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Expression of *Babesia gibsoni* thrombospondin-related adhesive protein in *Toxoplasma gondii* and evaluation of its antigenicity and immunogenicity

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ABSTRACT
A gene encoding *Babesia gibsoni* thrombospondin-related adhesive protein (BgTRAP), known as a vaccine candidate, was stably expressed in *Toxoplasma gondii* (Tg/BgTRAP). The molecular weight and the antigenic reaction of recombinant BgTRAP expressed by the Tg/BgTRAP were similar to the original ones expressed by *B. gibsoni*. To evaluate the antigenicity of the recombinant BgTRAP expressed by *T. gondii*, the lysates of the recombinant parasite tachyzoites were intraperitoneally injected into mice. The serum collected from Tg/BgTRAP-immunized mouse could react to *B. gibsoni* parasites, while the serum collected from wild-type *T. gondii* tachyzoites (Tg/wt)-immunized mice did not. These results indicate that *T. gondii* could provide a new tool to produce foreign antigens from other protozoan parasites and the recombinant BgTRAP expressed by *T. gondii* might be a useful antigen for developing a diagnostic reagent and vaccine to control canine babesiosis.

Keywords: *Toxoplasma gondii*, *Babesia gibsoni*, BgTRAP, expression, vaccine

INTRODUCTION

Babesia gibsoni is a tick-borne apicomplexan parasite that is thought to be the major cause of canine babesiosis. The disease is characterized by remittent fever, progressive anemia, hemoglobinuria, and marked splenomegaly and hepatomegaly; in addition, it sometimes causes death. *B. gibsoni* infection is endemic in many regions of Asia, Africa, Europe, and the Americas (Casapulla *et al.,* 1998). Recently, this disease has been found to occur frequently in companion animals and has become a significant problem from the clinical viewpoint (Boozer and Macintire, 2003). For the control of babesiosis, vaccination is considered to be one of the most effective means. However, no effective vaccines for canine babesiosis have been developed until now.

Thrombospondin-related adhesive proteins (TRAPs) are a conserved family identified in several apicomplexans, including *Plasmodium* spp. (Robson *et al.,* 1997; Templeton *et al.,* 1997; Trottein *et al.,* 1995; Templeton and Kaslow 1997), *Toxoplasma gondii* (Wan *et al.,* 1997), *Cryptosporidium parvum* (Spano *et al.,* 1998), *Eimeria tenella* (Tomley *et al.,* 1991), *Neospora caninum* (Lovett *et al.,* 2000), and *B. bovis* (Gaffar *et al.,* 2004). Previously, Zhou *et al.* (2006) identified and characterized a *B. gibsoni* TRAP (BgTRAP). The amino acid sequence of BgTRAP consists of several typical regions, including a signal peptide, a von Willebrand factor A domain, a thrombospondin type 1 domain, a transmembrane region, and a cytoplasmic C-terminus.

The BgTRAP showed bivalent cation-independent binding to canine RBC, and the specific antiserum was found to inhibit the growth of *B. gibsoni* in the infected severe combined immune deficiency mice given canine RBC. These results suggest that the BgTRAP is a new member of the TRAP family identified from *B. gibsoni* as well as functionally important in merozoite invasion and, therefore, might be a useful vaccine candidate against canine *B. gibsoni* infection (Zhou *et al.,* 2006).

T. gondii is an intracellular protozoan parasite capable of infecting most species of mammals and birds. Recently, *T. gondii* has been developed as an expression vector for foreign genes from protozoan parasites, which are difficult to cultivate *in vitro*, such as circumsporozoite proteins of *P. knowlesi* (Di Cristina *et al.,* 1999) and *P. yoelii* (Charest *et al.,* 2000) and P23 of *C. parvum* (Shirafuji *et al.,* 2005); all of these recombinant proteins yielded protective immunity in animal models.

In this study, BgTRAP was expressed in a *T. gondii* vector, and its antigenicity and immunogenicity were evaluated.

