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Recombinant proteins from new constructs of SAG1 and GRA7 sequences and their usefulness to detect acute toxoplasmosis

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Abstract. In this study we have cloned unreported gene fragments of *Toxoplasma gondii* GRA7 and SAG1 and expressed the corresponding recombinant proteins, followed by evaluation of their usefulness for the serological diagnosis of toxoplasmosis. Both recombinant proteins were expressed efficiently in insoluble form, purified by single step Ni-NTA affinity chromatography and their antigenicity to detect toxoplasma specific IgG antibodies were determined by immunoblotting. A total of 60 serum samples from three groups of individuals based on their anti-toxoplasma antibody profiles were tested, namely (I) IgM+, IgG+ (n=20), (II) IgM-, IgG+ (n=20) and (III) IgM-, IgG- (n=20). Both recombinant proteins exhibited high sensitivity (100%) with sera from Group I. rGRA7 and rSAG1 reacted 40% and 80% respectively with Group II sera. The specificity of the recombinant proteins based on reactivities with Group III sera were 100% and 80% with rGRA7 and rSAG1 respectively. Thus rGRA7 was found to be better at discriminating probable acute from chronic phases of toxoplasmosis, and it also showed higher specificity.

INTRODUCTION

The apicomplexan protozoa *Toxoplasma gondii* infects all warm-blooded animals including human beings. Approximately half of the world's population is infected with *T. gondii*. The course, symptoms, and consequences of *T. gondii* infection depend on the virulence and inoculum size of the parasite and genetic background and immune status of the infected host (Dlugonska, 2008). The infection is asymptomatic in immunocompetent hosts; but it can cause severe disease in immunocompromised subjects, like those with HIV/AIDS patients who usually suffer from toxoplasmic encephalitis. Another group of vulnerable people are fetuses who cannot develop an effective immune response against the parasite (Remington *et al.*, 2001), when it crosses the placenta during primary maternal infection. This can lead to spontaneous abortion, death *in utero*, or severe congenital defects, such as hydrocephaly, mental retardation, or chorioretinitis (Wong & Remington, 1994; Sukthana, 2006; Hurt & Tammaro, 2007).

Diagnosis of *T. gondii* infection relies mainly on serological tests enabling the detection of specific anti-*Toxoplasma* antibodies immunoglobulin G (IgG), IgM, IgA produced in the infected host. Detection of IgM and IgA could suggest an active *T. gondii* infection whereas IgG antibodies along with the absence of IgM and IgA leads to the diagnosis of chronic infection. These markers may present conflicting results since IgM antibodies to *T. gondii* can be detected for more than a year after initial infection (Wong & Remington, 1994). On the other hand, high IgG levels can be present after the onset of

infection (Camargo *et al.*, 1991). In recent years it has been demonstrated that IgG avidity status can be used to help differentiate acute from chronic infection (Pelloux *et al.*, 1998; Liesenfeld *et al.*, 2001; Pietkiewicz *et al.*, 2007). Although high avidity IgG denotes chronic infection, low IgG avidity do not necessarily confirm acute infection. Thus it is still useful to identify new diagnostic markers to differentiate acute and chronic infection.

Several recombinant proteins hold potential to be used for human toxoplasma diagnosis, among them dense granule antigens (GRAs) and major surface antigens (SAGs) have been shown to be good candidates for diagnosis (Kotresha & Noordin, 2010). In the present study we focused our efforts to clone two new constructs of SAG1 (P30) and GRA7 (P29) sequences, express and evaluate the diagnostic potential of the recombinant proteins, with the goal of developing an IgG assay that detects antibodies to antigens that appears primarily during acute stage of the infection.

MATERIALS AND METHODS

Construction of recombinant plasmids

The nucleotide sequence of the *T. gondii* RH genes encoding GRA7 (Accession No HM016952) and SAG1 (Accession No HM776940) proteins were obtained from the GenBank database submitted previously by our group. Total RNA was isolated from *T. gondii* RH strain by Trizol reagent (Invitrogen, USA), and the RNA was converted to cDNA and prepared for polymerase chain reaction (PCR) using Easy-A One-Tube RT-PCR system (Stratagene, USA) according to manufacturer's protocol. Primers designed for cloning and expression were from unreported gene fragments of GRA7 and SAG1.

