

Original Article

Development of a Lateral Flow Immunoassay Using Recombinant Dense Granular Antigen (GRA) 7 to Detect Anti-*Toxoplasma gondii* IgG Antibodies

Morovati ^{1,5}, H., Seyyed Tabaei ^{1,*}, S.G., Gholamzad ², M., Omidfar ³, K., Ahmadi ³,
A., Arab-Mazar ⁴, Z., Eshaghi ⁵, A., Sheikhsofla ⁶, F.

1. Department of Parasitology and Mycology, Faculty Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran

2. Department of Microbiology and Immunology, Islamic Azad University, Tehran Medical Branch, Tehran, Iran

3. Biosensor Research Center, Endocrinology and Metabolism Molecular-Cellular Sciences Institute, Tehran University of Medical Sciences, Tehran, Iran

4. Infectious Diseases and Tropical Medicine Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran

5. Department of Quality Control, Razi Vaccine and Serum Research Institute, Agricultural Research Education and Extension Organization, Karaj, Iran

6. Department of Technical and Research Lab, Arvin Pazoohan Noor (APN), Tehran, Iran

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Corresponding Author: Seyyedtabaei@sbmu.ac.ir

ABSTRACT

Toxoplasma gondii is an intracellular protozoan parasite that causes toxoplasmosis and is of medical importance in pregnant women and immunosuppressed patients. In recent years, many methods have been developed for the detection of infection caused by this parasite; however, most of the developed methods are not adequately sensitive. The dense granular antigen (GRA) 7 is a highly immunogenic protein that is used as a specific antigen for the diagnosis of toxoplasmosis. This study was designed to produce recombinant GRA7 (rGRA7) antigen in bacterial system in order to be applied as an antigen for developing a simple and rapid lateral flow immunoassay test strip using a gold nanoparticle-pAb conjugate probe to detect *Toxoplasma* IgG-specific antibodies in human sera. After the extraction of genomic DNA from RH strain tachyzoites, polymerase chain reaction (PCR) was performed using specific primers considering restriction sites and BglII and XhoI enzymes. Subsequently, the *GRA7* gene was cloned in pET-32a (+) expression vector, and then pET-32a(+)-*GRA7* was transformed into *E. coli* Rosetta (DE3). The induction of protein production was accomplished by IPTG, and the product was finally purified by Ni-NTA affinity chromatography. In order to make the strip test, the anti-human gold nanoparticle conjugate was applied on conjugate pad, rGRA7 antigen was immobilized to a nitrocellulose membrane as the capture agent, and sample and absorbance pads were assembled on a backing card. For the analysis of the sensitivity and specificity of the assay, the selected patients' sera samples were tested by standard chemiluminescence immunoassay (CLIA) method, and then compared with TOXO-IgG strip results. The findings showed that the use of rGRA7 is an accurate, sensitive, and inexpensive technique for the rapid detection of anti-*Toxoplasma* IgG in human sera. Therefore, rGRA7 can be applied as a diagnostic agent in laboratories.

Keywords: *Toxoplasma gondii*, rGRA7, Rosetta (DE3), pET-32a(+), lateral flow immunoassay (LFA)

Développement d'un Dosage Immunologique à Flux Latéral Utilisant l'Antigène GRA7 Recombinant pour Détecter les Anticorps IgG Anti-*Toxoplasma gondii*

Toxoplasma gondii est un parasite protozoaire intracellulaire responsable de la toxoplasmose avec une importance médicale pour les femmes enceintes et les patients immunodéprimés. Ces dernières années, de nombreuses méthodes ont été développées pour la détection des infections causées par ce parasite. Cependant, la plupart des méthodes développées ne sont pas suffisamment sensibles. La protéine antigène granulaire 7 (GRA7) est une protéine hautement immunogène utilisée comme antigène spécifique pour le diagnostic de la toxoplasmose. Cette étude a été conçue pour produire un antigène GRA7 (rGRA7) recombinant dans un système bactérien afin de l'appliquer en tant qu'antigène dans le développement d'une bandelette de test simple et rapide d'immunoessai à écoulement latéral à l'aide d'anticorps conjugués à des nanoparticules d'or (nanoparticle-pAb) afin de détecter les anticorps IgG spécifiques du *Toxoplasma* dans des sérums humains. Après l'extraction de l'ADN génomique des tachyzoïtes de la souche RH, une réaction en chaîne de la polymérase (PCR) a été effectuée à l'aide d'amorces spécifiques, tout en tenant compte des sites de restriction et des enzymes BglII et XhoI. Ensuite, le gène *GRA7* a été cloné dans le vecteur d'expression pET-32a (+), puis le pET-32a(+)-*GRA7* a été transformé en Rosetta (DE3). La production de protéines recombinantes a été induite par l'isopropyl beta-D-thiogalactopyranoside et purifiée par chromatographie d'affinité Ni-NTA. Afin de réaliser le test de bande, le conjugué anti-IgG humain et nanoparticules d'or a été ajouté à un tampon conjugué. L'antigène rGRA7 a été ensuite immobilisé sur une membrane de nitrocellulose en tant qu'agent de capture et des tampons d'échantillon et d'absorbance ont été assemblés sur une carte support. Pour l'analyse de la sensibilité et de la spécificité de l'analyse, les échantillons de sérum des patients sélectionnés ont été testés par la méthode standard d'immunoessai par chimiluminescence (CLIA), puis comparés aux résultats obtenus avec les bandelettes TOXO-IgG. Les résultats ont montré que l'utilisation de rGRA7 était une technique précise, sensible et peu coûteuse pour la détection rapide d'IgG *anti-Toxoplasma* dans des sérums humains. De ce fait, rGRA7 peut être utilisé comme agent de diagnostic dans les laboratoires.

