Expression of truncated *Babesia gibsoni* thrombospondin-related adhesive proteins in *Escherichia coli* and evaluation of their potential by enzyme-linked immunosorbent assay

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*Babesia gibsoni*のTRAPフラグメントの大腸菌による発現とELISA抗原としての有効性の評価

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Contents

Abbreviation

ABTS: 2,2'-azino-di-3-ethylbenzthiazoline-6-sulfonic acid

BgTRAP: *Babesia gibsoni* thrombospondin-related adhesive protein

BgTRAPc: *Babesia gibsoni* thrombospondin-related adhesive protein c terminal part

BgTRAPf: *Babesia gibsoni* thrombospondin-related adhesive protein full lenght

BgTRAPn: *Babesia gibsoni* thrombospondin-related adhesive protein n terminal part

bp: base pair

DNA: deoxyribonucleic acid

ELISA: enzyme-linked immunosorbent assay

GST: glutathione *S*-transferase

HRPO: horse radish peroxidase

IFAT: indirect fluorescent antibody test

IgG: immunoglobulin G

kDa: kilo Dalton

OD: optical density

ORF: open reading frame

PBS: phosphate-buffered saline

PBST: phosphate-buffered saline with 5% of Tween 20

PCR: polymerase chain reaction

SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis

SPF: specific pathogen-free

TRAP: thrombospondin related adhesive protein

TSP1: throbospondin 1

vWFA: von Willebrand factor A

Abstract

Among the previously established enzyme-linked immunosorbent assays (ELISAs), an ELISA using the full length of recombinant thrombospondin-related adhesive protein of *Babesia gibsoni* (rBgTRAPf) is considered as the most sensitive diagnostic method for the detection of antibody to *B. gibsoni* in dogs. However, the expression of rBgTRAPf in high concentration is poor and thus limits its usefulness as diagnostic antigen. To improve its expression level, we have truncated BgTRAPf into two fragments having either N- or C-terminus (BgTRAPn or BgTRAPc). The expression of BgTRAPc protein in *Escherichia coli* yielded adequate recombinant protein. The specificity and sensitivity of ELISAs with the truncated proteins were determined using *B. gibsoni*-experimentally infected dog sera and specific pathogen-free (SPF) dog sera. A total of 254 field dog sera were examined by the ELISA with rBgTRAPn, rBgTRAPc and rBgTRAPf as well as by indirect fluorescent antibody test (IFAT). The specificity of rBgTRAPc was highest (97.15%) and its kappa value (0.8003) was more than rBgTRAPn (0.7083). With sufficient level of expression as well as higher specificity and reliable sensitivity, rBgTRAPc appears to be a potential candidate antigen for serodiagnosis of *B. gibsoni* infection in dogs.

Introduction

Babesia gibsoni is a tick-borne apicomplexan parasite that causes piroplasmosis in domestic dogs and wild Canidae. Clinical manifestations of acute stage are characterized by remittent fever, progressive anemia, hemoglobinuria, and marked splenomegaly as well as hepatomegaly. Sometimes acute infections cause death, whereas chronic infections are often asymptomatic (Boozer et al., 2003; Wozniak et al., 1997).

The infection is endemic in many regions of Asia, Africa, Europe, and America, besides being emerging disease in many other countries. Even though this disease is transmitted by variety of well-described Ixodid vectors around the world, main reason of emerging pattern is due to non-vectored transmission by blood exchange during fighting and biting (Bostrom et al., 2008; Jefferies et al., 2007) as well as via the transplacental route to developing foetus (Fukumoto et al., 2005). Also it is speculated that *B. gibsoni* will be eventually reported from all countries where legal and illegal dog fighting is practiced (Irwin et al., 2009).

The diagnostic method of *B. gibsoni* infection is mainly by the microscopic examination of Giemsa-stained thin blood smear films. Although this method is simple, easy and appropriate for the diagnosis of *B. gibsoni* infection in the acute stage, it is difficult to detect *B. gibsoni* organisms in unapparent or chronic infections because of low levels of parasitemia (Verdida et al., 2004).

Recently polymerase chain reaction (PCR) has been used to diagnose *B. gibsoni* infection and higher diagnostic specificity and sensitivity have been achieved by using this method (Birkenheur et al., 2003; Bose et al., 1995; Fukumoto et al., 2001). Although PCR method can detect *B. gibsoni* infection with low parasitemia after clinical diagnosis (Inokuma et al., 2005), this method is limited by its ability to detect only current infection

in the peripheral blood, but not past infection (Miyama et al., 2006). Also survey in field sample is controversial and analyzing large numbers of sample is usually impossible due to expensive equipment and reagents.

Alternatively, the enzyme-linked immunosorbent assay (ELISA) and indirect fluorescent antibody test (IFAT) with infected erythrocytes as an antigen has been used for the serological diagnosis of *B. gibsoni* infection (Bose et al., 1995). These tests are useful for the identification of chronically infected dogs with significantly low parasitemia, especially those tests using recombinant proteins, which are pure avoiding the problem of false-positive reactions (Boonchit et al., 2006) and appropriate to conduct survey on large numbers of field sample.

Recombinant antigens namely, rBgP50, rBgSA1 and rBgP32, have been identified from *B. gibsoni* merozoite for the serological diagnosis of *B. gibsoni* infection (Aboge et al., 2007; Jia et al., 2006; Verdida et al., 2004). However, their sensitivities have not achieved perfect result for identifying naturally infected dogs with *B. gibsoni*.