The selection on the gene fragment was based on high antigenic regions using several free bioinformatic softwares (e.g http://immunax.dfci.harvard.edu/Tools/antigenic.html). GRA7 (corresponding to nucleotides 39-711) was amplified by PCR using the following gene specific primers, GRA7-Forward primer: 5'GCCGGATCC ATTTCCAAAATGGCCCGACACG-3' and GRA7-Reverse primer: 5'GCGGTACCAAGG CCAGTAGGATATGGGG-3'. The PCR product was inserted into TOPO cloning vector and transformed into *E. coli* TOP10' cells. Plasmid was extracted from positive colony and digested with *Eco*RI and the released GRA7 fragment was inserted into pProcEX HTa and transformed into BL21 (DE3) cells for protein expression.

Similarly, SAG1 (corresponding to nucleotides 42-927) was amplified by PCR using following gene specific primers, SAG1-Forward primer: 5'-GACGAGTATGTTT CCGAAGGCAGTGAGACG-3' and SAG1-Reverse primer: 5'-AGCCGATTTTGCTGA CCCTGCAGCCC-3'. The PCR product was inserted into TOPO cloning vector and transformed into *E. coli* TOP10' cells. Plasmid was extracted from positive colony and digested with *Pce* I and *Xba* I and released SAG1 fragment was inserted into specific sites of pProcEX HTb and transformed into BL21 (DE3) cells for protein expression.

Production and purification of recombinant His_6-tagged antigens

a) Expression of rGRA7 and rSAG1

Single colonies of *E. coli* BL21 (DE3), harbouring each of the plasmids pProcEX ΔGRA7 and pProcEX ΔSAG1, were grown (separately) in Terrific Broth (12g bacto-tryptone, 24 g yeast extract and 4 ml glycerol per liter of medium) containing 10% salt solution (125.4 g dipotassium hydrogen phosphate and 23.1 g potassium dihydrogen phosphate per liter of water) and 100 µg/ml ampicillin (Amresco, USA) overnight at 37ºC at 180 rpm. Cells were diluted 1:100 with fresh TB medium (1000 ml) supplemented with 100 µg/ml ampicillin and induced with 1mM IPTG (Fermentas, USA) after the cultures reached the $O.D_{600}$ of 0.4 and 0.6 for recombinant bacteria expressing rSAG1 and rGRA7 respectively. Subsequently recombinant bacteria expressing rGRA7 were incubated at 37ºC for 3 h each and that for rSAG1 was incubated at 30ºC at 200 rpm for 5 h. The cells were harvested by centrifugation and kept at -80ºC.

b) Fractionation of soluble and insoluble proteins

The cell pellets were each resuspended in 10 ml lysis buffer (50mM sodium dihydrogen phosphate, 500 mM sodium chloride, 10mM imidazole and 0.1% Triton-X 100), cocktail of protease inhibitor (Roche, Germany, 14.8 µg/ml) and lysozyme (Amresco, Solon, Ohio, 0.5 mg/ml), mixed well and incubated on ice for 30 minutes. The cells were lysed using French Press 40K (Thermo Spectronic, USA) followed by centrifugation at 12,000 x *g*, 4ºC for 30 minutes to separate the soluble (supernatant) and insoluble protein (pellet).