Mots-clés: *Toxoplasma gondii*, rGRA7, Rosetta (DE3), pET-32a(+), Dosage immunologique à flux latéral (LFA)

INTRODUCTION

Nowadays, the most important worldwide zoonosis that has drawn scientists' attention is toxoplasmosis, which is caused by *Toxoplasma gondii* (Drapala et al., 2014). Cats are the primary hosts of these microorganisms in which *T. gondii* can sexually reproduce to complete its lifecycle. This infection can occur in human or other warm-blooded hosts through the ingestion of unwashed fruits or raw vegetables as a result of the release of sporozoites from oocytes. Moreover, *T. gondii* persists in pigs, goats, and other mammals. Therefore, the consumption of undercooked meat, containing *T. gondii* tissue cysts, can be detrimental to humans. Another type of this infection is congenital toxoplasmosis, which is vertically transmitted from mother to her fetus (Pfrepper et al., 2005). Chorioretinitis, intracranial calcification, hydrocephalus, developmental delay, or other neurological disorders, particularly schizophrenia and bipolar disorders, are the

results of congenital toxoplasmosis (Drapala et al., 2014). The detection of antibodies in serum is the most common test for the diagnosis of toxoplasmosis, which is evidenced by the appearance of IgG in a week or enhancement of IgG titer. The IgG avidity test facilitates the distinction between acute and chronic infections (Drapala et al., 2014). The mice peritoneal cavity or host cells culture media-based tachyzoites are native antigen sources used in serological commercial assays. Despite the costliness and variable quality of *T. gondii* antigen production process, recombinant antigens are highly helpful. Moreover, the stage-specific antigens facilitate the establishment of a clear distinction between a recently acquired infection and an infection acquired in the past. A variety of recombinant antigens, produced in *Escherichia coli*, have been applied as the potential diagnostic markers of *T. gondii* infections in humans (Holec-Gasior, 2013). Some of these markers include dense granule proteins (e.g., recombinant granule antigen 1 [GRA1], rGRA2,

rGRA4, rGRA6, rGRA7, and rGRA8), rhoptry proteins (e.g., rROP1 and rROP2), matrix protein rMAG1, microneme proteins (e.g., rMIC2, rMIC3, rMIC4, and rMIC5), and surface antigens (e.g., rSAG1 and rSAG2). These recombinant proteins are mostly coated separately on the enzyme-linked immunosorbent assay (ELISA) plates in order to detect specific IgG and IgM antibodies in human serum. Recombinant antigens entail a number of advantages for the diagnosis of *T. gondii* infections as follows: a) antigen structure used in the assay is accurate and clear, b) they can be used for more than one antigen, and c) standardization of the process can be simply performed (Kotresha and Noordin, 2010). The GRA7 is expressed in all types of *Toxoplasma* infections; on the other hand, anti-GRA7 antibodies are generated in humans during the course of infection. The GRA7 induces the disposition of artificial liposomes and has been implicated in nutrient acquisition by the parasite via a mechanism that involves the sequestration of host endolysosomes (Holec-Gasior, 2013). The GRA7 is present in the parasitophorous vacuole and cytoplasm of the tachyzoite-infected host cell. Accordingly, the regular rupture of the cyst cells is followed by the invasion of the adjacent host cells, which facilitates the detection of this protein by the host immune system. Therefore, rGRA7 is the best candidate for recognition by IgG antibody. The use of several recombinant antigens, such as SAG1, GRA1, and GRA7, has been fully reported for differentiate between recent and past infections as compared to the native antigen in the assay (Holec-Gasior, 2013). According to the above, the present study was conducted to produce rGRA7 antigen in a bacterial system for developing a simple and rapid lateral flow immunoassay test strip using a gold nanoparticle-pAb conjugate probe.

