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Detection of anti-*Babesia gibsoni* heat shock protein 70 antibody and anti-canine heat shock protein 70 antibody in sera from *Babesia gibsoni*-infected dogs.

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ABSTRACT

Antibodies that recognized either *Babesia gibsoni* or canine red blood cell (RBC) 70-kilodalton (kDa) protein were detected in serum from acutely and chronically *B. gibsoni*-infected. In those sera, antibodies that reacted with recombinant *B. gibsoni* and canine heat shock protein 70 (rBgHsp70 and rcHsp70) were detected; therefore, *B. gibsoni* and canine RBC 70-kDa proteins seemed to be BgHsp70 and cHsp70, respectively. In infected dogs, the amounts of these antibodies increased after infection. Interestingly, polyclonal antibody raised against rBgHsp70 in two rabbits reacted not only with rBgHsp70 but also with rcHsp70 and native cHsp70 from canine RBCs. Because BgHsp70 showed high homology with cHsp70 (70.8%), anti-rBgHsp70 antibody might cross-react with cHsp70. Additonally, the localizations of both BgHsp70 and cHsp70 were observed by indirect fluorescence assay. As a result, cHsp70 was not found on the membrane surface of erythrocytes, suggesting that erythrocytes would not be targets of anti-cHsp70 antibody. Meanwhile, only exoerythrocytic parasites

were stained by anti-rBgHsp70 antibody. This result showed that BgHsp70 would be expressed on the surface of parasites during the exoerythrocytic stage. These results indicated that BgHsp70 was a highly immunogenic protein in canine *B. gibsoni* infection, and that exoerythrocytic parasites might be targets of anti-BgHsp70 antibody.

Keywords: *Babesia gibsoni*; heat shock protein 70; anti-heat shock protein 70 antibody

1. Introduction

Babesia gibsoni is a protozoan parasite that infects dogs and causes canine babesiosis. Previously, the sequence of the heat shock protein 70 (Hsp70) gene of *B. gibsoni* (BgHsp70) was analyzed (Yamasaki et al., 2002), and exposure of *B. gibsoni* to elevated temperature resulted in the increased expression of BgHsp70 (Yamasaki et al., 2008); however, the role and function of BgHsp70 remain largely unknown.

Hsp70, a 70-kDa Hsp, acts as a protein chaperone (Heike et al., 1996). In general, Hsp70 plays important roles in cell proliferation and the control of cellular functions (Lindquist, 1986). In a number of pathogens, Hsps are reported as immunoreactive molecules. In particular, characterization of recombinant *Mycobacterium tuberculosis* clones isolated from a *M. tuberculosis* library with polyclonal rabbit antisera revealed that clones expressing the *M. tuberculosis* 71-kDa antigen were frequently detected, which indicates that the hsp70 family of that species is highly immunogenic (Garsia et al., 1989). In *Babesia* parasites,

B. divergens hsp70 appears as an early antigen during the acute phase (Carcy et al., 1991). In the report of Terkawi et al. (2009), *B. gibsoni* HSP-70 protein had potent mitogenic effects on canine mononuclear cells, as evidenced by the high proliferative response and IFN-gamma production after stimulation. Immunization regimes in BALB/c and C57BL/6 mice using rBgHSP-70 elicited high antibody levels. Because of this immunogenicity, Hsp70 proteins are considered candidates for vaccine development; however, since the sequence of Hsp70 is well conserved between parasites and hosts, it is possible to induce autoreactive antibodies (Mattei et al. 1989). Indeed, antibodies recognizing human Hsp70 were detected in sera from humans infected with malaria. Similarly, it is possible that the structure of Hsp70 would be conserved between *B. gibsoni* and dogs, and that BgHsp70 would induce antibodies reacting with canine Hsp70. Because it was reported that the levels of anti-erythrocyte membrane antibodies and of erythrocyte-bound IgG were increased in dogs infected with *B. gibsoni* (Adachi and Makimura, 1992; Adachi et al., 1992; Adachi et al., 1994; Conrad et al., 1991; Farwell et al., 1982), antibodies reacting with

canine Hsp70 seem to be anti-erythrocyte membrane antibodies; therefore, the antigenecity of Hsp70s in *B. gibsoni*-infected dogs should be investigated. Accordingly, the objective of the present study was to study the antigenecity of BgHsp70 in canine *B. gibsoni* infection.

2. Materials and Methods

2.1. Experimental animals

Two adult male dogs were chronically infected with *B. gibsoni* (dog 1 and 2). These dogs had been inoculated with 1 x 10^9 parasites and splenectomized a few years before. They had mild anemia, but lived normal healthy lives. Two adult male dogs and three adult female dogs (dog 3-7) were used as acute *B. gibsoni*-infected dogs. These dogs were inoculated with 1 x 10^9 parasites obtained from dog 2. These experimentally inoculated dogs were monitored daily for the development of parasitemia by peripheral blood smear examination.

After the experiments, acutely infected dogs were maintained as chronically infected dogs in our laboratory. All dogs used were mongrels. The present study was carried out in accordance with Hokkaido University guidelines for the care and use of animals, which basically conform to the Association For Assessment and Accreditation of Laboratory Animal Care International (AAALAC). The present study was approved by the committee for Laboratory Animals, Graduate School of Veterinary Medicine, Hokkaido University (approval number: 9052).

2.2. Collection of blood from dogs

For the preparation of pre- and post-immune sera from *B. gibsoni*-infected dogs, blood was collected from the dogs described above. Pre-immune sera from infected dogs were collected before inoculation with the parasites. Post-immune sera from chronic *B. gibsoni*-infected dogs were collected when needed. Post-immune sera from acutely infected dogs were collected daily from day 1 to day 30 post-inoculation. In the present study, dog sera at day 16

post-innoculation were analyzed. All collected sera were frozen and stored at -30 ºC, and thawed once for use.

2.3. *in vitro* culture of *Babesia gibsoni*

The strain of *B. gibsoni* used in this study originated from a naturally infected dog in Nagasaki, Japan in 1973 and has been maintained in dogs and recently in *in vitro* culture as a laboratory-adapted parasite line as described previously (Yamasaki et al., 2003). The parasites are usually incubated at 37 ºC in a humidified atmosphere of 5% CO_2, 5% O_2, and 90% N_2 in a culture medium consisting of RPMI-1640 (Gibco BRL, Grand Island, New York), 20% dog serum, and dog RBCs sufficient to yield a packed cell volume of 5%. Every 24 hr, 60% of the culture supernatant is removed and replaced with an equal volume of fresh culture medium. Every 7 days, an equal volume of fresh dog RBC suspension is added to the cultured RBC suspension.

2.4. Cloning and sequencing of canine heat shock protein 70

Canine reticulocytes were collected as described previously (Yamasaki et al., 2000). Total RNA was extracted from canine reticulocytes using Trizol® (Molecular Research Center Inc., US) following the instruction manual supplied with the reagent. From the total RNA, cDNA was synthesized according to the method of Tajima et al. (1995) and used as a template for polymerase chain reaction (PCR).

The PCR primers used for amplification of the entire canine Hsp70 (cHsp70) gene (cHsp70F and cHsp70R; Table 1) were designed based on the reported sequence for canine Hsp70 from epithelial cells (GenBank accession number AB075027). PCR were performed as described previously (Yamasaki et al., 2002).

The nucleotide sequences of the amplification products were determined using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Tokyo, Japan) as described previously (Yamasaki et al., 2002). To compensate

for the possible misincorporation of nucleotides from the *Taq* polymerase, three amplified products were sequenced directly. As shown in Table 1, cHsp70F1-4 and cHsp70R1 were designed as sequencing primers based on the analyzed nucleotide sequence. Comparison and analysis of the nucleotide sequences were performed using GENETYX-MAC ver. 11.2 (Genetyx Co., Tokyo, Japan). The nucleotide sequence and predicted amino acid sequence of cHsp70 from canine reticulocytes were compared with those of the reported BgHsp70 gene (AB083510).