MATERIALS AND METHODS

Parasites and cell cultures

A *B. gibsoni* NRCPD strain isolated from a hunting dog in Japan was used (Fukumoto *et al.* 2001). The parasite stock was maintained by successive passage in splenectomized dogs. The RH strain of *T. gondii* and its recombinants were maintained in Vero cells cultured in Eagle's minimum essential medium (EMEM, Sigma) supplemented with 8% heat-inactivated fetal bovine serum (FBS). For the purification of tachyzoites, parasites and host cell debris were washed in cold phosphate-buffered saline (PBS), and the final pellet was resuspended in cold PBS and passed through a 27-gauge needle and a 5.0-μm pore filter (Millipore, USA).

Cloning of the BgTRAP gene

The fragment containing the open reading frame of the BgTRAP gene was amplified by PCR using a set of primers with introduced *Eco*RI sites (italics), 5'-AC *GAA TTC* CTG GCG AGG ATG AAG-3' and 5'-AC *GAA TTC* TCA GGC CCA CAT GGA TGA AG-3'. The PCR product was digested with *Eco*RI and then cloned into the *Eco*RI site of the cloning vector, pBluescriptSK (pBS) (Stratagene, La Jolla, CA, USA). The resulting plasmid was designated as pBS/BgTRAP.

Construction of a transfer vector for insertion of BgTRAP gene into *T. gondii*

The plasmid pBS/BgTRAP was cut with *Eco*RI, and then the fragment (2,227 bp) containing BgTRAP was blunt-ended using a Klenow fragment and cloned into the *Eco*RV site of pDMG (Nishikawa *et al.,* 2003). The BgTRAP gene is under the control of the *T. gondii* GRA1 promoter. The resulting plasmid was designated as pDMG/BgTRAP (Fig. 1).

Transfection and selection of *T. gondii* expressing recombinant BgTRAP (Tg/BgTRAP)

Electroporation of tachyzoites was performed as described previously (Sibley *et al.* 1994). Freshly lysed-out tachyzoites were washed and resuspended at 2-5×10^7/ml with 50 μg of pDMG/BgTRAP in a cytomix buffer (120 mM KCl, 0.15 mM $CaCl_2$, 10

mM K_2HPO_4-KH_2PO_4, 25 mM Hepes, 2 mM EDTA, 5 mM $MgCl_2$, pH 7.6) supplemented with 2 mM ATP and 5 mM glutathione. Cells were transferred to a 0.2 cm gap cuvette and electroporated with 2 KV at 25 μF and 50 Ω with Bio-Rad Genepulser II (Bio-Rad Laboratories, Hercules, CA, USA). After electroporation, parasites were allowed to recover for 15 min at room temperature and inoculated onto Vero cells grown in 25 cm^2 T-flasks. Transfected parasites were selected on Vero cells in the presence of pyrimethamine at a concentration of 1 μM. After incubation for 4 days, polyclonal transfected pyrimethamine-resistant tachyzoite cultures were subjected to plaque purification. The cultures were passaged three times in a medium containing 1% agarose, and a single green fluorescent plaque was obtained under a fluorescent microscope. The recombinant *T. gondii* clone expressing BgTRAP was designated as Tg/BgTRAP.

Expression and purification of recombinant BgTRAP expressed in *E. coli* (Ec/BgTRAP)

The expression and purification of the recombinant BgTRAP expressed in *E. coli* (Ec/BgTRAP) were carried out as described previously (Zhou *et al.,* 2006).

Production of anti-Ec/BgTRAP serum

Five hundred μg of the Ec/BgTRAP in Freund's complete adjuvant (Difco, Franklin Lakes, NJ, USA) was subcutaneously injected into rabbits (Japanese white rabbit; Clea Japan, Tokyo, Japan). The same antigen in Freund's incomplete adjuvant (Difco) was subcutaneously injected into the rabbits on day 14 and again on day 28. Sera from immunized rabbits were collected 14 days after the last immunization. In mouse, the anti-Ec/BgTRAP serum was raised according to the method reported previously (Fukumoto *et al.,* 2001).