MATERIAL AND METHODS

Proliferation of *Toxoplasma gondii* tachyzoites and DNA extraction. *Toxoplasma gondii* (RH strains) were obtained from the Quality Control Department of

Razi Vaccine and Serum Research Institute in Karaj, Iran. For the purpose of the study, 0.5 ml of mouse peritoneal fluid, containing 2×10^4 of fresh and active RH strains of *T. gondii* tachyzoites, were treated with 100 μ l/ml penicillin and injected into the peritoneal cavity of Swiss mice. After 4-5 days of injection, peritoneal fluid was obtained, washed with ice-cold phosphate buffered saline (PBS), and kept in a frozen state. DNA extraction was carried out using DNGTM-Plus kit (CinnaGen Inc, Tehran, Iran) according to the manufacturer's recommendation.

Amplification of GRA7 gene. Based on the nucleotide sequence of *T. gondii* RH strains, GRA7 gene, complete cds (accession number: DQ459443), the polymerase chain reaction (PCR) amplification was performed using the primers of 762 bp *GRA7* (i.e., forward: 5'-CCCAGATCTGATGGCACGACACGCAAT-3' and Reverse: 5'-GTGCTCGAGTTACTGGCGGCATCCTC-3'). The underlined letters in the sequence of the primers indicate the BglII and XhoI linker sites in the expression vector pET-32a (+) (Novagen, Germany). The PCR assay was performed in a 25 μ l reaction mixture composed of 12.5 μ l Master Mix (2x) (SinaClon, Iran), 1 μ l of each primer (0.5 pmol/ μ l), 2 μ l DNA (25 ng/ μ l), and 9.5 μ l double-distilled water (ddH₂O). The program of thermal cycler for PCR reaction included a 5-min initial denaturation at 96 °C, followed by 25 cycles of denaturation at 95 °C for 30 sec, annealing at 58 °C for 30 sec, extension at 72 °C for 60 sec, and a final extension of 10 min at 72°C. The amplified DNA was run on 1% (w/v) agarose gel (Fermentas), together with a DNA ladder (Fermentas, Germany). Total PCR product was loaded on agarose gel and purified for cloning using Silica Bead DNA Gel Extraction kit (Fermentas-Thermo Fisher Scientific).

Construction of Recombinant Vectors. According to the primer designing and restriction sites, the pET-32a(+) plasmid was selected and extracted from bacteria using DNA-SpinTM Plasmid DNA Purification kit (Intron-Biotechnology, Korea)

following the manufacturer's instruction. Subsequently, the PCR product and pET-32a(+) vectors were digested with both BglIII and XhoI enzymes. Both digested products were purified using Silica Bead DNA Gel Extraction kit (Fermentas-Thermo Fisher Scientific, USA) based on the protocol recommended by the manufacturer. The purified DNA was inserted into pET-32a(+) cloning vectors. In summary, 11 μ l (60 ng) of the purified PCR product was mixed with 2.5 μ l (150 ng) of pET-32a(+) plasmid, 2 μ l of 10x ligation buffer, 1 μ l (5 U/ μ L) of T4 DNA ligase, and 3.5 μ l of ddH₂O in a 1.5-mL microtube (a total of 20 μ l reaction mixture), and then incubated at 22 °C for 2 h. The ligation product was stored at -20 °C.

Transformation. *Escherichia coli* strain DH5 α was used as a competent cell. In this research, the transformation processes were performed using chemicals and the cold or heat shock. For transformation, 10 μ l of the ligation product (rGRA7-pET32a) was added to 50 μ l of *E. coli* strain DH5 α as competent cells, followed by gentle mixing with pipette tip stirring after a 30-min incubation on ice. In the next stage, the mixture was heat shocked for 90 sec in water bath at 42 °C, and then immediately placed on ice and incubated for 2 min. The transformed cells were cultivated on luria broth agar plates containing ampicillin (100 μ g/ml), and then incubated at 37 °C overnight.

Cloning verification and gene sequencing. The identification of transformed bacterial colonies containing recombinant plasmids was accomplished using Quick-Check (extraction by phenol and chloroform). The colony PCR was performed using a PCR condition similar to that of the first PCR reaction. The positive colonies were selected, and the presence of rGRA7-pET32a recombinant plasmids was verified and compared by restriction enzyme analysis using BglIII and XhoI in order to digest the DNA molecules at specific sites. Subsequently, they were subjected to 1% 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 (Macrogen Inc.) for gene sequencing. Finally, the similarities and differences of the cloned gene sequence with respect to the *GRA7* gene of *T. gondii* were evaluated on website: www.ncbi.nlm.nih.gov/blast.

