

OPTIMIZATION AND VALIDATION OF AN ELISA USING RECOMBINANT *TOXOPLASMA GONDII* MATRIX ANTIGEN 1 FOR SERODIAGNOSIS OF THE INFECTION

Buyannemekh Tumurjav^{1,2*}, Mohamad Alaa Terkawi¹, Houshuang Zhang¹, Guohong Zhang¹, Honglin Jia^{1,3}, Junya Yamagishi¹, Yoshifumi Nishikawa¹, Ikuo Igarashi¹, Chihiro Sugimoto³ and Xuenan Xuan^{1,*}

1-Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Japan

2-Institute of Veterinary Medicine, MULS, Mongolia

3-Research Center for Zoonosis Control, Hokkaido University, Sapporo, Japan

*-Corresponding author, E-mail: t.buyannemekh@yahoo.com

INTRODUCTION

Toxoplasma gondii infection can be diagnosed directly by polymerase chain reaction (PCR), hybridization and isolation of parasites and indirectly with serological methods [4; 5; 18]. Although all these tests have shortcomings, serological tests, particularly the enzyme-linked immunosorbent assay (ELISA), seem to be the most practical and economical. The crude antigen prepared from tachyzoites has been traditionally utilized for commercially serological detection kits. However the use of recombinant antigens can be alternative sources of antigens allowing better standardization of the tests and reducing the costs of production requires mass production of the parasite either from the peritoneal fluids of infected mice or from tissue cultures. In spite of the potential advantages of using recombinant antigens in serology tests, their sensitivities have not yet achieved perfect result; therefore, further research on new antigens is extremely desirable [10; 16; 17; 3]. In this context, the *Toxoplasma gondii* matrix antigen 1 (TgMAG1) known as 65-kDa protein abundantly expressed within the cyst and in the cyst wall surrounding the bradyzoites [15], has documented to be immunogenic during the infection with *T.gondii* in mouse model and promising reagent for serodiagnosis of toxoplasmosis in humans [15; 12; 6]. However, its usefulness has not yet been confirmed in animal toxoplasmosis.

In this study, the optimization and validation of E.coli-expressed rTgMAG1 as ELISA antigen were described.

MATERIALS AND METHODS

Parasite culture and purification

In this study, *Toxoplasma gondii* RH and PLK strains, and *N. caninum* Nc-1 strain were cultivated in Vero cells, and purified.

Expression and purification of recombinant TgMAG1

In this study, recombinant TgMAG1 was expressed

as the glutathione S-transferase (GST)-fusion protein.

The protein was purified using glutathione-Sepharose 4B beads (Amersham Pharmacia Biotech, USA). After dialysis and filtration, the purity and quality of purified protein were estimated by SDS-PAGE.

ELISA

Ninety-six-well plates (Nunc, Denmark) were coated with rTgMAG1 (1 µg/ml) and GST (1 µg/ml) diluted in the antigen coating buffer (0.05 M carbonate buffer, pH 9.6) at 4°C overnight. The plates were washed with washing solution (PBS containing 0.05% Tween 20) and then the wells were blocked with blocking solution (PBS containing 3% skim milk) at 37°C for 1 hr. After discarding the blocking solution the wells were incubated with the test sera (1:100) at 37°C for 1 hr. After washing the wells were incubated with horse radish peroxidase-conjugated anti-mouse IgG antibody (Bethyl, USA; 1:4,000) at 37°C for 1 hr. After washing the wells were incubated with substrate solution [0.1 M citric acid, 0.2 M sodium phosphate, 0.003% H₂O₂, 0.5 mg of 2,2'-azinobis (3-ethylbenzthiazoline sulfonic acid) per ml] at room temperature for 1 hr. The optical density (OD) was measured with the MTP-

500 microplate reader (Corona Electric, Japan) at 415 nm.

Experimental infection of mice

Eight-week-old female mice (ICR strain; n=10/group) were intraperitoneally inoculated with either *T.gondii* PLK strain (400 tachyzoites/mouse) or *N.caninum* Nc-1 strain (40,000 tachyzoites/mouse) with 0.2 ml of MEM. The blood samples were serially collected once a week from tail bleeds using capillary hematocrit tubes (Hirschmann Laborgerate, Germany). The mice were housed in a P2 level facility, and the experiments were conducted in accordance with the Stipulated Regulations for the Care and Use of Research Animals promulgated by the Obihiro University of Agriculture and Veterinary Medicine.