Detection of recombinant BgTRAP expressed in Tg/BgTRAP by immunofluorescence assay

Parasites were plated on Vero cells grown on 12-mm coverslips (approximately 2×10^5/well) in 24-well plates. After 24 hrs of incubation, coverslips were fixed with 3% paraformaldehyde in PBS for 15 min and then permeabilized with 0.3% Triton X-100 in PBS for 5 min. For staining in extracellular parasites, the purified tachyzoites were applied to poly-lysine-coated glass slides. The detection of recombinant BgTRAP expressed in Tg/BgTRAP was performed using the anti-Ec/BgTRAP serum as the primary antibody diluted 1:250 in PBS containing 3% bovine serum albumin (BSA). Goat anti-mouse Alexa 594 antibodies (Molecular Probes, Eugene, OR, USA) diluted 1:250 in PBS containing 3% BSA were used as secondary antibodies. Coverslips and glass slides were mounted with Mowiol (Calbiochem, San Diego, CA, USA) and observed using an epifluorescence microscope (Microphot FXA; Nikon, Tokyo, Japan). Images were captured with a CCD camera (Photometrics, Tucson, AZ, USA) and processed using Phase-3 Imaging System Software (Media Cybernetics, Rockville, MD, USA). For digital processing, Adobe Photoshop 5.5 Software (Adobe Systems, San Jose, CA, USA) was used.

SDS-polyacrylamide gel electrophoresis and Western blot analysis

The SDS-polyacrylamide gel electrophoresis and Western blot analysis were

carried out as described previously (Zhou *et al.,* 2006).

Immunization of mice with Tg/BgTRAP

Eight-week-old adult ddY female mice (Japan SLS, Shizuoka, Japan) were used. The lysate of Tg/BgTRAP tachyzoites (1×10^8/head) was intraperitoneally injected into mice with Freund's complete adjuvant (Sigma, Louis, MO, USA). The control group mice were inoculated with the lysate of *T. gondii* wild-type (Tg/wt) tachyzoites. On days 14, 28, 42, and 56, the same antigen was injected with Freund's incomplete adjuvant (Difco). The mouse sera were collected 3 weeks after the last immunization.

Indirect fluorescent antibody test (IFAT) for detection of anti-Tg/BgTRAP antibody produced in mice

Thin blood smear films of blood samples collected from a *B. gibsoni*-infected dog were fixed with methanol containing 2.5% acetone for 20 min. The diluted (appropriate dilutions were made in 10% fetal bovine serum in PBS (FBS–PBS)) Tg/BgTRAP-immunized mouse serum (1:100) as the first antibody was applied on the fixed smears and incubated for 30 min at 37°C. After three washings with PBS, Alexa-Fluor® 488-conjugated goat anti-mouse immunoglobin G (IgG) (Molecular Probes) was subsequently applied (1:1,000 dilution in FBS–PBS) as a secondary antibody and incubated for another 30 min at 37°C. After three washings with PBS, the glass slides were covered with a glass coverslip. The slides were examined under a fluorescent microscope.

Animal care and use

All animals described above were housed in a BSL2 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. The approval numbers are 21-18 and 23-71.

