Research paper

Babesia gibsoni: Detection in blood smears and formalin-fixed, paraffin-embedded tissues using deoxyribonucleic acid *in situ* hybridization analysis.

Masahiro Yamasaki[a,*], Yusuke Kobayashi[a], Kensuke Nakamura[a], Noboru Sasaki[a], Masahiro Murakami[a], Bandula Kumara Wickramasekara Rajapakshage[a], Hiroshi Ohta[a], Osamu Yamato[b], Yoshimitsu Maede[a], Mitsuyoshi Takiguchi[a]

[a]Laboratory of Internal Medicine, Department of Veterinary Clinical Sciences, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo 060-0818, Japan

[b]Laboratory of Clinical Pathology, Department of Veterinary Clinical Sciences, Faculty of Agriculture, Kagoshima University, 1-21-24 Kohrimoto, Kagoshima

890-0065, Japan

Abbreviations: ISH, *in situ* hybridization; RT-PCR, reverse transcriptase-polymerase chain reaction; BgHsp70, *Babesia gibsoni* heat shock protein 70

*Corresponding author. Tel.: +81-11-706-5224; fax: +81-11-709-7296

E-mail address: masayama@vetmed.hokudai.ac.jp (M. Yamasaki)

ABSTRACT

In the present study, we attempted to detect *Babesia gibsoni* in blood smears and formalin-fixed, paraffin-embedded tissues obtained from *B. gibsoni*-infected dogs using *in situ* hybridization. Using a digoxigenin-conjugated deoxyribonucleic acid (DNA) probe, both intraerythrocytic and exoerythrocytic parasites in the culture could be specifically stained in blood smears fixed with 4% phosphate-buffered paraformaldehyde. This indicated that genomic DNA extracted from the parasites could be detected using *in situ* hybridization. Moreover, the parasite could be specifically stained in paraffin-embedded spleen, lymph node, and kidney sections using *in situ* hybridization. Infected erythrocytes in blood vessels in the spleen and kidney, hemosiderin-laden macrophages in the spleen, and phagocytized erythrocytes, which seemed to be infected with the parasites, in lymph nodes were also specifically stained. This suggests that *in situ* hybridization can be utilized to investigate both the life cycle of *B. gibsoni* and the pathological condition of canine babesiosis.

Keywords: *Babesia gibsoni*; *in situ* hybridization; heat shock protein 70; blood smear; formalin-fixed, paraffin-embedded tissues

1. Introduction

Babesia gibsoni is a blood protozoan of dogs and a causative pathogen of canine babesiosis. In canine babesiosis, hemolytic anemia and thorombocytopenia develop even when the number of parasites in the peripheral blood is small after an acute infection. The problem is that the life cycle of the parasite and pathogenesis of the anemia and thrombocytopenia have not been sufficiently elucidated. The detection of Babesia parasites in tissue and in blood smears is relatively easy when large numbers of parasites are present. However, when the number of parasites is low, their detection is much more complicated. Therefore, for clinical veterinary and fundamental research, sensitive and reproducible methods for detecting parasites are needed.

The most commonly used technique for detection of Babesia in the vertebrate host is direct microscopy of blood smears stained with Giemsa stain. As described above, it is difficult to detect Babesia parasites in fixed tissue stained with hematoxylin and eosin (H&E) when their numbers are small.

Therefore, an immunohistochemical assay for detecting *Babesia* parasites in fixed tissues was reported (Torres-Velez, et al., 2003). This technique requires specific antibodies against each species of parasite. In addition, specific fluorescent dyes for deoxyribonucleic acid (DNA) or ribonucleic acid (RNA) such as acridine orange and Hoechst 33342, are utilized to stain malaria parasites, which are also blood protozoa (Shute and Sodeman, 1973; Rickman et al., 1989; Grimberg et al., 2007). However, these dyes used for staining are not specific for the parasite and also stain the cells of the host.

Previously, malaria-specific DNA and RNA probes were used in the diagnosis of malaria (McLaughlin et al., 1987; Lanar et al., 1989; Waters and McCutchan, 1989). These probes allow detection of low numbers of parasites, although the sensitivity of the DNA probes is not equal to that of direct microscopy of blood smears (Lanar et al., 1989). Since these methods require partial DNA or RNA purification, information about the number and the stage of development of individual parasites is lost. However, specific staining of the parasites by these probes in fixed tissues or in blood smears will greatly

contribute to their detection by microscopy. It was also reported that some *Babesia* parasites in blood smears were identified by using specific probes (Petchpoo et al., 1992; Loretti and Barros, 2005). If this technique allows the detection of *Babesia* parasites in fixed tissues, it is expected that it will contribute to studies about the life cycle of Babesia and the pathogenesis of babesiosis. However, staining of *Babesia* parasites in fixed tissues by specific probes remains to be developed. Therefore, in the present study, we attempted to detect *Babesia gibsoni* in blood smears and formalin-fixed, paraffin-embedded tissues obtained from a *B. gibsoni*-infected dogs by using DNA *in situ* hybridization (ISH) analysis with a digoxigenin-conjugated DNA probe.

2. Materials and Methods

2.1. in vitro culture of Babesia gibsoni

The strain of *B. gibsoni* used in this study originated from a naturally infected dog in the city of 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).

2.2. Experimental animals

Two adult female beagles were used in this study. These dogs were inoculated with 1 x 10^9 parasites obtained from an experimental chronically infected dog, and were splenectomized at day 11 postinoculation. These experimentally inoculated dogs were monitored daily for development of parasitemia by peripheral blood smear examination and were killed with

intravenous pentobarbital at the onset of clinically apparent lethargy (experimental endpoint). In the experimental protocols for animal care and handling, the investigators adhered to the guidelines of Hokkaido University, 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 the Laboratory Animal, Graduate School of Veterinary Medicine, Hokkaido University (approved number: 8099).

