Iran J Public Health, Vol. 45, No.7, Jul 2016, pp.911-916

Original Article

Immunoreactivity Analysis of *Toxoplasma gondii* Recombinant Antigen rSAG3 in Sera from Immunized BALB/c Mice and Toxoplasmosis Patients

Mohammad Hossein MOTAZEDIAN [1], ***Bahram KAZEMI*** [2], ***Bahador SHAHRIARI*** [1], ***Mojgan BANDEHPOUR*** [2], ****Khadijeh KHANALIHA*** [3]

1. *Dept. of Parasitology and Mycology, School of Medicine, Shiraz University of Medical Sciences, Shiraz, Iran*
2. *Cellular and Molecular Biology Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran*
3. *Research Center of Pediatric Infectious Diseases, Rasoul-e-Akram Hospital, Iran University of Medical Sciences, Tehran, Iran*

***Corresponding Author:** Email: khanaliha.kh@iums.ac.ir

(Received 14 Nov 2015; accepted 19 Feb 2016)

Abstract

Background: The coccidian protozoa *Toxoplasma gondii* is an obligate intracellular parasite of humans and other warm-blooded animals. Diagnosis of toxoplasmosis is of considerable medical importance for human, especially pregnant women and immunocompromised individuals. The apply of an *Escherichia coli* recombinant antigen(s) would be significantly useful in developing standardization of the diagnostic tests and reducing their costs. In this study, immunoreactivity of recombinant SAG3 against sera from immunized mice and human anti-*T. gondii* IgG positive patients was evaluated by western-blotting and enzyme immunoassay (EIA) in Department of Parasitology and Mycology, School of Medicine, Shiraz University of Medical Sciences in 2013.

Methods: Three inbreed BALB/c female mice were obtained**.** Two mice were injected with rSAG3 and one was remained untreated, as control. Sera from immunized mice and also pooled sera from IgG positive toxoplasmosis cases were evaluated with western-blotting. IgG antibody responses to recombinant SAG3 was measured by indirect ELISA against the negative control group.

Results: The rSAG3 protein reacted with sera of immunized mice and sera from patients with anti-*Toxoplasma* IgG antibodies in western-blot analysis. The result of ELISA showed that, there was marked differences in the absorbance values between the recombinant SAG3 immunized mice and control group.

Conclusion: The rSAG3 showed IgG reactivity with sera from immunized mice and anti-*Toxoplasma* IgG patients.

Keywords: Immunoreactivity*, Toxoplasma gondii,* Recombinant, SAG3, Mice

Introduction

Toxoplasma gondii is an obligate intracellular protozoan parasite with a global distribution among humans and mammals. Diagnosis of toxoplasmosis is of considerable medical importance for human, especially pregnant women and immunocompromised individuals (1).

T. gondii has a 43-kDa surface antigen (SAG3) that is exist on all of intrusive steps (2). SAG1 and SAG3 are similar with 24% amino acid integrity contain conservation of all of the cysteines in the mature protein and similar secondary and tertiary structure which suggests similar folding for SAG1 and SAG3 (2, 3). SAGl is a tachyzoite-specific antigen and highly immunogenic and is a good choice for vaccine (4). A strong immune response to SAG1 would allow the host to control the parasitic infection but cannot eliminate the bradyzoites (3).

SAG3 (P43), common tachyzoite and bradyzoite antigen exist in the surface of both tachyzoite and bradyzoite of *T. gondii* and similar to SAG1 anchored to the membrane with glycosyl-phosphatidylinositol groups (2). SAG3 is same as SAG1 in structure and function, and these two surface antigens have important roles in cellular invasion, attachment to cells and communication with host immune system (5). Tachyzoite mutants without SAG3 antigens is less virulent than wild-type parasite, this subject demonstrate that SAG3 is involved in the *T. gondii* infection process (6).
Immunized mice with recombinant SAG3 could produce much more specific IgG2a antibody against *T. gondii* (7). Although in some studies, rSAG1 detected IgG reactivity in the chronic phase of infection (8-10), but few reports exist about reactivity of r SAG3.
The aim of the present study was to investigate immunogenicity of *T. gondii* recombinant antigen, rSAG3 in BALB/c mice.