2.5. Expression and purification of recombinant *Babesia gibsoni* and canine heat shock protein 70

B. gibsoni Hsp70 (BgHsp70) was expressed and purified as described previously (Yamasaki et al., 2008). Recombinant cHsp70 protein was expressed and purified by the same procedure. The open reading frame (ORF) of the cHsp70 gene was amplified using PCR with cDNA as a template and specific

oligonucleotide primers (cHsp70F5 and cHsp70R2, Table 1). The ORF of the cHsp70 gene was ligated into the bacterial expression vector pCR® T7/NT-TOPO® (Invitrogen, Carlsbad, CA, USA) and then expressed as a HisG epitope fusion protein construct in *BL21(DE3)pLysS* chemically competent *Escherichia coli* (Invitrogen) as described previously (Yamasaki et al., 2008). The purified rBgHsp70 and cHsp70 were used as a sample for immunoblot analysis and rBgHsp70 was also used for development of anti-rBgHsp70 antibody. Recombinant glutathione S-transferase (GST) protein was used as a control irrelevant recombinant protein in the present study. This recombinant GST protein was a gift from Associate Professor Kota Sato, Laboratory of Molecular Medicine, Department of Veterinary Clinical Sciences, Graduate School of Veterinary Medicine, Hokkaido University.

2.6. Production of rabbit anti-rBgHsp70 polyclonal antibody

The purified rBgHsp70 protein was dialyzed in 100 times its volume of

dialysis buffer (10 mM Tris, 0.1% TritonX, pH 8.0) at 4 ºC for 16 hr. Two adult Japanese white rabbits (body weight 2.2 kg) were immunized on days 0, 14, 28, and 42 with 0.2 mg dialyzed rBgHsp70 protein. Sera were collected at day 0 (pre-immunization) and 60 (post-immunization), and checked for specific rabbit antibodies by immunoblot analysis. Rabbit anti-rBgHsp70 polyclonal antibodies were isolated from rabbit sera by affinity chromatography. This work was performed in accordance with the guidelines for the care and use of laboratory animals of Hokkaido System Science Co., Ltd (Sapporo, Japan).

2.7. Preparation of samples for immunoblotting

To prepare the parasite sample, canine RBCs infected with *B. gibsoni* were lysed by the addition of 5 mM phosphate buffer (PB, pH 7.4), and centrifuged at 15,000 g for 10 min at 4 ºC. After removal of the erythrocyte ghosts and lysate, the pelleted parasites were resuspended in M-PER™ Mammalian Protein Extraction Reagent (Pierce Chemical, Rockford, Illinois), and the protein

concentration of the suspension was measured with a commercial kit (Bio-Rad Protein Assay, Bio-Rad, Hercules, CA, USA) using the method of Bradford (1976). The suspension was mixed with sample buffer (62.5 mM Tris-HCl, 2% SDS, 5% beta-mercaptoethanol, 10% glycerol, and 0.02% bromphenol blue, pH 6.8) to yield a final concentration of 18 µg/µL, and boiled at 95 ºC for 5 min. An aliquot of 5 µL (90 µg) of the parasite sample was used for a single analysis.

To prepare the RBC membrane sample, whole blood was collected from an uninfected dog in a heparinized syringe. The blood was centrifuged at 900 g for 5 min at room temperature. After removal of the supernatant plasma and buffy coat, packed erythrocytes were washed three times with 10 mM phosphate-buffered saline (PBS, pH 7.4). After washing, packed RBCs were resuspended in dog plasma to yield a packed cell volume of 50%, filtered through an alpha-cellulose/microcrystalline cellulose column to remove leukocytes and platelets (Beutler et al., 1976), and washed in PBS 3 times. Washed RBCs were resuspended in PBS to yield a cell count 1 x 10^7 cells/10 µL. The RBC suspension was mixed with an equal volume of hypoosmotic solution

(5 mM Tris-HCl. 1 mM ethylenediaminetetraacetic acid [EDTA], pH 7.8) containing 0.8 mM phenylmethylsulfonyl fluoride (PMSF) for the preparation of hemoglobin-depleted RBC membrane ghosts. The lysed RBC suspension was centrifuged at 18,000 g for 10 min at 4 ºC. The resultant pellet was washed in 250 mM sucrose and 1 mM EDTA solution at pH 7.4 by centrifuging at 18,000 g for 10 min at 4 ºC. The obtained RBC membrane ghosts were dissolved in the sample buffer described above. The entire volume (1 x 10^7 cells) of the RBC membrane sample was used for a single analysis as an RBC membrane sample.

2.8. Immunoblot analysis

Immunoblot analysis was performed by the procedure described previously (Yamasaki et al., 2008). The parasite sample, RBC membrane sample, and rBgHsp70, rcHsp70, and recombinant GST proteins were subjected to SDS-PAGE (Laemmli, 1970) and immunoblot analysis (Towbin et al., 1979).

For immunoblot analysis, the membranes were incubated for 1 hr at room temperature with a suitable primary antibody described below; 200 µL pre- and post-immune dog sera, 50 µL pre- and post-immune rabbit sera (pre- and post-immunization) or diluted rabbit anti-rBgHsp70 polyclonal antibodies (1:1000) in Tris-buffered saline/Tween 20 (TBST) (10 mM Tris-HCl, 150 mM NaCl, and 0.05% Tween 20 [ICN Biomedicals, Inc., Costa Mesa, CA, USA], pH 7.5), and incubated for 30 min at room temperature with the suitable secondary antibody described below; diluted alkaline phosphatase-conjugated rabbit anti-dog IgG (H&L) antibody (ROCKLAND, Gilbertsville, PA, USA) (1:500) in TBST, or diluted goat F(ab')2 anti-rabbit IgG(H+L) alkaline phosphatase-conjugated (Invitrogen) in TBST in the present study. Nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phoshate (BCIP) were used for color development. We calculated and compared the intensity of anti-rBgHsp70 and anti-rcHsp70 antibodies in pre- and post-immune dog sera by a densitometer (LumiVision PRO400EX; AISIN, Aichi, Japan).

2.9. Indirect fluorescence assay

Thin smears of cultured RBCs infected with *B. gibsoni* were placed on an MAS-coated slide (S9443; MATSUNAMI, Osaka, Japan) and unfixed. The smears were blocked for 30 min in 3% BSA in PBS. After 3 washes, either diluted rabbit anti-rBgHsp70 polyclonal antibody (1:50) in 3% BSA or diluted mouse anti-human Hsp70 antibody (SPA-810; Stressgen Biotechnologies, Victoria, Canada) (1:200) in 3% BSA was applied and incubated for 1hr at room temperature. After washing twice in PBS, either the diluted fluorescein-conjugated sheep IgG fraction to rabbit IgG (Cappel Research Products, Durham, NC, USA) (1:100) in 3% BSA or diluted goat anti-mouse IgG (H&L) F(ab')2 fragments conjugated to fluorescein (American Qualex, San Clemente, CA, USA) (1:100) in 3% BSA, and Hoechst 33342 (Wako Pure Chemical Industries, Osaka, Japan) (0.01 mg/mL; DNA stain) were added. Thin smears were incubated for 30 min in a moist dark environment, then washed three times in PBS and allowed to dry in the dark. Thin smears were mounted

using 90% glycerin in PBS and visualized using a fluorescence microscope (ECLIPSE E800; Nikon, Tokyo, Japan). Indirect fluorescence assay using sera from a dog with idiopathic immune-mediated hemolytic anemia (IMHA) and diluted rabbit anti-dog IgG (Fc) antibody conjugated to rhodamine red (CORTEX BIOCHEM, CA, USA) (1:100) in 3% BSA was conducted as a positive control. Indirect fluorescence assay either without the first and/or second antibodies or with pre-immune rabbit sera was also conducted as a negative control.