c) Purification of insoluble rGRA7 and rSAG1

The above pellet was dissolved in 10 ml denaturing lysis/binding buffer (7 M Urea, 100 mM NaH_2PO_4, 10 mM Tris-HCl, pH 8.0) and vortexed until it was completely resuspended. The sample was centrifuged to sediment the insoluble debris and the supernatant was used for further purification steps. After DNase I (Fermentas, USA) treatment, the supernatant was incubated with 1 ml of washed Ni-NTA resin (Qiagen, Germany) for one hour at 4ºC. The resin was loaded into purification columns pre-equilibrated with denaturing lysis buffer. The columns were washed thrice with 10 bed volumes of denaturing washing buffer (8 M urea, 100 mM NaH_2PO_4, 10 mM Tris-HCl, pH 6.3) and five times with 10 ml of denaturing buffer (8 M urea, 100 mM NaH_2PO_4, 10 mM Tris-HCl, pH 5.9). The recombinant proteins were eluted as 500 µl fraction with 10 ml denaturing elution buffer (8M urea, 100mM NaH2PO4, 10mM Tris-Cl, pH 4.5) and checked for the protein content by BioRad assay (Bio-Rad, USA). The purified fractions were pooled and elution buffer was exchanged with storage buffer using spin column (3000 dalton molecular weight cut-off (Vivapsin, Sartorius, USA)). The buffer exchange process was repeated five times until the concentration of urea is sufficiently reduced and concentrated proteins were stored at -80ºC for further use.

Electrophoresis and Western-blot analysis

The recombinant proteins were subjected to 10% SDS–polyacrylamide gel electrophoresis and stained with Coomassie blue. Western blot analysis was performed using His-Tag HRP conjugated antibody (Novagen, Germany) at 1: 2000 dilution.

For immunoblotting, the recombinant proteins (3 ug/well) were separated with 10% SDS-PAGE and the proteins were transferred onto 0.45 µm nitrocellulose membrane (Bio-Rad, France) The membranes were blocked with 5% fat-free milk in PBS for 1 h and then washed thrice with wash buffer TBS-T (50 mM Tris, 150 mM NaCl, 0.05% Tween-20). The membranes strips were then incubated for 2 h with human serum samples (1:400) in the TBS for 2 h followed by washing with TBS-T and incubation with peroxidase conjugated mouse anti-human IgG (Invitrogen, USA) for 45 min at a dilution of 1:10,000. Following three washing steps, the membrane strips were developed using chemiluminescence blotting substrate (Thermo Scientific, USA) and Kodak films (Kodak, USA).

Human serum samples

A total of 60 serum samples were used in this study to assess the diagnostic efficiency of rSAG1 and rGRA7. Serum samples were divided into three groups on the basis of serological profiles:

Group I: This group consisted of 20 serum samples from patients clinically suspected of toxoplasmosis, and who were positive by both anti-Toxoplasma IgM and IgG; ten (50%) of these had low avidity IgG. The presence of specific IgM and IgG antibodies and the IgG avidity status were determined by Platelia kits (Bio-Rad, France).

Group II : This group consisted of 20 human serum samples from patients who were positive for anti-Toxoplasma IgG positive but negative for anti-Toxoplasma IgM, thus these were from individuals who acquired the infection in the distant past (chronic toxoplasmosis). All serum samples had high IgG avidity.

Group III: This comprised 20 human serum samples from individuals who were seronegative for IgM and IgG antibodies to *T. gondii*.

RESULTS

Cloning and expression in *E. coli* host were performed for the two *Toxoplasma gondii* recombinant proteins i.e. rGRA7 (P29) and rSAG1 (P30). They were successfully expressed in insoluble form and purified by one step Ni-NTA chromatographic method.

Reactivities of rGRA7 and rSAG1 with anti-*T. gondii* IgG antibodies in serum samples

Figures 1 and 2 show the results of the Western blot using anti-His-HRP and representative serum samples using rGRA7 and rSAG1 respectively. A total of 60 serum samples belonging to the three groups of individuals were examined in immunoblots to determine the reactivities of IgG antibodies to the two recombinant proteins. As shown in Table 1, rGRA7 and rSAG1 showed variations in reactivities to anti-Toxoplasma IgG antibody with sera from the three groups of individuals. For sera from Group 1, both rGRA7 and rSAG1 showed 100% reactivities, this group included ten low IgG avidity serum samples. Out of 20 sera from Group II, sixteen (80%) exhibited IgG reactivity against rSAG1 and only eight sera (40%) showed the reactivity towards rGRA7 antigen. Group III sera were from people not infected with *T. gondii*, thus these were tested to evaluate the specificities of the recombinant proteins. The results showed that rSAG1 and rGRA7 were 80% and 100% specific respectively.

