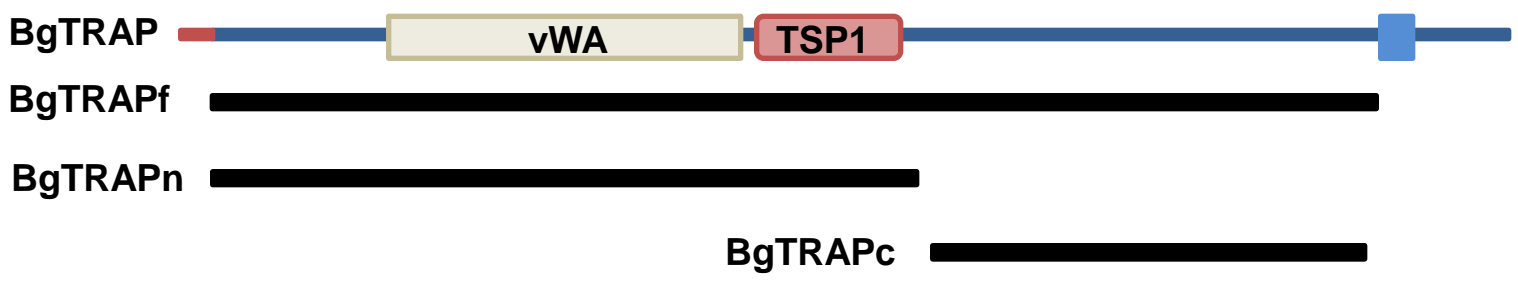




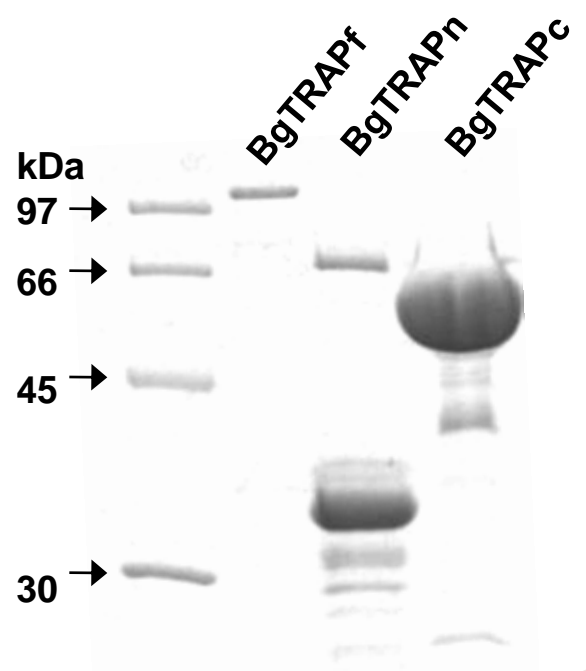
Expression of truncated Babesia gibsoni thrombospondin-related adhesive proteins in Escherichia coli and evaluation of their diagnostic potential by enzyme-linked immunosorbent assay

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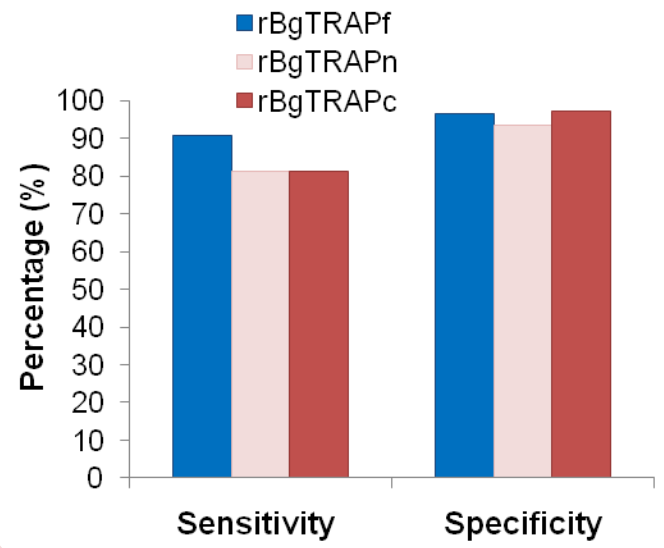
Diagram of BgTRAPf, BgTRAPn and BgTRAPc



Comparison of expression level



Sensitivity and specificity of ELISA with three antigens for *B. gibsoni* infection



The BgTRAP was truncated two parts, BgTRAPn and BgTRAPc. In addition to higher expression level of rBgTRAPc compared to that of rBgTRAPf, ELISA with rBgTRAPc has the highest specificity and reliable sensitivity. The 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.

1 **Title**

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

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1 **ABSTRACT**

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

1

2 **Keywords**

3 *Babesia gibsoni*, diagnosis, enzyme-linked immunosorbent assay (ELISA),
4 thrombospondin-related adhesive protein (TRAP).

5

6 **1. Introduction**

7 *Babesia gibsoni* is a tick-borne apicomplexan parasite that causes
8 piroplasmosis in domestic dogs and wild Canidae. Clinical manifestations of
9 acute stage are characterized by remittent fever, progressive anemia,
10 hemoglobinuria, and marked splenomegaly as well as hepatomegaly.
11 Sometimes acute infections cause death, whereas chronic infections are often
12 asymptomatic (Wozniak et al., 1997; Boozer et al., 2003). The infection is
13 endemic in many regions of Asia, Africa, Europe, and America, besides being
14 emerging disease in many other countries. Even though this disease is
15 transmitted by variety of well-described Ixodid vectors around the world, main
16 reason of emerging pattern is due to non-vectored transmission by blood
17 exchange during fighting and biting (Jefferies et al., 2007; Bostrom et al., 2008)
18 as well as via the transplacental route to developing foetus (Fukumoto et al.,

1 2005). Also it is speculated that *B. gibsoni* will be eventually reported from all
2 countries where legal and illegal dog fighting is practiced (Irwin et al., 2009).

