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ORIGINAL RESEARCH

Development, optimization, and validation of an in-house Dot-ELISA rapid test based on SAG1 and GRA7 proteins for serological detection of *Toxoplasma gondii* infections

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Background: The aim of the present study was to develop a simple, portable, and rapid assay for serodiagnosis of toxoplasmosis based on recombinant *Toxoplasma gondii* (*T. gondii*) SAG1 (rSAG1) and GRA7 (rGRA7) proteins.

Methods: The rSAG1 and rGRA7 proteins were expressed in *Escherichia coli* (*E. coli*) and purified in a single step by immobilized metal ion affinity chromatography. The immunoreactivity of the recombinant antigens was tested in an in-house IgG and IgM Dot enzyme-linked immunosorbent assay (Dot-ELISA) for potential use in serodiagnosis of *T. gondii* infection.

Results: Results from the comparison of in-house rSAG1-Dot-ELISA with ELISA for the detection of anti-*Toxoplasma* IgG and IgM include sensitivity of 83.7% and 81.2%, specificity of 90.2% and 89.3%, positive predictive values of 85.9% and 68.4%, and negative predictive values of 88.6% and 94.3%, respectively. Sensitivity of 66.2%, specificity of 81.2%, positive predictive values of 71.6%, and negative predictive values of 77.1% were concluded from in-house IgG rGRA7-Dot-ELISA. The sensitivity and specificity of IgM rGRA7-Dot-ELISA included 87.5% and 83.9%, respectively. Sensitivity and specificity of in-house Dot-ELISA for a combination of rSAG1 and rGRA7 included 87.5% and 91.1% for IgG and IgM, respectively. Sensitivity and specificity of a combination of rSAG1 and rGRA7 for the detection of IgM in suspected sera to acute toxoplasmosis were higher than those for the detection of IgG in sera with chronic infections (90.6% and 92% instead of 86.2% and 91.6%, respectively).

Conclusion: The highlighted parameters of combined recombinant proteins were more significant than those of single recombinant proteins in in-house Dot-ELISA. These data suggest that the in-house Dot-ELISA based on rSAG1 and rGRA7 combination is a promising diagnostic tool with a similar sensitivity to the native antigens of *T. gondii*, which can be used for the serodiagnosis of toxoplasmosis in fields as well as less equipped laboratories.

Keywords: *Toxoplasma gondii* RH strain, in-house Dot-ELISA, rSAG1, rGRA7, soluble tachyzoite antigen (STAg), recombinant proteins

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Introduction

Toxoplasma gondii (*T. gondii*; agent of toxoplasmosis) is a ubiquitous intracellular parasite capable of infecting a broad range of warm-blooded animals and humans.[1] Humans can be infected through ingestion of contaminated food and water with oocysts and consumption of undercooked/raw meats containing tissue *T. gondii*

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cysts. Vertical transmission of rapidly dividing *T. gondii* tachyzoites from pregnant mothers to developing fetuses is another route of human infection.[2] Generally, *T. gondii* infection is asymptomatic in immune-competent individuals. However, the infection can result in serious diseases in fetuses and immunocompromised patients, including those with HIV/AIDS, cancer, or organ transplantation.[3] To date, various methods have been used in the diagnosis of toxoplasmosis, including microbial isolation, protein analysis, immunological (serological) and molecular techniques. Of these methods, serological methods are most commonly used for the detection of specific antibody classes or antigens against toxoplasmosis.[4] Most of the available commercially serological kits for the diagnosis of toxoplasmosis use total parasite antigens prepared from tachyzoites in mice and/or tissue cultures in vitro and possibly contain varying quantities of extra parasitic material.[5,6] However, inter-assay variability is sometimes seen due to the lack of standard antigens or proper protocols for the preparation of these antigens. Usually, preparation of these antigens is expensive. Furthermore, preparations commonly include host cell-derived components. In addition, use of live parasites in antigen preparation can result in serious health problems. To solve these problems, antigens are now produced using recombinant DNA technology.[7] Relatively, target genes from *T. gondii* have recently been cloned and expressed using various systems. Of these recombinant proteins, surface antigens (SAGs), matrix antigens (MAGs), microneme proteins (MICs), rhoptry proteins (ROPs), and dense granule antigens (GRAs) are the most commonly used proteins in literature.[8] Studies have described the successful use of recombinant antigenic proteins to detect *T. gondii*-specific antibodies. These studies analyzed recombinant antigens separately and in combination with each other to increase diagnostic sensitivity.[9,10] In the current study, SAG1 was chosen because it is one of the most immunogenic and stage-specific *T. gondii* antigens, present in tachyzoites but not in bradyzoite.[11] Another chosen proteins included GRA7 because it causes a powerful antibody response in acute phases of the infection.[12]

Nowadays, enzyme-linked immunosorbent assays (ELISAs) are widely used in routine diagnoses and seroepidemiological investigations using purified recombinant proteins. However, the assay is expensive, laborious and time-consuming and requires expert operators, special materials and equipment.[13,14] These requirements are sometimes unavailable in resource-limited countries. Hence, an easily operated, cost-effective, and rapid assay such as Dot-ELISA is necessary for the early diagnosis of toxoplasmosis in clinics and fields. Dot-ELISA, as a modified ELISA technique in which the antigen–antibody interaction is performed on nitrocellulose (NC) membranes, has been developed to detect antigens or antibodies.[15] In 1983, Pappas et al have first introduced Dot-ELISA for the serodiagnosis of human visceral leishmaniasis (VL) and later standardized the assay.[16] Dot-ELISA has been used for the diagnosis of helminths such as *Fasciola gigantica* in experimentally infected sheep, *Trichinella spiralis* in swine, and *Haemonchus contortus* in sheep sera. Moreover, this technique has been widely used for the detection of infections such as those by *Toxocara canis, Dirofilaria immitis, Leishmania infantum, Babesia bovis, B. bigemina, Anaplasma marginale*, and *T. gondii*.[17]

To date, a very few studies have been carried out to detect *T. gondii* antigens or antibodies using Dot-ELISA.[15,18,19] To the best of the authors' knowledge, no studies have been performed to assess Dot-ELISA based on recombinant *T. gondii* SAG1 (rSAG1) and GRA7 (rGRA7) antigens in human sera. Therefore, the aim of the present study was to assess an in-house Dot-ELISA based on various *T. gondii* antigens such as rSAG1, rGRA7, combination of rSAG1 and rGRA7, and soluble tachyzoite antigens (STAg) for the serodiagnosis of human toxoplasmosis. Results were compared to those from standard ELISA.