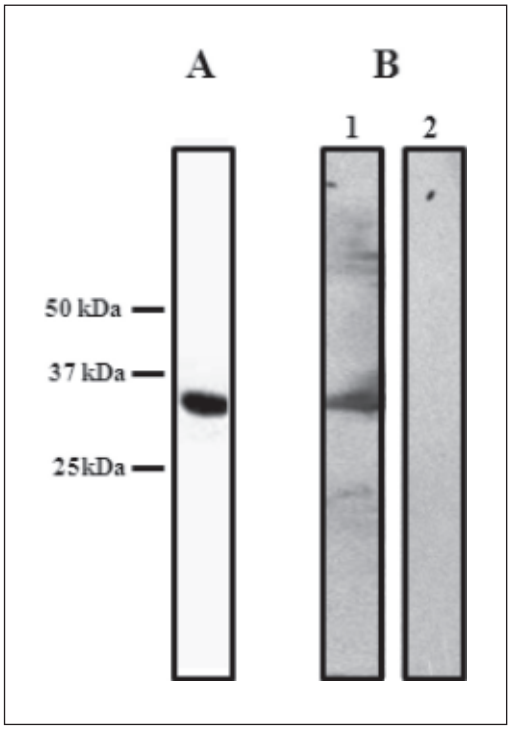

Figure 1. Western-blot analysis of rGRA7 purified by Ni-NTA affinity chromatography; (A) anti-His antibody, (B) Reactivity with human sera; Lane 1: Group 1 serum sample, Lane 2: Group III serum sample

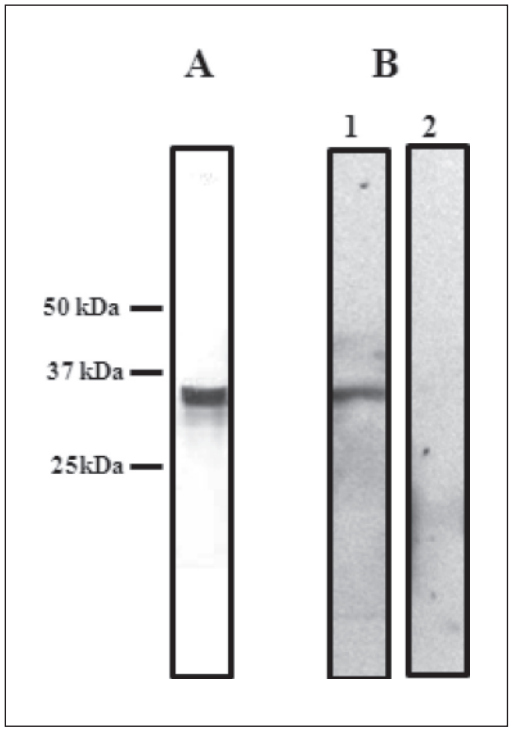

Figure 2. Western-blot analysis of rSAG1 purified by Ni-NTA affinity chromatography; (A) anti-His antibody (B) Reactivity with human sera; Lane 1: Group 1 serum sample, Lane 2: Group III serum sample

Table 1. Results of anti-Toxoplasma IgG immunoblots using recombinant proteins GRA-1, GRA-7 and SAG-1 and serum samples from three groups of individuals

Group I: IgM +ve, IgG +ve

Serum code n=20	IgG avidity	rGRA7	rSAG1
Tg59	Low	+	+
Tg66	Low	+	+
Tg61	Low	+	+
Tg83	Low	+	+
Tg30	Low	+	+
Tg03	Low	+	+
Tg19	Low	+	+
Tg21	Low	+	+
Tg22	Low	+	+
Tg26	Low	+	+
Tg01	High	+	+
Tg02	High	+	+
Tg04	High	+	+
Tg06	High	+	+
Tg12	High	+	+
Tg55	High	+	+
Tg80	High	+	+
Tg113	High	+	+
Tg10	High	+	+
Tg37	High	+	+
% Sensitivity		20/20 (100%)	20/20 (100%)