Expression and purification of recombinant antigens. To obtain the rGRA7, *E. coli* Rosetta (DE3) strain with the rGRA7-pET32a was propagated in LB broth, incubated at 37 °C, and kept in the media to obtain an optical density of 0.5 at 600 nm. In order to obtain the highest expression level of GRA7, the expression conditions, including isopropyl β -D-1-thiogalactopyranoside (IPTG) concentration, temperature, and time, were optimized. Furthermore, to establish the best or more effective cultivation conditions, a successful recombinant protein induction was achieved using 1.0 mM IPTG at 37 °C for 8 h. The IPTG-induced samples were screened and examined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE; Mini-PROTEAN® 3 system; Bio-Rad) with 12.5% resolving gel, and then subjected to Coomassie Brilliant Blue G-250 staining. For the preparation of cleared lysates under denaturing condition, following incubation, the culture-induced bacteria were centrifuged at 8000 \times g for 1 min. The pellet was resuspended in lysis buffer (50 mM phosphate buffer [pH 8.0] and 0.1% Triton X-100), and the cells were disrupted by freeze-thaw method; thereafter, the cell pellet was resuspended in 6 M Gu-HCl. Insoluble debris was removed by centrifugation to save the supernatant (cleared lysate). The purification was carried out under denaturing condition using Ni-NTA purification system (GE Healthcare, Germany) according to the manufacturer's protocol. Briefly, 1 ml 50% Ni-NTA agarose bead was mixed with each 5 ml of lysate, and then incubated at room temperature on a rotary shaker for 1 h. The mixture was loaded on a column, and resin was washed twice with the volumes of buffers C and D, and finally with that of buffer E. The eluted proteins were analyzed using spectrophotometry.

Western blot analysis. After performing electrophoresis on 12.5% polyacrylamide gel, the proteins were transferred onto the nitrocellulose membranes (porablot, NCP, MN, Germany) in a transfer cell (Mini-Protean Tetra system, Biorad) at 100 V/h in order to evaluate immunoreactivity using Western blot analysis (Mahmood and Yang, 2012). For this purpose, the samples were prepared by boiling the cell suspension, containing SDS-PAGE sample buffer, for 5 min. The transferred protein was used as antigen for immunoblotting assay using positive control human serum (Diamex, Germany), containing anti-*Toxoplasma gondii* IgG antibodies at the dilutions of 1:10 and 1:100.

Dot blotting analysis. The verification of rGRA7 immunogenicity was performed using dot blotting analysis. The antigens (2 μ l) were transferred onto nitrocellulose membrane and cut into strips. The membrane strips were blocked for 1 h at 37 °C with shaking in a blocking buffer, contain 1% bovine serum albumin (BSA) in PBS-0.05% Tween 20 (PBS-T). Each membrane strip was washed twice with PBS-T. Subsequently, at the dilutions of 1:10 and 1:100 human sera in PBS-T, BSA 0.25% and skim milk 5% (dilution buffer) were used as dilution buffers for 45 min at 37 °C. Each membrane strip was washed twice with PBS-T. In the next stage, the membrane strips were incubated with horseradish peroxidase-conjugated goat anti-human IgG (Dako, Denmark) diluted 1:1000 in dilution buffer for 60 min at 37 °C. The nitrocellulose membrane was washed twice with washing buffer, PBS-T. Finally, the antigen-antibody complex was detected by enzyme substrate-chromogen reaction. Hydrogen peroxide and 3,3'-Diaminobenzidine (DAB) (Sigma-Aldrich, MO, USA) were used as enzyme substrate and chromogen, respectively.

Indirect enzyme-linked immunosorbent assay for *Toxoplasma gondii*-specific IgG. For the detection of *T. gondii*-specific IgG, ELISA test was carried out as described previously (Marcolino et al., 2000). The high protein-binding capacity 96-well ELISA plates

(MaxiSorp, Nunc, Denmark) were coated with 100 ng of rGRA7 antigens per well in 0.06 M carbonate buffer with a pH of 9.6 overnight at refrigerator temperature. The plates were washed five times with washing solution prepared by PBS-PBS-T and blocked with BSA 1% in PBS-T overnight at 4 °C. The plates were washed five times with (PBS-T). Subsequently, the wells were incubated with positive and negative samples diluted at 1:100 in BSA 0.25% and skim milk 5% in PBS-TM for 1 h at 37 °C. After washing for five times with PBS-T, horseradish peroxidase (HRP)-conjugated goat anti-human IgG antibody diluted at 1:6000 was incubated for 1 h at 37 °C. After washing for five times with PBS-T, the unbound conjugate, TMB substrate solution (3,3',5,5'-tetramethylbenzidine) was added for color development, and the plate was covered with a black cover and incubated at room temperature for 30 min. The reaction was stopped by adding 100 μ l of 1 N H₂SO₄ to each well. The absorbance of tests was determined within 30 min at 450 nm by means of a photometer (DYNEX, USA). The cutoff values were calculated using the mean optical density of negative control sera plus three standard deviations. Antibody titers were expressed as sample optical density (OD)/cutoff OD, as previously described (Silva et al., 2002).