Methods

Bacterial strains, plasmids, enzymes, and reagents

The *E. coli* BL21 (DE3) pLysS (Promega, USA) was used to express recombinant antigens. The pET28a plasmid (Novagen, USA) was used to construct an expression system. The *E. coli* cells with plasmids were cultured aerobically at 30°C in Luria-Bertani (LB) media supplemented with 50 µg/ml of kanamycin and 50 µg/mL of chloramphenicol. Restriction enzymes were purchased from New England Biolabs (USA) and reagents of polymerase chain reaction (PCR) from CinnaGen (CinnaGen, Iran). Purification system using nickel nitrilotriacetic acid (Ni-NTA) resin (Qiagen, Germany) was provided by Invitrogen (USA). Isopropyl-D-thiogalactopyranoside (IPTG), agarose, and reagents of protein purification were purchased from Sigma-Aldrich (USA). The NC membrane was purchased from Bio-Rad (USA). Goat anti-human IgG and IgM horseradish peroxidase (HRP)

labeled conjugates, diaminobenzidine substrate (DAB), and prestained protein markers were purchased from Sigma-Aldrich (USA).

Ethical consideration

The study was carried out according to the ethical standards by institutional and/or national research committees and the Helsinki Declaration, 1964. Animal procedures were carried out according to Guidelines for the Care and Use of Laboratory Animals published by the United States National Institutes of Health and approved by the Ethical Committee of Tehran University of Medical Sciences, Tehran, Iran. The current study was approved by the Ethics Committee of Tehran University of Medical Sciences on April 11, 2016 (Approval No. 29,999). Furthermore, written informed consents were signed by the participants before starting the study. In case the person is illiterate, informed consent can be given by thumbprint and a signature of an impartial witness. Parental consent was obtained from the parent or guardian for participants less than 16 years old included in this study.

Study design and clinical sample collections

Human serum samples were provided by various clinical laboratories in Tehran and Shahriar, Iran, from May 2016 to November 2017. In the current retrospective study, human serum samples were tested using commercial *Toxoplasma* IgG and IgM ELISA kits (Trinity Biotech, USA) as reference method for *T. gondii*-specific IgG (Toxo IgG) and IgM (Toxo IgM) detections according to the manufacturer's instructions. ELISA was chosen as reference, as it is the classical and most widely used test for detection of *T. gondii*-specific antibodies in patient sera and the diagnostic standard test in our laboratory.[19] A total of 224 sera were divided into 3 major groups according to clinical and serological criteria,[20] as follows: 1) Group I, chronic infection sera (CIS) including 80 samples positive for anti-*T. gondii* IgG and negative for anti-*T. gondii* IgM with a follow-up sample indicating no increase in IgG or presence of IgM; 2) Group II, acute infection sera (AIS) including 32 samples positive for anti-*T. gondii* IgM and sera were also had very low avidity in avidity assay (VIDAS Toxo IgG Avidity, bioMerieux, France) and all these patients had the sign of lymphadenopathy; and 3) Group III, negative infection sera (NIS) including 112 samples negative for anti-*T. gondii* IgG and IgM antibodies. Four samples were excluded from further analysis, due to positive for anti-*T. gondii* IgM and had high or equivocal avidity in avidity assay without any sign of lymphadenopathy, suggesting that IgM alone is not an accurate acute-phase marker. To ensure a blinded analysis, each sample was given a unique identification code. Samples from each setting (CIS, AIS, NIS) were then used for the performance assessment of in-house Dot-ELISA strips with various antigens for the detection of human *Toxoplasma* specific antibodies according to the identification number. For cross-reactivity studies, in-house Dot-ELISA strips were tested against human sera corresponding to parasitic infections other than toxoplasmosis, including *F. hepatica* (n=3), *L. infantum* (n=3), *Echinococcus granulosus* (n=4), and malaria (n=5).

Enzyme-linked immunosorbent assay (ELISA)

The ELISA commercial kits (Trinity Biotech Captia, USA) were used to detect Toxo IgG and IgM according to the manufacturer's instructions. First, serum specific antibodies against *T. gondii* were bound with *T. gondii* coated antigen on the surface of reagent wells to form antigen–antibody complexes. Goat anti-human IgG or IgM labeled with HRP was used as the secondary antibody to the antigen–antibody complex. After incubation and wash, a chromogen substrate of 3,3′,5,5′-tetramethylbenzidine (TMB) was added to each well to detect HRP activity. The process was stopped by addition of stop solution (1-N H_2SO_4) and optical density (OD) was recorded using an automated ELISA reader (Biotek, USA) at 450 nm. Calibrator and control sera were used in each test set. Immune status ratio (ISR) of the samples was calculated by dividing the sample OD by the cutoff value (cutoff=mean OD of the calibrators×correction factor). Results were interpreted as follows according to the manufacturer's recommendations: sera <0.9 ISR were reported as nonreactive, 0.91–1.09 as equivocal, and >1.1 as reactive.[19]

Expression of recombinant proteins

Recombinant expression plasmids were successfully constructed and purified in the laboratory as described previously.[21,22] Briefly, PCR amplicons were amplified using DNA extracted from RH strains of *T. gondii*. Then, PCR products were purified and cloned into pET28a vectors at specified restriction sites. Resulting recombinant

plasmids containing SAG1 and GRA7 genes were tagged pET28a/SAG1 and pET28a/GRA7, respectively. The *E. coli* strain BL21 (DE3) pLysS transformed with the pET28a/SAG1 or pET28a/GRA7 was overnight grown in LB broth supplemented with 50 µg/mL of kanamycin and 50 µg/mL of chloramphenicol at 37°C with a 200 rpm shake. Then, 100 mL of LB media, supplemented with the same antibiotics, were inoculated with 2 mL of the overnight culture. Temperature decreased 30°C with vigorous shaking until an OD of 0.5–0.6 was achieved at 600 nm. Protein production was then induced using IPTG to a final concentration of 1 mM. Culture was grown at 30°C for 16 hrs with a 200 rpm shake. Cells were harvested using centrifugation at 5000× g for 5 mins and subjected to protein purification.