Positive result : + Negative result : –

Group II: IgM –ve, IgG +ve

Serum code n=20	rGRA7	rSAG1
SG1	+	–
SG2	–	–
S220	–	+
S223	–	+
S2	–	+
1907	+	+
9693	+	+
1992	+	+
8301	–	+
8594	–	+
Tg107	–	+
Tg108	+	–
Tg110	+	+
Tg112	–	+
Tg115	–	+
Tg117	+	–
Tg118	–	+
Tg119	+	+
Tg120	–	+
Tg121	–	
% Sensitivity	8/20 (40%)	16/20 (80%)

Positive result : + Negative result : –

Group III: IgM -ve, IgG ve

Serum code n=20	rGRA7	rSAG1
Tg 191	–	+
Tg175	–	–
Tg168	–	–
Tg192	–	–
Tg171	–	–
Tg172	–	–
Tg173	–	–
Tg174	–	–
Tg175	–	+
Tg178	–	–
Tg 179	–	–
Tg 180	–	–
Tg 182	–	+
Tg 183	–	–
Tg 188	–	+
Tg 189	–	–
Tg 190	–	–
Tg 191	–	–
Tg 192	–	–
Tg 193	–	–
	20/20 (100%)	16/20 (80%)

Positive result : + Negative result : –

DISCUSSION

To date, most of the commercially available serological kits for diagnosis of *T. gondii* infections use whole cell extracts of tachyzoites grown in mice or in tissue culture which are often contaminated with extra parasitic material (Wu *et al.*, 2009). This results in inter-assay variability and pseudo positive concordance among the various kits (Buffolano *et al.*, 2005). For differential diagnosis of acute and chronic infection, the determination of anti-Toxoplasma IgM status and the IgG avidity index has been shown to be very useful (Liesenfeld, *et al.*, 2001; Sadeghiani *et al.*, 2009). To increase the accuracy of diagnosis of acute stage infection, it is useful to identify *T. gondii* antigens, especially recombinant proteins, that induce an early immune response with anti-Toxoplasma antibodies in patients' serum samples, and has low reactivity to antibodies in chronic infections.

Thus in this regard, the use of recombinant proteins not only allows for more accurate standardization of tests but may also have the potential to be used in the creation of reliable, specific and standard tests to differentiate acute and chronic infections (Jenum *et al.*, 1997; Buffolano *et al.*, 2005). For diagnostic applications, target genes like SAGs and GRAs have been cloned and expressed. However the reported variations in sensitivities and specificities of the recombinant proteins from one study to another was probably due to differences in the selected gene fragments and cloning strategies, as well as the panel of serum samples used in their evaluations.

In this study, we evaluated the efficiency of two recombinant proteins from new sequence constructs to detect early stage toxoplasma specific IgG in human sera. DNA sequences encoding fragments GRA7 (14-326 amino acids) and SAG1 (14-336 amino acids) from cDNA of *T. gondii* RH strain were used, cloned into pProcEX HT vector and expressed in *E. coli* system. The selected gene fragments corresponding of the three recombinant proteins were different from those previously reported for rGRA7 (Jacobs *et al.*, 1998; 1999; Aubert *et al.*, 2000; Nigro *et al.*, 2003; Pfrepper *et al.*, 2005; Velmurugan *et al.*, 2008) and rSAG1 (Jenum *et al.*, 1997; Jacobs *et al.*, 1999; Nigro *et al.*, 2003; Pietkiewicz *et al.*, 2004; Buffolano *et al.*, 2005; Pfrepper *et al.*, 2005; Altcheh *et al.*, 2006). The recombinant proteins were expressed at comparatively higher level in insoluble form (than soluble form) and purified under denaturing condition. The method applied was very efficient and rapid without the need for multiple dialysis steps.