2.3. Preparation of thin smears

Thin smear samples of *B. gibsoni*-infected erythrocytes and erythrocyte-free parasites were prepared using *B. gibsoni* cultured described above. For the preparation of erythrocyte-free parasites, hemolysin purified from the *Aeromonas hydrophila* strain Ah-1 was used. The purified hemolysin was a gift from Prof. C. Sugimoto, Department of Collaboration and Education, Research Center for Zoonosis Control, Hokkaido University, and was used

according to the method of Sugimito et al. (1991) with some modifications. *B. gibsoni*-infected erythrocytes from the culture were centrifuged at 1,000 g for 10 min. After removal of the culture supernatant, erythrocytes were resuspended in 10 mM Tris-HCl, 150 mM NaCl buffer (pH 7.4). After two washes, the erythrocytes were resuspended in the Tris-NaCl buffer at a concentration of 50% (by vol.). Ah-1 hemolysin was added to the erythrocyte suspension to a final concentration of 300 hemolytic units (HU)/mL. After incubation at 37 C for 10 min, the erythrocyte lysate was cooled on ice. An ethylenediaminetetraacetic acid (EDTA) solution (0.5 M, pH 9) was added to the lysate to a final concentration of 5 mM. The erythrocyte lysate was centrifuged at 10,000 g for 5 min. After removal of the erythrocyte lysate and membrane, the gray pellet was collected as erythrocyte-free parasites and resuspended in 10 mM phosphate-buffered saline (PBS, pH 7.4). After two washes, the erythrocyte-free parasites were smeared on MAS-coated glass slides (S9443 micro slide glass, Matsunami, Tokyo, Japan) using a Cytospin 2 (Shandon Southern Products, Ltd., England). *B. gibsoni*-infected erythrocytes were also smeared on MAS-coated glass slides

using the Cytospin 2. Smears were air-dried, and fixed with either methanol or 4% paraformaldehyde (PFA) for 10 min. The fixed smears were stained with 10% Giemsa solution, or utilized for ISH analysis.

2.4. Preparation of formalin-fixed, paraffin-embedded tissues

The experimentally *B. gibsoni*-infected dogs described above were necropsied following a standard protocol. In the present study, the spleen, liver, kidney, and lymph nodes were obtained from experimentally infected dogs. Tissues were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 3-5 μm, and mounted on MAS-coated glass slides. Specimens were stained with Harris hematoxiylin and eosin (H&E) using standard laboratory procedures, or utilized for ISH analysis.

2.5. Preparation of digoxigenin-labeled probes.

For hybridization with genomic DNA of *B. gibsoni*, a 516 bp fragment of the *B. gibsoni* heat shock protein 70 (BgHsp70) gene was used. The reverse transcriptase-polymerase chain reaction (RT-PCR) was used for producing DNA fragments (Yamasaki et al., 2002). The PCR primers used for the amplification of the partial BgHsp70 gene (GenBank accession number AB083511, Yamasaki et al., 2002) are shown in Table 1. The cDNA synthesized from total RNA of *B. gibsoni* was amplified in a reaction mix (*Ex*Taq polymerase, Takara, Tokyo, Japan) by 35 cycles (94 C, 1min; 55 C 1min; 72 C, 1min) in an AB-1820 thermocycler (ATTO, Tokyo, Japan). The 516 bp DNA fragment of BgHsp70 was labeled with digoxigenin by using a DIG DNA Labeling kit (Roche Diagnostics Corporation, IN, USA) according to the supplied protocol. Moreover, the nucleotide sequence of this 516 bp DNA fragment of BgHsp70 was compared with that of *B. canis canis* (GenBank accession number: AB248734), *B. canis rossi* (AB248736), *B. canis vogeli* (AB248733), *B. bovis* (AF107118), *B. ovis* (AB248741), *B. caballi* (AB248742), *B. odocoilei* (AB248740), *B. divergens* (AB248739), *T. annulata* (AB248746), *T.equi* (AB248743), *T. cervi* (AB248748),

T. orientalis (D12692), *T. ovis* (AB248747), *B. microti* (AB248744) and *B. rodhaini* (AB103587) using GENETYX-MAC ver. 11.2 (Genetyx Co., Tokyo, Japan).

A 489 bp DNA fragment of canine 18S rDNA was also prepared by RT-PCR. The PCR primers used for the amplification of the partial rDNA were supplied in QuantumRNA^{TM} classic 18S Internal Standards (Ambion Inc., Texas, USA). The cDNA synthesized from total RNA of canine white blood cells was used as a template for PCR. The nucleotide sequence of the amplification product was determined using the BigDye Terminator® Cycle-Sequencing kit v 3.1 (Applied Biosystem Japan, Tokyo, Japan), and was compared with that of Homo sapiens RNA, 18S ribosomal 1 (RN18S1), ribosomal RNA (NR_003286) and Human 18S rRNA gene (M10098), exhibiting 99.6% identity, respectively. Therefore, this amplification product was determined as a partial fragment of canine 18S rDNA. This DNA fragment of canine 18S rDNA was labeled with digoxigenin as described above.