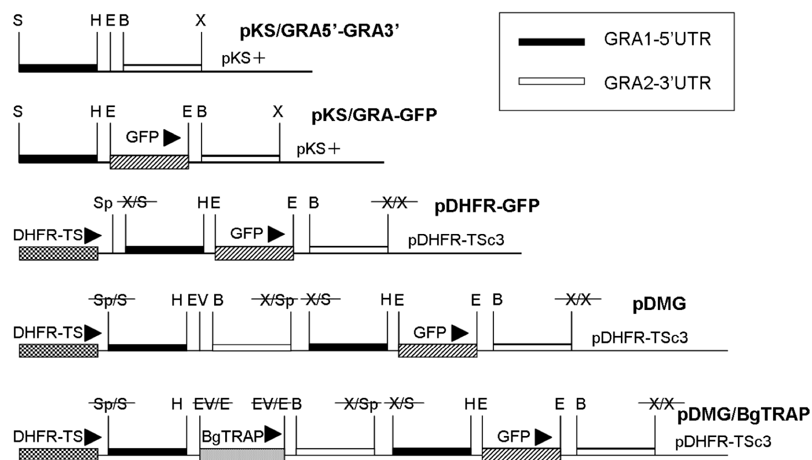

Fig. 1. Plasmid map of transfer vectors. Coding sequences are shown as wide rectangles, untranslated regions (UTR) used to drive expression, as narrow rectangles, and pKS+ or DHFR-TSc3 vector sequences, as solid lines. pDHFR-TSc3 is a minigene in which genomic 5' flanking sequences were fused to a full-length cDNA clone of DHFR-TS. The site shown by a strikethrough line was destroyed by filling and blunt-end ligation. B stands for the restriction site for *Bam*HI, E for *Eco*RI, EV for *Eco*RV, H for *Hind*III, S for *Sal*I, Sp for *Spe*I, and X for *Xba*I.

RESULTS

The recombinant *T. gondii* expressing both BgTRAP and GFP was generated using a plasmid construct, pDMG/BgTRAP (Fig. 1). After transfecting pDMG/BgTRAP into *T. gondii* tachyzoites by electroporation, drug-resistant and green fluorescent parasites were selected on Vero cells in the presence of pyrimethamine and subjected to plaque purification under fluorescent microscopy. The recombinant *T. gondii* clone expressing recombinant BgTRAP was designated as Tg/BgTRAP.

To detect the recombinant BgTRAP expressed in Tg/BgTRAP tachyzoites, IFAT was performed (Fig. 2). Tg/BgTRAP tachyzoites incubated with anti-Ec/BgTRAP-specific mouse serum showed specific fluorescence, which was mainly detected in the endoplasmic reticulum and the apical end of the tachyzoites (Fig. 2B). However, specific fluorescence was not detected on Tg/wt tachyzoites (data not shown). On the other hand, the GFP expressed in Tg/BgTRAP parasites was present within the parasite cytosol and cellular nuclei (Fig. 2A).

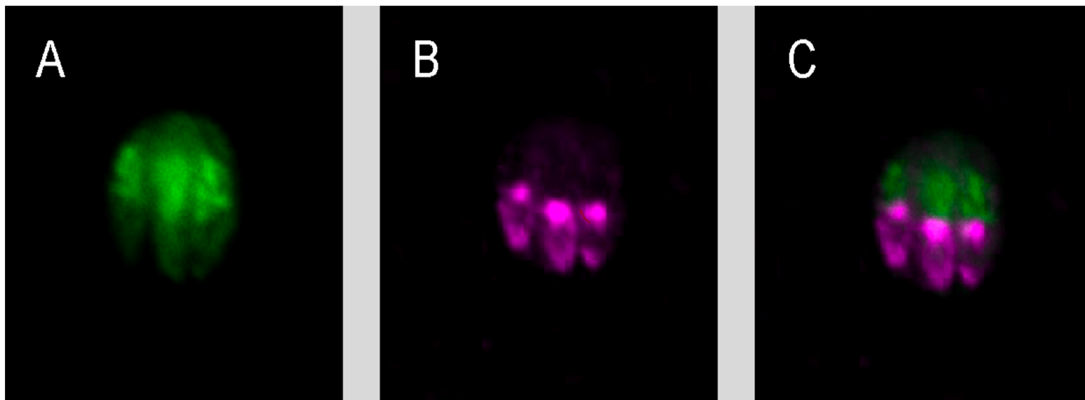

Fig. 2. Indirect fluorescent antibody test of recombinant BgTRAP expressed in *T. gondii*. Tg/BgTRAP tachyzoites were allowed to infect Vero cells, and the test was performed 24 hr post-infection with mouse anti-BgTRAP serum followed by Alexa Flour-594-conjugated secondary antibodies. Alexa Fluor-594-stained proteins are visualized in magenta. Panel A shows a GFP image, B shows an Alexa Fluor-594 image of the same field, and C shows a merged image of A and B.