2.10. Incubation of canine red blood cells in the medium containing rabbit anti-rBgHsp70 polyclonal antibodies

Canine RBCs were cultured without parasites for 1 week by the same procedure as the culture of *B. gibsoni*. Uninfected and infected RBCs were incubated in culture medium containing 1.2 mg/mL rabbit anti-rBgHsp70 polyclonal antibodies for 1 hr at room temperature to observe the hemolytic effects of these antibodies on canine RBCs. After incubation, RBC suspensions

were centrifuged at 4,000 g for 5 min at room temperature. The hemoglobin concentrations of the resultant supernatant were measured using the hemoglobin test (Wako Pure Chemical Industries). Uninfected and infected RBCs were also incubated in culture medium containing 1.2 mg/mL mouse anti-human apolipoprotein AI antibody (American Research Products, Inc., MA, USA) as a control.

2.11. Statistical analysis

Statistical analysis was performed using Student's *t*-test.

3. Results

3.1. Detection of antibodies reacting with *Babesia gibsoni* or canine red blood cell 70-kilodalton proteins from *B. gibsoni*-infected dogs.

The parasite sample and RBC membrane sample were subjected to

electrophoresis and stained with Coomassie blue (Fig. 1A). Major RBC membrane proteins, such as protein 4.1 and protein 4.2, were stained with Coomassie blue (Fig. 1A). The electrophoretic pattern of the parasite sample was different from that of the RBC membrane sample.

Pre- and post-immune dog sera from chronic *B. gibsoni*-infected dogs were analyzed using immunoblot analysis. As a result, post-immune dog sera contained a number of antibodies reacting with the antigens included in the parasite sample, although the pre-immune sera had few antibodies reacting with the *B. gibsoni* antigens (Fig. 1B). In particular, post-immune sera from chronically infected dogs had antibodies reacting with *B. gibsoni* 70-kDa protein (Fig. 1B). Moreover, post-immune dog sera contained a number of antibodies reacting with antigens in the RBC membrane sample, although pre-immune sera had few antibodies reacting with the RBC membrane antigens (Fig. 1C). Interestingly, antibodies reacting with canine RBC 70-kDa protein were detected in post-immune sera from chronically infected dogs (Fig. 1C).

Pre- and post-immune sera from acutely infected dogs were also analyzed

(Fig. 2). In those dogs, many antibodies reacting with antigens in the parasite sample were detected in post-immune sera, though there were some antibodies in pre-immune sera (Fig. 2A). In particular, antibodies reacting with *B. gibsoni* 70-kDa protein were clearly detected in post-immune sera from those dogs (Fig. 2A). Post-immune sera from those dogs also contained many antibodies reacting with antigens in the RBC membrane sample (Fig. 2B), although there were some antibodies in pre-immune sera (Fig. 2B). Interestingly, antibodies reacting with canine RBC 70-kDa protein were clearly detected in post-immune sera from those dogs (Fig. 2B).

3.2. Comparison of cHsp70 with BgHsp70

The cHsp70 gene obtained from canine reticulocytes was analyzed. According to nucleotide sequencing analysis, the amplified product was 2,027 bp long, and encoded a 642 amino acid peptide. The nucleotide sequence of the cHsp70 gene was compared with that of the BgHsp70 gene, and was found to

show 67.4% identity. The full length of predicted amino acid sequence of cHsp70 was also compared with that of BgHsp70, and was found to have 70.8% identity (Fig. 3). If the aligned amino acid sequences are divided into two domains, 76.5% of the first 529 amino acids are identical, while the C-terminal domain (113 amino acids) is only 44.8% identical.

3.3. Detection of antibodies reacting with recombinant BgHsp70 and cHsp70 from *B. gibsoni*-infected dogs.

In chronically infected dogs, antibodies reacting with rBgHsp70 were clearly detected in post-immune sera (Fig. 4A), and those antibodies were also detected in pre-immune sera in trace proportions. Antibodies reacting with rcHsp70 were also clearly detected in post-immune sera (Fig. 4B). In pre- and post-immune sera from a chronically infected dog, antibodies reacting with recombinant GST were not detected (Fig. 4C).

In acutely infected dogs, antibodies reacting with rBgHsp70 were detected

in both pre- and post-immune sera (Fig. 5A). In all acutely infected dogs, antibodies reacting with rcHsp70 were detected in both pre- and post-immune sera (Fig. 5B). Although, the number of antibodies reacting with rBgHsp70 and rcHsp70 in pre-immune sera varied, they seemed to increase after the infection (Fig. 5); therefore, we calculated and compared the intensity of bands by a densitometer. The intensity of anti-rBgHsp70 antibodies in post-immune sera from all seven infected dogs (average ± S.D. = 80,651,492.7 ± 39,145,582.3) was significantly ($P < 0.001$) higher than in pre-immune sera (12,124,832.6 ± 11,893,468.4). The intensity of anti-rcHsp70 antibodies in post-immune sera from all seven infected dogs (46,285,985.0 ± 37,483,891.2) was significantly ($P < 0.05$) higher than in pre-immune sera (14,190,153.4 ± 9,974,237.8).

3.4. Development of rabbit anti-rBgHsp70 polyclonal antibodies

Rabbit anti-rBgHsp70 polyclonal antibodies were developed in two rabbits by immunization with rBgHsp70 (Fig. 6A). Antibodies reacting with rBgHsp70

were detected in post-immune rabbit sera (Fig. 6A), although no antibody reacting with rBgHsp70 was detected in pre-immune rabbit sera (Fig. 6A). Rabbit anti-rBgHsp70 polyclonal antibodies were purified from post-immune rabbit sera by affinity chromatography (Fig. 6A).

Interestingly, antibodies reacting with rcHsp70 were detected in post-immune rabbit sera (Fig. 6B), although no antibodies reacting with rcHsp70 was detected in pre-immune rabbit sera (Fig. 6B). Moreover, purified rabbit anti-rBgHsp70 polyclonal antibodies reacted with rcHsp70 (Fig. 6B) and also reacted with native cHsp70 in the canine RBC membrane (Fig. 6C). Antibodies reacting with recombinant GST were detected in pre- and post-immune rabbit sera (Fig. 6D). The amount of those antibodies was not increased by the immunization with rBgHsp70, and purified rabbit anti-rBgHsp70 polyclonal antibodies could not react with recombinant GST (Fig. 6D).

3.5. Detection of BgHsp70 and cHsp70 on the membrane surface of canine red blood cells and *Babesia gibsoni*

Neither BgHsp70 nor cHsp70 could be detected on the surface of infected or uninfected RBCs using mouse anti-human Hsp70 monoclonal antibody and rabbit anti-rBgHsp70 polyclonal antibodies by the indirect fluorescence assay (Fig. 7 and 8). Only a few RBCs were stained using mouse anti-human Hsp70 monoclonal antibody, and stained strongly by Hoechst 33342, which was used for staining the nuclei of the parasites (Fig. 7A, 7G). Some antigens on the surface of canine RBCs were stained using serum from a dog with idiopathic IMHA, although intraerythrocytic parasites were not stained (Fig. 7C and 7I).

Additionally, intraerythrocytic parasites were observed as oval and flat structures within canine RBCs (Fig. 8D, 8E and 8F), and exoerythrocytic parasites were observed as oval and centroclinal structures by phase-contrast microscopy (Figs. 7E and 8D). The nuclei of these parasites were stained by Hoechst 33342 (Figs. 7B, 8B and 8C). Only exoerythrocytic parasites were stained using rabbit anti-rBgHsp70 polyclonal antibodies (Fig. 8A and 8G). Pre-immune rabbit sera could not stain either cHsp70 or BgHsp70 on the

surface of the RBC membrane and the parasites (Fig. 8C and 8I).