Purification of recombinant proteins

The histidine-tagged recombinant protein was purified through purification system using Ni-NTA resin according to the manufacturer's instructions under denaturing and native conditions for SAG1 and GRA7 proteins, respectively. Briefly, cell pellets were resuspended in lysis buffer A1 for His6-SAG1 protein purification (8 M of urea, 20 mM of NaH_2PO_4, 500 mM of NaCl [pH 8], 1 mg/mL of lysozyme and protease inhibitor cocktail) or lysis buffer A2 for His6-GRA7 (50 mM of NaH_2PO_4, 500 mM of NaCl [pH 8], 1 mg/mL of lysozyme and protease inhibitor cocktail) (Roche Applied Science, Switzerland). Suspensions were sonicated for 30 pulses for at least 15 times at 1 min intervals in an ice–water mixture bath using microtip (Branson Ultrasonic Corporation, Danbury, USA). Soluble fractions were collected using centrifugation at 8000× g for 30 mins at 4°C. Washed pellets containing insoluble inclusion body proteins were extracted using 8 M of urea in extraction buffer (20 mM of NaH_2PO_4 and 500 mM of NaCl, pH 6.3) supplemented with a protease inhibitor cocktail. Solubilized proteins were incubated with Ni-NTA resin for 30–60 mins using gentle agitation to keep the resin suspended in lysate solution at room temperature. Recombinant antigens were washed twice with 8 mL of washing buffer B20 (buffer A1 or A2 containing 20 mM of imidazole) and twice with washing buffer B50 (buffer A1 or A2, containing 50 mM of imidazole). Recombinant proteins were eluted with 8–12 mL elution buffer C (buffer A1 or A2, containing 250 mM of imidazole). Eluted fractions were dialyzed against a phosphate-buffered saline (PBS) buffer (1% of NaCl w/v, 0.075% of KCl w/v, 0.14% of Na_2HPO_4 w/v, and 0.0125% of KH_2PO_4 w/v). The sizes of the expressed target proteins were determined using 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and staining with Coomassie blue. The concentration of the purified recombinant proteins was assessed using Bradford assay (Bio-Rad, USA) and bovine serum albumin (BSA) as standard. Protein solutions were adjusted to 2 mg/mL, divided into small aliquots and stored at −80°C until use. Purified denatured SAG1 was refolded through urea gradient dialysis before assessment of the protein content using Bradford assay. Proteins were loaded into a dialysis bag with a membrane molecular mass cutoff of 13,000 Da and dialyzed against 100 volumes of buffer (20 mM of NaH_2PO_4 and 500 mM of NaCl, pH 8) at 4°C for nearly 20 hrs. Denaturants were slowly removed using serial dialysis with descending concentration of urea from 6 to 3 and to 1.5 and then to 0 M in 10 mmol/L of PBS, pH 7.[23,24]

Western blot analysis of recombinant proteins

The immunoreactivities of recombinant proteins were determined using Western blot analysis. After electrophoresis, proteins were transferred to NC membrane. Membranes were blocked with PBS containing 5% of skimme milk (PBS-M 5%) for 1 hr at room temperature and then washed 3 times with PBS containing 0.1% of Tween 20 (PBS-T 0.1%) for 5 mins. Then, membranes were incubated with positive *T. gondii* human sera diluted 1:100 in blocking solution for 2 hrs at room temperature. After three washes, membranes were incubated with anti-human IgM peroxidase-labeled conjugate diluted 1:2000 in skimmed milk for 1 hr with shaking. Then, membranes were washed and proteins were detected using DAB as chromogenic substrate. Prestained protein markers were used for SDS-PAGE and Western blotting.

Preparation of soluble antigens of *T. gondii*

The STAg was prepared from tachyzoites of *T. gondii* as previously described.[25] Briefly, RH tachyzoites of *T. gondii* were intraperitoneally inoculated into BALB/c mice. At day 5 after infection, tachyzoites were collected from the abdominal cavity of the mice using peritoneum wash with sterile PBS (pH 7.4). Tachyzoites were washed 3 times with PBS and then sonicated and centrifuged at 14,000× g for 1 hr at 4°C. Supernatants were filtered through Whatman® No. 1 paper filters and then divided into small aliquots and stored at −20°C until use. Protein quantity of the samples was subsequently assessed using Bradford assay.

Preparation of the strips and optimization of in-house Dot-ELISA

Dot-ELISA was carried out based on the standardized protocols described previously with optimization of the recombinant antigen concentrations, dilution of serum samples and secondary antibody conjugates.[26] Generally, NCs with 0.22 μm pores were cut into strips of 0.8 mm wide and 5 cm long. Then, various quantities of STAg, purified rSAG1 and rGRA7 from *T. gondii* and combinations of rSAG1 and rGRA7 (1:1, 2:1 and 1:2) were dotted on NCs followed by incubation for 45 mins at room temperature. After drying, non-specific protein binding sites were blocked by addition of PBS-M 5% (pH 7.4) before the incubation with serum samples. Then, serum samples were diluted with PBS-M 3% and added to strips. After incubation at 37°C for 45 mins, strips were washed 3 times in PBS-T 0.1%. Bound human IgG was detected by adding goat anti-human IgG HRP-labeled conjugates to the strips. Human IgM was detected using goat anti-human IgM conjugated with HRP. Following incubation at 37°C for 45 mins, strips were washed 3 further times and soaked with a fresh solution of DAB and incubated for 10 mins, rinsed with distilled water (DW) and blotted dry. Each in-house Dot-ELISA strip was tested using serum from each category. Samples with brown spots (compared to negative and positive control sera, antigens, and secondary antibody controls) were reported as positive. The optimum concentration of each antigen used in the test was assessed using four concentrations of antigens (1.25, 2.5, 3.75, and 5 μg/mL) and tested using positive and negative control sera.