To determine the specificity and sensitivity of each digoxigenin-labeled

probe, probe assays were performed. Four hundred nanogram samples of genomic DNAs extracted from *B. gibsoni* and normal canine white blood cells (WBC) were applied on Hybond^{TM}-N (GE Healthcare UK Ltd., Buckinghamshire, England), and were exposed to 0.6 J/m^2 of ultraviolet light. The membranes were incubated in hybridization buffer composed of 25% formamide, 2% blocking solution (Roche Diagnostics Corporation, IN, USA), 1.25 x SSC, 0.1% sodium *N*-lauroyl salcosinate, and 0.1% SDS for 2 hr for prehybridization. The labeled BgHsp70 probe was diluted in the hybridization buffer and boiled at 100 C for 5 min, and hybridization was performed overnight at 42 C. Membranes were rinsed at 37 C with 5 x SSC for 5 min, washed once in 2 x SSC, 50% formamide for 30 min at 37 C, and washed twice in 2 x SSC for 20 min at 37C. After preincubation with 1% blocking solution (Roche Diagnostics Corporation, IN, USA) for 30 min at RT, membranes were incubated with alkaline phosphatase-conjugated sheep anti-digoxigenin Fab fragments (Roche Diagnostics Corporation, IN, USA) diluted 1:2000 with a solution containing 1% blocking solution, 0.1 M maleic acid, 0.15 M NaCl, and 0.3% Tween 20 (pH 7.5)

for 1h at RT. After two rinses with a solution containing 0.1 M maleic acid, 0.15 M NaCl, and 0.3% Tween 20 (pH 7.5) for 15 min, they were incubated with NBT/BCIP (Roche Diagnostics Corporation, IN, USA) in 0.1 M Tris-HCl, 0.1 M NaCl (pH 9.5) in a dark room for 4 hr at RT. The color development was stopped rinsing with distilled water. The same probe assay was performed for the canine 18S rDNA probe.

2.6. DNA in situ hybridization for thin smears

The fixed smears were air-dried. After one wash in PBS for 10 min, they were digested with 0.1 mg/mL proteinase K in 10 mM Tris-HCl, 1 mM EDTA (pH 8.0) for 15 min at 37 C, refixed with 4% PFA in 10 mM PBS for 10 min at room temperature (RT), and rinsed with 10 mM PBS for 10 min at RT. The refixed smears were put in 0.2 M HCl for 10 min at RT, rinsed with 10 mM PBS for 10 min at RT, put in 2 mg/mL glycine in 10 mM PBS for 10 min at RT, rinsed with 10 mM PBS for 10 min at RT, and then air-dried. The labeled BgHsp70 probe was

diluted in the hybridization buffer. After boiling the probe solution at 100 C for 5 min, 50 µL of probe solution was applied on the dried smear and hybridization was performed overnight at 37 C. After the hybridization, slides were washed and color developed by using the same procedure as for the probe assay. The period of incubation with NBT/BCIP depended on the degree of the color development. The smears were rinsed with distilled water, and finally mounted with 50% glycerin.

Positive control hybridization was performed with a labeled canine 18S rDNA probe using the procedure described above. For negative control hybridization, the smears were blocked in a 10-fold excess amount of an unlabeled BgHsp70 probe in 1% blocking solution for 1 hr at 37 C before the hybridization.

2.7. DNA in situ hybridization for the formalin-fixed, paraffin-embedded tissues

The sections prepared above were deparaffinized in xylene, and

rehydrated to 70% ethanol in a graded ethanol series. The rehydrated sections were digested, refixed, and put in 0.2 M HCl and 2 mg/mL glycine using the same procedure as for the thin smears. After washing in 10 mM PBS for 10 min at RT, the sections were dehydrated to 100% ethanol in a graded ethanol series. Hybridization, detection of digoxigenin-labeled probes, and color development were performed using the same procedure as for the thin smears. The sections were rinsed with distilled water and finally mounted with glycerin. Positive and the negative control hybridization were performed using the procedure described above.

3. Results and discussion

3.1. Probe assay

The partial specificity of both digoxigenin-labeled BgHsp70 and canine 18S rDNA probes were observed (Fig. 1). The labeled BgHsp70 probe hybridized well with genomic DNA from *B. gibsoni*, but could not hybridize the genomic DNA from a normal dog (Fig. 1). These results indicated that this DNA probe detected only genomic DNA extracted from *B. gibsoni*. Moreover, the labeled canine 18S rDNA probe hybridized well with genomic DNA extracted from normal canine WBC, but could not hybridize the genomic DNA from *B. gibsoni* (Fig. 1). These results indicated that this DNA probe detected only genomic DNA extracted from the normal dog, suggesting that this DNA probe could be utilized as a positive control in the present study.

In addition, the nucleotide sequence of BgHsp70 probe was compared with that of other *Babesia* and *Theileria* parasites. It is reported that the 3'-terminal side of the nucleotide sequences of Hsp70 gene from piroplasms were

characteristic for each species (Yamasaki et al., 2007). Therefore, BgHsp70 probe was located on the 3'-terminal side of BgHsp70 gene. However, the nucleotide sequence of that had 85.0, 84.4, 84.1, 82.5, 82.4, 80.8, 80.7 and 80.0% identity, respectively, for Hsp70 genes of *B. canis rossi, B. canis vogeli*, *B. canis canis*, *B. odocoilei*, *B. divergens*, *B. ovis*, *B. bovis* and *B. caballi*. It was supposed that the BgHsp70 probe could hybridize with Hsp70 gene from those *Babesia* parasites. The nucleotide sequence of BgHsp70 probe was also compared with that of *T. orientalis*, *T.ovis*, *T. annulata*, *T. equi*, *B. rodhaini*, *T. cervi*, and *B. microti*, and found to have 68.2, 67.8, 65.5, 65.2, 64.4, 64.2, and 61.2% identity, respectively. Because the identity of the BgHsp70 probe with canine Hsp70 gene was 61.9%, it is considered that this probe could not hybridize with Hsp70 gene from those *Babesia* and *Theileria* parasites. Additionally, experimental dogs were not infected with those piroplasms including *B. canis canis*, *B. canis rossi*, and *B. canis vogeli*, in the present study. From these results, the digoxigenin-labeled BgHsp70 probe could detect only genomic DNA from *B. gibsoni*.