3.6. Effect of rabbit anti-rBgHsp70 polyclonal antibodies on canine red blood cells

The hemolytic effect of rabbit anti-rBgHsp70 polyclonal antibodies was observed. The extracellular hemoglobin concentrations of both uninfected and infected RBC suspension incubated with anti-rBgHsp70 antibodies were not siginificantly different from without antibodies (Table 2). The extracellular hemoglobin concentrations of both uninfected and infected RBC suspension with anti-human apolipoprotein AI antibody were not significantly different from without antibodies (Table 2).

4. Discussion

In the present study, antibodies reacting with the *B. gibsoni* 70-kDa

protein were detected in post-immune sera from four infected dogs. It is reported that a 75-kDa *P. falciparum* protein, which is a family of 70-kDa heat-shock proteins, is a target of the host's immune response (Ardeshir et al., 1987). Garsia et al. (1989) reported that the gene coding for part of the highly immunogenic 70-kDa protein antigen isolated from a *Mycobacterium laprae* genomic library was found to be a member of the heat-shock protein 70 family genes. Shinnick et al. (1988) showed that a 65-kDa mycobacterial protein expressed as a heat-shock protein is a highly immunoreactive molecule; therefore, we expected that this *B. gibsoni* 70-kDa protein would be a member of Hsp70. Moreover, antibodies reacting with canine RBC 70-kDa protein were also detected in post-immune dogs. Mattei et al. (1989) analyzed the humoral immune response to a 72-kDa heat shock-like protein of *P. falciparum* using human immune sera. As a result, sera from humans infected with malaria recognized human Hsp70, indicating that autoantibodies directed against host Hsp70 can be induced by homologous parasite protein. Accordingly, we also expected that this canine RBC 70-kDa protein would be cHsp70, and that antibodies reacting with cHsp70

could be induced by infection with *B. gibsoni*.

Consequently, anti-rBgHsp70 and anti-rcHsp70 antibodies were detected in both pre- and post-immune dog sera from all seven infected dogs. The amount of both anti-rBgHsp70 and anti-cHsp70 antibodies were significantly increased after infection, indicating that both anti-BgHsp70 and anti-cHsp70 antibodies were induced by infection with *B. gibsoni*. Interestingly, antibodies reacting with BgHsp70 and cHsp70 were detected in pre-immune dog sera from all dogs. Young et al. (1988) suggested that an antigen from one pathogen could immunize against another pathogen, because the stress response is common to prokaryotes and eukaryotes, and some stress proteins are highly conserved in sequence. From the present results and the previous report, it is considered that the antibodies induced by homologous proteins of Hsp70 in either a vaccine or pathogen in pre-immune dog sera might react with BgHsp70 and cHsp70.

Because the nucleotide and the predicted amino acid sequence of cHsp70 had high homology with BgHsp70, it was possible that BgHsp70 could induce antibodies reacting with both BgHsp70 and cHsp70. In fact, rabbit

anti-rBgHsp70 polyclonal antibodies, which developed in rabbits by immunization with rBgHsp70, reacted not only with rBgHsp70 but also with rcHsp70 and native cHsp70 in canine RBC. These results indicated that the anti-cHsp70 antibodies in *B. gibsoni*-infected dog sera should be induced by BgHsp70 during infection with the parasites. Increased levels of anti-erythrocyte membrane antibodies and erythrocyte-bound IgG in dogs infected with *B. gibsoni* have been reported (Adachi and Makimura, 1992; Adachi et al., 1992; Adachi et al., 1994; Conrad et al., 1991; Farwell et al., 1982). Because cHsp70 was expressed in canine RBC, it was possible that anti-cHsp70 antibodies in infected dog sera would be anti-erythrocyte membrane antibodies; however, it is necessary for cHsp70 molecules to be expressed on the surface of the RBC membrane to combine with anti-cHsp70 antibodies.

Therefore, unfixed RBCs were stained using either mouse anti-human Hsp70 antibody or rabbit anti-rBgHsp70 antibodies. Both infected and uninfected RBCs could not be stained by either anti-BgHsp70 or anti-cHsp70 antibodies, suggesting that BgHsp70 and cHsp70 are not expressed on the surface of

canine RBCs. In addition, rabbit anti-rBgHsp70 polyclonal antibodies could not enhance the hemolysis of either *B. gibsoni*-infected or uninfected RBCs, indicating that BgHsp70 and cHsp70 were not expressed on the membrane surface of infected RBCs. These results showed that antibodies reacting with both BgHsp70 and cHsp70 in infected dog sera were not anti-erythrocyte membrane antibodies.

Meanwhile, only a few RBCs, containing a number of parasites, expressed Hsp70s on their surface. Moreover, only exoerythrocytic parasites, which seemed to be merozoites, expressed Hsp70s on their surface. Accordingly, it was supposed that Hsp70s would be expressed on the surface of the parasite or RBC membrane when merozoites broke away from RBCs, and invaded RBCs. Similarly, in the development cycle of merozoites of *P. falciparum*, it is possible that p75, which is homologous with the conserved hsp70 family, might be cytoplasmic during the early stages and might adhere to the surface of merozoites after they have formed, or during cell lysis (Ardeshir et al, 1987). It was speculated that merozoites of *B. gibsoni* might be targets of anti-BgHsp70

antibody; however, Terkawi et al. (2009) also reported that anti-rBgHSP-70 IgG could not inhibit the growth of *B. gibsoni* in the absence of immune cells *in vitro*, and that anti-rBgHSP-70 IgG was not protective. In the lifecycle of *B. gibsoni*, the roles of BgHsp70 have not been clarified. Further study concerning the role of BgHsp70 in the development and proliferation of parasites is therefore necessary. In contrast, it was demonstrated that HSP-70 protein could induce cellular immunity, known to play a more crucial role in controlling intra-erythrocytic parasites than humoral immunity (Terkawi et al., 2009); therefore, Terkawi et al. emphasized the potential of HSP-70s as a molecular adjuvant vaccine. Since *B. divergens* Hsp70 appears as an early antigen during the acute phase, it is considered a candidate for an anti-babesiosis vaccine (Carcy et al., 1991). It is reported that p75 in *P. falciparum* is also a possible vaccine component (Ardeshir et al., 1987); however, since there is a high degree of sequence conservation between the parasite and host Hsp70, it follows that an immune response could induce autoreactive antibodies (Mattei et al., 1989). Indeed, rBgHsp70 could induce anti-cHsp70 antibodies in rabbits in the present

study. This should be taken into account in the design of recombinant or synthetic peptides used as vaccines. Nevertheless, since it is known that the C-terminal portion of Hsp70s is relatively poorly conserved among organisms (Hunt and Morimoto, 1985), Ardeshir et al. (1987) investigated whether this region of p75 from *P. falciparum* contained antigenic determinants that are conserved among *P. falciparum* isolates but are not shared with the Hsp70s of the human host. Similarly, because the C-terminal portion of the BgHsp70 was poorly conserved with cHsp70, it is possible that the C-terminal portion of BgHsp70 could be an adjuvant vaccine.

In conclusion, BgHsp70 is a highly immunogenic protein in canine *B. gibsoni* infection, and could induce antibodies reacting with both BgHsp70 and cHsp70; however, these antibodies could not enhance hemolytic anemia in canine babesiosis.

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Table 1. Oligonucleotides for analysis of the cHsp70 gene.

Name	Sequence	Location[a]
cHsp70F	5’-cgt cgc gga gcc cga ccg cct t-3’	1-21
cHsp70R[b]	5’-cac aaa cag gaa caa tct ggg-3’	2008-2028
cHsp70F1	5’-gcg ttc tac ccc gag gag at-3’	409-428
cHsp70F2	5’-aga agg aca tca gcc aga aca-3’	821-841
cHsp70F3	5’-ctc cac cat ccc cac caa gca-3’	1323-1343
cHsp70F4	5’-cag ggt gtc tgc caa gaa cg-3’	1674-1693
cHsp70R1[b]	5’-cac ctt ggg ctt gtc ccc gt-3’	362-381
cHsp70F5	5’-atg gct aag agc gcg gcc atc gg-3’	73-95
cHsp70R2[b]	5’-cta atc cac ctc ctc gat ggt ggg gc-3’	1973-1998

cHsp70F1, cHsp70F2, cHsp70F3, cHsp70F4 and cHsp70R1 primers were utilized for cycle-sequencing reactions.

cHsp70F5 and cHsp70R2 primers were utilized for the expression of recombinant cHsp70

[a]Locations indicate nucleotide numbers of the *Babesia gibsoni* heat shock

protein 70 gene determined in the present study.