3 The diagnostic method of *B. gibsoni* infection is mainly by the microscopic
4 examination of Giemsa-stained thin blood smear films. Although this method is
5 simple, easy and appropriate for the diagnosis of *B. gibsoni* infection in the acute
6 stage, it is difficult to detect *B. gibsoni* organisms in unapparent or chronic
7 infections because of low levels of parasitemia (Verdida et al., 2004). Recently
8 polymerase chain reaction (PCR) has been used to diagnose *B. gibsoni* infection
9 and higher diagnostic specificity and sensitivity have been achieved by using
10 this method (Bose et al., 1995; Fukumoto et al., 2001; Birkenheur et al., 2003).
11 Although PCR method can detect *B. gibsoni* infection with low parasitemia after
12 clinical diagnosis (Inokuma et al., 2005), this method is limited by its ability to
13 detect only current infection in the peripheral blood, but not past infection
14 (Miyama et al., 2006). Also survey in field sample is controversial and analyzing
15 large numbers of sample is usually impossible due to expensive equipment and
16 reagents. Alternatively, the enzyme-linked immunosorbent assay (ELISA) and
17 indirect fluorescent antibody test (IFAT) with infected erythrocytes as an antigen
18 has been used for the serological diagnosis of *B. gibsoni* infection (Bose et al.,

1 1995). These tests are useful for the identification of chronically infected dogs
2 with significantly low parasitemia, especially those tests using recombinant
3 proteins, which are pure avoiding the problem of false-positive reactions
4 (Boonchit et al., 2006) and appropriate to conduct survey on large numbers of
5 field sample.

6 Thrombospondin-related adhesive proteins (TRAPs) are a conserved family
7 identified in several apicomplexan parasites. A number of studies have
8 suggested that the TRAP plays a crucial role in host cell invasion. Recently,
9 Zhou et al. (2006) identified the TRAP of *B. gibsoni* (BgTRAP) as a leading
10 immunoreactive antigen. In addition, the recombinant antigens namely, rBgP50,
11 rBgSA1 and rBgP32, have been identified from *B. gibsoni* merozoite for the
12 serological diagnosis of *B. gibsoni* infection (Verdida et al., 2004; Jia et al., 2006;
13 Aboge et al., 2007). However, their sensitivities have not achieved perfect result
14 for identifying naturally infected dogs with *B. gibsoni*. The full length of
15 recombinant BgTRAP (rBgTRAPf) has so far shown the best sensitivity by using
16 ELISA with regard to detection and differentiation of naturally infected and
17 healthy dogs (Goo et al., 2008). Despite this achievement, expressing rBgTRAPf
18 is quite difficult resulting in low expression level, which limits its usefulness in

1 ELISA.

2 Therefore, we truncated full length of BgTRAPf into two fragments. The first
3 one was a 1,158-bp fragment of BgTRAPn encoding N-terminus, which included
4 the vWFA and TSP1 domains. The other one was an 816-bp fragment of
5 BgTRAPc encoding cytoplasmic C-terminus without a specific domain. We
6 expressed the truncated rBgTRAPs in *Escherichia coli* and evaluated their
7 diagnostic potentials in an ELISA using 254 dog sera collected from Pet Health
8 Examination Centers around Japan.

9

10 **2. Materials and methods**

11 2.1. Parasite

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

17

18 2.2. Cloning of the truncated BgTRAPn and BgTRAPc genes into the

1 pGEX-4T-3 vector

2 The pGEX-4T-3 vector (GE healthcare, UK) containing an open reading frame

3 (ORF) encoding glutathione S-transferase (GST)-fusion protein was used.

4 Oligonucleotide prime sets including *EcoRI* and *XhoI* restriction enzyme sites

5 were designed and used to clone the truncated genes, encoding either 43-kDa

6 protein for BgTRAPn without signal peptide (forward primer,

7 5'-GTCGAATTCCGCGATGGCGATGGAGATGAAAAG-3'; reverse primer,

8 5'-GAACTCGAGGCTGCTGTGGTGCATTGTATT-3') or 30-kDa protein for

9 BgTRAPc without transmembrane region (forward primer,

10 5'-GGCGAATTCTACCCAGCGAATAACAATGCAC-3'; reverse primer,

11 5'-GCGCTCGAGATCTTCGTCTTCTGAGTAATC-3'). Plasmid including

12 BgTRAPf was used as a template DNAs. Amplification was performed in 50 µl of

13 1xPCR buffer (Roche, Switzerland) containing 2.5 U of Taq polymerase, 2 µl of

14 DNA template, 10 pmol of each primer and 2 mM concentration of each

15 deoxynucleoside triphosphate. The PCR for BgTRAPn was performed as

16 follows; initial denaturation at 96°C for 5 min, followed by 35 amplification cycles

17 (96°C for 30 s, 55°C for 30 s, 72°C for 30 s) and final extension step at 72°C for

18 5 min. The PCR for BgTRAPc involved initial denaturation at 96°C for 5 min,

1 followed by 35 amplification cycles (96°C for 30 s, 57°C for 30 s, 72°C for 30 s)
2 and final extension step at 72°C for 5 min. Subsequently, the PCR products were
3 cloned into *EcoRI* and *XhoI* restriction enzyme sites of the pGEX-4T-3 vector.
4 Resulting plasmids were checked for accurate insertion by restriction enzyme
5 and nucleotide sequencing.