3.2. DNA in situ hybridization for thin smears

In the smears fixed by methanol, exoerythrocytic parasites were well stained with Giemsa stain (Fig. 2a). The size of erythrocyte-free parasites were between approximate 2.0 and 4.0 μm in diameter, and some of them seemed to have two nuclei (Fig. 2a). However, it was impossible to detect the parasites by using the labeled BgHsp70 probe in the smears fixed with methanol (Fig. 2c). Moreover, the nuclei of white blood cells in smears fixed with methanol could not be detected by using the labeled canine 18S rDNA probe (Fig. 2e). These results showed that *B. gibsoni* could not be detected in the smears fixed with methanol by DNA ISH analysis, though it was reported that the genes of parasites were detected in smears fixed with methanol (Ge et al., 1995). In general, because of the mechanism of the fixation of methanol, it is speculated that the nuclic acids would not be fixed strongly in the cell. In the report about estrogen receptor mRNA in rhesus monkey uterus, the fixation with PFA for 20 min was much

better for ISH analysis than other fixation agents (Koji and Brenner, 1993). Therefore, we used PFA for fixing the smears.

In the smears fixed with PFA, both exoerythrocytic and intraerythrocytic parasites were indistinctly stained with Giemsa stain (Fig. 2b, 3a). Following DNA ISH using the labeled BgHsp70 probe for thin smears fixed with PFA, exoerythrocytic and intraerythrocytic *B. gibsoni* gave specific signals after 16 hrs of hybridization (Fig. 2d, 3b). Labeling was restricted to the nuclei of parasites. The staining was sufficient to detect parasites at magnifications of x 400 and x 1,000 in the thin smears. On the other hand, nuclei of canine white blood cells were stained by the labeled canine 18S rDNA probe as positive controls (Fig. 2f). and neither exoerythrocytic nor intraerythrocytic parasites were detected by this probe (Fig. 2f, 3c). Specific signals of *B. gibsoni* disappeared when the smears were blocked with the unlabeled BgHsp70 probe before the hybridization as negative controls (data not shown). These results showed that both exoerythrocytic and intraerythrocytic *B. gibsoni* in blood smears were specifically stained, and that the BgHsp70 gene could be detected in blood smears by DNA

ISH analysis. It is reported that certain genes from *Anaplasma marginale* (Ge et al., 1995), *Plasmodium berghei* (Thompson et al., 1999; Thompson, 2002; Van der Berg et al., 1991), *P. falciparum* (Jambou et al., 1995), and *Toxoplasma gondii* (Kohler et al., 1997) within host cells or tissues are detected by ISH analysis. ISH analysis is not only utilized as a specific detection procedure for *A. marginale* (Ge et al., 1995) and *P. berghei* (Van der Berg et al., 1991), but also for observing the changes of expression of the certain genes in *P. berghei* (Thompson et al., 1999), *P. falciparum* (Jambou et al., 1995), and *T. gondii* (Kohler et al., 1997). In the present study, because the specific signals were observed on nuclei of the parasites, the DNA probe seemed to hybridize only with the BgHsp70 gene in genomic DNA. This suggested that the specific detection of *B. gibsoni* would be possible by our DNA ISH analysis.

3.3. DNA in situ hybridization for the formalin-fixed, paraffin-embedded tissues

DNA ISH detection of *B. gibsoni* in formalin-fixed, paraffin-embedded

sections of tissues such as spleen, lymph node, and kidney, obtained from the experimental acutely infected dogs was possible with the protocol developed using the labeled BgHsp70 probe (Fig. 4c-4f, 5b, and 5f). On the other hand, nuclei of cells in those tissues were not stained by using the labeled BgHsp70 probe (Fig. 4c-4f, 5b, 5f). Moreover, cells in those tissues of dogs were stained by the labeled canine 18S rDNA probe used as a positive control (Fig. 4g, 5c and 5g), indicating that DNA ISH for those tissues should be successful. In addition, the signals of the parasites disappeared when the sections were blocked with the unlabeled BgHsp70 probe before the hybridization as a negative control (Fig. 4h, 5d and 5h). These results suggested that the BgHsp70 gene could be detected in formalin-fixed, paraffin-embedded sections of those tissues by DNA ISH analysis.

In spleen sections stained with H&E, hemosiderosis (Fig. 4a, 4b) and small numbers of infected erythrocytes (Fig. 4b) were observed in red pulp. The same areas as for hemosiderosis (Fig. 4c, 4e) and infected erythrocytes (Fig. 4f) in red pulp were specifically stained by DNA ISH using the labeled BgHsp70 probe.