Antibody conjugation to gold nanoparticles. Based on the common protocol (Gholamzad et al., 2015), goat anti-human antibodies (JieYi Biotech, China) were prepared at various concentrations (i.e., 10, 20, 30, 40, 50, 60, 70, and 100 μ g/ml) in Borax buffer. The pH of colloidal gold solution (40 nm; Arista Biological Inc. USA) was adjusted at 9 using 0.2 M potassium carbonate. In the next step, 1 ml of gold nanoparticles with a pH of 9 was added to 100 μ l of each antibody dilution and then incubated for 10 min at room temperature. Subsequently, 100 μ l of 10% sodium chloride was added to each vial. The optimum protein concentration for stabilizing the colloidal gold was determined by calculating OD at 540 nm (Ultrospec 3000, Pharmacia Biotech, Piscataway, NJ, USA; Figure 1).

Conjugate stabilizing buffer. In order to stabilize the conjugated antibody, gold-conjugated antibody was diluted in a buffer containing 50 mM phosphate with a pH of 9, 0.1% of Tween 20, and 1% BSA, and then stored at 4 °C.

Assembling of pads and membrane to make immunochromatographic strip. Different layers of the test strip, including the cellulose fiber as sample pad, glass fiber as conjugate pad, high-flow nitrocellulose membrane, and absorbance pad (Millipore, Bedford, MA) were placed on a backing card (Millipore, Bedford, MA) with 2 mm overlap (Figure 1). The conjugate pad was sprayed with 2 $\mu\text{l}/\text{cm}$ of conjugated antibody diluted in 20 mM phosphate buffers using a quantitative air jet dispenser (BioDot, Irvine, CA.). Then, it was dried at 37 °C for 30 min. On the high-flow nitrocellulose membrane (Millipore, Bedford, MA), 1 $\mu\text{l}/\text{cm}$ of the recombinant *Toxoplasma* antigen GRA7 (1 mg/ml), diluted in PBS (0.01 M, pH 7) and 0.5 $\mu\text{l}/\text{cm}$ mouse anti-goat antibodies (1 mg/ml) (JieYi Biotech, China), was dispensed in the test and control lines, respectively. The nitrocellulose membrane was dried at room temperature. Assembled sheets were cut into strips of 5 mm. The strips were placed in aluminum foil with desiccant and kept at room temperature until use. Figure 1 shows a schematic presentation of assay principle for TOXO-IgG strip test. More specifically, 100 μl of diluted human sera was dropped onto the sample pad. The solution migrated toward the absorbent pad via capillary force and rehydrated the gold nanoparticle conjugated with goat anti-human IgG. If the testing sera contained anti-*Toxoplasma*-specific IgG antibodies, they were detected and captured by the gold nanoparticle conjugated with goat anti-human IgG probes. When the complexes reached the T-line, the immobilized rGRA7 antigens were interacted with the captured anti-*Toxoplasma*-specific IgG antibodies. Furthermore, when they reached the C-line, the immobilized mouse anti-goat antibody captured the excess gold conjugated anti-human IgG probes. Therefore, two characteristic red bands emerged within

10 min. However, negative sera without anti-*Toxoplasma*-specific IgG antibodies resulted in the emergence of one red band on the C-line.

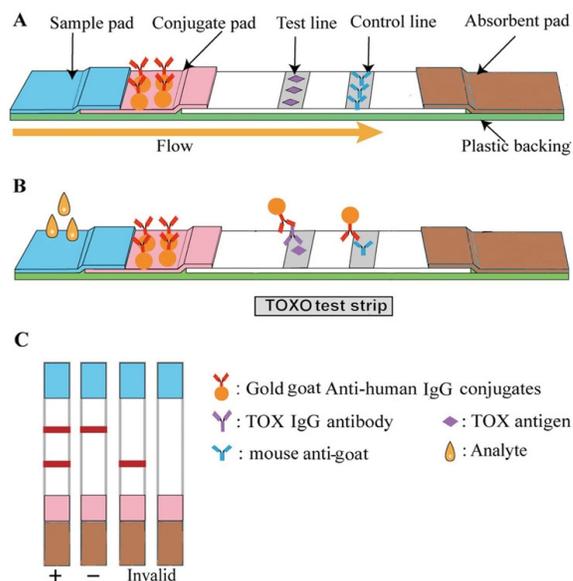


Figure 1. A schematic presentation of TOXO-IgG strip test (edited by author).

Sample preparation. The proposal was approved by the Ethics Committee of Shahid Beheshti University of Medical Sciences, Tehran, Iran. The sera were obtained voluntarily with consent from Razi Medical Laboratory of Karaj, Iran, in accordance with the Helsinki Declaration. The study population corresponded to a group of 91 males and females with the age range of 25-50 years who were sera positive (n=61) or sera negative (n=30) and referred to the medical diagnostic laboratory. The serum aliquots were stored at -20 °C. The samples were tested by LIAISON Toxo IgG II (Diasorin, Italy) assay that is based on chemiluminescence immunoassay (CLIA) technology.