Evaluation of the in-house Dot-ELISA strip

Sensitivity, specificity, positive predictive value, negative predictive value, validity, and relative agreement of in-house Dot-ELISA strips were calculated for each antigen as follows: sensitivity=TP/TP+FN×100, specificity=TN/TN+FP×100, positive predictive values=TP/TP+FP×100, negative predictive values=TN/TN+FN×100, validity=sensitivity+specificity/2 and relative agreement=TP+TN/TP+TN+FP+FN×100; when TP was true positive (No. of samples positive with both tests), FP was false positive (No. of samples positive with in-house Dot-ELISA strips and negative with ELISA), TN was true negative (No. of samples negative with both tests) and FN was false negative (No. of samples negative with in-house Dot-ELISA strips and positive with ELISA).

Stability of the in-house Dot-ELISA strips

Stability of the strips was assessed using standard positive and negative sera. Ten strips were, respectively, stored for 2, 4, and 6 months at room temperature. Stored strips were re-tested for specificity and sensitivity with known *T. gondii* positive or negative human sera.

Statistical analysis

Statistical analysis was carried out using SPSS software v.20 (IBM Analytics, USA). Data were reported using calculation of frequencies (%) and 95% confidence intervals. Furthermore, Kappa test (κ) was used to calculate the degree of agreement between the in-house Dot-ELISA and Trinity ELISA according to a method originally described by Landis and Koch.[27] The strength of agreement was assessed by κ, with values interpreted as poor ($\kappa \leq 0$), slight ($0 < \kappa \leq 0.20$), fair ($0.21 < \kappa \leq 0.40$), moderate ($0.41 < \kappa \leq 0.60$), substantial ($0.61 < \kappa \leq 0.80$), and near-perfect agreement ($0.81 < \kappa \leq 1.0$). Accuracy of in-house Dot-ELISA strips for the detection of exposures to *T. gondii* was assessed using sensitivity and specificity. Positive and negative predictive values were reported according to a method by Jacobson.[28] *P*-values less than 0.05 were considered statistically significant.

Results

Cloning of the SAG1 and GRA7 genes into pET28a vectors

The SAG1 (1011 bp) and GRA7 (711 bp) genes were amplified from tachyzoite genomic DNA and successfully cloned into pET28a vectors. Positive colonies were verified using PCR with gene-specific primers, restriction enzymes, and sequencing. Digestion of pET28a/SAG1 with *Eco*RI and *Xho*I endonucleases produced two bands of 5369 and 1011 bp for pET28a and SAG1, respectively (Figure 1A). After digestion of pET28a/GRA7 using *BamH*I and *Not*I nucleases, two bands of 5369 bp for pET28a and 711 bp for GRA7 were produced (Figure 1B). Sequencing results revealed 100% similarity with the previously recorded SAG1 and GRA7 gene sequences in GenBank database (Accession Nos. MK250980 and MK250981). Verified recombinant plasmids were transformed into BL21 (DE3) pLysS expression bacterial system.

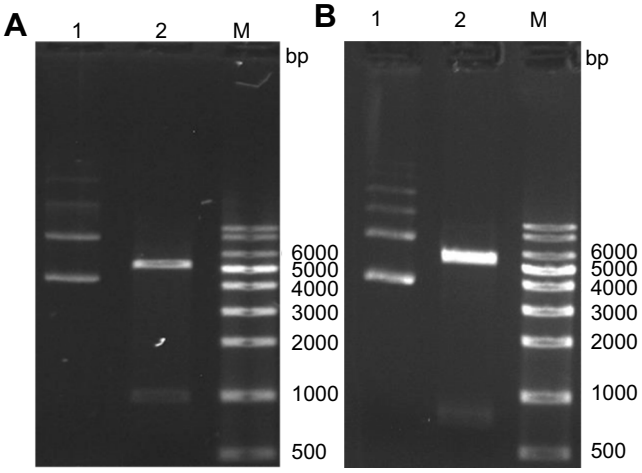

Figure 1 The 1.5% agarose gel electrophoreses of digested (**A**) recombinant pET28a/SAG1 and (**B**) recombinant pET28a/GRA7. (**A**) Lane 1, undigested pET28a/SAG1; Lane 2: *EcoR*I and *Xho*I digested pET28a/SAG1; Lane M, 1-kb DNA ladder. (**B**) Lane 1, undigested pET28a/GRA7; Lane 2, *BamH*I and *Not*I digested pET28a/GRA7; Lane M, 1-kb DNA ladder.

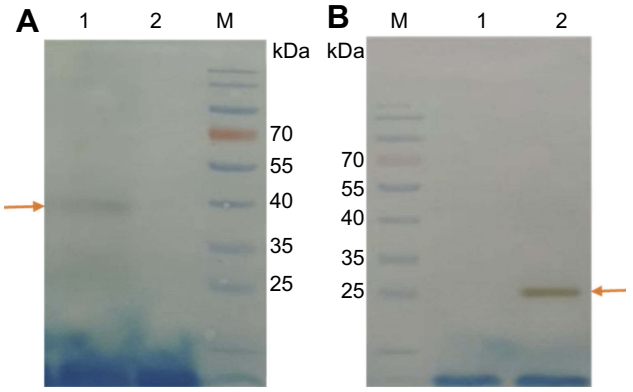

Figure 2 Western blot probed with toxoplasmosis patient sera. (**A**) Lane 1, purified rSAG1 protein; Lane 2, BL21 lysates containing pET28a vector only; Lane M, 10–180 kDa protein marker. (**B**) Lane 1, BL21 lysates containing pET28a vector only; Lane 2, purified rGRA7 protein; Lane M: 10–180 kDa protein marker. Arrows indicate purified rSAG1 and rGRA7 proteins at approximately 37 and 26 kDa, respectively.