6

7 2.3. Expression and purification of rBgTRAPf, rBgTRAPn and rBgTRAPc

8 The truncated BgTRAPn and BgTRAPc genes in the pGEX-4T-3 vector were
9 transformed into *E. coli* (GE healthcare, UK). The BgTRAPn and BgTRAPc
10 genes were expressed in the *E. coli* BL21 (DE3) strain as GST-fusion proteins
11 according to the manufacturer's instructions (GE healthcare, UK). The resulting
12 *E. coli* cells were washed three times with phosphate-buffered saline (PBS),
13 lysed in 1% Triton X-100-PBS, sonicated and then centrifuged at 12,000 × *g* for
14 10 min at 4°C. Supernatants containing the either soluble BgTRAPn or
15 BgTRAPc were purified by glutathione-Sepharose 4B beads (GE healthcare,
16 UK) according to the manufacturer's instruction. The expression of the
17 recombinant protein was confirmed by performing sodium dodecyl
18 sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) using a 12%

1 polyacrylamide gel. A previously reported rBgTRAPf was expressed in *E. coli*
2 and purified according to the described methods (Zhou et al., 2006).

3

4 2.4. SDS-PAGE and Western blotting

5 Protein expressions were verified by SDS-PAGE after staining with Coomassie
6 blue. Thereafter, Western blot analysis was done to determine the antigenicity of
7 rBgTRAPf and truncated rBgTRAPs for application in serodiagnostic methods.
8 Recombinant BgTRAPf, rBgTRAPn and rBgTRAPc were loaded in a 12%
9 polyacrylamide gel of SDS-PAGE and then electro-blotted on an Immobilon-P
10 transfer membrane (Millipore, USA). After blocking with 5% skim milk dissolved
11 in PBS, the membrane was incubated with primary antibodies (*B.*
12 *gibsoni*-infected dog serum and specific pathogen free (SPF) dog serum were
13 diluted 1:100 times) for 1 h at 37°C, then washed with PBS containing 0.5% of
14 Tween 20 (PBST). Then, the membrane was soaked in peroxidase-conjugated
15 goat anti-dog immunoglobulin G (IgG; 1:4,000) and incubated 1 h at 37°C. After
16 washing with PBST, the membrane was reacted with 3, 3'-diaminobenzidine
17 tetrahydrochloride (Nacalai Tesque, Inc., Japan) and H₂O₂ to detect rBgTRAPf,
18 rBgTRAPn and rBgTRAPc.

1

2 2.5. Enzyme-linked immunosorbent assay (ELISA)

3 The ELISA with rBgTRAPf, rBgTRAPn or rBgTRAPc was carried out according
4 to a previously described method (Fukumoto et al., 2004). GST-rBgTRAPf (50
5 ng/well), GST-rBgTRAPn (50 ng/well), GST-rBgTRAPc (50 ng/well) and GST (50
6 ng/well) diluted in an antigen coating buffer (0.05 M carbonate-bicarbonate buffer,
7 pH 9.6) were coated on 96-well microplates (Nunc, Denmark) overnight at 4°C
8 and the plate was blocked with a 3% skim milk solution for 1 h at 37°C. After
9 washing, the plate was incubated with sera at a dilution of 1:100. The binding of
10 second antibody was detected by treatment with horseradish
11 peroxidase-conjugated anti-dog IgG (Bethyl Laboratories, Inc., USA) (1:2,000)
12 and ABTS [2,2'-asinobis (3-ethylbenzthiazolne sulfonic acid)] (Sigma-Aldrich,
13 USA). The optical density (OD) was measured using the MTP-500 microplate
14 reader (Corona Electric, Japan) at 415 nm. The ELISA titer was expressed as
15 the reciprocal of the maximum dilution that showed an ELISA value equal to or
16 greater than 0.32, 0.29 and 0.19 for rBgTRAPf, rBgTRAPn and rBgTRAPc,
17 respectively, which is the difference of absorbance between antigens
18 (GST-rBgTRAPf, GST-rBgTRAPn or GST-rBgTRAPc) and GST. The OD values

1 (0.32, 0.29 and 0.19) were calculated by obtaining the mean OD value plus
2 3-fold standard deviations of 20 SPF dog sera.

3

4 2.6. Indirect fluorescent antibody test (IFAT)

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

12

13 2.7. Polymerase chain reaction (PCR)

14 DNA of 254 field dog blood samples were extracted from canine whole-blood
15 samples using QIAamp DNA blood Mini-kit according to the manufacturer's
16 instruction (QIAGEN, Germany). PCR was performed to detect parasite DNA
17 using method described by Fukumoto et al. (2001). Two oligonucleotide primers
18 (d3; TCCGTTCCCACAACACCAGC and d4;

1 AGGAGGAGTAGTAGGAGTAAGC) targeting 182-bp fragment which encodes *B.*
2 *gibsoni* P18 gene were used for the implication. Amplification was performed in
3 50- μ l reaction mixture containing about 1 μ g of template DNA, 50 pmol of each
4 primer, 200 μ M of dNTPs and 1.25 U of Taq Gold DNA polymerase (ABI, USA) in
5 1x buffer ((10 mM Tris-HCl, 1.5 mM MgCl₂, 0.001% gelatin) ABI, USA). PCR
6 was performed for 10 min at 95°C to activate the Taq Gold DNA polymerase, and
7 then the reaction was repeated for 40 cycles under the following conditions: 30
8 sec of denaturation at 94°C, 1 min of annealing at 54°C and 1 min of extension
9 at 72°C. Five microliters of the PCR products were electrophoresed in a 1.5%
10 agarose gel and the PCR bands were detected after staining the gel with
11 ethidium bromide.