Assay procedure. For the identification of the anti-*Toxoplasma* IgG antibody using strip test, 100 μL of 1:8 diluted human serum samples was transferred into a microtube. Subsequently, the test strips were placed in this liquid from the sample pad side to allow the liquid to move along. The test strips were oriented flatwise at ambient temperature, and the results were read after 10

min. The positive and negative sera were evaluated by test strip, and phosphate buffer was used as negative control. Additionally, in order to estimate the sensitivity of the strip tests, serial dilutions of the standard positive control were prepared, and the strips were assayed. The emergence of two bands (i.e., control line and test line) on the strip was interpreted as a positive result, while a single band in the control band was interpreted as a negative result.

RESULTS

Molecular Results. Specific primers were used for the amplification of GRA7 fragment by the PCR of genomic DNA extracted from the tachyzoites assay that presented 726 bp with a size similar to that of the *GRA7* gene of *T. gondii* using agarose gel electrophoresis (Figure 1). Agarose gel electrophoresis analysis on the constructed pET-32a (+) GRA recombinant plasmids following BglII and XhoI restriction enzymatic digestion revealed two different bands. The first band, which was heavier, indicated linear plasmids, and the second, a lighter 726 bp band, indicated the *GRA7* gene (Figure 2). These findings revealed that the *GRA7* gene was correctly cloned into pET-32 a (+) plasmids. Analysis of sodium dodecyl sulfate-polyacrylamide gel electrophoresis showed that *E. coli* Rosetta DE3 optimized the efficient production of the recombinant protein when induced with 1.0 mM IPTG at 37°C for 8 h (Figure 3). Western blot analysis of transfected bacterial culture medium using positive human serum confirmed the presence of rGR7 in bacterial culture medium supernatant with a weight of 42 KD (Figure 4A).

Immunoassay Results

Antigenicity and immunogenicity evaluation of rGRA7. Immunoreactivity of rGRA7 against the serum containing anti-*Toxoplasma*-specific IgG antibodies was confirmed by dot blot and western blot analyses. In the blot, rGRA7 antigen reacted with various dilutions of the serum that was positive for anti-*Toxoplasma*-specific IgG antibodies. However, it showed no

reaction with the serum-negative samples for anti-*Toxoplasma*-specific IgG antibodies. There was no reactivity in the dot blot strip immunobilized with BSA as a negative antigen control (Figure 4B). The rGRA7 protein also reacted with the sera of the patients with anti-*Toxoplasma*-specific IgG antibodies in western blot analysis with a band of almost 42 kDa (Figure 4A).

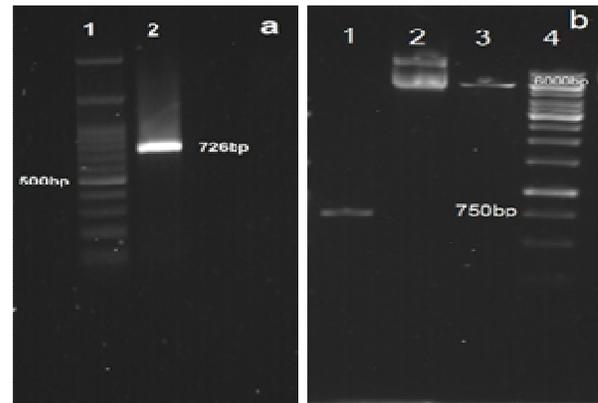


Figure 2. Agarose 1% gel electrophoresis analysis; a) *GRA7*, lane 1) 100 bp DNA ladder, lane 2) polymerase chain reaction product of *GRA7* gene; (b) recombinant plasmid, lane 1) *GRA7* fragment after digestion, lane 2) pET32a(+) plasmid, lane 3) pET32a(+) plasmid following BglII and XhoI enzymatic digestion, lane 4) 1 Kb DNA ladder.

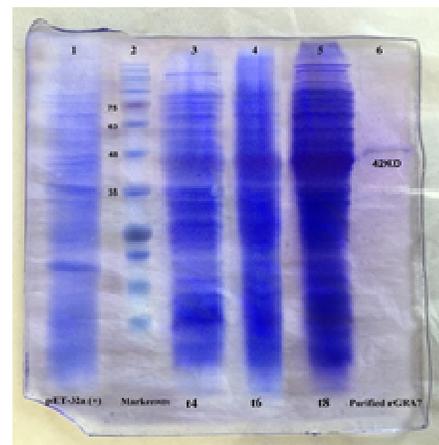


Figure 3. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis of the expression of pET-32a (+) *GRA7* protein in *E. coli* Rosetta (DE3): SDS-PAGE analysis and Coomassie brilliant blue staining; lane 1) Rosetta (DE3) containing pET-32a (+) plasmid without insertion, lane 2) protein marker, lanes 3-5) Rosetta (DE3) containing recombinant pET-32a (+) plasmid 4, 6, and 8 h after induction, respectively, lane 6) purified rGRA7 42-KD *GRA7*.