Expression and purification of the recombinant antigens

Expression of rSAG1 and rGRA7 were induced using 1 mM of IPTG when cells grew to an OD of 0.6 at 600 nm for 16 hrs. Recombinant *T. gondii* SAG1 and GRA7 antigens containing 6 histidyl residues at either N- and C-terminals were expressed in *E. coli* with calculated molecular masses of 37 and 26 kDa, respectively. These recombinant proteins were successfully purified using one-step chromatography and Ni-NTA resin. Furthermore, purified recombinant proteins were verified using Western blot with human sera from *Toxoplasma* infected patients. In each case, 37 and 26 kDa bands were seen for SAG1 and GRA7 proteins, respectively. No bands were detected in vector control lanes (Figure 2).

Optimization of in-house Dot-ELISA strips for detection of anti-*T. gondii* antibodies

In the present study, optimum concentrations of STAg and recombinant antigens included 2.5 µg for the detection of Toxo IgG and 3.75 µg for the detection of Toxo IgM used in tests appropriate for coating NC strips. The optimum dilution of conjugates included 1:2000 using checker-board titration. During optimization of antigens and conjugates, brown dots on NC strips at antigen-coated sites were clearly visible up to 1:50 dilution values of the positive control serum samples and hence a 1:50 dilution of sera was used for further in-house Dot-ELISA tests.

Reactivity of human serum samples against various antigens of in-house Dot-ELISA strips

Positive reactions were visualized as brown spots. Results were reported as unreactive when no colors were seen (Figure 3). Positive and negative samples of in-house Dot-ELISA strips with various antigens for the detection of Toxo IgG and IgM alone and combined Toxo IgG and IgM antibodies are shown in Tables 1, 2, and 3, respectively. Moreover, intrinsic parameters including sensitivity and specificity, positive and negative predictive values, validity, relative agreement, and kappa (κ) statistics were calculated for each antigen of in-house Dot-ELISA strips, compared to those of commercial ELISA for the detection of anti-*Toxoplasma* antibodies. Table 4 shows results of in-house Toxo IgG Dot-ELISA strips with various antigens, compared to those of ELISA. Results from the current study on group I sera (CIS) showed that the highest sensitivities were achieved from STAg and combined rSAG1 and rGRA7 antigens of strips for the Toxo IgG with 88.7% and 86.2%, respectively. Furthermore, the highest specificity, validity, relative agreement, and kappa value were achieved from combined rSAG1 and rGRA7 antigens of strips with 91.1%, 88.6%, 89%, and 0.77, respectively (Table 4). Sensitivity achieved with GRA7 antigen was the lowest (66.2%) for Toxo IgG in CIS. Results from in-house IgM Dot-ELISA strips with various antigens are

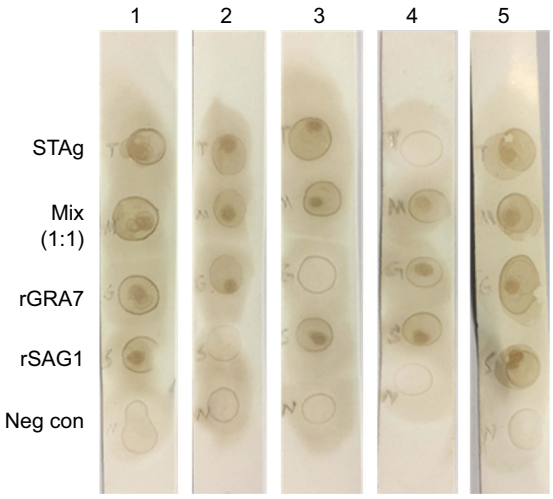

Figure 3 Reactivity patterns of in-house Dot-ELISA strip with various antigens for the detection of anti-*T. gondii,* antibodies. Five strips are shown in the figure as examples. All directions are from top to bottom. Strip 1, positive reaction of STAg, combination of rSAG1 and rGRA7 (1:1), rGRA7 and rSAG1 and negative reaction of negative control with patient sera. Strip 2, positive reaction of STAg, combination of rSAG1 and rGRA7 (1:1), rGRA7 and negative reaction of rSAG1 and negative control with patient sera. Strip 3, positive reaction of STAg, combination of rSAG1 and rGRA7 (1:1), rSAG1 and negative reaction of rGRA7and negative control with patient sera. Strip 4, positive reaction of combination of rSAG1 and rGRA7 (1:1), rSAG1, rGRA7 and negative reaction of STAg and negative control with patient sera. Strip 5, positive reaction of STAg, combination of the rSAG1 and rGRA7 (1:1), rGRA7 and rSAG1 and negative reaction of negative control with patient sera.

summarized in Table 5. Results of the study on AIS group showed that for detection of Toxo IgM, STAg alone and combined rSAG1 and rGRA7 resulted in a 90.6% sensitivity while GRA7 and SAG1 antigens alone showed 87.5% and 81.2% sensitivity, respectively (Table 5). The relative sensitivity, specificity, validity, and agreement for combined rSAG1 and rGRA7 using in-house Dot-ELISA strip to detect Toxo IgM were 90.6%, 92%, 91.3%, and 91.7%, respectively (Table 5). Results from the comparison of various antigens of in-house Dot-ELISA with ELISA for Toxo IgG and IgM are presented in Table 6. For the detection of Toxo IgG and IgM, STAg alone and a combination of the two antigens (rSAG1+rGRA7) were further sensitive (89.3% and 87.5% respectively). Totally, the following intrinsic parameters were calculated for Toxo IgG and IgM rSAG1+rGRA7 of in-house Dot-ELISA strips, compared to those of ELISA: sensitivity of 87.5%, specificity of 91.1%, validity of 89.3% and further PPV and NPV of 90.7% and 87.9%, respectively (Table 6). No non-Toxo parasitic infection sera reacted with various antigens of in-house Dot-ELISA strips.