The reactivities of the recombinant proteins by IgG immunoblot assays were evaluated using sera from three groups of individuals. The first group comprised 20 serum samples with anti-toxoplasma IgM+, IgG+; ten of them had low IgG avidity index. Low IgG avidity antibodies are indicative of infection acquired within the past three months i.e. acute infection (Abdullah, 2010). In this study rGRA7 with amino acids residues of 14-326 showed 100% sensitivity in detecting Group I sera, and low sensitivity (40%) in detecting Group II sera which comprised chronically infected individuals. In terms of specificity, the rGRA7 was not reactive with any of the sera from Group III. Thus these results showed that rGRA7 produced in this study is potentially a good diagnostic reagent for detection of acute toxoplasmosis. The results showed agreement with several previous studies which showed that sensitivity of rGRA7 was higher with sera from acute cases than chronic cases. Pfrepper *et al.* (2005), reported that IgG antibodies to rGRA7 was exclusively present at the beginning of IgG response. Aubert *et al.* (2000), showed by IgG-ELISA that rGRA7 reacted more with acute (97.7%) sera as compared with chronic (67%) sera. Similarly, Nigro *et al.* (2003), also obtained much higher sensitivity of rGRA7 with serum samples from recently infected individuals (75%) compared with those with chronic infection (36.3%).

rSAG1 produced in this study showed good sensitivity (100%) and specificity (80%). However but it did not seem to show significant difference in reactivity between acute and chronic sera groups, with 80%

positivity with the latter (Group II sera). Thus rSAG1 did not seem to be suitable for use in discriminating acute and chronic infection. A study performed by Pfrepper *et al.* (2005), using rSAG1 (49-323 amino acids) reported immunoreactivities which ranged from 9.1% – 60% using sera from three different phases of acute infection and 91.5% reactivity with sera from chronic infection. Nigro *et al*. (2003), had reported IgG-ELISA results from a study using SAG1m (49-336 amino acids) and SAG1 ct (171-336 amino acids). They showed that rSAG1m was not recognized by any of the serum samples, suggesting an incorrect folding and rSAG1ct did not show difference in reactivity patterns between acute and chronic groups of serum samples. They stated that the difference in reactivities may be due to variations in preparations of this very complex molecule leading to presentation of different epitopes. In the present study, the preparation of rSAG1 antigen was found to be good since it showed high reactivity with Group I sera. Chen *et al*. (2001) cloned truncated (78-306 amino acids) region of SAG1 in pET-30 expression vector and produced high level expression of insoluble form of the recombinant protein which was then refolded and developed into an ELISA that was well-recognized by antibodies from patients with toxoplasmosis. Subsequently, Wu *et al*. (2009) subcloned the same region of SAG1 into pET-32 which contain thioredoxin and resulted in a soluble protein which also exhibited specific immunoreactivity.

This study showed that the two recombinant proteins were highly sensitive in IgG immunoblots using serum samples from *T. gondii* infected individuals. However, only rGRA7 is deemed suitable to be used in discriminating between probable acute and chronic cases of *T. gondii* infection. In addition rGRA7 showed higher specificity than rSAG1. Therefore rGRA7 produced in this study holds promise to be used for diagnosis of acute toxoplasma infection. GRA7 is a secretary protein and is found in the parasitophorus vacuole and the cytoplasm of the tachyzoite-infected host cells, and thus not surprisingly it produces strong antibody reaction in early phase of infection (Li *et al*., 2000).

This report and other previous reports (as described above) showed rather consistent evidences of the usefulness of rGRA7 to diagnose early stage infection with *T. gondii*, albeit using different sequence constructs. Thus it would be useful to perform side by side comparisons of the rGRA7 produced from different constructs using the same panel of serum samples to determine the best rGRA7 for development of an IgG test for acute infection, preferably in the form a rapid lateral flow test. Such a test can then form part of the panel of tests needed in the confirmation of acute toxoplasmosis.

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