[b]Antisense primer.

Table 2. Influence of rabbit anti-recombinant *Babesia gibsoni* heat-shock protein 70 (rBgHsp70) antibody on extracellular hemoglobin concentration as an indication of hemolysis of erythrocyte suspension. Extracellular hemoglobin concentrations of *B. gibsoni*-infected and uninfected erythrocyte suspension were measured 1 hr after erythrocytes were exposed to 1.2 mg/ml antibodies. Data are expressed as the mean ± SD (n = 3).

	Control	Anti-rBgHsp70 Ab*	Anti-ApoAI Ab**
Uninfected RBCs†	0.91 ± 0.23	1.18 ± 0.68	1.96 ± 1.71
B. gibsoni-infected RBCs†	1.44 ± 0.23	1.57 ± 0.39	1.57 ± 0.39

* Rabbit anti-rBgHsp70 antibody was added to *B. gibsoni*-infected and uninfected erythrocyte suspensions.

** Mouse anti-human apolipoprotein AI antibody was added to *B. gibsoni*-infected and uninfected erythrocyte suspensions as a irrelevant control antibody.

† red blood cells

Figure captions

Fig. 1. SDS-PAGE and Immunoblot analysis of the lysate of *Babesia gibsoni* (parasite sample) and canine RBC membrane (RBC membrane sample). (A) SDS-PAGE of the parasite sample and RBC membrane sample. Protein bands were stained with Coomassie blue. Lane 1, molecular marker; Lane 2, parasite sample; Lane 3, RBC membrane sample. Protein 4.1 and protein 4.2 were observed around 70 kDa in the RBC membrane sample. (B) Immunoblot analysis of parasite sample. (C) Immunoblot analysis of RBC membrane sample. Pre-immune sera (Lane 4 and 6) and post-immune sera (Lane 5 and 7) from two chronically infected dogs (Dog 1 [Lane 4 and 5] and 2 [Lane 6 and 7]) were used as the primary antibody. Arrow shows *B. gibsoni* 70-kDa protein. Arrowhead shows canine RBC 70-kDa protein. Bg (+) is post-immune dog sera. Bg (-) is pre-immune dog sera.

Fig. 2. Immunoblot analysis of the lysate of *Babesia gibsoni* (parasite sample)

and canine RBC membrane (RBC membrane sample) with sera from acutely *B. gibsoni*-infected dogs. (A) Immunoblot analysis of parasite sample. (B) Immunoblot analysis of RBC membrane sample. Pre-immune (Lane 1 and 3) and post-immune sera (Lane 2 and 4) from two acutely infected dogs (Dog 3 [Lane 1 and 2] and 6 [Lane 3 and 4]) were used as the primary antibody. Arrow shows *B. gibsoni* 70-kDa protein. Arrowhead shows canine RBC 70-kDa protein. Bg (+) is post-immune dog sera. Bg (-) is pre-immune dog sera.

Fig. 3. Alignment of the predicted amino acid sequence of heat-shock protein 70 from *Babesia gibsoni* (BgHsp70) and canine (cHsp70). Identical residues are denoted by periods (.), while additions and gaps in the sequences are indicated by dashes (-); Amino acids conserved among *B. gibsoni* and dogs, are marked by asterisks (*).

Fig. 4. Immunoblot analysis of recombinant *Babesia gibsoni* heat shock protein 70 (rBgHsp70) and recombinant canine Hsp70 (rcHsp70) with sera from

chronically *B. gibsoni*-infected dogs. (A) Immunoblot analysis of rBgHsp70. (B) Immunoblot analysis of rcHsp70. (C) Immunoblot analysis of recombinant glutathione S-transferase (GST). Pre-immune (Lane 1 and 3) and post-immune sera (Lane 2 and 4) from two chronically infected dogs (Dog 1 [Lane 1 and 2] and 2 [Lane 3 and 4]) were used as the primary antibody. Arrow shows rBgHsp70 protein. Arrowhead shows cHsp70 protein. Open arrowhead shows recombinant GST protein. Bg (+) is post-immune dog sera. Bg (-) is pre-immune dog sera.

Fig. 5. Immunoblot analysis of recombinant *Babesia gibsoni* heat shock protein 70 (rBgHsp70) and recombinant canine Hsp70 (rcHsp70) with sera from acutely *B. gibsoni*-infected dogs. (A) Immunoblot analysis of rBgHsp70. (B) Immunoblot analysis of rcHsp70. Pre-immune (Lane 1, 3, 5, 7 and 9) and post-immune sera (Lane 2, 4, 6, 8 and 10) from five acutely infected dogs (Dog 3 [Lane 1 and 2], 4 [Lane 3 and 4], 5 [Lane 5 and 6], 6 [Lane 7 and 8] and 7 [Lane 9 and 10]) were used as the primary antibody. Arrow shows rBgHsp70 protein. Arrowhead shows

cHsp70 protein. Bg (+) is post-immune dog sera. Bg (-) is pre-immune dog sera.

Fig. 6. Immunoblot analysis of recombinant *Babesia gibsoni* heat shock protein 70 (rBgHsp70), recombinant canine Hsp70 (rcHsp70), and native canine Hsp70 (cHsp70) with rabbit sera and isolated rabbit anti-rBgHsp70 polyclonal antibodies. (A) Immunoblot analysis of rBgHsp70. (B) Immunoblot analysis of rcHsp70. (C) Immunoblot analysis of native cHsp70 in the canine RBC membrane. (D) Immunoblot analysis of recombinant glutathione S-transferase (GST). Rabbits were immunized with 0.2 mg rBgHsp70 protein. Pre-immune (Lane 1) and post-immune sera (Lane 2), and isolated rabbit anti-rBgHsp70 polyclonal antibodies (Lane 3) were used as the primary antibody. Arrow shows rBgHsp70 protein. Arrowhead shows cHsp70 protein. Open arrowhead shows recombinant GST protein.

Fig. 7. Indirect fluorescence assay of canine red blood cells (RBCs) cultured with *Babesia gibsoni*. Mouse anti-human heat-shock protein 70 (Hsp70) antibody (A,

B, G and H) and serum from a dog with idiopathic immune-mediated hemolytic anemia (IMHA) (C and I) was used as a primary antibody. Specific reaction of the antigen and mouse anti-human Hsp70 antibody is green by FITC (A) and the nucleus is blue by Hoechst 33342 (A, B and C). Specific reaction of the antigen and serum from a dog with idiopathic IMHA is red by Rhodamine Red (C). Smears were observed using phase-contrast microscopy (D, E and F). Image of fluorescent green, blue and red staining is overlaid on phase-contrast images (G, H and I). Arrows show RBCs stained by both mouse anti-human Hsp70 antibody and Hoechst 33342. Arrowheads show exoerythrocytic parasites stained by Hoechst 33342. Open arrowheads show RBCs stained by both serum from a dog with idiopathic IMHA and Hoechst 33342. Bar = 10 μm.

Fig. 8. Indirect fluorescence assay of canine red blood cells (RBCs) cultured with *Babesia gibsoni*. Rabbit anti-recombinant *B. gibsoni* heat-shock protein 70 (rBgHsp70) antibodies (A, B, G and H) and rabbit sera obtained before immunization with rBgHsp70 (C and I) were used as the primary antibody. The

specific reaction of the antigen and rabbit anti-rBgHsp70 antibody is green by FITC (A) and the nucleus is blue by Hoechst 33342 (A, B and C). Smears were observed using phase-contrast microscopy (D, E and F). Image of fluorescent green and blue staining is overlaid on phase-contrast images (G, H and I). Arrows show exoerythrocytic parasites stained by both rabbit anti-rBgHsp70 polyclonal antibodies and Hoechst 33342. Arrowheads show intraerythrocytic parasites stained by Hoechst 33342. Bar = 10 μm.