From these results, it was inferred that macrophages containing hemosiderin would also have the genomic DNA from *B. gibsoni* in their cytoplasm, and that those macrophages might phagocytose the infected erythrocytes. In lymph node sections stained with H&E, large numbers of erythrocytes were phagocytosed by macrophages (Fig. 5a). Many of the phagocytosed erythrocytes seemed to be infected with the parasites (Fig. 5a). Following DNA ISH using the labeled BgHsp70 probe, those phagocytosed erythrocytes were specifically stained deep purple (Fig. 5b). These results suggested that the erythrocytes infected with *B. gibsoni* would be actively phagocytosed by macrophages in lymph nodes during the acute stage of canine babesiosis. It is reported that macrophages phagocytose infected erythrocytes *in vitro* (Murase et al., 1996). The present results in spleen and lymph node tissues agreed with this previous report. Moreover, since the BgHsp70 gene in macrophages containing hemosiderin was detected by DNA ISH analysis, the genomic DNA from *B. gibsoni* might remain in the cytoplasm of macrophages after the death of the parasites. However, we could not determine how long the genomic DNA from the parasites remained in

macrophages in the present study.

In addition, erythrocytes in blood vessels within red pulp of the spleen were stained by the labeled BgHsp70 probe (Fig. 4c, 4d), though it was difficult to detect the parasites using sections stained with H&E (Fig. 4a, 4b). In the kidney, erythrocytes in blood vessels were specifically stained by the labeled BgHsp70 probe (Fig. 5f), though it was also difficult to detect the parasites using sections stained with H&E (Fig. 5e). These results suggested that a number of infected erythrocytes would stay in blood vessels in red pulp of the spleen and kidney. It is known that erythrocytes infected either with *P. falciparum* or *B. bovis* adhere to the endothelium in blood vessels in the brain (O'Connor et al., 1999; Aikawa et al., 1992), and erythrocytes infected either with *B. bigemina* or *B. rodhaini* adhere to thrombospondin (Parrodi et al., 1990). We hypothesized that erythrocytes infected with *B. gibsoni* could also adhere to endothelium in blood vessels in the spleen and kidney. However, further study will be necessary to confirm this hypothesis. Since it was difficult to detect infected erythrocytes in specimens stained with H&E, DNA ISH analysis seemed to be more sensitive to

detect parasitized erythrocytes in tissues. Therefore, it is considered that DNA ISH analysis would be a useful technique for studying both the life cycle of *B. gibsoni* and the pathological condition of canine babesiosis.

In liver tissue, macrophage-like cells were rarely stained by the labeled BgHsp70 probe (Fig. 5j). Moreover, hepatocytes were hardly stained by the labeled canine 18S rDNA probe, though macrophages in liver were detected by that probe (Fig. 5k). Meanwhile, positive cells disappeared when the sections were blocked with the unlabeled BgHsp70 probe before the hybridization (negative control, Fig. 5l). These results suggested that DNA ISH analysis in liver according to our ISH protocol developed in the present study would not be successful, and that our ISH protocol should be improved depending on the tissue. Additionally, because the purpose of the present study was to develop an ISH protocol for detecting *B. gibsoni* in both thin smears and formalin-fixed, paraffin-embedded sections, we utilized only four tissues for the ISH analysis. Further ISH analysis using other tissues such as brain and bone marrow will be necessary to clarify the life cycle of the parasites and pathological condition of

canine babesiosis. Moreover, the expression of rRNA genes from *P. berghei* were examined in infected mosquito using ISH analysis (Thompson et al., 1999). Therefore, it would be possible that the genes from *B. gibsoni* might be detected in infected ticks using ISH analysis. However, because tissues and cells of ticks seem to be different from those of dogs, it might be necessary to determine the suitable experiment condition for ticks.

As described above, *B. gibsoni* was detected in formalin-fixed, paraffin-embedded sections of tissues by our DNA ISH analysis. These results suggested that the DNA probe used in the present study could hybridize with genomic DNA or mRNA from the parasites in formalin-fixed, paraffin-embedded sections of tissues. Since ISH analysis targeting genomic DNA could detect the DNA from even dead and degraded parasites, we would be able to observe the eliminaion mechanism, the localized pattern and the latent infection of *B. gibsoni* in infected dogs. In the present study, we could detect the infected erythrocytes, which were phagocytosed by macrophages. In contrast, ISH analysis targeting mRNA could be more beneficial in detecting living parasites. Using ISH analysis

targeting mRNA, we could also examine the transcriptional activity of certain genes during their life cycle. Moreover, it is recently reported that the GFP-expressing *B. bovis* merozoites have been developed and that those parasites were easily detected by fluorescence microscopy (Suarez and McElwain, 2009). Those methods would contribute to track the parasites within mammalian and tick hosts, and to analyze the function of certain genes of the parasites. It is expected that the similar technique would be developled for *B. gibsoni*.

In the present study, *B. gibsoni* was detected by ISH analysis both in blood smears and in formalin-fixed, paraffin-embedded sections of tissues. Further observation of other tissues obtained from infected dogs by ISH analysis will contribute to clarification of the life cycle of *B. gibsoni* and the pathological condition of canine babesiosis.

Acknowledgement

This work was supported in part by a grant from the Science Research Fund of the Ministry of Education, Culture, Sports, Science and Technology of Japan.

References

Aikawa, M., Pongponratn, E., Tegoshi, T., Nakamura, K., Nagatake, T., Cochrane, A., Ozaki, L.S. 1992. A study on the pathogenesis of human cerebral malaria and cerebral babesiosis. Memorias do Instituto Oswaldo Cruz, Rio de Janeiro 87, suppl. III, 297-301.

Ge, N.L., Kocan, K.M., Murphy, G.L., Blouin, E.F. 1995. Detection of *Anaplasma marginale* DNA in bovine erythrocytes by slot-blot and *in situ* hybridization with a PCR-mediated digoxigenin-labeled DNA probe. Journal of Veterinary Diagnostic Investigation 7, 465-472.