12

13 2.8. Sera and blood samples

14 In this study, we used a total of 344 sera and 254 blood samples. The canine
15 sera used for the evaluation of the ELISA with truncated rBgTRAPn and
16 rBgTRAPc were as follows: 10 sera from dogs experimentally infected with *B.*
17 *gibsoni*; 20 sera from SPF dogs; 3 sera from dogs experimentally infected with *B.*
18 *canis canis*; 3 sera from dogs experimentally infected with *B. canis vogeli*; 3 sera

1 from dogs experimentally infected with *B. canis rossi*; 3 sera from dogs
2 experimentally infected with *Leishmania infantum*; 48 sequential sera (from day
3 0 to 541 days post-infection) from a dog experimentally infected with *B. gibsoni*;
4 254 blood samples from Pet Health Examination Centers in Japan.

5

6 2.9. Statistical analysis

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

11

12 2.10. Ethical guideline

13 The experimental animals were housed, fed and given clean drinking water in
14 accordance with the stipulated rules for the Care and Use of Research Animals
15 Promulgated by the Obihiro University of Agriculture and Veterinary Medicine,
16 Hokkaido, Japan.

17

18 **3. Results**

1 3.1. Characterization of rBgTRAPf, rBgTRAPn and rBgTRAPc

2 In order to improve the expression level of rBgTRAP, rBgTRAP was truncated
3 into two fragments based on their hydropathy plot and antigenic index. The
4 fragments included BgTRAPn with vWFA and TSP1 domains as well as
5 BgTRAPc with C-terminus (Fig. 1). Hydrophilic and antigenic characteristics of
6 BgTRAP were evaluated by using DNASTAR analyzer program (Netwell, Japan).
7 The values and frequency obtained were higher in BgTRAPc than BgTRAPn.
8 After confirming the successful expression of these truncated recombinant
9 proteins as 69-kDa and 56-kDa soluble GST-fusion proteins in *E. coli*, three
10 proteins, rBgTRAPf, rBgTRAPn and rBgTRAPc, were expressed from the same
11 volume of medium. Thereafter, the expression levels of the products were
12 compared by SDS-PAGE. As shown in (a) of Fig. 2, the rBgTRAPc was
13 expressed at a higher concentration and purity, 1:50 times better than that of
14 rBgTRAPf. Therefore, this truncation solved the problem of low expression level.
15 Although, rBgTRAPn was expressed at a relatively lower concentration than
16 rBgTRAPc, another band of approximately 35-kDa possibly from a degraded
17 protein of BgTRAPn was expressed at a concentration 1:20 times higher than
18 that of rBgTRAPf (Fig. 2). Thereafter, to evaluate whether those recombinant

1 proteins have antigenicity to be used for serodiagnostic test, rBgTRAPf,
2 rBgTRAPn and rBgTRAPc were probed with *B. gibsoni*-infected dog serum after
3 diluting each proteins in appropriate concentration to be observed in Western
4 blotting. As shown in (b) of Fig. 2, 108-kDa, 69-kDa and 56-kDa bands for
5 BgTRAPf, BgTRAPn and rBgTRAPc, respectively, were detected after screening
6 with *B. gibsoni*-infected dog serum but not by SPF dog serum. Moreover, the
7 degraded protein of BgTRAPn reacted with *B. gibsoni*-infected dog serum.

8

9 3.2. Evaluation of the potential of rBgTRAPn and rBgTRAPc as serodiagnostic
10 candidates in the ELISA

11 In order to evaluate performances of the purified rBgTRAPn and rBgTRAPc as
12 candidates for serodiagnostic method of *B. gibsoni* infection, the proteins were
13 coated on ELISA plate. Sera from dogs experimentally infected with *B. gibsoni*,
14 sera from SPF dogs and sera from dogs experimentally infected with parasites
15 closely related to *B. gibsoni* were used for ELISA with these purified two
16 BgTRAPs. As shown in Fig. 3, 10 sera from dogs infected with *B. gibsoni* were
17 positive for the parasite antibodies when rBgTRAPf, rBgTRAPn and rBgTRAPc
18 were used as antigens in an ELISA system (lanes 1-3, optical density; >0.32,

1 >0.29 and >0.19). On the other hand, 20 sera from SPF dogs (lanes 4-6), 3 sera
2 each from dogs infected with *B. canis canis* (lanes 7-9), *B. canis vogeli* (lanes
3 10-12), *B. canis rossii* (lanes 13-15) and *L. infantum* (lanes 16-18) were negative
4 for the parasite antibodies (optical density; <0.32, <0.29 and <0.19 for rBgTRAPf,
5 rBgTRAPn and rBgTRAPc, respectively). As shown in Fig. 4, sequential sera
6 from a dog experimentally infected with *B. gibsoni* developed high titer of
7 antibody response to BgTRAPf, BgTRAPn and BgTRAPc from 8 days
8 post-infection. The high antibody titer was maintained until 541 days
9 post-infection even when the dog became chronically infected with low level of
10 parasitemia.

11

12 3.3. Diagnosis of *B. gibsoni* infection in field dog samples by ELISAs with
13 rBgTRAPf, rBgTRAPn and rBgTRAPc, IFAT and PCR

14 A total of 254 sera collected from dogs in Pet Health Examination Centers
15 around Japan were tested for antibodies to *B. gibsoni* by ELISAs using
16 rBgTRAPf, rBgTRAPn and rBgTRAPc. As shown in Table 1, 46 (18.1%), 49
17 (19.3%) and 41 (16.1%) of the tested samples were positive by ELISA with
18 rBgTRAPf, rBgTRAPn and rBgTRAPc, respectively. Moreover, after analyzing all

1 the 254 blood samples by PCR, the parasite DNA was detected in 37 (16.1%)
2 samples. For reference test, the 254 dog sera were screened by using IFAT and
3 43 (16.9%) samples were found to be positive for the parasite antibodies.