Material and Methods

P43 gene

SAG3 antigen has been cloned in pGEMEX1 plasmid and sequenced in previous study in Cellular and Molecular Biology Research Center, Shahid Beheshti University of Medical Science in 2007(11). Recombinant pGEMEX-1 expression vector including P43 gene was confirmed by PCR, using specific primers and restriction analysis by PstI restriction enzyme. PCR products were electrophoresed on 1.5% agarose gel and pGEMEX-1 containing the SAG3 insert (1158bp) was observed (12).

Protein expression and purification

The recombinant pGEMEX-1 plasmid was transformed into *Escherichia coli* JM109 competent cells according to Hanahan method (13).A single colony was inoculated in LBmedium (Merck, Germany) containing 100 µg/ml ampicillin and incubated overnight at 37 °C. Cells was subcultured, using10-fold fresh LB medium containing 100 µg/ml ampicillin and shacked at 200 rpm in 37 °C until the OD600=0.7. The plasmid promoter was induced by 1 mM Isopropyl β- D-1-thiogalactopyranoside (IPTG) for 5 h.
Sampling was done before and after induction in one hour intervals and finally the bacterial cells were harvested by centrifugation at 8000 rpm for 15 min.
Recombinant protein was purified, using The T7•Tag Affinity Purification Kit (Novagen, Madison Wisconsin, USA) according to the manufacturer's method with some modifications that described before (12).

Preparation of anti SAG3 mouse hyper immune serum

Three inbreed BALB/c female mice, 6-8 wk old and 18-22 g weight, were obtained from the animal house of Shiraz University of Medical Sciences. Two mice were injected with rSAG3 and one was remained untreated, as control. The BALB/c mice were intraperitoneally injected with 50 µgr of purified rSAG3 in sterile PBS three times with one week interval. Complete Freund's adjuvant was used for the first injection and incomplete Freund's was used in subsequent injections. Mice sera were collected one week after the last injection.
Serum samples were collected from different laboratories around Shiraz and Tehran and confirmed by using commercial IgG ELISA kits (Euroimmun, Lubeck, Germany). The pooled sera was prepared and used for western blotting analysis.
This study was approved by the Ethics Committee of Shiraz University of Medical Sciences (Ethic no.9153) in accordance with Helsinki Declaration and guidelines.

Western blotting and dot blotting analysis

Immunoreactivity of recombinant SAG3 against sera from immunized mice and sera from anti-*T. gondii* IgG positive cases was evaluated with western-blotting.
Recombinant SAG3 proteins was subjected to 12% gradient SDS-PAGE with the molecular protein marker (Fermentas, Lithuania cat) (12), and western blot, was performed. The antigen was transferred from the gel into nitro-cellulose membrane (Schleicher and Schuell). All of trans-

ferred bands were checked by staining the membrane with Ponceau S stain (0.001 g/ml in 3% trichloroacetic acid). The membranes with blotted antigen were cut into strips and blocked with 5% (w/v) of skimmed milk in washing buffer (10 mM Tris, 150 mM NaCl, and 0.05% Tween 20; pH 7.4) for 2 h. The strips were incubated with sera obtained injected mice and human anti-*T. gondii* IgG positive cases (1/100 dilution in washing buffer with 1% bovine serum albumin, BSA) for 2 h at room temperature. After 3 washes (each 15 min), the strips were incubated with HRP-conjugated goat anti-mouse IgG (Abcam, USA) and HRP-conjugated anti-human IgG (Sigma, USA) at a dilution of 1/2000 (in washing buffer+1% BSA) for 2 h at room temperature. The washes were done as described before, antigen bounds were developed using diaminobenzidine (DAB) substrate (0.1% H2O2+ 10 mg DAB in 10 ml (Tris-HCl, 10 mM, pH 7.4).