Grimberg, B.T., Udomsangpetch, R., Xainli, J., McHenry, A., Panichakul, T., Sattabongkot, J., Cui, L., Bockarie, M., Chitnis, C., Adams, J., Zimmerman, P.A., King, C.L. 2007. *Plasmodium vivax* invasion of human erythrocytes inhibited by antibodies directed against the duffy binding protein. PLoS Medicine 4, 1940-1948.

Jambou, R., Hatin, I., Jaureguiberry, G. 1995. Evidence by in situ hybridization

for stage-specific expression of the ATP/ADP translocator mRNA in *Plasmodium falciparum*. Experimental Parasitology 80, 568-571.

Kohler, S., Delwiche, C.F., Denny, P.W., Tilney, L.G., Webster, P., Wilson, R.J.M., Palmer, J.D., Roos, D.S. 1997. A plastid of probable green algal origin in Apicomplexan parasites. Science 275, 1485-1489.

Koji, T., Brenner, R.M. 1993. Localization of estrogen receptor messenger ribonucleic acid in rhesus monkey uterus by nonradioactive in situ hybridization with digoxigenin-labeled oligodeoxynucleotides. Endocrinology 132, 382-392.

Lanar, D.E., McLaughlin, G.L., Wirth, D.E., Barker, R.F., Zolg, W.E., Chulay, J.D. 1989. Comparison of thick films, *in vitro* culture and DNA hybridization probes for detecting *Plasmodium falciparum* malaria. The American Journal of Tropical Medicine and Hygiene 40, 3-6.

Loretti, A.P., Barros, S.S. 2005. Hemorrhagic disease in dogs infected with an unclassified intraendothelial piroplasm in southern Brazil. Veterinary Parasitology 134, 193-213.

McLaughlin, G.L., Collins, W.E., Campbell, G.H. 1987. Comparison of genomic, plasmid, synthetic and combined DNA probes for detecting *Plasmodium falciparum* DNA. Journal of Clinical Microbiology 25, 791-795.

Murase, T., Ueda, T., Yamato, O., Tajima, M., Maede, Y. 1996. Oxidative damage and enhanced erythrophagocytosis in canine erythrocytes infected with *Babesia gibsoni*. Journal of Veterinary Medical Science 58, 259-261.

O'Connor, R.M., Long, J.A., Allred, D.R. 1999. Cytoadherence of *Babesia bovis*-infected erythrocytes to bovine brain capillary endothelial cells provides an in vitro model for sequestration. Infection and Immunity 67, 3921-3928.

Parrodi, F., Wright, I.G., Kerr, J.D., Dobson, D. 1990. *In vitro* adherence of erythrocytes infected with *Babesia bigemina* and *Babesia rodhaini* to thrombospondin. International Journal for Parasitology 20, 899-903.

Petchpoo, W., Tan-ariya, P., Boonsaeng, V., Brockelman, C.R., Wilairat, P., Panyim, S. 1992. A specific DNA probe which identifies *Babesia bovis* in whole blood. Veterinary Parasitology 42, 189-198.

Rickman, L.S., Oberst, R., Sangalang, R., Chulay, J.D., Long, G.W., Cabanban, A., Smith, J.I., Hoffman, S.L. 1989. Rapid diagnosis of malaria by acridine orange staining of centrifuged parasites. Lancet 1, 68-71.

Shute, G.T., Sodeman, T.M. 1973. Identification of malaria parasites by fluorescence microscopy and acridine orange staining. Bulletin of World Health Organization 48, 591-596.

Suarez, C.E., McElwain, T.F. 2009. Stable expression of a GFP-BSD fusion protein in *Babesia bovis* merozoites. International Journal for Parasitology 39, 289-297.

Sugimoto, C., Sato, M., Kawazu, S., Kamio, T., Fujisaki, K. 1991. Purification of merozoites of *Theileria sergenti* from infected bovine erythrocytes. Parasitology Research 77, 129-131.

Thompson, J., Van Spaendonk, R.M.L., Choudhuri, R., Sinden, R.E., Janse, C.J., Waters, A.P. 1999. Heterogeneous ribosome populations are present in *Plasmodium berghei* during development in its vector. Molecular Microbiology 31, 253-260.

Thompson, J. 2002. *In situ* detection of RNA in blood- and mosquito-stage malaria parasites. Methods in Molecular Medicine 72, 225-233.

Torres-Velez, F.J., Nace, E.K., Won, K.Y., Bartlett, J., Eberhard, M., Guarner, J. 2003. Development of an immunohistochemical assay for the detection of babesiosis in formalin-fixed, paraffin-embedded tissue samples. American Journal of Clinical Pathology 120, 833-838.

Van den Berg, F.M., Van Amstel, P.J., Janse, C.J., Meis, J.F.G.M., Mons, B. 1991. Detection of different developmental stages of malaria parasites by non-radioactive DNA *in situ* hybridization. Histochemical Journal 23, 109-115.

Waters, A.P., McCutchan, T.F. 1989. Rapid, sensitive diagnosis of malaria based on ribosomal RNA. Lancet 2, 1343-1346.

Yamasaki, M., Tajima, M., Lee, K.W., Jeong, J.R., Yamato, O., Maede, Y. 2002. Molecular cloning and phylogenetic analysis of *Babesia gibsoni* heat shock protein 70. Veterinary Parasitology 110, 123-129.

Yamasaki, M., Hossain, M.A., Jeong, J.R., Chang, H.S., Satoh, H., Yamato, O.,

Maede, Y. 2003. *Babesia gibsoni*-specific isoenzymes related to energy metabolism of the parasites in infected erythrocytes. Journal of Parasitology 89, 1142-1146.