4 In addition, IFAT results were used to evaluate sensitivity and specificity of
5 ELISA with rBgTRAPf, rBgTRAPn and rBgTRAPc. In Table 2, concordance
6 between the results of IFAT and those of ELISA with BgTRAPn or BgTRAPc
7 indicated that 35 (13.7%) sera were positive for both tests. Furthermore, 39
8 (15.35%) sera showed positive results by IFAT and ELISA with rBgTRAPf. The
9 data showed that the sensitivities of both truncated proteins were the same
10 (81.39%) whereas the sensitivity of rBgTRAPf was 90.69% (Table 4). On the
11 other hand, 11 (4.3%), 22 (8.6%) and 14 (5.4%) sera revealed distinct results
12 between IFAT and ELISA based on rBgTRAPf, rBgTRAPn or rBgTRAPc. In
13 addition, 7 (2.7%), 14 (5.5%) and 6 (2.3%) sera that were negative for IFAT, were
14 found to be positive for ELISAs with rBgTRAPf, rBgTRAPn or rBgTRAPc,
15 respectively. As shown in Table 4, the specificity of rBgTRAPf was 96.68%, while
16 those of rBgTRAPn and rBgTRAPc were 93.36% and 97.15%, respectively.
17 Furthermore, 4 (1.6%), 8 (3.1%) and 8 (3.1%) sera were positive on IFAT, but
18 negative on ELISA using rBgTRAPf, rBgTRAPn or rBgTRAPc, respectively. The

1 results of PCR were compared with those of ELISA using rBgTRAPf, rBgTRAPn
2 and rBgTRAPc. Thirty four (13.4%), 29 (11.4%) and 29 (11.4%) samples were
3 positive for PCR and for each of the ELISAs, respectively (Table 3). Three
4 (1.2%) samples were negative for ELISA using rBgTRAPf while 8 (3.1%)
5 samples were negative for ELISA based on rBgTRAPn and rBgTRAPc. However,
6 the negative serum samples were positive on PCR analysis. On the other hand,
7 12 (4.7%) samples were positive for ELISA with rBgTRAPf while 20 (7.8%) were
8 positive for rBgTRAPn-ELISA. The other 12 (4.7%) were positive on ELISA
9 using the rBgTRAPc antigen. These seropositive samples were all negative for
10 the parasite DNA on PCR analysis.

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

17

18 **4. Discussion**

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

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

17 Subsequently, to evaluate whether the ELISA using truncated rBgTRAPs
18 could detect the *B. gibsoni* specific antibodies including chronic infections,

1 another ELISA was performed using sequential sera from a dog experimentally
2 infected with *B. gibsoni* applying either rBgTRAPn or rBgTRAPc as antigens.
3 Significant level of antibody titer was developed from 8 days post-infection and
4 the high level of antibody titer was maintained until 541 days post-infection, even
5 when the dog was chronically infected by *B. gibsoni* with a significantly low level
6 of parasitemia. This result indicates that the truncated rBgTRAPn and
7 rBgTRAPc could induce strong humoral immunity in both the acute and the
8 chronic stages of *B. gibsoni* infection in dogs, similar to that of rBgTRAPf.
9 Therefore, these recombinant antigens could be used for serodiagnostic
10 methods for detection of acute and chronic stage of *B. gibsoni* infection in dogs.

11 In this study, we used sera collected from clinically infected dogs for the
12 screening in order to determine whether rBgTRAPn or rBgTRAPc could be used
13 as the antigen in ELISA with field samples. Next, the sensitivity and specificity of
14 ELISA with either rBgTRAPn or rBgTRAPc were compared with those of ELISA
15 using rBgTRAPf, IFAT and PCR results. As shown in Table 1, IFAT and ELISA
16 detected more positive samples than PCR, implying that these serodiagnostic
17 methods are more sensitive than the detection of parasite DNA in peripheral
18 blood by PCR. From the result of ELISA and IFAT in Table 2, sensitivity and

1 specificity were calculated (Table 4). The sensitivity of the ELISA with rBgTRAPf,
2 rBgTRAPn and rBgTRAPc were 90.69%, 81.39% and 81.39%, respectively. In
3 addition, ELISA with BgTRAPc showed highest specificity (97.15%) while
4 specificity of either rBgTRAPf or rBgTRAPn was less moderate (96.68%) or
5 quite low (93.36%), suggesting that N-terminal part is responsible for the lower
6 specificity of rBgTRAPf. Supporting to this data, 41 (16.1%) field dog sera were
7 positive by ELISA with rBgTRAPc, which is closest to IFAT result that detected
8 43 (16.9%) positive sera, whereas 49 (19.3%) and 46 (18.1%) sera were positive
9 by ELISA with rBgTRAPn and rBgTRAPf, respectively. Therefore, we could
10 confirm that BgTRAPn fragment having vWFA and TSP1 domains resulted in
11 lower specificity because of their conservative characteristics with other
12 protozoan species. On the other hand, BgTRAPc fragment without specific
13 domain specifically detected *B. gibsoni* infection. Taken together, ELISA with
14 rBgTRAPc is likely to be the most specific among all ELISAs based on the three
15 rBgTRAPs.

16 Moreover, some 12 (4.7%) samples that were negative for PCR were positive
17 for *B. gibsoni* antibodies by ELISA with rBgTRAPf and rBgTRAPc. In contrast, 20
18 (7.9%) samples were PCR negative but were positive by ELISA with rBgTRAPn.