RESULTS

The rTgMAG1 was expressed as the GST-fusion protein with a molecular mass of 91 kDa. Sera from mice experimentally infected with *T.gondii* PLK strain specifically reacted with the recombinant TgMAG1 but not with GST protein by Western blot analysis, suggesting that rTgMAG1 has good antigenicity for detecting a specific antibody to *T.gondii*. The anti-rTgMAG1 serum produced in mice did not cross-react with *N.caninum* and specifically recognized 65 kDa protein on the *T.gondii* lysate by Western blot analysis. The potential of the recombinant protein as a diagnostic antigen was then evaluated in an ELISA using sera from mice experimentally infected with *T.gondii* and *N. caninum*. Consistent with results of Western blot analysis, sera from mice infected with *T.gondii* (2 months post-infection) demonstrated strong

reactivity (optical density, >0.1), contrary to sera from either mice experimentally infected with *N.caninum* or non-uninfected mice that showed very low OD values (Fig. 1). Furthermore, the sensitivity of rTgMAG1 was evaluated in ELISA with sera collected serially from experimentally *T.gondii*-infected mice. As shown in Figure 2, the antibody responses to rTgMAG1 were detected in serial serum samples as early as the second week post-infection and maintained in a high level until 24 weeks post-infection, indicating that the ELISA with rTgMAG1 could detect antibodies to *T.gondii* in both acute and chronic stages. These data suggest the successful optimization and standardization of ELISA using rTgMAG1 for detection of *T. gondii* infection.

DISCUSSION

The detection of *T.gondii* infection with high sensitivity and specificity is crucial in the management of the disease. Toxoplasmosis is generally diagnosed by demonstrating specific antibodies to *T.gondii* antigens in the patient's serum sample. Most of the commercially available tests use *T.gondii* native antigens and display wide variations in test accuracy. A large number of different recombinant antigens were produced in *E.coli* and studied for their potential to serve as diagnostic

reagents for detection of *T.gondii* infection in human and animals; these included dense granule proteins GRA1, GRA2, GRA4, GRA6, GRA7, GRA8; surface antigen SAG1 and SAG2; rhoptry antigens ROP1 and ROP2; microneme proteins MIC3 and MIC5 [14; 11; 19; 8; 10; 1; 2]. Although this approach would allow detection on antibody to a single protein of the parasites, the high productivity and purity of recombinant proteins coupled with specificity suggest their potential as replacements

for tachyzoite materials in serodiagnosis. Despite the potential advantages of these recombinant antigens in serodiagnosis, only few commercial diagnostic kits are based on recombinant antigens. Thus, the discovery of new infection markers and development of improved diagnostic assays using recombinant antigens are needed [9].

The sufficient production of recombinant TgMAG1 expressed in *E.coli* as GST-fusion protein and its high immunological activity with infected sera were promising to develop an ELISA based on rTgMAG1 to detect an antibody to *T.gondii* infection in mouse models. The rTgMAG1 reacted with sera from mice experimentally infected with *T.gondii*, and showed significantly higher OD values than those from *N.caninum*-infected or non-infected mice, which reveals the success of ELISA as a tool for detection of infection. Next, the sensitivity of the test was evaluated with mice experimentally infected with *T.gondii*. The detectable antibody responses to rTgMAG1 were observed as early as the second week post-infection and maintained these responses until 24 weeks post-infection. No significant difference in OD values between sera collected from acute and chronic infection stages. These results indicated that the ELISA with rTgMAG1 could detect antibodies to *T.gondii* in both acute and

chronic infection stages. However, rTgMAG1 was initially reported to be expressed specifically during bradyzoite development and localized to the ground substance of the tissue cyst; but it was subsequently reported that MAG1 was expressed during both tachyzoite and bradyzoite development stages and is not a bradyzoite-specific protein [13; 7]. On the other hand, it was found that IgG reactivity to rTgMAG1 could be highly detected in acute infection [6]. Pfrepper et al. [17] reported that rTgMAG1 was weakly reactive in acute and highly reactive in chronic infection. The variations in these results may be due to the differences of the protein fragment, expression system and serum samples studied. However, in this study rTgMAG1 has shown high reactivity with sera from both acutely and chronically *T.gondii*-infected mice.

Taken together, these data coupled with the unique characteristics of TgMAG1 being an immunogenic and conserved protein among different strains suggest that the ELISA using rTgMAG1 is promising tool for detection of infection and can be further used for routine test or for screening a mass of samples to detect the presence of antibody for toxoplasmosis.

SUMMARY

Toxoplasma gondii matrix antigen 1 (TgMAG1), known as the 65 kDa protein that abundantly expressed in both bradyzoites and tachyzoites, was evaluated as a candidate for the development of a diagnostic reagent in mouse models.