Thrombospondin-related adhesive proteins (TRAPs) are a conserved family identified in several apicomplexan parasites. A number of studies have suggested that the TRAP plays a crucial role in host cell invasion. Recently, Zhou et al. (2006) identified the TRAP of *B. gibsoni* (BgTRAP) as a leading immunoreactive antigen. The full length of recombinant BgTRAP (rBgTRAPf) has so far shown the best sensitivity by using ELISA with regard to detection and differentiation of naturally infected and healthy dogs (Goo et al., 2008). Despite this achievement, expressing rBgTRAPf is quite difficult resulting in low expression level, which limits its usefulness in ELISA.

Therefore, i truncated full length of BgTRAPf into two fragments. The first one was a 1,158-bp fragment of BgTRAPn encoding N-terminus, which included the vWFA and TSP1 domains. The other one was an 816-bp fragment of BgTRAPc encoding cytoplasmic

C-terminus without a specific domain. I expressed the truncated rBgTRAPs in *Escherichia coli* and evaluated their diagnostic potentials in an ELISA using 254 dog sera collected from Pet Health Examination Centers around Japan.

Materials and methods

1. Parasite

B. gibsoni was initially isolated from a hunting dog of Hyogo Prefecture in Japan. This parasite was designated as the NRCPD strain, and it was maintained in splenectomized beagles as described earlier (Fukumoto et al., 2001). The *B. gibsoni*-infected dog erythrocytes were collected from an experimentally infected dog at peak parasitemia of 14% and stored at -80°C.

2. Cloning of the truncated BgTRAPn and BgTRAPc genes into the pGEX-4T-3 vector

The pGEX-4T-3 vector (GE healthcare, UK) containing an open reading frame (ORF) encoding glutathione *S*-transferase (GST)-fusion protein was used. Oligonucleotide prime sets including *Eco*RI and *Xho*I restriction enzyme sites were designed and used to clone the truncated genes, encoding either 43-kDa protein for BgTRAPn without signal peptide (forward primer, 5′-GTCGAATTCCGCGATGGCGATGGAGATGAAAAG-3′; reverse primer, 5′-GAACTCGAGGCTGCTGTGGTGCATTGTATT-3′) or 30-kDa protein for BgTRAPc without transmembrane region (forward primer, 5′-GGCGAATTCTACCCAGCGAATACAATGCAC-3′; reverse primer, 5′-GCGCTCGAGATCTTCGTCTTCTGAGTAATC-3′). Plasmid including BgTRAPf was used as a template DNA. Amplification was performed in 50 μl of 1xPCR buffer (Roche, Switzerland) containing 2.5 U of Taq polymerase, 2 μl of DNA template, 10 pmol of each primer and 2 mM concentration of each deoxynucleoside triphosphate. The PCR for

BgTRAPn was performed as follows; initial denaturation at 96°C for 5 min, followed by 35 amplification cycles (96°C for 30 s, 55°C for 30 s, 72°C for 30 s) and final extension step at 72°C for 5 min. The PCR for BgTRAPc involved initial denaturation at 96°C for 5 min, followed by 35 amplification cycles (96°C for 30 s, 57°C for 30 s, 72°C for 30 s) and final extension step at 72°C for 5 min. Subsequently, the PCR products were cloned into *Eco*RI and *Xho*I restriction enzyme sites of the pGEX-4T-3 vector. Resulting plasmids were checked for accurate insertion by restriction enzyme and nucleotide sequencing.

3. Expression and purification of rBgTRAPf, rBgTRAPn and rBgTRAPc

The truncated BgTRAPn and BgTRAPc genes in the pGEX-4T-3 vector were transformed into *E. coli* (GE healthcare, UK). The BgTRAPn and BgTRAPc genes were expressed in the *E. coli* BL21 (DE3) strain as glutathione *S*-transferase (GST)-fusion proteins according to the manufacturer's instructions (GE healthcare, UK). The resulting *E. coli* cells were washed three times with phosphate-buffered saline (PBS), lysed in 1% Triton X-100-PBS, sonicated and then centrifuged at 12,000 × *g* for 10 min at 4°C. Supernatants containing the either soluble BgTRAPn or BgTRAPc were purified by glutathione-Sepharose 4B beads (GE healthcare, UK) according to the manufacturer's instruction. The expression of the recombinant protein was confirmed by performing sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) using a 12% polyacrylamide gel. A previously reported rBgTRAPf was expressed in *E. coli* and purified according to the described methods (Zhou et al., 2006).

4. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting

Protein expressions were verified by SDS-PAGE after staining with Coomasie blue. Thereafter, Western blot analysis was done to determine the antigenicity of rBgTRAPf and truncated rBgTRAPs for application in serodiagnostic methods. Recombinant BgTRAPf, rBgTRAPn and rBgTRAPc were loaded in a 12% polyacrylamide gel of SDS-PAGE and then electro-blotted on an Immobilon-P transfer membrane (Millipore, USA). After blocking with 5% skim milk dissolved in PBS, the membrane was incubated with primary antibodies (*B. gibsoni*-infected dog serum and specific pathogen free (SPF) dog serum were diluted 1:100 times) for 1 h at 37°C, then washed with PBS containing 0.5% of Tween 20 (PBST). Then, the membrane was soaked in peroxidase-conjugated goat anti-dog immunoglobulin G (IgG; 1:4,000) and incubated 1 h at 37°C. After washing with PBST, the membrane was reacted with 3, 3′-diaminobenzidine tetrahydrochloride (Nacalai Tesque, Inc., Japan) and H_2O_2 to detect rBgTRAPf, rBgTRAPn and rBgTRAPc.