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

10 The kappa value and strength of agreement between IFAT and ELISA with
11 rBgTRAPf, rBgTRAPn and rBgTRAPc revealed that all three recombinant
12 proteins have substantial indications (0.8502, 0.7083 and 0.8003, respectively)
13 as well as high percentage of agreement (92.17%, 87.01% and 90.72%,
14 respectively). Although ELISA with rBgTRAPf had the highest kappa value and
15 percentages of agreement with IFAT, rBgTRAPc appeared to be a better
16 candidate than rBgTRAPf for serodiagnosis of *B. gibsoni* infection because of its
17 high specificity as well as expression level. Since rBgTRAPc was expressed at
18 high level, it is possible that this antigen could be used to develop other

1 serodiagnostic methods such as immunochromatographic test. This is a quick
2 and simple diagnostic method, which have not tried with rBgTRAPf due to its low
3 expression level.

4

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9

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13

14 **Figure legends**

15

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

18 (a) Diagram of specific domains of BgTRAP (red block; signal peptides, vWA;

1 von Willebrand A domain, TSP1; thrombospondin 1, blue block; transmembrane
2 domain). (b) Kyte-Doolittle hydrophathy plot of BgTRAP. (c) Jameson-Wolf
3 antigenic index of BgTRAP.

4

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

7 (a) Comparison of expression level of rBgTRAPf, rBgTRAPn and rBgTRAPc in
8 same volume of medium. The rBgTRAPs expressed in *E. coli* were loaded in
9 12% polyacrylamide gel and stained with Coomassie Blue (M, Molecular weight
10 marker). (b) Western blot analysis of rBgTRAPs with *B. gibsoni*-infected dog
11 serum. The size of each recombinant protein is indicated and each recombinant
12 protein was diluted in appropriate concentration to be detected in Western
13 blotting (lane 1, rBgTRAPf-GST; lane 2, rBgTRAPn-GST; lane 3,
14 rBgTRAPc-GST; lane 4, GST protein).

15

16 Fig. 3. Detection of cross-reactivity by ELISA with rBgTRAPf, rBgTRAPn and
17 rBgTRAPc. Lane 1-3, *B. gibsoni*-infected dog sera (n=10); lane 4-6, SPF dog
18 sera (n=20); lane 7-9, *B. canis canis*-infected dog sera (n=3); lane 10-12, *B.*

1 *canis vogeli*-infected dog sera (n=3); lane 13-15, *B. canis rossi*-infected dog sera
2 (n=3); lane 16-18, *L. infantum*-infected dog sera (n=3).

3

4 Fig. 4. Comparison of the specific antibody level to *B. gibsoni* among rBgTRAPf
5 (blue empty triangle), rBgTRAPn (black empty diamond) and rBgTRAPc (red
6 square) using sequential sera from a dog experimentally infected with *B. gibsoni*.
7 The parasitemia levels (%) were determined by Giemsa-staining of thin blood
8 smears and observation by microscopy.

Fig. 1. Sandagdorj et al.

