands, however, were observed with healthy sera. The results of our studies showed that the recombinant GRA2 was sufficiently specific to detect human anti-*Toxoplasma* IgG and IgM antibodies.

The recombinant GRA2 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. In addition, it can be used in all in-house ELISA diagnosis kit to detect toxoplasmosis in infected patients.

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