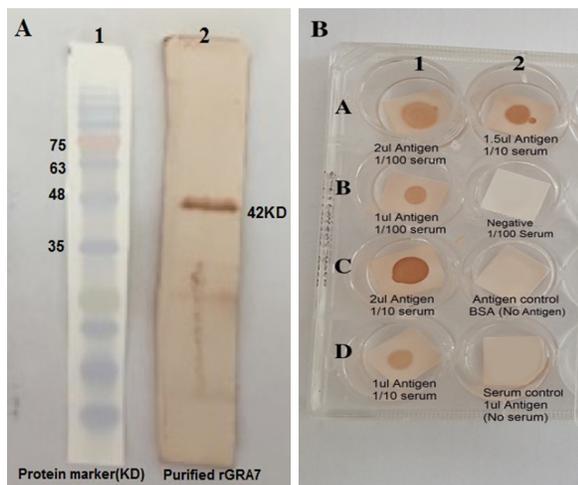


Figure 4. Western blotting (A) and dot blotting (B) after the purification of rGRA7 antigen; lane 1) protein marker, lane 2) 1:10 diluted sera sample using purified rGRA7 dotted onto nitrocellulose membrane; B: A1 and B1) 1:100 diluted serum positive sample using 2 and 1 µl antigens, respectively, C2 and D2) 1:10 diluted serum positive sample using 2 and 1 µl antigens, respectively, A2) 1:10 diluted serum positive sample using 1.5 µl antigens, B2) 1:100 diluted serum negative sample using 1 µl antigens, C2) antigen control using bovine serum albumin instead of serum, D2) serum control using no serum with antigen.

Optimization of anti-human antibody conjugation to colloidal gold solution. To ensure that enough anti-human antibody was used to conjugate with the gold nanoparticles and stabilize the colloidal gold, 5 µg/ml was considered as the optimum concentration of purified antibody for conjugation to gold nanoparticles (Figure 5).

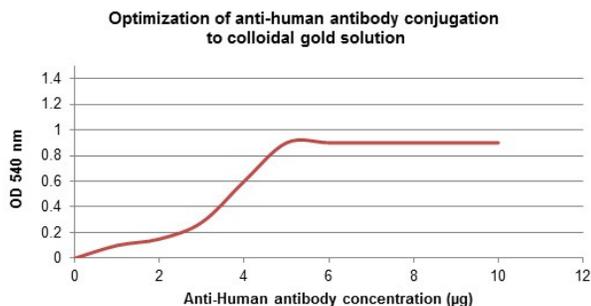


Figure 5. Optimum antibody concentration for colloidal gold conjugation (Minimum antibody concentration that causes no color change after NaCl addition is suitable. Use of 5 µg antibody for 1 ml colloidal gold conjugation is suitable.)

Sensitivity and specificity of TOXO-IgG strip. A total of 91 samples, including 61 positive sera and 30

negative sera, were examined by the strip tests. To this end, all serum samples were subjected to CLIA as a golden method. Based on the results, the sensitivity and specificity of the test strip were 100% and 96.7% (only one false-positive result), respectively. The measurement of the limit of detection was accomplished by comparing the visual signal obtained from the strip test through running 100 µl of the standard sample pool in dilution ratios of 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, and 1:128 (Figures 6-7). Each dilution was examined with three test strips to achieve reliable results. The Toxo-IgG strip test showed a remarkable color in the control line. However, such color intensity did not appear in the test line at dilutions greater than 1:8. Serum sample with a dilution higher than 1:8 showed a visible color in the control and test lines. The detection limit of the Toxo-IgG strip test was evaluated with a serum dilution of 1:8. Possible cross-reactions ruled out using such materials as IgM antibody, and BSA solution was added to the positive samples.

Stability of the test strip. Strip stability was determined by accelerated study method. For this purpose, the test strips were incubated at 37 °C. Based on an established document, one-week stability at 37 °C is equal to two-month stability at 4 °C. The stability of the strips at 4 °C was estimated at about 16 months. Recent studies have shown that the use of test strip under this condition can lead to successful results without compromising the test performance.

DISCUSSION

Dense granule antigens of *T. gondii* play a key role in parasite survival and virulence. They form a significant part of antigens during the early steps following infection. Although anti-rGRA1 was considered as an assay marker of chronic infection, the other GRA proteins, such as rGRA5, rGRA6, rGRA7, and rGRA8, have been reported as the markers of recent infection and potential serodiagnostic assays (Holec-Gasior, 2013; Arab-Mazar et al., 2016). The vacuole and cytoplasm of tachyzoite-infected host cell contain GRA7; therefore, the host immune system well

recognizes this protein once the cyst cells rupture during infection (Jongert et al., 2007). Regarding this, rGRA7 is believed to be a good diagnostic marker when applied by IgG antibody. The IgG antibodies against GRA7 and/or GRA8 solely occur in the earliest stage of immunological responses (Dunn et al., 2008). The purified rGRA7 was applied as an antigen for developing a simple and rapid lateral flow immunoassay test strip using a gold nanoparticle-pAb conjugate probe to detect anti-*Toxoplasma*-specific IgG antibodies in human sera. According to Hiszczyńska-Sawicka et al. (2003), there are several reports on the recombinant production process of *T. gondii* antigens.