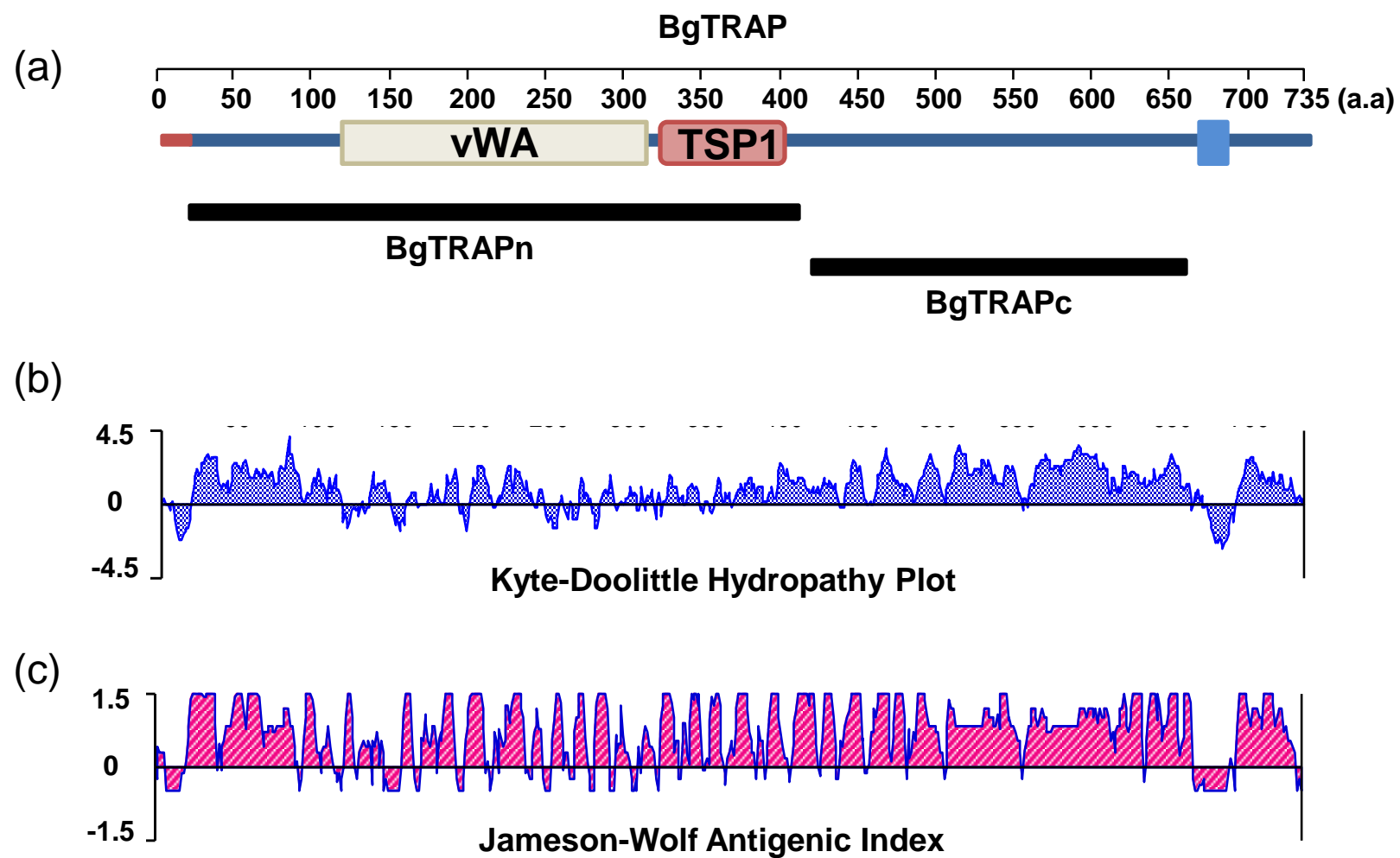


Fig. 2. Sandagdorj et al.

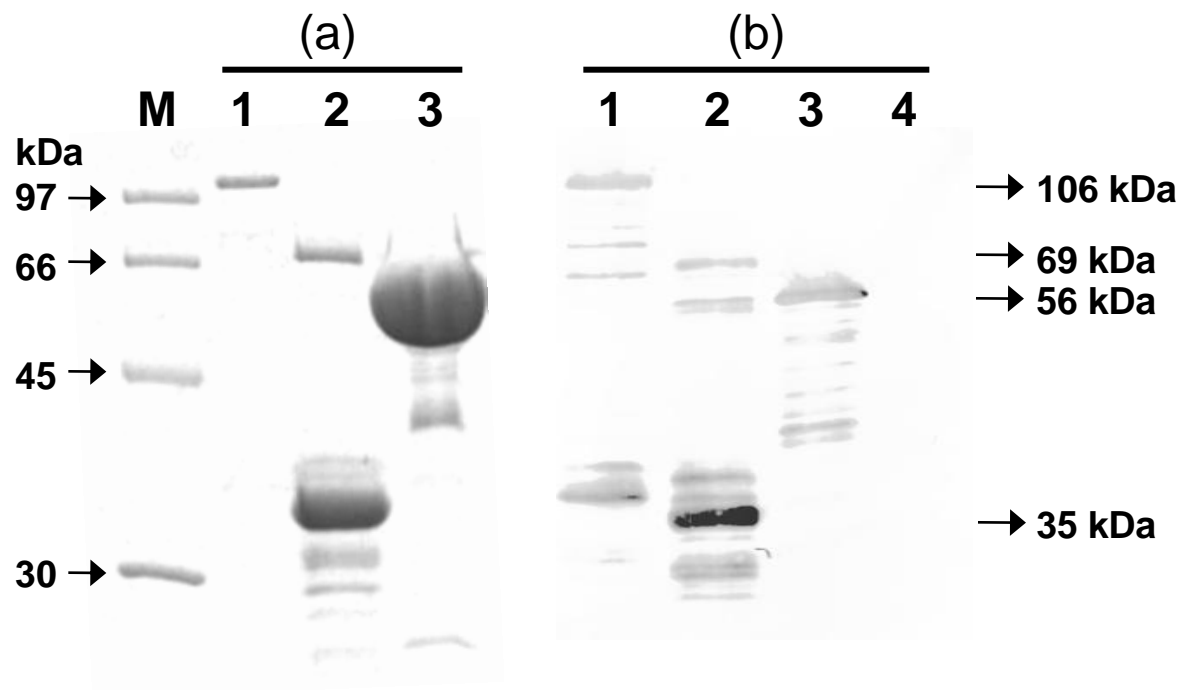


Fig. 3. Sandagdorj et al.