The recombinant BgTRAP expressed in Tg/BgTRAP tachyzoites and its culture supernatant was examined by Western blot analysis using a mouse antiserum against the Ec/BgTRAP. A specific band with the molecular mass of 121 kDa was determined in the *B. gibsoni* merozoite lysate and the Tg/BgTRAP tachyzoite lysate as well as in their supernatants with the molecular mass of 80-82 kDa (Fig. 3).

To examine the immunogenicity of recombinant BgTRAP expressed in Tg/BgTRAP, five mice were inoculated intraperitoneally with lysed Tg/BgTRAP tachyzoites, and five other mice were inoculated with lysed Tg/wt tachyzoites 4 times at 2-week intervals with an adjuvant. Serum samples collected from these mice were subjected to IFAT to measure the antibodies against *B. gibsoni* parasites (Fig. 4). Serum collected from mice inoculated with lysed Tg/BgTRAP specifically reacted to *B. gibsoni* merozoites (Fig. 4C). This reaction was similar to that of *B. gibsoni* merozoites detected by serum samples collected from mice inoculated with Ec/BgTRAP, which

were used as a positive control (Fig. 4A). In contrast, sera collected from mice inoculated with lysed Tg/wt did not react to *B. gibsoni* merozoites (Fig. 4D).

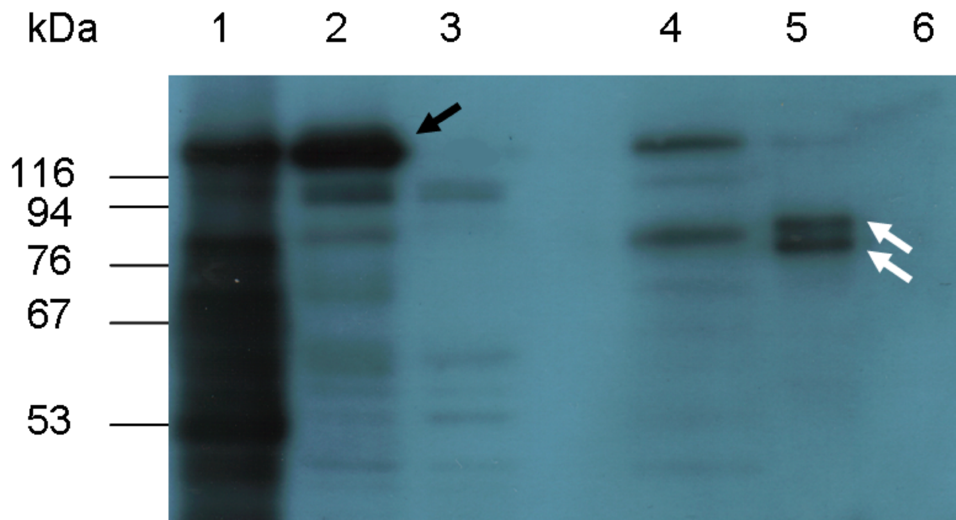

Fig. 3. Western blot analysis of recombinant BgTRAP expressed in *T. gondii*. The *B. gibsoni* merozoite lysate (lane 1), Tg/BgTRAP tachyzoite lysate (lane 2), Tg/GFP tachyzoite lysate (lane 3), *B. gibsoni* culture supernatant (lane 4), Tg/BgTRAP culture supernatant (lane 5), and Tg/wt culture supernatant (lane 6) were separated by SDS-polyacrylamide gel electrophoresis following Western blotting analysis using mouse anti-BgTRAP serum as the primary antibody. The black arrow indicates the position of the recombinant BgTRAP expressed in Tg/BgTRAP tachyzoites, and the white arrow indicates the position of the recombinant BgTRAP secreted from Tg/BgTRAP.