Stability of in-house Dot-ELISA strips

Test results were similar from months 2 to 6 with all known *T. gondii* positive sera being positive and all known *T. gondii* negative sera being negative. False positives or negatives were not detected. These results suggest that in-house Dot-ELISA strips can be stored at room temperature for at least 6 months without losing their sensitivity or specificity.

Discussion

Diagnosis of toxoplasmosis is most commonly based on the detection of anti-*Toxoplasma* specific antibodies in infected sera. Although Dye test (DT) is the gold standard and ELISA is one of the most reliable methods for the detection of specific antibodies against *Toxoplasma* in sera, use of DT is complicated due to the need of live parasites and ELISA is time-consuming, laborious, expensive, and impractical in fields.[29] Available immunoassays are mainly based on tachyzoite lysate (TLA) antigens as coating antigens, which are highly sensitive and specific diagnostic tools.[30] However, it is well known that whole somatic antigen-based assays may vary significantly between the laboratories or batches and hence be difficult to standardize. An alternative approach is the use of recombinant antigenic proteins with advantages of precise antigens and easy standardization.[10] However, no effective

Table 1 Summary of results from in-house Dot-ELISA strips with various antigens for the detection of anti-*T. gondii* IgG in 192 sera, compared to results from ELISA

Result	No. of positive and negative sera using in-house Dot-ELISA strip							
	rSAG1		rGRA7		rSAG1 + rGRA7 (1:1)		STAg	
ELISA	Pos	Neg	Pos	Neg	Pos	Neg	Pos	Neg
Pos	67	13	53	27	69	11	71	9
Neg	11	101	21	91	10	102	20	92
Total	78	114	74	118	79	113	91	101

Abbreviations: ELISA, enzyme-linked immunosorbent assay; rSAG1, recombinant SAG1 protein; rGRA7, recombinant GAR7 protein; STAg, soluble tachyzoite antigen; Pos, positive; Neg, negative.

Table 2 Summary of results from in-house Dot-ELISA strips with various antigens for the detection of anti-*T. gondii* IgM in 144 sera, compared to results from ELISA

Result	No. of positive and negative sera using in-house Dot-ELISA strip							
	rSAG1		rGRA7		rSAG1 + rGRA7 (1:1)		STAg	
ELISA	Pos	Neg	Pos	Neg	Pos	Neg	Pos	Neg
Pos	26	6	28	4	29	3	29	3
Neg	12	100	18	94	9	103	18	94
Total	38	106	46	98	38	106	47	97

Abbreviations: ELISA, enzyme-linked immunosorbent assay; rSAG1, recombinant SAG1 protein; rGRA7, recombinant GAR7 protein; STAg, soluble tachyzoite antigen; Pos, positive; Neg, negative.

Table 3 Summary of results from in-house Dot-ELISA strips with various antigens for detection of anti-*T. gondii* IgG and IgM in 224 sera, compared to results from ELISA

Result	No. of positive and negative sera using in-house Dot-ELISA strip							
	rSAG1		rGRA7		rSAG1 + rGRA7 (1:1)		STAg	
ELISA	Pos	Neg	Pos	Neg	Pos	Neg	Pos	Neg
Pos	93	19	81	31	98	14	100	12
Neg	12	100	21	91	10	102	20	92
Total	105	119	102	122	108	116	120	104

Abbreviations: ELISA, enzyme-linked immunosorbent assay; rSAG1, recombinant SAG1 protein; rGRA7, recombinant GAR7 protein; STAg, soluble tachyzoite antigen; Pos, positive; Neg, negative.

Table 4 Assessment of in-house Dot-ELISA strips with various antigens for the detection of anti-*T. gondii* IgG in 192 sera, compared to that of ELISA

Result	In-house Dot-ELISA strip			
	rSAG1 (%)	rGRA7 (%)	rSAG1+rGRA7 (1:1) (%)	STAg (%)
Sensitivity	83.7	66.2	86.2	88.7
Specificity	90.2	81.2	91.1	82.1
PPV	85.9	71.6	87.3	78
NPV	88.6	77.1	90.3	91.1
Validity	86.9	73.7	88.6	85.4
Relative agreement	87.5	75	89	84.9
Kappa (95% CI)	0.74 (0.64–0.83)	0.48 (0.35–0.6)	0.77 (0.68–0.86)	0.69 (0.59–0.79)

Abbreviations: *T. gondii, Toxoplasma gondii*; ELISA, enzyme-linked immunosorbent assay; rSAG1, recombinant SAG1 protein; rGRA7, recombinant GAR7 protein; STAg, soluble tachyzoite antigen; Pos, positive; Neg, negative; PPV, positive predictive values; NPV, negative predictive values; CI, confidence interval.

and convenient methods are available for the detection of *T. gondii* in fields. Therefore, the development of diagnostic kits with affordable prices that do not require special equipment is favorable. In recent years, interests have been increased in immunodiagnosis of microbial and parasitic diseases using fast and inexpensive methodologies with high sensitivity and specificity. Relatively, Dot-ELISA assay has been introduced as an appropriate method for the assessment of antibodies or antigens for human and animal infectious diseases and for the development of seroepidemiological surveys in rural areas and less equipped laboratories. For example, the assay has been used to detect human leptospirosis and mycobacteria antigens in patients with pulmonary tuberculosis.[31,32] Furthermore, Dot-ELISA has been shown as a quick and reliable assay for the detection of other infectious diseases

Table 5 Assessment of in-house Dot-ELISA strips with various antigens for the detection of anti-*T. gondii* IgM in 144 sera, compared to that of ELISA

Result	In-house Dot-ELISA strip			
	rSAG1 (%)	rGRA7 (%)	rSAG1+rGRA7 (1:1) (%)	STAg (%)
Sensitivity	81.2	87.5	90.6	90.6
Specificity	89.3	83.9	92	83.9
PPV	68.4	60.9	76.3	61.7
NPV	94.3	95.9	97.2	96.9
Validity	85.2	85.7	91.3	87.2
Relative agreement	87.5	84.7	91.7	85.4
Kappa (95% CI)	0.66 (0.51–0.8)	0.6 (0.45–0.74)	0.77 (0.65–0.89)	0.64 (0.5–0.77)

Abbreviations: *T. gondii, Toxoplasma gondii*; ELISA, enzyme-linked immunosorbent assay; rSAG1, recombinant SAG1 protein; rGRA7, recombinant GAR7 protein; STAg, soluble tachyzoite antigen; Pos, positive; Neg, negative; PPV, positive predictive values; NPV, negative predictive values; CI, confidence interval.