5. Enzyme-linked immunosorbent assay (ELISA)

The ELISA with rBgTRAPf, rBgTRAPn or rBgTRAPc was carried out according to a previously described method (Fukumoto et al., 2004). GST-rBgTRAPf (50 ng/well), GST-rBgTRAPn (50 ng/well), GST-rBgTRAPc (50 ng/well) and GST (50 ng/well) diluted in an antigen coating buffer (0.05 M carbonate-bicarbonate buffer, pH 9.6) were coated on 96-well microplates (Nunc, Denmark) overnight at 4°C and the plate was blocked with a 3% skim milk solution for 1 h at 37°C. After washing, the plate was incubated with sera at a dilution of 1:100. The binding of second antibody was detected by treatment with HRPO

(horse radish peroxidase)-conjugated anti-dog IgG (Bethyl Laboratories, Inc., USA) (1:2,000) and ABTS [2,2'-asinobis (3-ethylbenzthiazolne sulfonic acid)] (Sigma-Aldrich, USA). The optical density (OD) was measured using the MTP-500 microplate reader (Corona Electric, Japan) at 415 nm. The ELISA titer was expressed as the reciprocal of the maximum dilution that showed an ELISA value equal to or greater than 0.32, 0.29 and 0.19 for rBgTRAPf, rBgTRAPn and rBgTRAPc, respectively, which is the difference of absorbance between antigens (GST-rBgTRAPf, GST-rBgTRAPn or GST-rBgTRAPc) and GST. The OD values (0.32, 0.29 and 0.19) were calculated by obtaining the mean OD value plus 3-fold standard deviations of 20 SPF dog sera.

6. Indirect fluorescent antibody test (IFAT)

Thin blood smear films of *B. gibsoni*-infected blood samples collected from an *in vitro* culture were fixed with methanol for 30 min at -20°C. The slides were incubated with 254 field dog sera diluted in PBS containing 10% of fetal calf serum for 30 min at 37°C. After washing with PBST, FICT-conjugated goat anti-dog IgG was applied on slides and incubated for 30 min at 37°C. After washing with PBST and PBS, the slide was covered with a glass cover slip. The slides were examined under a fluorescent microscope (Nikon, Japan).

7. Polymerase chain reaction (PCR)

DNA of 254 field dog blood samples were extracted from canine whole-blood samples using QIAamp DNA blood Mini-kit according to the manufacturer's instruction (QIAGEN, Germany). PCR was performed to detect parasite DNA using method described by

Fukumoto et al. (2001). Two oligonucleotide primers (d3; TCCGTTCCCACAACACCAGC and d4; AGGAGGAGTAGTAGGAGTAAGC) targeting 182-bp fragment which encodes *B. gibsoni* TRAP gene were used for the implication. Amplification was performed in 50µl reaction mixture containing about 1 µg of template DNA, 50 pmol of each primer, 200 µM of dNTPs and 1.25 U of Taq Gold DNA polymerase (ABI, USA) in 1x buffer ((10 mM Tris-HCl, 1.5 mM MgCl2, 0.001% gelatin) ABI, USA). PCR was performed for 10 min at 95°C to activate the Taq Gold DNA polymerase, and then the reaction was repeated for 40 cycles under the following conditions: 30 sec of denaturation at 94°C, 1 min of annealing at 54°C and 1 min of extension at 72°C. Five microliters of the PCR products were electrophorosed in a 1.5% agarose gel and the PCR bands were detected after staining the gel with ethidium bromide.

8. Sera and blood samples

In this study, we used a total of 344 sera and 254 blood samples. The canine sera used for the evaluation of the ELISA with truncated rBgTRAPn and rBgTRAPc were as follows: 10 sera from dogs experimentally infected with *B. gibsoni*; 20 sera from SPF dogs; 3 sera from dogs experimentally infected with *B. canis canis*; 3 sera from dogs experimentally infected with *B. canis vogeli*; 3 sera from dogs experimentally infected with *B. canis rossi*; 3 sera from dogs experimentally infected with *Leishmania infantum*; 48 sequential sera (from day 0 to 541 days post-infection) from a dog experimentally infected with *B. gibsoni*; 254 blood samples from Pet Health Examination Centers in Japan.

9. Statistical analysis

The results of ELISAs were compared with those of IFAT to calculate the sensitivity, specificity, percentage of agreement and kappa values. Kappa values were evaluated as: fair (0.21-0.40), moderate (0.41-0.60), and substantial (above 0.61). (http://faculty.vassar.edu/lowry/VassarStats.html).

Results

1. Characterization of rBgTRAPf, rBgTRAPn and rBgTRAPc

In order to improve the expression level of rBgTRAP, rBgTRAP was truncated into two fragments based on their hydropathy plot and antigenic index. The fragments included BgTRAPn with vWFA and TSP1 domains as well as BgTRAPc with C-terminus (Fig. 1). Hydrophilic and antigenic characteristics of BgTRAP were evaluated by using DNASTAR analyzer program (Netwell, Japan). The values and frequency obtained were higher in BgTRAPc than BgTRAPn. After confirming the successful expression of these truncated recombinant proteins as 69-kDa and 56-kDa soluble GST-fusion proteins in *E. coli*, three proteins, rBgTRAPf, rBgTRAPn and rBgTRAPc, were expressed from the same volume of medium. Thereafter, the expression levels of the products were compared by SDS-PAGE. The rBgTRAPc was expressed at a higher concentration and purity, better than that of rBgTRAPf. Therefore, this truncation solved the problem of low expression level. Although, rBgTRAPn was expressed at a relatively lower concentration than rBgTRAPc, another band of approximately 35-kDa possibly from a degraded protein of BgTRAPn was expressed in higher concentration than that of rBgTRAPf (Fig. 2). Thereafter, to evaluate whether those recombinant proteins have antigenicity to be used for serodiagnostic test, rBgTRAPf, rBgTRAPn and rBgTRAPc were probed with *B. gibsoni*-infected dog serum. As shown in Fig. 2, 108-kDa, 69-kDa and 56-kDa bands for BgTRAPf, BgTRAPn and rBgTRAPc, respectively, were detected after screening with *B. gibsoni*-infected dog serum but not by SPF dog serum (data and picture not shown). Moreover, the degraded protein of BgTRAPn strongly reacted with *B. gibsoni*-infected dog serum.