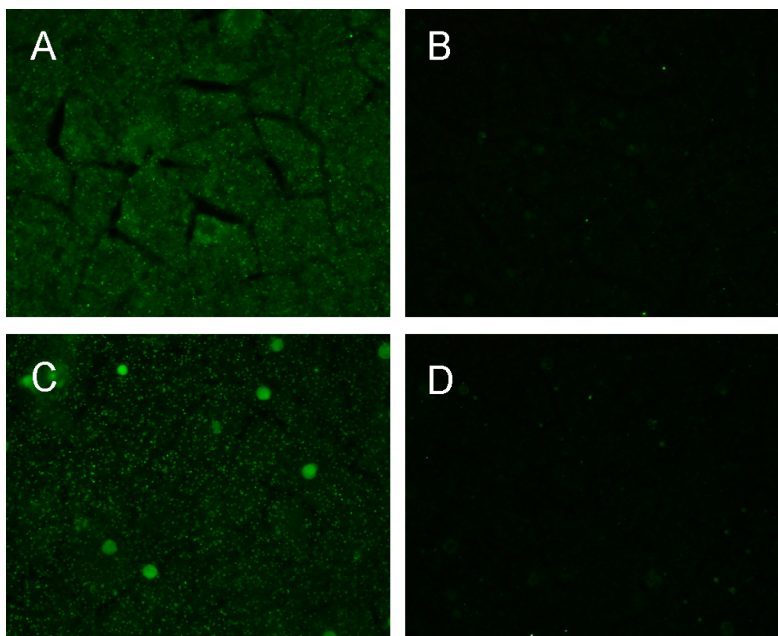

Fig. 4. Detection of the antibodies against BgTRAP by IFAT. As an antigen, *B. gibsoni*-infected blood samples collected from a *B. gibsoni*-infected dog were used. Serum samples collected from mice inoculated with BgTRAP expressed in *E. coli* by a pGEX-4T vector (Ec/BgTRAP) were used as positive controls of the first antibody, and normal mouse serum was used as a negative control of the first antibody. The panels show fluorescence images of *B. gibsoni* merozoites recognized by Ec/BgTRAP-immunized mouse serum (A), normal mouse serum (B), Tg/BgTRAP tachyzoite-immunized mouse serum (C), and Tg/wt tachyzoite-immunized mouse serum (D).

DISCUSSION

In this study, a *T. gondii* vector expressing BgTRAP (Tg/BgTRAP) was constructed, and its antigenicity and immunogenicity were verified. There are many potential merits for using *T. gondii* as a vector for expressing BgTRAP: 1) both *B. gibsoni* and *T. gondii* are closely related apicomplexans, and the antigenic structures of recombinant BgTRAP expressed in *T. gondii* might mimic those of the original BgTRAP expressed in *B. gibsoni*; 2) a *T. gondii* vector expressing BgTRAP might be used as bivalent vaccine against both *B. gibsoni* and *T. gondii* infections in dogs; 3) live *T. gondii* could induce strong cell-mediated immunity against itself as well as nonspecific resistance against other pathogens and tumors (Mahmoud *et al.,* 1976; Reyes *et al.,* 1987; Denkers *et al.,* 1998).

Immunofluorescence analyses showed that BgTRAP was expressed only in Tg/BgTRAP but not in Tg/wt and was mainly detected in the endoplasmic reticulum and the apical end of the tachyzoites (Fig. 2). BgTRAP was first expected to be expressed in the microneme of tachyzoites because it shares amino-acid homology with TgMIC2, which is also one of the TRAP family members conserved in the microneme of *T. gondii* tachyzoites and has been reported to contain five copies of the conserved thrombospondin (TSP)-like motif (Wan *et al.,* 1997). If it is assumed that the post-translation modification of BgTRAP is similar to that of TgMIC2, BgTRAP might be accumulated in the microneme after having been processed in the endoplasmic reticulum. There were a small quantity of BgTRAP expressed in the microneme and a large quantity expressed in the endoplasmic reticulum. A generalization of these conceptions suggests that it is possible that the precursor of BgTRAP remains in the endoplasmic reticulum.