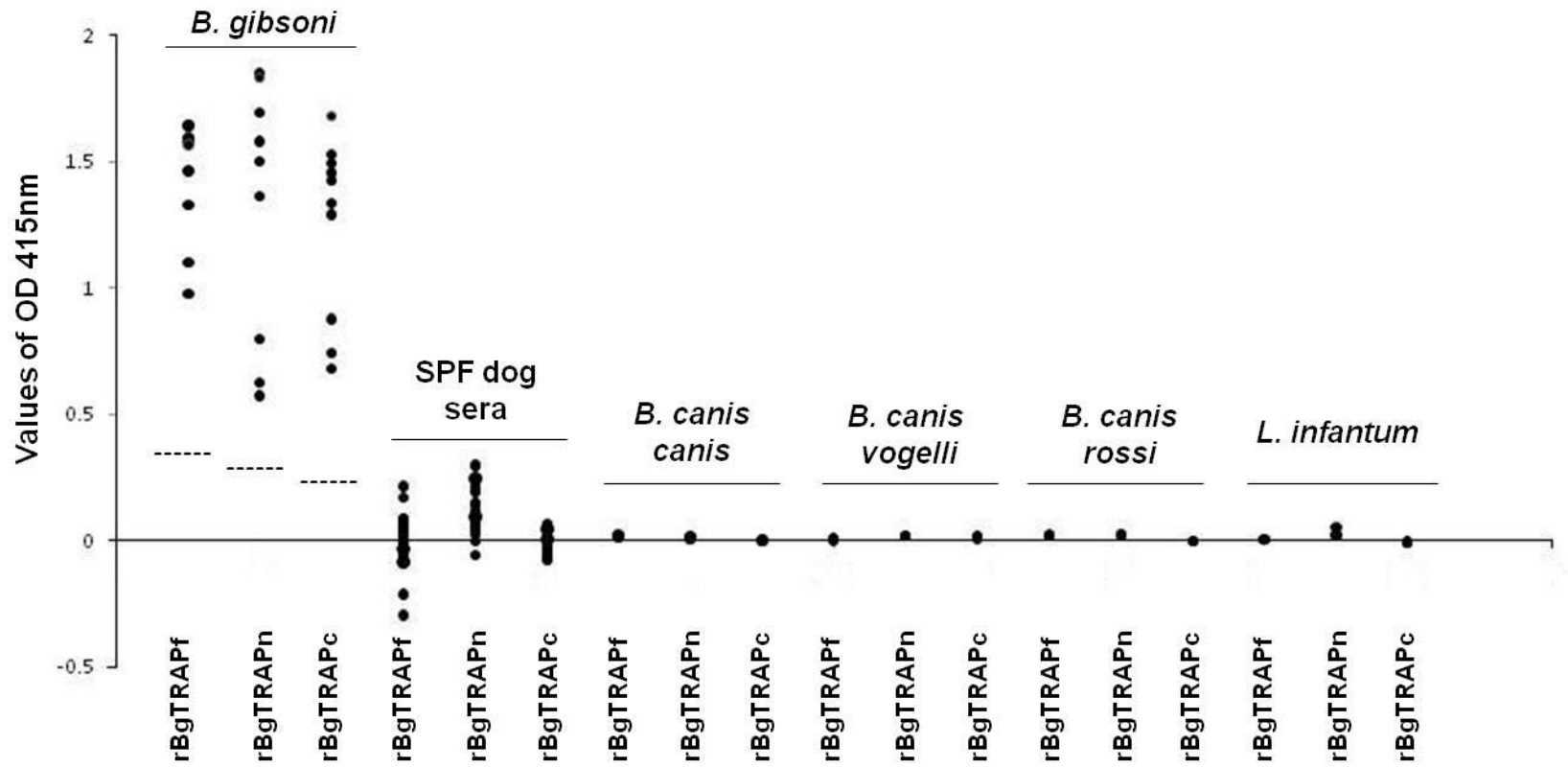


Fig. 4. Sandagdorj et al.

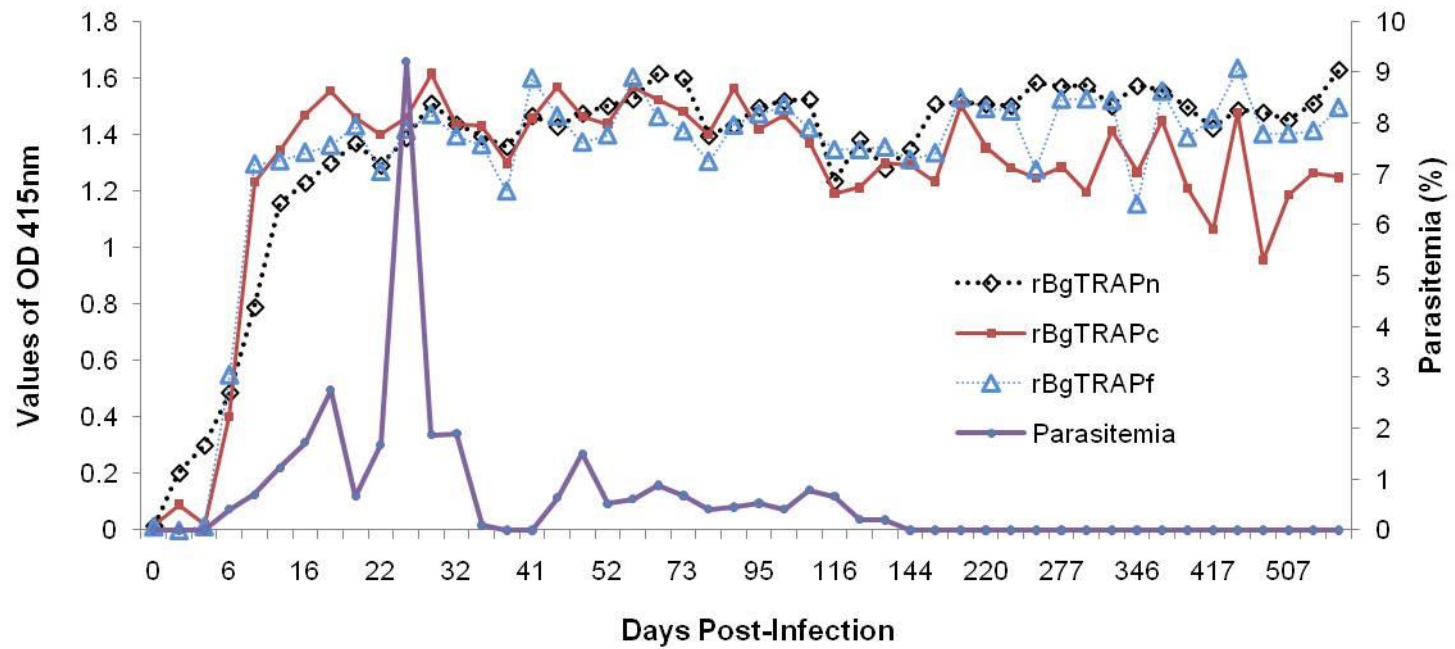


Table 1. Overall results of *B. gibsoni* detection in field dog sera by PCR, IFAT and ELISA with rBgTRAPf, BgTRAPn or rBgTRAPc.

	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 or 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