2. Evaluation of the potential of rBgTRAPn and rBgTRAPc as serodiagnotic candidates in the ELISA

In order to evaluate performances of the purified rBgTRAPn and rBgTRAPc as candidates for serodiagnostic method of *B. gibsoni* infection, the proteins were coated on ELISA plate. Sera from dogs experimentally infected with *B. gibsoni*, sera from SPF dogs and sera from dogs experimentally infected with parasites closely related to *B. gibsoni* were used for ELISA with these purified two BgTRAPs. As shown in Fig. 3, 10 sera from dogs infected with *B. gibsoni* were positive for the parasite antibodies when rBgTRAPf, rBgTRAPn and rBgTRAPc were used as antigens in an ELISA system (lanes 1-3, optical density; >0.32, >0.29 and >0.19). On the other hand, 20 sera from SPF dogs (lanes 4-6), 3 sera each from dogs infected with *B. canis canis* (lanes 7-9), *B. canis vogeli* (lanes 10-12), *B. canis rossi* (lanes 13-15), and *L. infantum* (lanes 16-18) were negative for the parasite antibodies (optical density; <0.32, <0.29 and <0.19 for rBgTRAPf, rBgTRAPn and rBgTRAPc, respectively). As shown in Fig. 4, sequential sera from a dog experimentally infected with *B. gibsoni* developed high titer of antibody response to BgTRAPf, BgTRAPn and BgTRAPc from 8 days post-infection. The high antibody titer was maintained until 541 days post-infection even when the dog became chronically infected with low level of parasitemia.

3. Diagnosis of B. gibsoni infection in field dog samples by ELISAs with rBgTRAPf, rBgTRAPn and rBgTRAPc, IFAT and PCR

A total of 254 sera collected from dogs in Pet Health Examination Centers around Japan were tested for antibodies to *B. gibsoni* by ELISAs using rBgTRAPf, rBgTRAPn and

rBgTRAPc. As shown in Table 1, 46 (18.1%), 49 (19.3%) and 41 (16.1%) of the tested samples were positive by ELISA with rBgTRAPf, rBgTRAPn and rBgTRAPc, respectively. Moreover, after analyzing all the 254 blood samples by PCR, the parasite DNA was detected in 37 (16.1%) samples. For reference test, the 254 dog sera were screened by using IFAT and 43 (16.9%) samples were found to be positive for the parasite antibodies.

In addition, IFAT results were used to evaluate sensitivity and specificity of ELISA with rBgTRAPf, rBgTRAPn and rBgTRAPc. In Table 2, concordance between the results of IFAT and those of ELISA with BgTRAPn or BgTRAPc indicated that 35 (13.7%) sera were positive for both tests. Furthermore, 39 (15.35%) sera showed positive results by IFAT and ELISA with rBgTRAPf. The data showed that the sensitivities of both truncated proteins were the same (81.39%) whereas the sensitivity of rBgTRAPf was 90.69% (Table 4). On the other hand, 11 (4.3%), 22 (8.6%) and 14 (5.4%) sera revealed distinct results between IFAT and ELISA based on rBgTRAPf, rBgTRAPn or rBgTRAPc. In addition, 7 (2.7%), 14 (5.5%) and 6 (2.3%) sera that were negative for IFAT, were found to be positive for ELISAs with rBgTRAPf, rBgTRAPn or rBgTRAPc, respectively. As shown in Table 4, the specificity of rBgTRAPf was 96.68%, while those of rBgTRAPn and rBgTRAPc were 93.36% and 97.15%, respectively. Furthermore, 4 (1.6%), 8 (3.1%) and 8 (3.1%) sera were positive on IFAT, but negative on ELISA using rBgTRAPf, rBgTRAPn or rBgTRAPc, respectively. The results of PCR were compared with those of ELISA using rBgTRAPf, rBgTRAPn and rBgTRAPc. Thirty four (13.4%), 29 (11.4%) and 29 (11.4%) samples were positive for PCR and for each of the ELISAs, respectively (Table 3). Three (1.2%) samples were negative for ELISA using rBgTRAPf while 8 (3.1%) samples were negative for ELISA based on rBgTRAPn and rBgTRAPc. However, the negative serum samples were positive on PCR analysis. On the other hand, 12 (4.7%) samples were positive for ELISA

with rBgTRAPf while 20 (7.8%) were positive for rBgTRAPn-ELISA. The other 12 (4.7%) were positive on ELISA using the rBgTRAPc antigen. These seropositive samples were all negative for the parasite DNA on PCR analysis.

Also as shown in Table 4, kappa values of rBgTRAPf, rBgTRAPn and rBgTRAPc were 0.8502, 0.8003 and 0.7083, respectively, indicating that all three recombinant antigens revealed substantial results. The percentages of the tests agreement between IFAT and ELISA with rBgTRAPf, rBgTRAPn as well as rBgTRAPc, at 95% of confidence interval, were 92.17%, 87.01% and 90.72%, respectively.