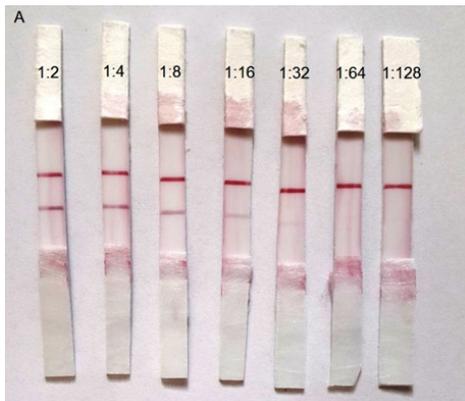


Figure 6. Results of TOXO test strips after running 100 μ l of the TOXO-IgG standard positive sample in different dilution ratios.

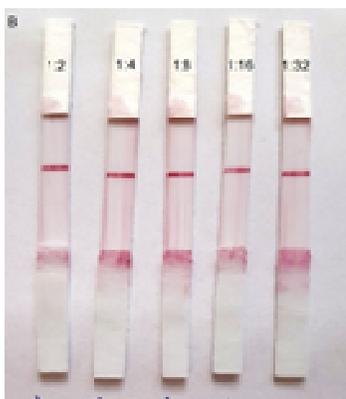


Figure 7. Results of TOXO test strips after running 100 μ l of TOXO-IgG standard negative sample in different dilution ratios.

They used expression and purification process of FLK-BLV cells, *Saccharomyces cerevisiae*, and insect cells by recombinant baculovirus. *Toxoplasma gondii* infection has been detected by many diagnostic methods, including immunologic and molecular techniques. The GRA7 is one of the recombinant proteins that is commonly applied as an antigen in ELISA and CLIA for the detection of specific IgG and IgM, and is usually coated alone on the ELISA plates (Kotresha and Noordin, 2010). Purification of the rGRA7 protein allowed the researchers to use this antigen as a diagnostic marker (Arab-Mazar et al., 2016). The use of *T. gondii* recombinant protein is still preferable to the application of lysed host cell *T. gondii* tachyzoites. It is also necessary to develop new approaches for the improvement of recombinant protein production in *E. coli* expression system. The pET-32a (+) vector fused with Trx-Tag thioredoxin protein was used to improve recombinant protein production to obtain more soluble and stable proteins and purification, and increase the immunoreactivity of antigen. Moreover, the Rosetta (DE3) is used as an expression system that can express rare human codon, thereby having a higher expression ability. Similar immunoreactivity results were obtained for the antigen after 8 months of protein production, which were indicative of a reasonable stability of antigen. Arab-Mazar et al. (2016) adopted a different cloning (pTZ57RT) and expression system (pGEX-6p-1) in their produced rGRA7 proteins. However, they exhibited lower sensitivity (92%) and specificity (94%), compared with those obtained in the current study. Lateral flow immunoassay or immunochromatographic assay is a rapid, accurate, sensitive, and inexpensive diagnostic technique that facilitates the detection of IgG antibodies against *T. gondii* in human serum using rGRA7 protein. Gold nanoparticles are the most commonly used labels in rapid strip tests due to having a strong color that is clearly visualized and does not disappear on the white paper. Gold Colloid particles are made of golden elemental nucleus with a double-

layered negative coating. Immunoglobulins are coupled directly to the surface of gold nanoparticles during the conjugation process using a non-covalent reaction (Goudarzi et al., 2015). In this study, a gold-pAb probe was used to detect IgG antibodies and rGRA7 antigen as a capture agent for trapping a Toxo-IgG-specific antibody. The test was performed by placing 100 μ L of 1:8 diluted serum on the sample pad, and the sample was moved along the nitrocellulose membrane path. The Toxo-specific IgG was bonded to the gold-Ab probe in the conjugate pad on the one side and attached to the Toxo-antigen on the other side that was immobilized in the test line. When the sample solution complex reached the control line, a non-specific reaction was captured because the mouse anti-goat secondary antibody was coated in this section. The experiment results were recorded with the naked eye within 10 min. Meanwhile, the ability of this test was examined on a patient with cervical lymphadenopathy caused by *T. gondii*. This patient was referred to an oncology specialist physician to undergo medical examination in order to rule out cancer.

Ethics

We hereby declare all ethical standards have been respected in preparation of the submitted article.

Conflict of Interest

The authors declare that they have no conflict of interest.

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