Table 1. Oligonucleotides for analysis of the cHsp70 gene.

Name	Sequence	Location[a]
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cHsp70F1	5’-gcg ttc tac ccc gag gag at-3’	409-428
cHsp70F2	5’-aga agg aca tca gcc aga aca-3’	821-841
cHsp70F3	5’-ctc cac cat ccc cac caa gca-3’	1323-1343
cHsp70F4	5’-cag ggt gtc tgc caa gaa cg-3’	1674-1693
cHsp70R1[b]	5’-cac ctt ggg ctt gtc ccc gt-3’	362-381
cHsp70F5	5’-atg gct aag agc gcg gcc atc gg-3’	73-95
cHsp70R2[b]	5’-cta atc cac ctc ctc gat ggt ggg gc-3’	1973-1998

cHsp70F1, cHsp70F2, cHsp70F3, cHsp70F4 and cHsp70R1 primers were utilized for cycle-sequencing reactions.

cHsp70F5 and cHsp70R2 primers were utilized for the expression of recombinant cHsp70

[a]Locations indicate nucleotide numbers of the *Babesia gibsoni* heat shock

protein 70 gene determined in the present study.

[b]Antisense primer.

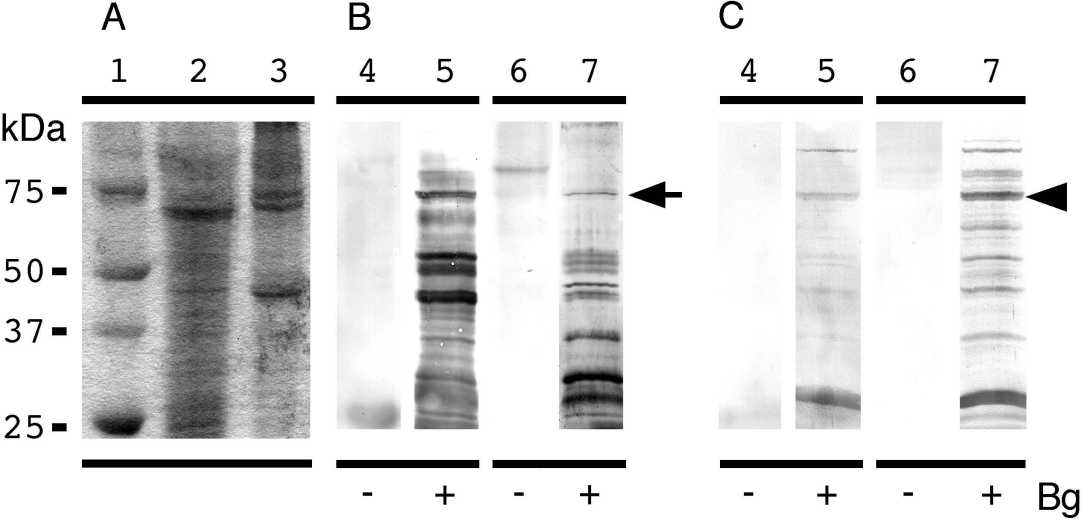
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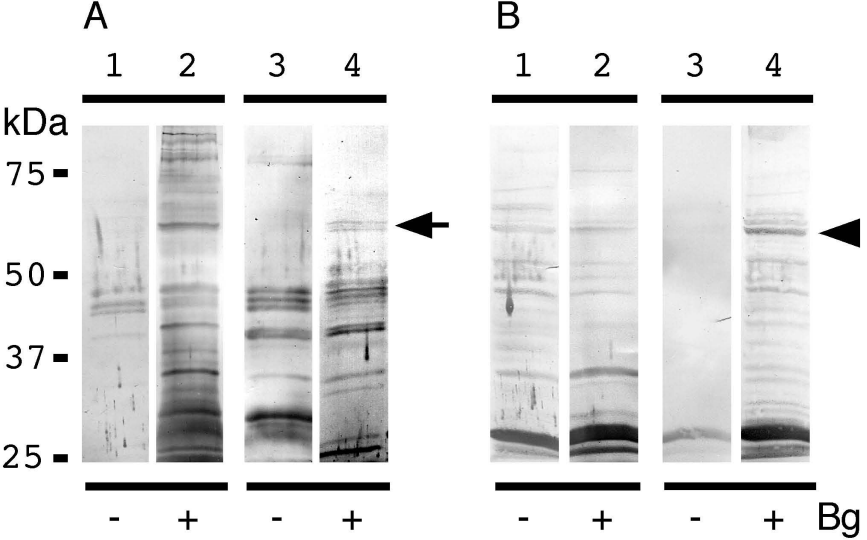
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† red blood cells