Discussion

In this study, i have successfully expressed sufficient quantities of a truncated rBgTRAPf and solved the problem of low expression level of the non truncated rBgTRAPf, which was previously considered as the best antigen for ELISA (Goo et al., 2008). The expression level of truncated proteins namely, rBgTRAPs, rBgTRAPn and rBgTRAPc, in *E. coli* expression system was better especially for rBgTRAPc, which was predicted to have higher hydrophilic characteristics (Kyte-Doolittle) as well as antigenic index (Jameson-Wolf) than rBgTRAPn by DNASTAR software analysis (NetWell, Japan).

The ELISA with either rBgTRAPn or rBgTRAPc clearly differentiated between *B. gibsoni*-infected dog sera and SPF dog sera. In addition, there was no cross-reaction with sera from dogs experimentally infected with closely related parasites, such as *B. canis canis*, *B. canis vogeli*, *B. canis rossi*, and *L. infantum* for both truncated rBgTRAPs on ELISA. These results indicated that the rBgTRAPn and rBgTRAPc are *B. gibsoni*-specific antigens, although the TRAPs are known as genetically conserved molecules among apicomplexan parasites (Zhou et al., 2006).

Subsequently, to evaluate whether the ELISA using truncated rBgTRAPs could detect the *B. gibsoni* specific antibodies including chronic infections, another ELISA was performed using sequential sera from a dog experimentally infected with *B. gibsoni* applying either rBgTRAPn or rBgTRAPc as antigens. Significant level of antibody titer was developed from 8 days post-infection and the high level of antibody titer was maintained until 541 days post-infection, even when the dog was chronically infected by *B. gibsoni* with a significantly low level of parasitemia. This result indicates that the truncated rBgTRAPn and rBgTRAPc could detect antibodies in both the acute and the chronic stages of *B. gibsoni* infection in dogs, similar to that of rBgTRAPf. Therefore,

these recombinant antigens could be used for serodiagnostic methods for detection of acute and chronic stage of *B. gibsoni* infection in dogs.

In this study, i used sera collected from clinically infected dogs for the screening because sera from dogs experimentally infected with *B. gibsoni* did not yield good results when rBgTRAPn and rBgTRAPc were used as antigens in ELISAs. Next, the sensitivity and specificity of ELISA with either rBgTRAPn or rBgTRAPc were compared with those of ELISA using rBgTRAPs, IFAT and PCR results. As shown in Table 1, IFAT and ELISA detected more positive samples than PCR, implying that these serodiagnostic methods could detect antibodies in carrier animal. From the result of ELISA and IFAT in Table 2, sensitivity and specificity were calculated (Table 4). The sensitivity of the ELISA with rBgTRAPf, rBgTRAPn and rBgTRAPc were 90.69%, 81.39% and 81.39%, respectively. In addition, ELISA with BgTRAPc showed highest specificity (97.15%) while specificity of either rBgTRAPf or rBgTRAPn was less moderate (96.68%) or quite low (93.36%), suggesting that N-terminal part is responsible for the lower specificity of rBgTRAPf. Supporting to this data, 41 (16.1%) field dog sera were positive by ELISA with rBgTRAPc, which is closest to IFAT result that detected 43 (16.9%) positive sera, whereas 49 (19.3%) and 46 (18.1%) sera were positive by ELISA with rBgTRAPn and rBgTRAPf, respectively. Therefore, i could confirm that BgTRAPn fragment having vWFA and TSP1 domains resulted in lower specificity because of their conservative characteristics with other protozoan species. On the other hand, BgTRAPc fragment without specific domain specifically detected *B. gibsoni* infection. Taken together, ELISA with rBgTRAPc is likely to be the most specific among all ELISAs based on the three rBgTRAPs. Moreover, some 12 (4.7%) samples that were negative for PCR were positive for *B. gibsoni* antibodies by ELISA with rBgTRAPf and rBgTRAPc. In contrast, 20 (7.9%) samples were PCR negative but were positive by ELISA with rBgTRAPn. This suggests that ELISA with

rBgTRAPc is more specific than ELISA with rBgTRAPn. In addition, it is possible that the low numbers of positives on PCR analysis was due to the time of sample collection, especially at chronic stage of infection. At this stage, parasites may have been removed from peripheral blood but antibodies still remained. Moreover, some 3 (1.2%), 8 (3.1%) and 8 (3.1%) samples were positive for PCR and negative for ELISA with rBgTRAPf, rBgTRAPn and rBgTRAPc, respectively. These samples represent sera and blood samples collected from dogs with acute infections when there is still no production of specific antibodies.

The kappa value and strength of agreement between IFAT and ELISA with rBgTRAPf, rBgTRAPn and rBgTRAPc revealed that all three recombinant proteins have substantial indications (0.8502, 0.7083 and 0.8003, respectively) as well as high percentage of agreement (92.17%, 87.01% and 90.72%, respectively). Although ELISA with rBgTRAPf had the highest kappa value and percentages of agreement with IFAT, rBgTRAPc appeared to be a better candidate than rBgTRAPf for serodiagnosis of *B. gibsoni* infection because of its high specificity as well as expression level. Since rBgTRAPc was expressed at high level, it is possible that this antigen could be used to develop other serodiagnostic methods such as immunochromatographic test. This is a quick and simple diagnostic method, which have not tried with rBgTRAPf due to its low expression level.

In conclusion, i have demonstrated that ELISA with rBgTRAPc has the highest specificity and reliable sensitivity. In addition, the expression level and purity of the recombinant protein was 50 times higher than that of ELISA with rBgTRAPf. This suggests that ELISA with rBgTRAPc could be the most promising serodiagnostic antigen compared to the other ELISAs previously developed and used for the detection of antibodies to *B. gibsoni* in dogs.