Table 6 Assessment of in-house Dot-ELISA strips with various antigens for the detection of anti-*T. gondii* IgG and IgM in 224 sera, compared to that of ELISA

Result	In-house Dot-ELISA strip			
	rSAG1 (%)	rGRA7 (%)	rSAG1+rGRA7 (1:1) (%)	STAg (%)
Sensitivity	83	72.3	87.5	89.3
Specificity	89.3	81.2	91.1	82.1
PPV	88.6	79.4	90.7	83.3
NPV	84	74.6	87.9	88.5
Validity	86.1	76.7	89.3	85.7
Relative agreement	86.2	76.8	89.3	85.7
Kappa (95% CI)	0.72 (0.63–0.81)	0.54 (0.42–0.64)	0.79 (0.7–0.86)	0.71 (0.62–0.8)

Abbreviations: *T. gondii, Toxoplasma gondii*; ELISA, enzyme-linked immunosorbent assay; rSAG1, recombinant SAG1 protein; rGRA7, recombinant GAR7 protein; STAg, soluble tachyzoite antigen; Pos, positive; Neg, negative; PPV, positive predictive values; NPV, negative predictive values; CI, confidence interval.

caused by bacteria, viruses, and parasites such as Chagas, syphilis, *Toxocariasis canis, Neospora caninum*, chronic *Schistosomiasis japonica*, cystic echinococcosis, malaria, and neurocysticercosis.[33–38]

In the present study, the method was developed based on the successful amplification, cloning, and expression of rSAG1 and rGRA7. An expression vector with a short His fusion tag to the recombinant protein was used to avoid possible nonspecific reactions of recombinant proteins with serum proteins in in-house Dot-ELISA rapid tests. Therefore, SAG1 and GRA7 genes of *T. gondii* RH strain were cloned using T7 promoter-based pET28a vectors and highly expressed in BL21 (DE3) pLysS *E. coli* systems. These vectors included multiple advantages such as common restriction sites, strong T7 promoters, medium copy numbers, reasonable yields in mini preparations, and His Tag sequences to assay expression levels and purify proteins using independent strategies. The His Tag sequence binds to divalent cations (eg, Ni_2^+) immobilized on His bind metal chelation resins.[39,40] In the current study, recombinant proteins were successfully purified using one-step chromatography procedure and Ni-NTA resins, verified by Western blot. Previously, several *Toxoplasma* recombinant proteins have been prepared in *E. coli* and assessed for their potential as diagnostic antigens in detection of infections in humans.-[9,10] Selection of *T. gondii* antigens with conserved T/B cell epitopes is essential for the successful use of these protein epitopes in diagnosis.[41] The SAG1 has been used extensively and demonstrated as a good serological marker for the detection of anti-*T. gondii* antibodies in acute and chronic infections.[9,10] Furthermore, studies have demonstrated that GRA7 is a novel vaccine antigen and diagnostic tool.[10] These explain the reasons for the selection of SAG1 and GRA7 as antigen candidates in the current study.

Results from the in-house Dot-ELISA using rSAG1 antigen showed a higher sensitivity to sera from humans

with chronic toxoplasmosis than sensitivity to sera from humans in AIS group (83.7 instead of 81.2%; Table 4,5). Studies have shown that rSAG1 can detect IgG reactivity in chronic phase of the infection. However, in other studies, no reactivity was seen in chronic but seen in acute phases of the infection.[5,9] These differences in detection levels of rSAG1 in human sera possibly occur due to differences in selected gene fragments, cloning strategies, and preparations of this complex molecule in various studies. Therefore, various epitopes may present depending on the cloning and preparation techniques. The SAG1 protein includes a native structure of six intramolecular cysteine bridges that form immunologically conformational epitopes.[42,43] However, the rSAG1 antigen was expressed in the form of insoluble inclusion bodies. This phenomenon possibly occurred because of saturated host cell folding machinery, cofactor deficiency, or rare codons affecting rapid mRNA decay, which could downregulate the expression rate of foreign genes in heterologous systems.[44,45] Furthermore, irregular interactions of the multiple disulfide bonds might result in misfolded insoluble proteins as well as persistence of rSAG1 C-terminal hydrophobic region (acceptor of GPI group).[46,47] In the current study and after one-step chromatographic purification of rSAG1 under denaturing conditions, a cost-saving dialysis procedure was used for refolding the proteins.[23] Therefore, the rSAG1 ELISA was more sensitive than other assays, as sensitive as soluble rSAG1-based assays.[7,47,48] The correct refolding procedure used in the current study may improve the specific immunoreactivity of this highly complex molecule. In this study, in-house Dot-ELISA using rGRA7 antigen showed a considerably higher sensitivity to sera from patients with IgM acute toxoplasmosis than to sera from CIS group (87.5% instead of 66.2%; Table 4,5). When GRA7 is secreted from tachyzoites and bradyzoites, it directly contacts with the host immune system inducing strong antibody and cell-mediated responses in acute and chronic infections and can hence be used to detect anti-*T. gondii* antibodies in early and late stages of the infection, more associated to acute infections.[12,49–51] In humans with *T. gondii*, GRA7 can be detected much earlier than other antigens such as SAG1 and MAG1. Therefore, some epitopes of GRA7 seem to play important roles in human antibody responses in acute toxoplasmosis.[52,53]