Yamasaki, M., Inokuma, H., Sugimoto, C., Shaw, S.E., Aktas, M., Yabsley, M.J. Yamato, O., Maede, Y. 2007. Comparison and phylogenetic analysis of the heat shock protein 70 gene of Babesia parasites from dogs. Veterinary Parasitology 145, 217-227.

Figure captions

Fig. 1. Probe assay for the digoxigenin-labeled BgHsp70 and canine 18S rDNA probes. Probes for either BgHsp70 or canine 18S rDNA were hybridized to genomic DNA extracted from either *Babesia gibsoni* or canine white blood cells. Spots were observed when hybridization occurred between the probe and genomic DNA. *Bg = *B. gibsoni*. †WBC = canine white blood cells.

Fig. 2. Observation of exoerythrocytic *Babesia gibsoni* parasites in blood smears. Smears were fixed with methanol (a, c, e) or 4% paraformaldehyde (b, d, f). Smears were stained with Giemsa (a, b), by *in situ* hybridization with a digoxigenin-labeled BgHsp70 probe (c, d) or with a digoxigenin-labeled 18S rDNA probe (e, f). Arrowhead shows *B. gibsoni* parasites including two nuclei. Arrow shows a nuclei of a canine white blood cell. Bar = 10 µm.

Fig. 3. Observation of intraerythrocytic *Babesia gibsoni* parasites in blood

smears. Smears were fixed with 4% paraformaldehyde. Smears were stained with Giemsa (a), by *in situ* hybridization with the digoxigenin-labeled BgHsp70 probe (b) or the digoxigenin-labeled 18S rDNA probe (c). Bar = 10 μm.

Fig. 4. Observation of formalin-fixed, paraffin-embedded section of spleen obtained from an experimentally acute *Babesia gibsoni*-infected dog. Section stained with H&E at x 400 (a) and x 1,000 (b) magnification. Section stained by *in situ* hybridization with the digoxigenin-labeled BgHsp70 probe at x 400 (c) and x 1,000 (d, e, and f) magnification. Section stained by *in situ* hybridization with the digoxigenin-labeled canine 18S rDNA probe as a positive control (g). Section blocked in a 10-fold excess amount of an unlabeled BgHsp70 probe before the hybridization as a negative control (h). Arrowheads show *B. gibsoni*-infected erythrocytes in blood vessels within red pulp of spleen. Arrows show hemosiderosis. Bar = 50 μm.

Fig. 5. Observation of formalin-fixed, paraffin-embedded section of a lymph node

(a, b, c, d), kidney (e, f, g, h), and liver (i, j, k, l) obtained from an experimental acutely *B. gibsoni*-infected dog. Section stained with H&E at x 1,000 (a) and x 400 (e, i) magnification. Section stained by *in situ* hybridization with the digoxigenin-labeled BgHsp70 probe at x 1,000 (b) and x 400 (f, j) magnification. Section stained by *in situ* hybridization with the digoxigenin-labeled canine 18S rDNA probe as a positive control (c, g, k). Section blocked in a 10-fold excess amount of the unlabeled BgHsp70 probe before the hybridization as a negative control (d, h, l). Closed Arrows show erythrocytes phagocytosed by macrophages. Closed arrowheads show *B. gibsoni*-infected erythrocytes phagocytosed by macrophages. Open arrowheads show *B. gibsoni*-infected erythrocytes in blood vessels within the kidney. Open arrow show stained cells. Bar = 100 μm.

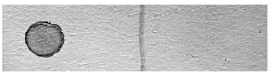
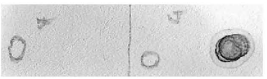
Table 1.

Oligonucleotides for the amplification of the BGHsp70 gene.