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Figures

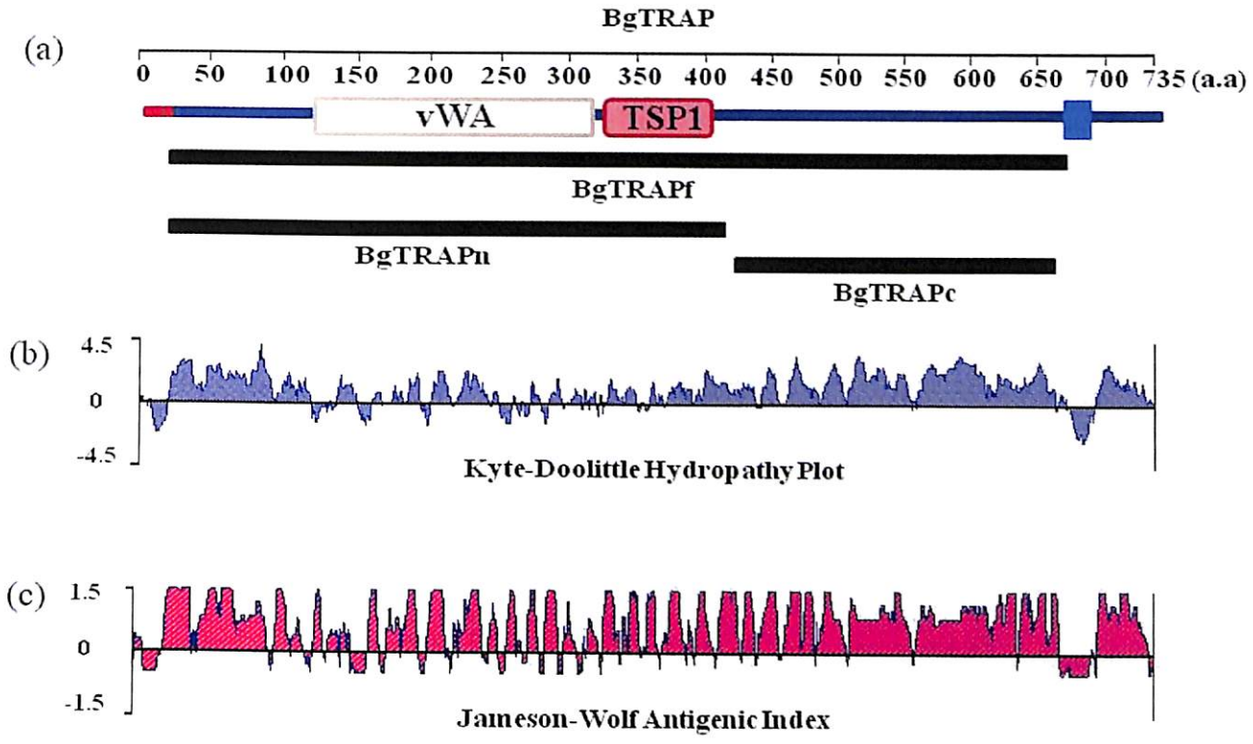

Fig. 1. Schematic representation of protein structure showing hydropathy plot and antigenic index of BgTRAP.

(a) Diagram of specific domains of BgTRAP (red block; signal peptides, vWA; von Willebrand A domain, TSP1; thrombospondin 1, blue block; Transmembrane domain). (b) Kyte-Doolittle hydropathy plot of BgTRAP. (c) Jameson-Wolf antigenic index of BgTRAP.

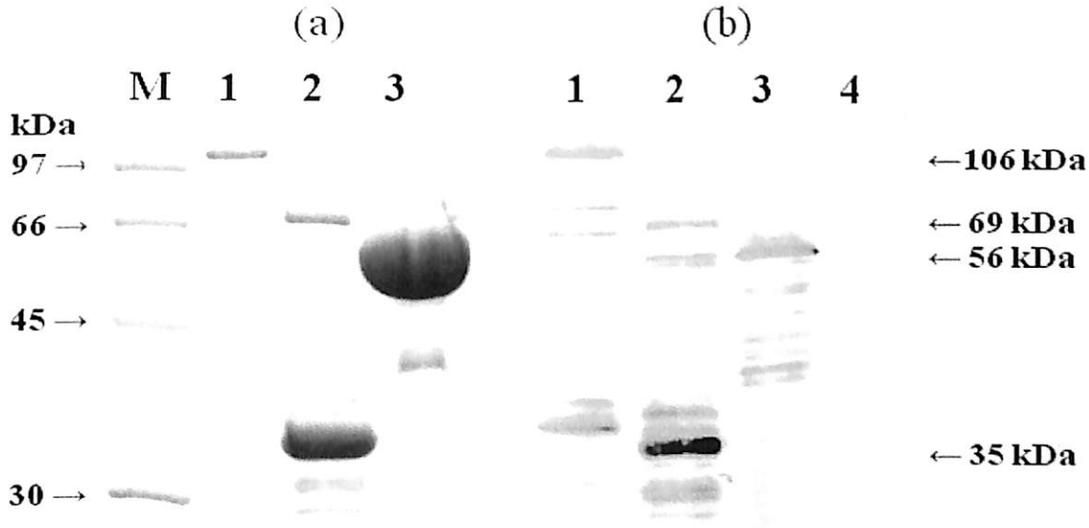

Fig. 2. SDS-PAGE and Western blot analysis of rBgTRAPf, rBgTRAPn and rBgTRAPc.