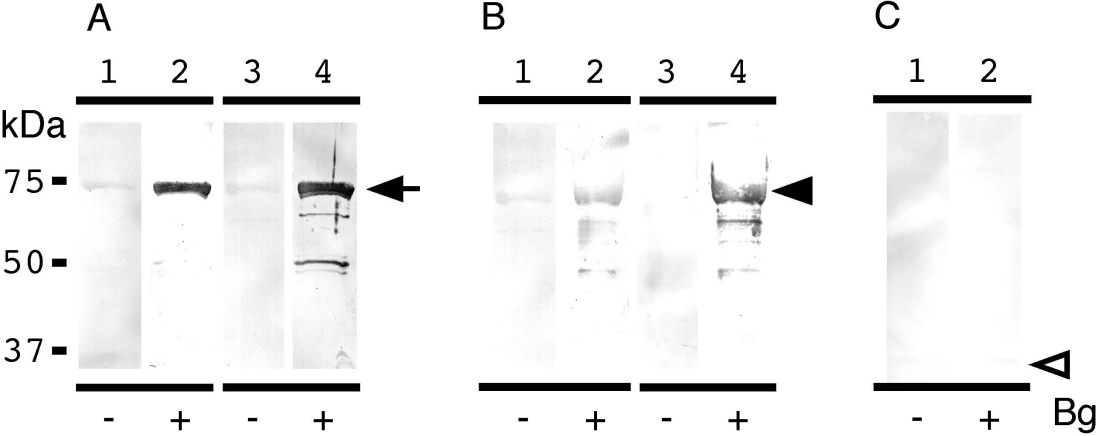
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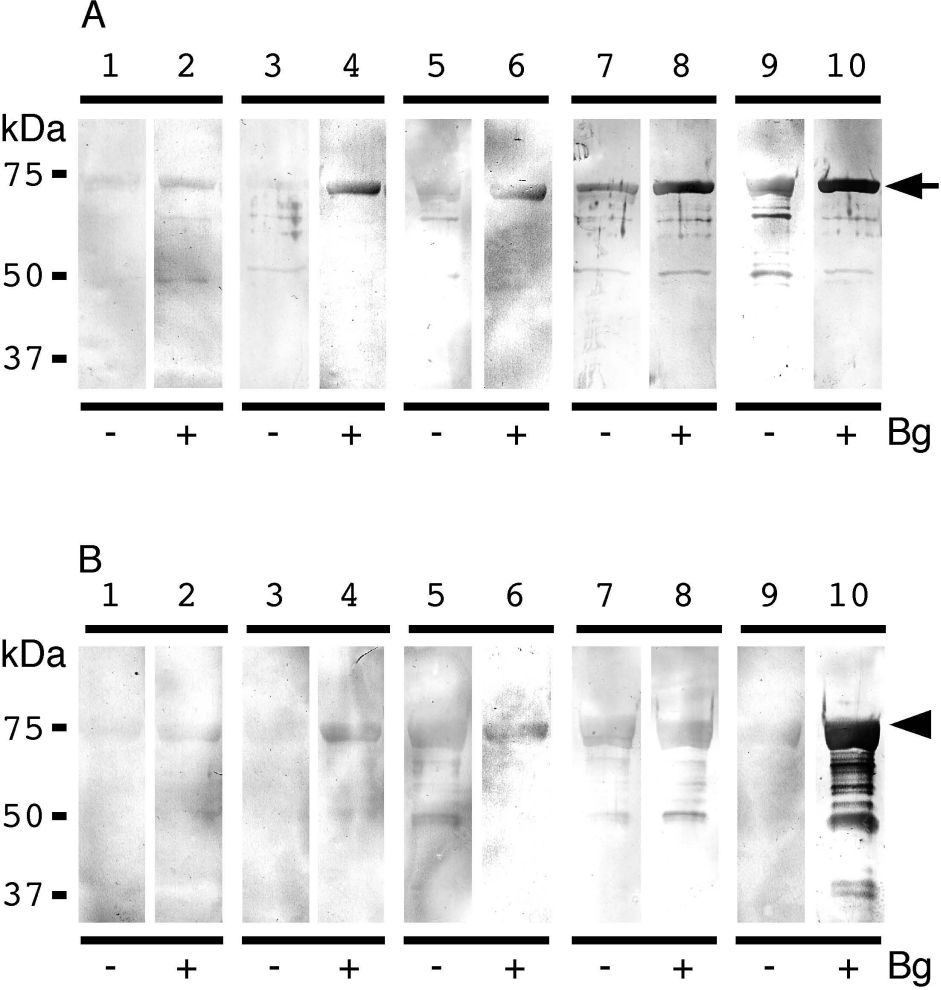
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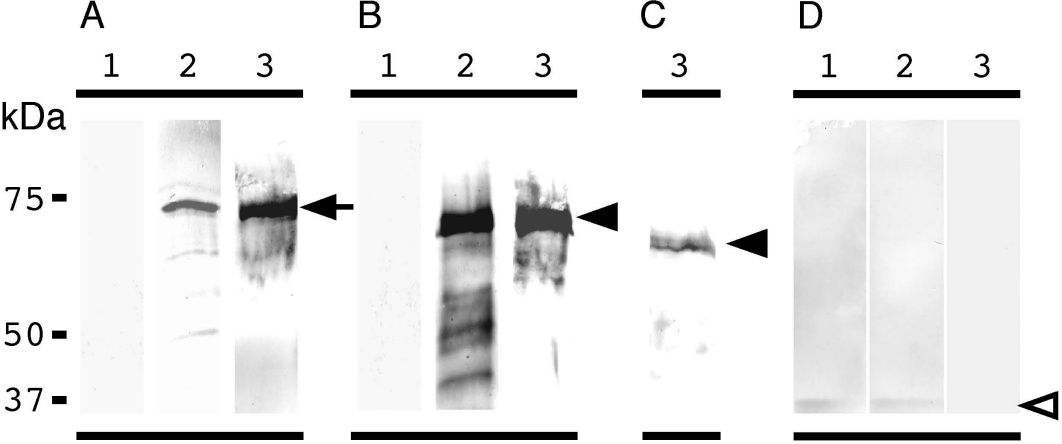
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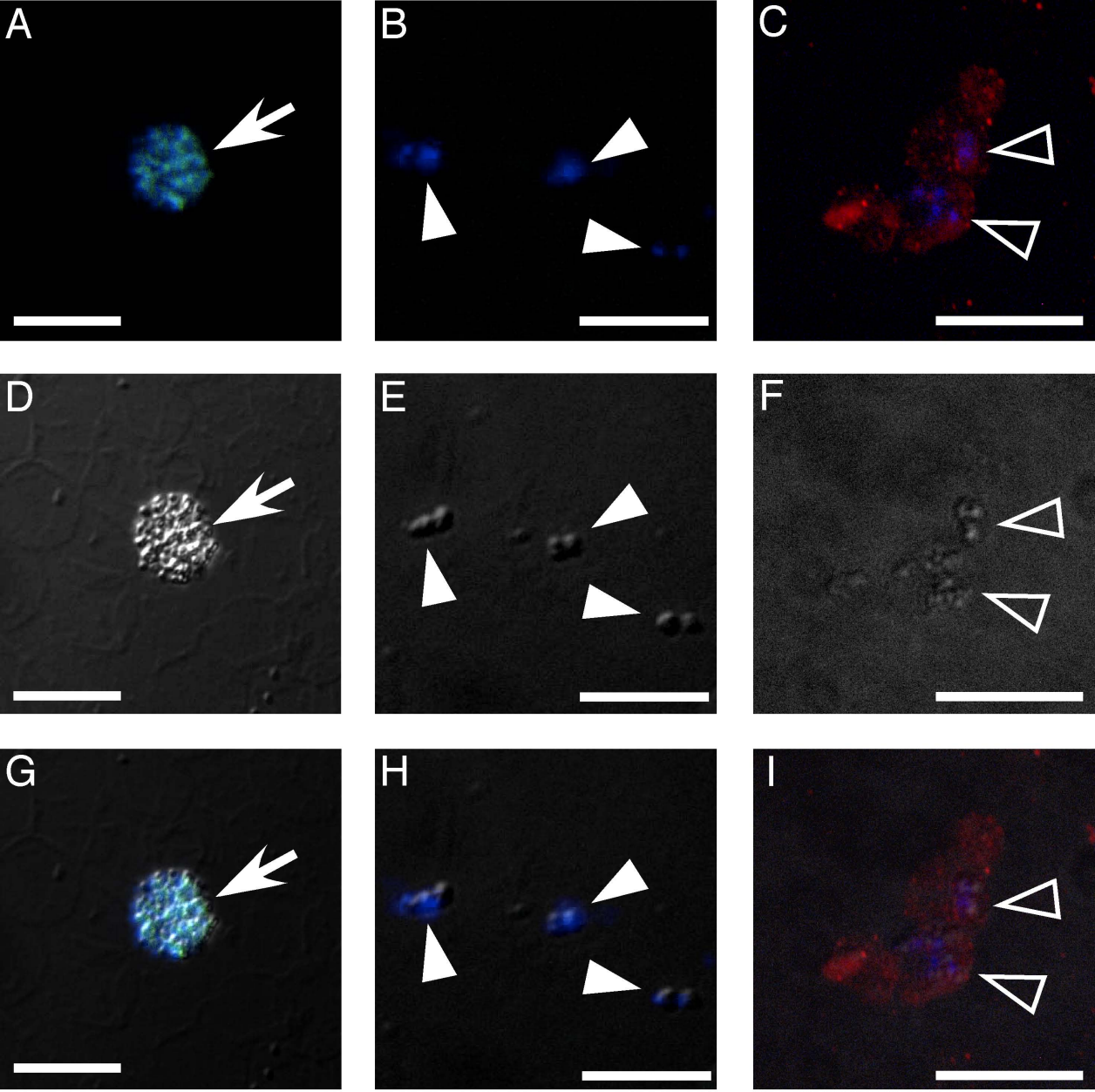
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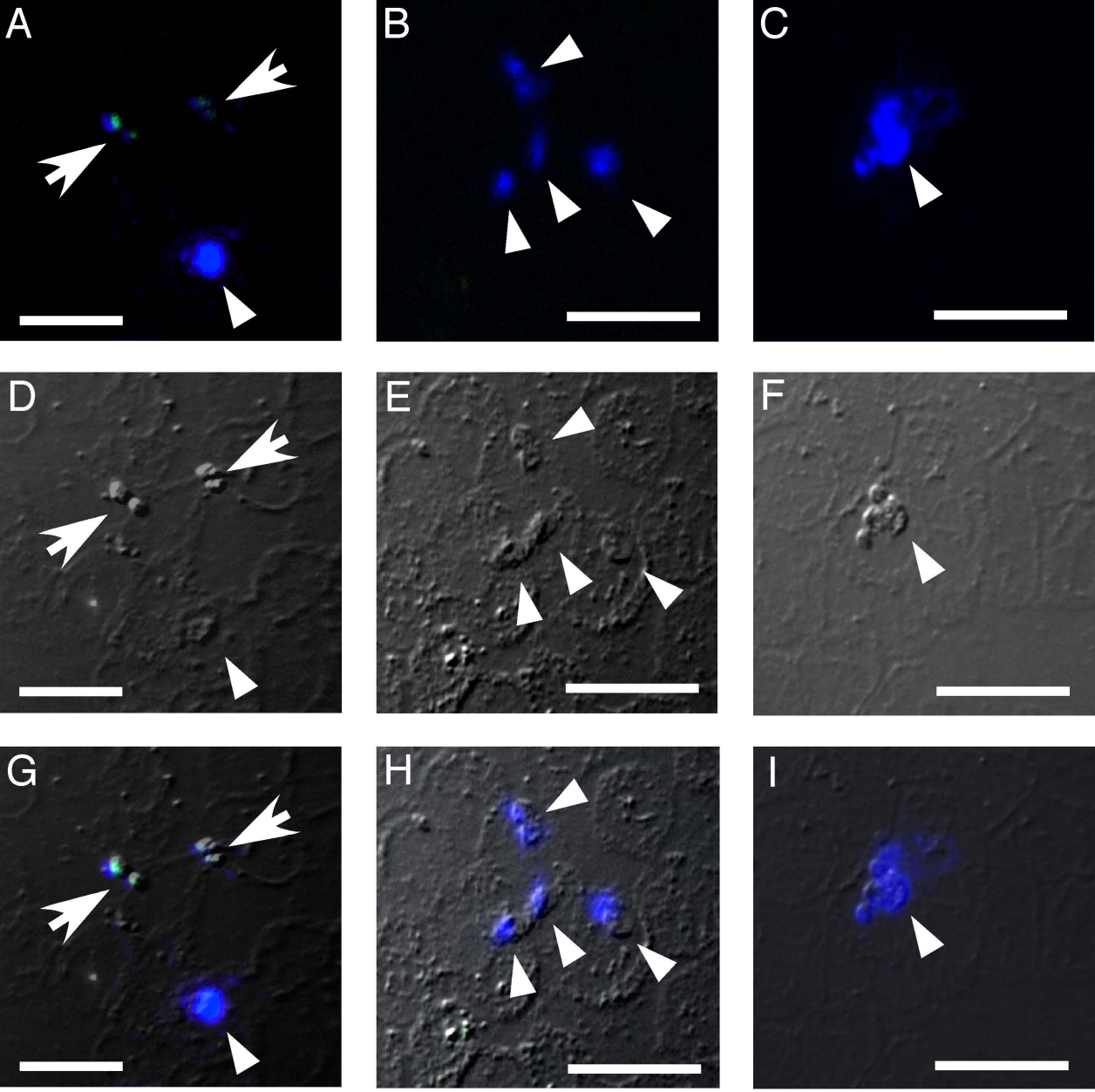
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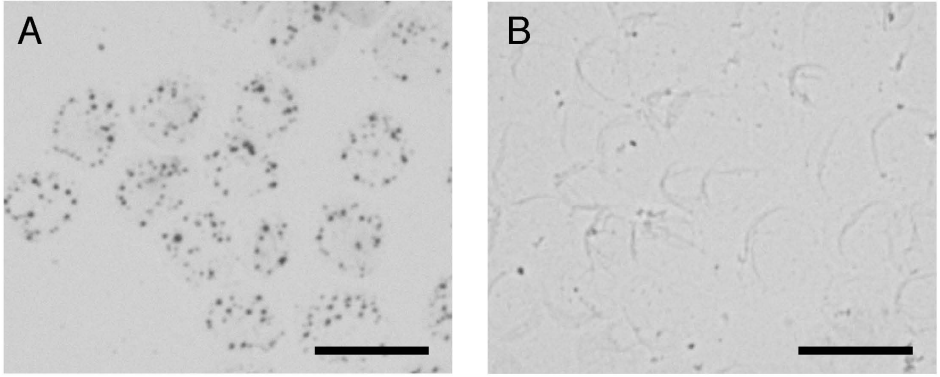
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YamasakiM. Fig. 7