The ELISA with rTgMAG1 showed a highly specific reaction with sera from mice experimentally

infected with *T.gondii* but not with the closely related *Neospora caninum*. Moreover, the antibodies to rTgMAG1 were detectable from the acute to the chronic infection stages. These results suggest that rTgMAG1 could be used as a reliable antigen for the detection of *T.gondii* infection in other animals.

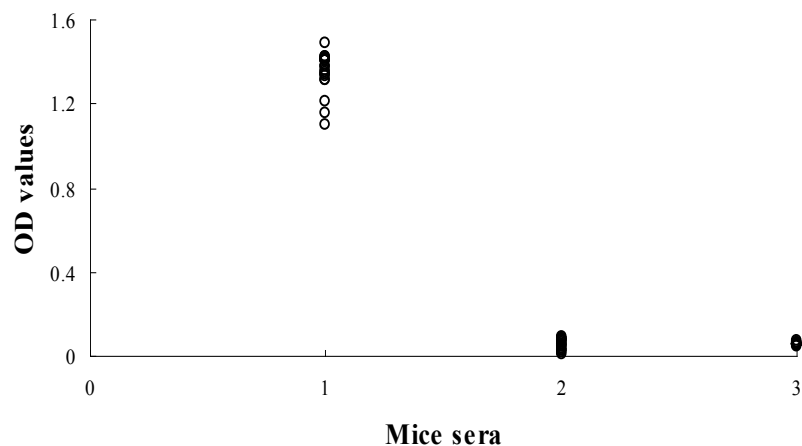


Figure 1. Specificity of *T. gondii* by the ELISA with rTgMAG1. Lane 1, sera from mice infected with *T. gondii* (n=10); lane 2, sera from mice infected with *N. caninum* (n=10); lane 3, sera from uninfected mice (n=10).

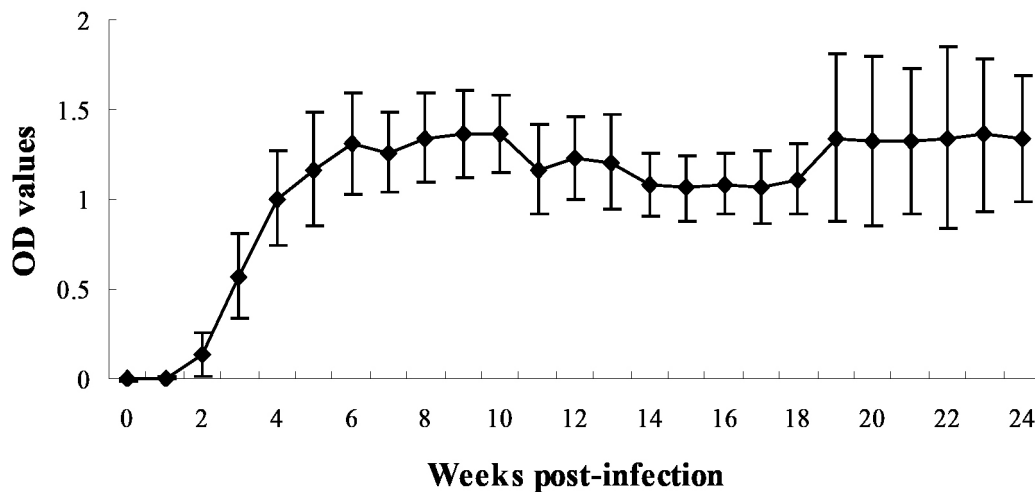


Figure 2. Sensitivity of the ELISA with rTgMAG1 in detection of *T.gondii* infection. Serial serum samples from mice experimentally infected with *T.gondii* (n=10).