Dot blot of recombinant SAG3 with sera obtained from injected mice and untreated mouse was carried out to evaluate serum samples and conjugate dilution was used as same as described for western blotting analysis**,** recombinant SAG3 boiled with loading buffer (SDS 0.003 M, Beta-mercaptoethanol 0.5 M, Tris (pH=6.8) 0.5 M, 5ml Glycerol) and was blotted on nitro-cellulose membrane, after drying blocked with 5% (w/v) of skimmed milk and the strips were incubated with sera obtained from injected and untreated mice with 1/100 serum dilution in washing buffer with 1% bovine serum albumin (BSA) for 2 h at room temperature. The nitrocellulose strips were washed as it described before. The strips were incubated with HRP-conjugated goat anti-mouse IgG (Abcam, USA) at a dilution of 1/2000 (in washing buffer+1% BSA) for 2 h at room temperature. After washing, antigen bounds were developed using diaminobenzidine (DAB) substrate.

Enzyme immunoassay

IgG antibody responses to recombinant SAG3 was measured by indirect ELISA against the negative control group**.**

Wells of ELISA micro plate were coated by 3 µg/ml of recombinant SAG3 protein. Wells were washed three times with PBS containing 0.05% Tween 20 (PBST) and blocked with 3% skimmed milk in PBS-T. The mice sera samples diluted (1/100 and1/50 in PBST) and were added to the plate and incubated for 1.5 h. One hundred µL of HRP-conjugated goat anti-mouse IgG (Abcam, USA) was added to the palate and the plate was incubated for 1 h. The plate was washed as before and 100 µL of o-phenylenediamine dihydrochloride (OPD) (0.4 mg/ ml buffer) was added to the plate. Finally, the optical density was measured after 30 min at 490 nm wave length, using an automatic ELISA reader (Bio-TEK).

Results

Immunoreactivity of rSAG3 with serum of immunized mice was confirmed by dot blot, and western- blot analysis and ELISA. Result of dot blot of recombinant SAG3 with sera of immunized mice are shown in (Fig. 1).

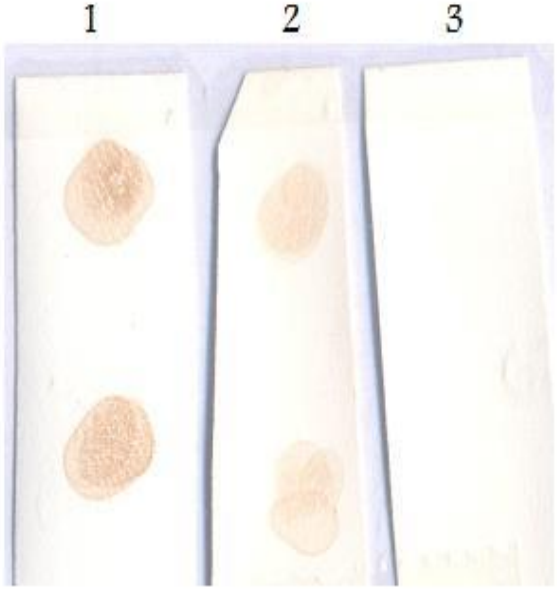

Fig. 1: Result of dot blot of recombinant SAG3 with sera obtained injected mice
1-Injected mouse; 2-Injected mouse; 3-Untreated mouse

As it can be seen in the blot, recombinant SAG3 reacted with sera of immunized mice but not reacted with the serum of control mouse.
The rSAG3 protein also reacted with sera of immunized mice and sera from patients with IgG anti *Toxoplasma* antibodies in western-blot analysis and a band of almost 70 kDa (for SAG3, 43kDa and T7 gen 10 as fusion protein, almost 30kDa) and a band of almost 100 kDa were detected by rSAG3-immunized mice and also sera of patients with IgG anti *Toxoplasma* antibody while these bands were not present in any of either mice or human control sera, (Fig. 2).
The result of ELISA showed that, there was marked differences in the absorbance values between the recombinant SAG3 immunized mice and control group. Mean of optical density in immunized mice was 0.78 while the mean value of control group was 0.23, (Fig. 3).