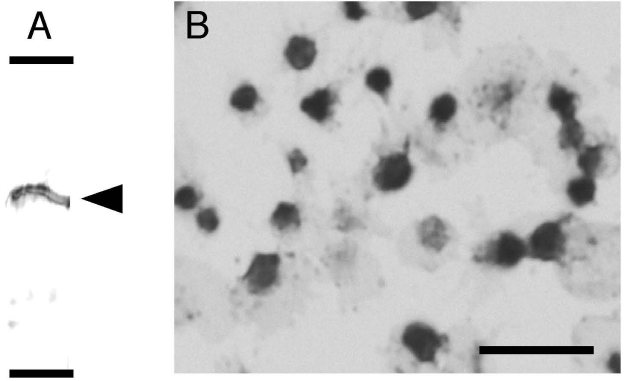
YamasakiM. Fig. 8



Supplemental Fig. 1 Rabbit anti-recombinant *B. gibsoni* heat shock protein 70 (BgHsp70) polyclonal antibodies could stain native canine Hsp70 (cHsp70) in canine red blood cells (RBCs) by immuno staining.

Thin smears of washed RBCs were made on the MAS coated slide (S9443, MATSUNAMI, Osaka, Japan) and fixed using 6% paraformaldehyde in PBS for 20 min. After washing 3 times in PBS, the smears were pretreated in 1% Triton X-100 in PBS, washed 3times, and blocked for 30 min in 3% BSA in PBS. After 3 washes, the smears were incubated with diluted rabbit anti-rBgHsp70 antibodies (1:200) in 3% BSA for 1hr at room temperature, washed 3times, incubated with diluted goat F(ab')2 anti-rabbit IgG(H+L) alkaline phosphatase-conjugated (1:200) in 3% BSA for 30 min at room temperature, and again washed 3times. NBT and BCIP were used for color development. Immunostaining without first and/or second antibodies or with pre-immune rabbit sera was also conducted as negative controls.

Native cHsp70 in canine RBCs was stained by rabbit anti-rBgHsp70 polyclonal antibodies (A), though pre-immune rabbit sera could not stain native cHsp70 (B). Bar = 10 μm.



Supplemental Fig. 2. Rabbit anti-recombinant *B. gibsoni* heat shock protein 70 (BgHsp70) polyclonal antibodies could stain native canine Hsp70 (cHsp70) in canine platelets (PLTs) by immunoblot analysis and immuno staining.

To prepare canine PLTs, citrated blood (blood : 3.8% sodium citrate = 9 : 1) was collected, and centrifuged at 130 g for 15 min at room temperature. The resultant supernatant was collected as PLT rich plasma, and centrifuged at 800 g for 5 min at room temperature. The resultant pellet was washed 3 times in PBS.

The obtained PLT was dissolved in the sample buffer to yield a cell count of 2.5 x 10^7 cells/10 μL, and subjected to immunoblot analysis according to the method described our manuscript. The primary antibody was diluted rabbit anti-rBgHsp70 antibodies (1:1000). The secondary antibody was diluted goat F(ab')2 anti-rabbit IgG(H+L) alkaline phosphatase-conjugated (1:200).

Thin smears of washed PLTs were stained by rabbit anti-rBgHsp70 antibodies according to the method described supplemental Figure 1.

Rabbit anti-rBgHsp70 antibodies reacted with native cHsp70 in canine PLTs by immunoblot analysis (A). Native cHsp70 in canine PLTs were stained by rabbit anti-rBgHsp70 antibodies (B). Bar = 10 μm.