REFERENCES

- Aubert, D., Maine, G.T., Villena, I., Hunt, J.C., Howard, L., Sheu, M., Brojanac, S., Chovan, L.E., Nowlan, S.F., Pinon, J.M. 2000. Recombinant antigens to detect *Toxoplasma gondii*-specific immunoglobulin G and immunoglobulin M in human sera by enzyme-linked immunosorbent assay. *J. Clin. Microbiol.*, 38: 1144-1150.
- Beghetto, E., Neilsen, H.V., Del Porto, P., Buffolano, W., Guglietta, S., Felici, F., Petersen, E., Gargano, N. 2005. A combination of antigenic regions of *Toxoplasma gondii* microneme proteins induces protective immunity against oral infection with parasite cysts. *J. Infect. Dis.*, 191: 637-645.
- Calderaro, A., Peruzzi, S., Piccolo, G., Gorrini, C., Montecchini, S., Rossi, S., Chezzi, C., Dettori, G. 2009. Laboratory diagnosis of *Toxoplasma gondii* infection. *Int. J. Med. Sci.*, 6: 135-136.
- Dubey, J.P., Emond, J.P., Desmonts, G., Anderson, W.R. 1987. Serodiagnosis of postnatally and prenatally induced toxoplasmosis in sheep. *Am. J. Vet. Res.*, 48: 1239-1243.
- Hunter, D., Chadwick, P., Balfour, A.H., Bridges, J.B. 1982. Examination of ovine foetal fluid for antibodies to *Toxoplasma gondii* by the dye test and an indirect immunofluorescence test specific for IgM. *Br. Vet. J.*, 138: 29-34.
- Holec, L., Hisczynska-Sawicka, E., Gasior, A., Brillowska-Dabrowska, A., Kur, J. 2007. Use of MAG1 recombinant antigen for diagnosis of *Toxoplasma gondii* infection in humans. *Clin. Vac. Immunol.*, 14: 220-225.
- Ferguson, J.P., Parmley, S.F. 2002. *Toxoplasma gondii* MAG1 protein expression. *Trends. Parasitol.*, 18: 482-483.
- Jacobs, D., Dibremetz, J.F., Loyens, A., Bosman, F., Saman, E. 1998. Identification and heterologous expression of a new dense granule protein (GRA7) from *Toxoplasma gondii*. *Mol. Biochem. Parasitol.*, 91: 237-249.
- Kotresha, D., Rahmah, R. 2010. Recombinant proteins in the diagnosis of toxoplasmosis. *APMIS*. In press. DOI 10.1111/j.1600-0463.2010.02629.x.
- Li, S., Galvan, F., Araujo, G., Suzuki, Y., Remington, J.S., Parmley, S. 2000. Serodiagnosis in recently acquired *Toxoplasma gondii* infection using a enzyme-linked immunosorbent assay with a combination of recombinant antigens. *Clin.Diagn. Lab. Immunol.*, 7: 781-787.
- Мйвйлйс, М.Н., Chardйс, Т., Mercereau-Puijalon, O., Bourguin, I., Achbarou, A., Dubremetz, J.F., Bout, D. 1992. Molecular cloning of GRA4, a *Toxoplasma gondii* dense granule protein recognized by mucosal IgA antibodies. *Mol. Biochem. Parasitol.*, 56: 227-238.
- Nielsen, H.V., Di Cristina, M., Beghetto, E., Spadoni, A., Petersen, E., Gargano, N. 2006. *Toxoplasma gondii*: DNA vaccination with

- bradyzoite antigens induces protective immunity in mice against oral infection with parasite cysts. *Exp. Parasitol.*, 112: 274-279.
13. Parmley, S.F., Yang, S., Harth, G., Sibley, L.D., Sucharczuk, A., Remington, J.S. 1994. Molecular characterization of a 65-kilodalton *Toxoplasma gondii* antigen expressed abundantly in the cyst matrix of tissue cysts. *Mol. Biochem. Parasitol.*, 66: 283-296.
 14. Prince, J.B., Araujo, F.G., Remington, J.S., Burg, J.L., Boothroyd, J.C., Sharma, S.D. 1989. Cloning of cDNAs encoding a 28 kDa antigen of *Toxoplasma gondii*. *Mol. Biochem. Parasitol.*, 34: 3-14.
 15. Parmley, S.F., Slifer, T., Araujo, F. 2002. Protective effects of immunization with a recombinant cyst antigen in mouse models of infection with *Toxoplasma gondii* tissue cysts. *J. Infect. Dis.*, 15: 90-95.
 16. Pietkeiwicz, H., Hiszczyńska-Sawicka, E., Kur, J., Petersen, E., Neilsen, H.V., Stankiewicz, M., Andrzejewska, I., Myjak, P. 2004. Usefulness of *Toxoplasma gondii*-specific recombinant antigens in serodiagnosis of human toxoplasmosis. *J. Clin. Microbiol.*, 42: 1779-1781.
 17. Pfrepper, K.I., Enders, G., Gohl, M., Krczal, D., Hlobil, H., Wassenberg, D., Soutschek, E. 2005. Seroactivity to and avidity for recombinant antigens toxoplasmosis. *Clin. Diagn. Lab. Immunol.*, 12: 977-982.
 18. Tenter, A.M., Vietmeyer, C., Johnson, A.M. 1992. Development of ELISAs based on recombinant antigens for the detection of *Toxoplasma gondii*-specific antibodies in sheep and cats. *Vet. Parasitol.*, 43: 189-201.
 19. Redlich, A., Miller, W.A. 1998. Serodiagnosis of acute toxoplasmosis using a recombinant form of the dense granule antigen GRA6 in enzyme-linked immunosorbent assay. *Parasitol. Res.*, 84: 700-706.