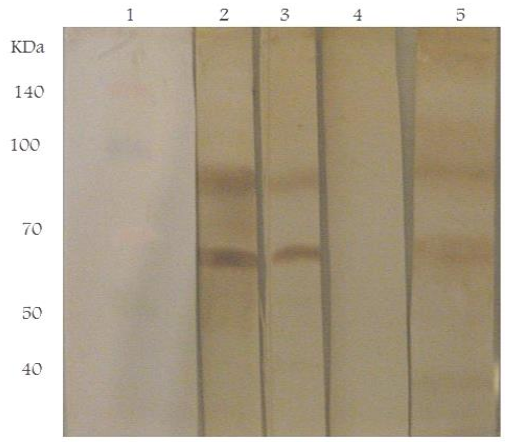

Fig. 2: Result of western blot using rSAG3 and sera obtained injected mice and human anti *Toxoplasma* sera; 1-marker; 2, 3 sera from injected mice; 4-serum from untreated mouse, 5- pooled sera from IgG anti Toxoplasma antibody

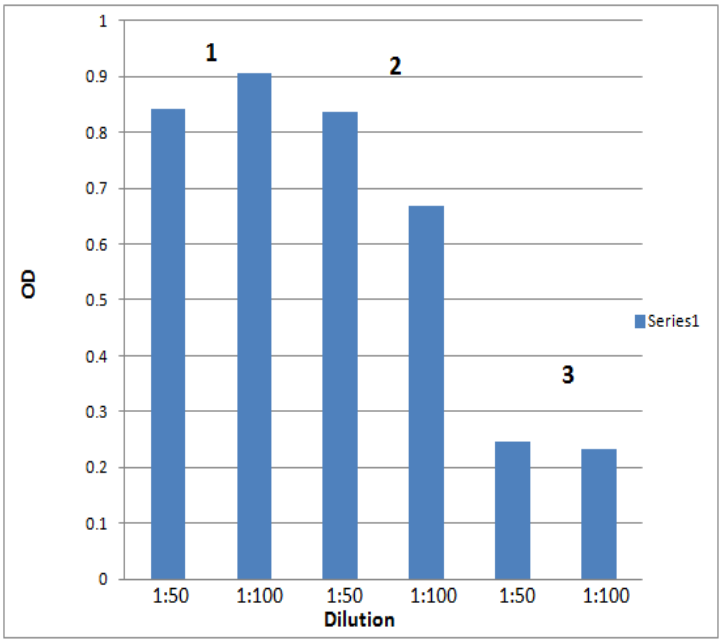

Fig. 3: Result of IgG-ELISA using rSAG3 and sera obtained from injected mice ;1- serum obtained from injected mouse with 1:50 ,1:100 dilution;2- serum obtained from injected mouse with 1:50 ,1:100 dilution;3- serum obtained from untreated mouse

Discussion

In this study we expressed and purified recombinant SAG3 as a fusion protein with T7 gen10 in *Escherichia coli* and then BALB/c mice were injected with the recombinant SAG3 protein in order to evaluate Immunoreactivity of this antigen with sera obtained from injected mice and

pooled sera from patients with IgG anti *Toxoplasma* antibodies.

In the western blot analysis two bands (70 KDa and 100 KDa) were seen in injected rSAG3 mice group and patients with IgG anti *Toxoplasma* antibody but it is absent in untreated mouse as negative control. Recombinant SAG3 was produced in *E. coli* (JM109) seem to be as a mixture of monomer and dimmer forms in this study.

The existence of both recombinant SAG1 monomer and dimmer forms were reported in a study (13).