(a) Comparison of expression level of rBgTRAPf, rBgTRAPn and rBgTRAPc in same volume of medium. The rBgTRAPs expressed in *E. coli* were loaded in 12% polyacrylamide gel and stained with Coomassie Blue (M, Molecular weight marker; lane 1, rBgTRAPf; lane 2, rBgTRAPn; lane 3, rBgTRAPc).

(b) Western blot analysis of rBgTRAPs with *B. gibsoni*-infected dog serum. The size of each recombinant protein is indicated (lane 1, rBgTRAPf-GST 1:150; lane 2, rBgTRAPn-GST 1:200; lane 3, rBgTRAPc-GST 1:4000; lane 4, GST protein).

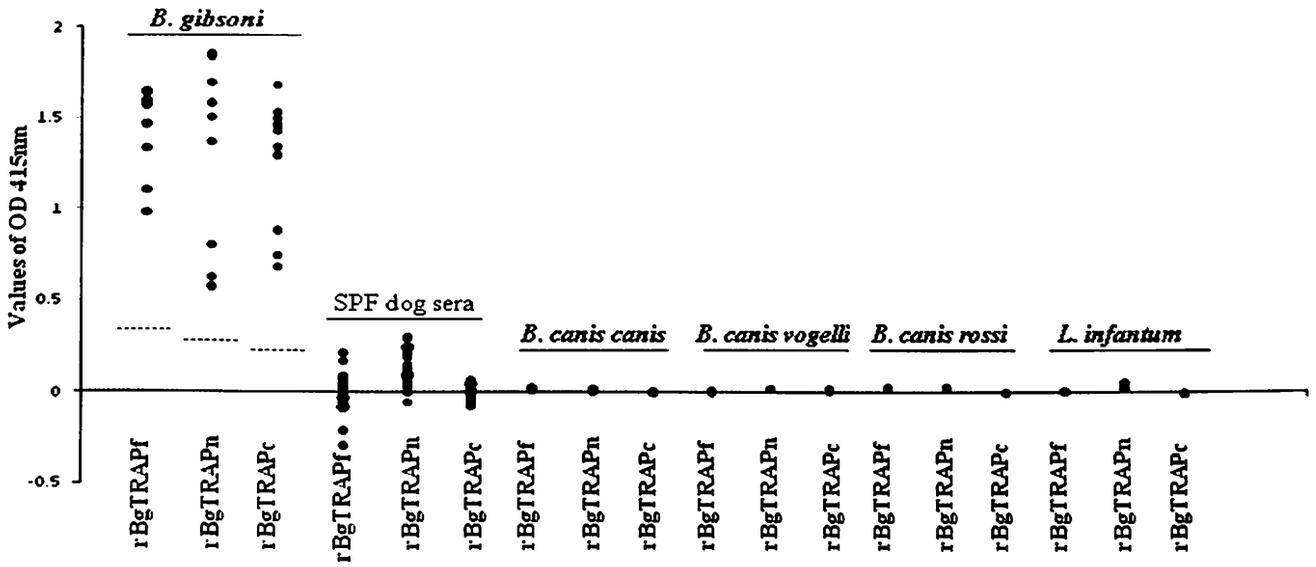

Fig. 3. Detection of cross-reactivity by ELISA with rBgTRAPf, rBgTRAPn and rBgTRAPc.

Lane 1-3, *B. gibsoni*-infected dog sera (n=10); lane 4-6, SPF dog sera (n=20); lane 7-9, *B. canis canis*-infected dog sera (n=3); lane 10-12, *B. canis vogeli*-infected dog sera (n=3); lane 13-15, *B. canis rossi*-infected dog sera (n=3); lane 16-18, *L. infantum*-infected dog sera (n=3).

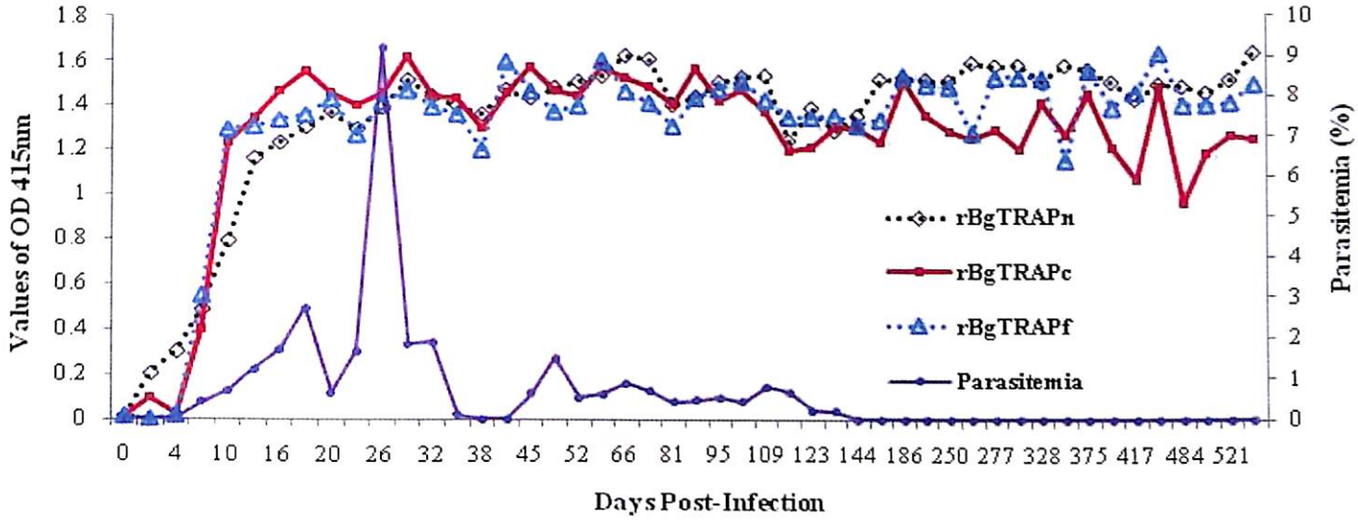

Fig. 4. Comparison of the specific antibody level to *B. gibsoni* among rBgTRAPf (blue empty triangle), rBgTRAPn (black empty diamond) and rBgTRAPc (red square) using sequential sera from a dog experimentally infected with *B. gibsoni*.