In most studies, recombinant proteins are used as ELISA antigens to detect specific IgG and IgM; mostly coated on ELISA plates alone. However, these proteins may be coated in various combinations including 2–3 proteins.[9,10] In a study by Johnson et al (1992), ELISAs based on the combination of two recombinant antigens of H4/GST and H11/GST resulted in a higher sensitivity (81.3%) than that IgM ELISA did with either H4/GST or H11/GST (54% and 61%, respectively).[54,55] After initial introductions, a combination of recombinant proteins including GRA7, GRA8, and ROP1 was then reported as an antigen preparation for the detection of IgM against *T. gondii* in human sera. Similarly, a combination of rGRA7, rGRA8, and rSAG1 was studied for the detection of IgG antibodies.[5] Data showed the potential use of two or three complementary recombinant antigens to achieve sensitivity rates comparable to that achieved with a crude antigen preparation. Therefore, these recombinant proteins can be used together for the serodiagnosis of toxoplasmosis. Moreover, several combinations of the recombinant proteins have been suggested for the detection of IgG antibodies against *T. gondii*. Some of these combinations include GRA7, GRA8, and SAG1; GRA7, GRA8, SAG2, and H4; SAG1, GRA1, and GRA7; SAG1 and GRA5 mixed with MAG1, GRA2, or ROP1.[9,10] Results from many studies have revealed that combining complementary recombinant *T. gondii* antigens improves relative sensitivity of the test. The highlighted combinations of the recombinant antigens which included at least one of GRA7, GRA8, SAG2, and H4 proteins could be used in the differentiation of recent and past infections.[56] Results of the in-house Dot-ELISA strips in CIS group showed that the highest sensitivities achieved using STAg and combined rSAG1 and rGRA7 antigens for the Toxo IgG included 88.7% and 86.2%, respectively. However, the highest specificity, validity, relative agreement, and kappa value were achieved using a combination of rSAG1 and rGRA7 antigens. In AIS group, the highest sensitivity was seen for STAg (90.6%) and a combination of rSAG1 and rGRA7 (90.6%) antigens for the detection of Toxo IgM. However, GRA7 or SAG1 alone showed 87.5% and 81.2% of sensitivity, respectively. In the present study, the overall diagnostic performance observed for rSAG1 and rGRA7 combination in in-house Dot-ELISA strips was achieved in AIS at maximum values of 90.6%, 92%, 76.3%, 97.2%, 91.3%, and 91.7% for diagnostic sensitivity, specificity, PPV, NPV, validity, and relative agreement, respectively. In general, STAg and a combination of rSAG1 and rGRA7 were more sensitive (89.3% and 87.5%, respectively) for the detection of Toxo IgG and IgM. The current results have shown that no serologic cross-reactivity is seen

between the rSAG1 and rGRA7 of in-house Dot-ELISA strips and the non-Toxo parasitic infection sera. Some epitopes featured in the native antigen seem that have not been presented in recombinant proteins and hence cannot be recognized by *T. gondii* or cross-react with other antibodies. Furthermore, immune diversity can affect epitope diagnostic values.[57]

Whole sequences of SAG1 and GRA7 used in this work were assessed using ELISA in previous studies by the authors with good results.[21,22] Results showed sensitivity and specificity values of rSAG1 as 87% and 95% for acute phase and 93% and 95% for chronic phase sera, respectively. Sensitivity and specificity of rGRA7 included 96% and 90% for acute and 89% and 90% for chronic phase sera, respectively. Based on results from the current study, it can be concluded that the transfer of the recombinant antigens from ELISA microplates to this new diagnostic procedure (in-house Dot-ELISA) was successful. In the previous study, recombinant antigens were coated separately in ELISA microplates for the detection of *T. gondii* antibodies in human sera.[21,22] In the current study, antigens were used separately and in combination forms for the detection of specific antibodies against *T. gondii* in human sera. Using other antigens (separately or combined) and/or deepening the optimization of the technique can increase sensitivity and specificity values. Moreover, assays including use of NC membranes have multiple advantages over the classic ELISA since protein absorption is higher in membranes.[58,59] The current study was a pilot study for designing a diagnostic kit using the highlighted protocol. The Dot-ELISA method is easily used as all incubation steps can be carried out at room temperature or 37°C with mild agitation. Moreover, results can be verified by naked eyes and the method does not need the use of expensive equipment or highly trained researchers. The Dot-ELISA assay greatly simplifies diagnostic procedures for the detection of *T. gondii* infections, compared to that commercially available ELISA kits do. Furthermore, the protocol used in this study is less expensive and can produce much recombinant proteins. Stability of the in-house Dot-ELISA strips has suggested that the validity period of these strips includes at least 6 months at room temperature with no loss of sensitivity and specificity. Stability of antigens in NC strips has been assessed in other studies. The NC membranes with antigens can be stored for up to 5 months at room temperature without any reactivity changes.[38]

Conclusion

The rSAG1 and rGRA7 antigens can be described as valuable diagnostic markers of toxoplasmosis. However, rGRA7 was more appropriate for IgM Dot-ELISA than IgG Dot-ELISA. For the detection of Toxo IgG and IgM, the highlighted parameters of combined recombinant proteins were more significant than those of single recombinant proteins in in-house Dot-ELISA with a similar sensitivity to the native antigens of *T. gondii*. Therefore, combination of these recombinant proteins can replace soluble extracts of tachyzoites in serologic tests. Indeed, the Dot-ELISA rapid test kit described in this study is fast and simple to use. The prepared antigen NC strips can be stored at room temperature for at least 6 months without antigenic changes. Therefore, strips can be used for the serodiagnosis of toxoplasmosis in fields as well as less equipped laboratories, where the use of major immunodiagnostic assays is limited by their dependency on electricity, refrigeration, or laboratory-grade water. In conclusion, data from the current study suggest that the in-house Dot-ELISA rapid test based on rSAG1 and rGRA7 combination is a promising diagnostic tool, which can be used as a serological screening test for toxoplasmosis.

Data availability statement

All data generated during this study are included in this published article and the datasets analyzed during the current study are publicly available in the Figshare repository, https://figshare.com/s/bad4c70e468cb86aedea. Furthermore, the SAG1 and GRA7 sequencing data are available at GenBank under accession numbers MK250980 and MK250981, respectively.

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Disclosure

The authors declare no conflicts of interest in this work.

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