In a study, serum with the high titer of IgG_2 from mice infected with the C56, C37 virulent and non-virulent strain of *Toxoplasma gondii* immunoprecipitated with P43 and P27 surface antigens. P43 was the prominent band precipitated by serum from mice infected after 8 d with the C56 strain of *T.gondii* (14).

We obtained similar bands in sera obtained from injected recombinant SAG3 mice as a positive control after 3 wk and pooled sera from patients with IgG anti Toxoplasma antibody.

Vaccination of mice with rSAG3 can induce partial immunity against *T. gondii* infection concluded from the induction of Th1 type immune responses and production of IgG2a antibody (7).

In another study a DNA vaccine was produced by using two surface antigens SAG1 and SAG3 from T. gondii and A_2/B subunit of cholera toxin, as a genetic adjuvant and finally this vaccine was injected to BALB/c mice. Results of this study showed quiet more production of IgG antibodies, proliferation of lymphocyte and production of IFN-γ from spleen cells were induced in mice immunized with PSAG1/SAG3 compared to mice immunized with PSAG1 alone that result of this study demonstrates the role of SAG3 in combination with SAG1 in this vaccine (15).

In other study, evaluate recombinant SAG1 for diagnosing of toxoplasmosis. Sensitivity and specificity of the recombinant SAG1 for the detection IgG anti- *Toxoplasma* antibodies were 93% and 95%, and the sensitivity and specificity of this recombinant antigen for the detection of IgM were 87% and 95% in comparison with commercial ELISA respectively (16). The sensitivity and specificity of the recombinant GRA7 for diagnosing toxoplasmosis were 89% and 90%, for the detection of IgG anti- *Toxoplasma* antibodies and were 96% and 90% for the detection of IgM respectively in another study (17).

Diagnostic usefulness of three recombinant antigens (SAG1, SAG2, and SAG3) of *Toxoplasma gondii* were evaluated and compared with commercial ELISA kit .The results showed sensitivity and specificity of recombinant surface antigens for the detection of anti-*Toxoplasma* IgG in comparison with commercially ELISA were as follows: SAG1 (93.6% and 92.9%), SAG2 (100.0% and 89.4%), and SAG3 (95.4% and 91.2%), respectively. Sensitivity and specificity of these recombinant antigens for the detection of anti-*Toxoplasma* IgM were SAG1 (39.3% and 80%), SAG2 (64.3% and 83.3%) and SAG3 (17.9% and 76.7%) respectively (18). These results showed rSAG3 had better performance for diagnosing of anti-Toxoplasma IgG than IgM.

Production of IgG antibodies was induced in mice immunized with recombinant SAG3 and the result of IgG ELISA shows immunoreactivity of recombinant SAG3 in sera obtained from injected recombinant SAG3 mice and sera from IgG positive toxoplasmosis patients .It seems recombinant SAG3 has the good performance in detecting anti-*Toxoplasma* IgG.

Conclusion

In the current study, rSAG3 showed IgG reactivity with sera from immunized mice and *T.gondii* infected patients.

Ethical Considerations

The study was performed in compliance with current national laws and regulations.

Ethical issues (Including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.) have been completely observed by the authors.

Acknowledgments

This study was financially supported by the office of Vice-chancellor for research of Shiraz University of Medical Sciences (Grant No. 89-5229). The authors appreciate the generous cooperation of Dr. Hamid Reza Rahimi and Dr. Tahereh Mohammadzadeh. The authors declare that there is no conflict of interests.