The parasitemia levels (%) were determined by Giemsa-staining of thin blood smears and observation by microscopy.

Tables

Table 1. Overall results of *B. gibsoni* detection in field dog sera by PCR, IFAT, ELISA with rBgTRAPf, BgTRAPn and rBgTRAPc.

	PCR	IFAT	ELISA		
			rBgTRAPf	rBgTRAPn	rBgTRAPc
Number (%) positive	37 (16.1%)	43 (16.9%)	46 (18.1%)	49 (19.3%)	41 (16.1%)
Number (%) negative	217 (83.9%)	208 (81.9%)	208 (82.9%)	205 (80.7%)	213 (83.9%)

Table 2. Diagnosis of *B. gibsoni* infection in field dog sera by IFAT and ELISA with rBgTRAPf, rBgTRAPn and rBgTRAPc.

IFAT	ELISA					
	rBgTRAPf		rBgTRAPn		rBgTRAPc	
	Positive	Negative	Positive	Negative	Positive	Negative
Number (%) positive	39 (15.3%)	4 (1.6%)	35 (13.7%)	8 (3.1%)	35 (13.7%)	8 (3.1%)
Number (%) negative	7 (2.7%)	204 (80.4%)	14 (5.5%)	197 (77.7)	6 (2.3%)	205 (80.9%)

Table 3. Compared results of PCR and ELISA with rBgTRAPf, BgTRAPn and rBgTRAPc.

PCR	ELISA					
	rBgTRAPf		rBgTRAPn		rBgTRAPc	
	Positive	Negative	Positive	Negative	Positive	Negative
Number (%) positive	34 (13.4%)	3 (1.2%)	29 (11.4%)	8 (3.1%)	29 (11.4%)	8 (3.1%)
Number (%) negative	12 (4.7%)	205 (80.7%)	20 (7.9%)	197 (77.6%)	12 (4.7%)	206 (80.8%)

Table 4. The sensitivity, specificity, kappa value and strength of agreement ELISAs with IFAT.

Name	Sensitivity (%)	Specificity (%)	Kappa value	Strength of agreement (%)
rBgTRAPf	90.69	96.68	0.8502	92.17
rBgTRAPn	81.39	93.36	0.7083	87.01
rBgTRAPc	81.39	97.15	0.8003	90.72

Summary in Japanese

*Babesia gibsoni*のTRAPフラグメントの大腸菌による発現とELISA抗原としての有効性の評価

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【背景と目的】イヌバベシア原虫感染症は、バベシア原虫の赤血球内寄生によって引き起こされるマダニ媒介性疾患である。バベシア原虫に感染した犬は、重度な溶血性貧血を引き起こし、死に至る場合も多い。日本を含めアジア地域に広く流行しているのは*Babesia gibsoni*感染症であり、その被害は非常に深刻とされるが、いまだに有効な診断法が開発されていないのが現状である。これまでに幾つかの診断用抗原遺伝子が同定され、組換え抗原を用いたELISA法が検討されているが、そのなかで最も効果的な抗原としてBgTRAPが知られている。しかし、組換えBgTRAPの大腸菌における発現量が非常に低いことが難題として残されていた。そこで、本研究では組換えBgTRAPの大腸菌における発現量の改善を試みた。

【材料と方法】完全長BgTRAPf遺伝子をほぼ中央で切断し、二つのフラグメント（N末端側とC末端側をそれぞれBgTRAPnとBgTRAPcと称する）としてそれぞれ大腸菌発現用ベクターpGEXに組み込み、GST融合タンパク質として発現した。これら３種類の組換え抗原の発現量の比較と、それぞれの抗原を用いたELISA法の有効性の評価を行った。

【結果と考察】BgTRAPf遺伝子、BgTRAPn遺伝子フラグメント、TgTRAPc遺伝子フラグメントをそれぞれ大腸菌で発現したところ、組換えTgTRAPcの発現量の上昇が顕著で、組換えTgTRAPfと比較して約50倍以上の上昇が認められた。一方、組換えTgTRAPnの発現量も少々の上昇はあったものの、発現産物の著しい分解が認められた。次に、これら３種類の組換えタンパク質を抗原としたELISA法を確立し、実験感染犬と野外感染犬の血清を用いてそれぞれの有効性を評価した。BgTRAPn-ELISA、BgTRAPn-ELISA、BgTRAPc-ELISAの検出感度はそれぞれ90.69%、81.39%、81.39%であった。また、特異性はそれぞれ96.68%、93.36%、97.15%であった。即ち、BgTRAPc-ELISAの感度はBgTRAPf-ELISAには少々及ばないものの、もっとも優れた特異性を有することが分かった。これらの結果より、組換えBgTRAPcは大腸菌における大量発現が可能で、なおかつ、優れた感度と特異性を有する有望な診断抗原になりうることが示唆された。