The molecular bands of recombinant BgTRAP expressed in *B. gibsoni* merozoites and Tg/BgTRAP tachyzoites were both mainly 121 kDa, indicating that BgTRAP is processed similarly in both *B. gibsoni* merozoites and Tg/BgTRAP tachyzoites. Similarly, the molecular weight of BgTRAP expressed in the supernatant of the *B. gibsoni* merozoite culture was 121 kDa. On the other hand, the molecular weight of BgTRAP in the supernatant of the Tg/BgTRAP tachyzoite culture differed from that of *B. gibsoni*. This might be due to the difference of post-translation modification between *B. gibsoni* and *T. gondii*.

Serum collected from mouse immunized with Tg/BgTRAP detected *B. gibsoni* merozoites by IFAT and showed that anti-*B. gibsoni* antibodies were induced (Fig. 4). Nevertheless, anti-*B. gibsoni* antibodies were not detected by ELISA with Ec/BgTRAP as an antigen (data not shown). The antibody obtained by Tg/BgTRAP immunization might be induced against conformational epitope(s) on BgTRAP and, therefore, could not recognize the epitope(s) on BgTRAP expressed in *E. coli*.

Because the *T. gondii* RH strain is highly virulent and leads to lethal infections in mice, induction of immune responses by using Tg/BgTRAP as a live vaccine could not be attempted in this experiment. Thus, the use of an avirulent *T. gondii* strain is needed for actual use of the live vaccine. Temperature-sensitive 4 (Ts-4) is a mutant strain of the *T. gondii* RH strain. Unlike the RH strain, ts-4 is avirulent for immunocompetent mice and does not form tissue cysts even in doses up to 10^3 to 10^5 tachyzoites (Pfefferkorn and Pfefferkorn 1976). Ts-4 grows more slowly *in vitro* at 37°C than the wild-type RH parental strain, and its growth becomes progressively slower at 38°C, 39°C, and 40°C (Pfefferkorn and Pfefferkorn 1976). Furthermore,

infection with ts-4 induces strong protection against rechallenge with virulent *T. gondii* in mice by involving strong Th1 cytokine and $CD8^{+}$ T cell responses (Charest *et al.,* 2000). In practice, ts-4 has been utilized as an expression system of heterologous proteins, and induction of protective immunity by ts-4 has been confirmed in mice (Charest *et al.,* 2000; Ramirez *et al.,* 2002) and monkeys (Di Cristina *et al.,* 1999). In addition, attenuated *T. gondii* strain 48 (s48) is also a good candidate for expressing BgTRAP. S48 is already commercially available as a live vaccine against sheep toxoplasmosis (Toxovax®, Intervet, New Zealand).

The key point of immunization against *B. gibsoni* infection is commonly believed to be protecting erythrocytes from merozoite invasion and eliminating infected erythrocytes. The former is assumed to consist of antibody responses, and the latter, of phagocytosis by macrophages. Because BgTRAP is expected to be important for erythrocyte invasion by *B. gibsoni* merozoites (Zhou *et al.,* 2006), inducing antibodies to BgTRAP by Tg/BgTRAP could be useful for host immunity. Moreover, a Th1-type cytokine, such as IFN-γ or IL-2, might be induced by *T. gondii* itself (Reyes *et al.,* 1987; Denkers *et al.,* 1998; Charest *et al.,* 2000). Thereafter, macrophages and NK cells will be activated by these cytokines, and hemophagocytosis will occur.

Collectively, evaluating the results of this experiment, the use of a live *T. gondii* vaccine against *B. gibsoni* infection in dogs appears promising. Further studies must be conducted using an attenuated *T. gondii* strain, and the immune responses must be evaluated by immunizing dogs.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

SUBMISSION DECLARATION AND VERIFICATION

This manuscript is approved by all authors, and if accepted, it will not be published elsewhere in the same form, in English or in any other language, including electronically without the written consent of the copyright-holder.

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