References

1. Dai J, Jiang M, Wang Y, Qu L, Gong R, Si J (2012). Evaluation of a recombinant multiepitope peptide for serodiagnosis of *Toxoplasma gondii* infection. *Clin Vaccine Immunol*, 19(3):338-42.
2. Cesbron-Delauw M-F, Tomavo S, Beauchamps P, et al. (1994). Similarities between the primary structures of two distinct major surface proteins of *Toxoplasma gondii*. *J Biol Chem*, 269(23):16217-22.
3. Boothroyd JC, Hehl A, Knoll LJ, Manger ID (1998). The surface of *Toxoplasma*: more and less. *Int J Parasitol*, 28(1):3-9.
4. Manger ID, Hehl AB, Boothroyd JC (1998). The surface of *Toxoplasma* tachyzoites is dominated by a family of glycosylphosphatidylinositol-anchored antigens related to SAG1. *Infect Immun*, 66(5):2237-44.
5. Jacquet A, Coulon L, De Nève J et al. (2001). The surface antigen SAG3 mediates the attachment of *Toxoplasma gondii* to cell-surface proteoglycans. *Mol Biochem Parasitol*, 116(1): 35-44.
6. Tomavo S (1996). The major surface proteins of *Toxoplasma gondii*: structures and functions. *Curr Top Microbiol Immunol*, 219: 45-54.
7. Lee Y-H, Shin D-W, Lee J-H, Nam H-W, Ahn M-H (2007). Vaccination against murine toxoplasmosis using recombinant *Toxoplasma gondii* SAG3 antigen alone or in combination with Quil A. *Yonsei Med J*, 48(3):396-404.
8. Aubert D, Maine G, Villena I, Hunt J, Howard L, Sheu M, et al. (2000). Recombinant antigens to detect *Toxoplasma gondii*-specific immunoglobulin G and immunoglobulin M in human sera by enzyme immunoassay. *J Clin Microbiol*, 38(3):1144-50.
9. Pfrepper K-I, Enders G, Gohl M et al. (2005). Seroreactivity to and avidity for recombinant antigens in toxoplasmosis. *Clin Diagn Lab Immunol*, 12(8):977-82.
10. Gatkowska J, Hiszczynska-Sawicka E et al. (2006). *Toxoplasma gondii*: an evaluation of diagnostic value of recombinant antigens in a murine model. *Exp Parasitol*, 114(3):220-7.
11. Kazemi B, Maghen L, Bandehpour M, Shahabi S, Haghighi A (2007). Gene cloning of p43 surface protein of *Toxoplasma gondii* tachyzoite and bradyzoite (SAG3). *Gene Ther Mol Biol*, 11:113-6.
12. Khanaliha K, Motazedian M, Sarkari B, Bandehpour M, Sharifnia Z, Kazemi B (2012). Expression and purification of P43 *Toxoplasma gondii* surface antigen. *Iran J Parasitol*, 7(3):48-53.
13. He X-l, Grigg ME, Boothroyd JC, Garcia KC (2002). Structure of the immunodominant surface antigen from the *Toxoplasma gondii* SRS superfamily. *Nat Struct Biol*, 9(8):606-11.
14. Handman E, Remington JS (1980). Antibody responses to toxoplasma antigens in mice infected with strains of different virulence. *Infect Immun*, 29(1):215-20.
15. Cong H, Zhang M, Xin Q, Wang Z, Li Y, Zhao Q, et al. (2013) . Compound DNA vaccine encoding SAG1/SAG3 with A2/B subunit of cholera toxin as a genetic adjuvant protects BALB/c mice against *Toxoplasma gondii*. *Parasit Vectors,* 6(63): 10.1186/1756-3305-6-63.
16. Selseleh M, Keshavarz M, Mohebali M et al. (2012). Production and evaluation of *Toxoplasma gondii* recombinant surface antigen 1 (SAG1) for serodiagnosis of acute and chronic *Toxoplasma* Infection in human sera. *Iran J Parasitol*, 7(3):1-9.
17. Selseleh M , Keshavarz M, Mohebali M et al. (2012). Production and evaluation of *Toxoplasma gondii* recombinant GRA7 for serodiagnosis of human infections. *Korean J Parasitol*, 50(3): 233- 38.
18. Khanaliha K, Motazedian MH, Kazemi B, Bandehpour M, Sharifniya Z (2014). Evaluation of recombinant SAG1, SAG2, and SAG3 antigens for serodiagnosis of toxoplasmosis. *Korean J Parasitol*, 52(2): 137-42.