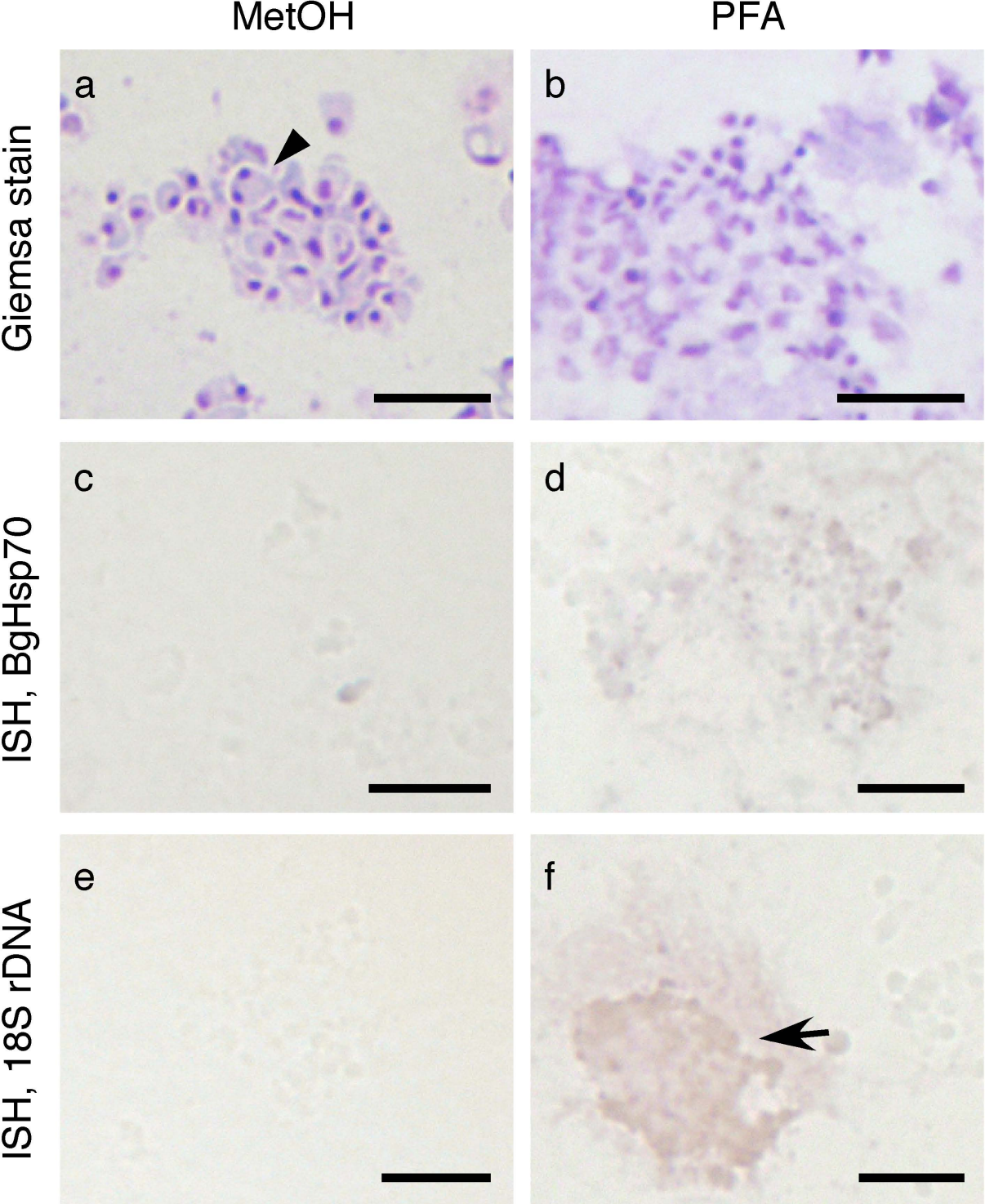
Name	Sequence	Location[a]
BGHsp70F4	5’-gat cga ggt tac ctt cga ta-3’	1422-1441
BGHsp70R[b]	5’-cwt gtg htt agt caa cyt cct cwa c-3’	1924-1938

[a]Locations indicate nucleotide numbers of the *Babesia gibsoni* heat shock protein 70 gene reported previously.

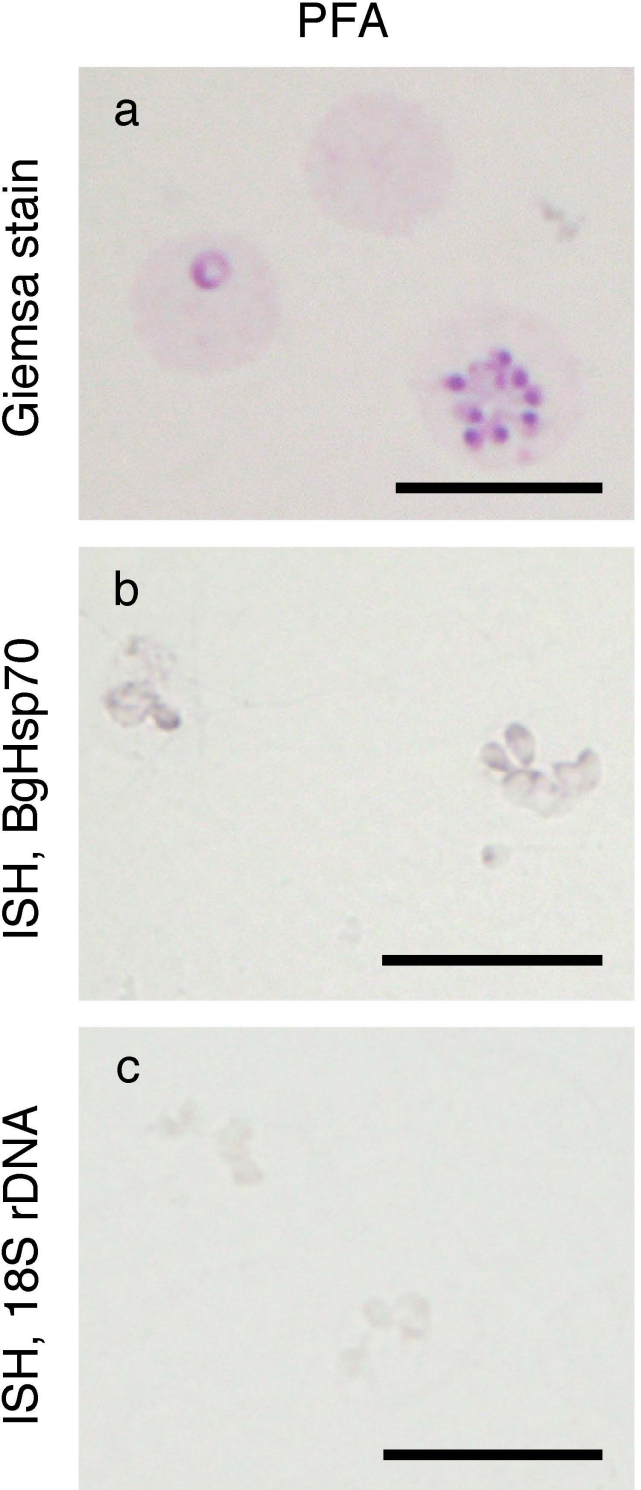
[b]Antisense primer.

		genomic DNA	
		Bg*	WBC†
probe	BgHsp70		
	18S rDNA		

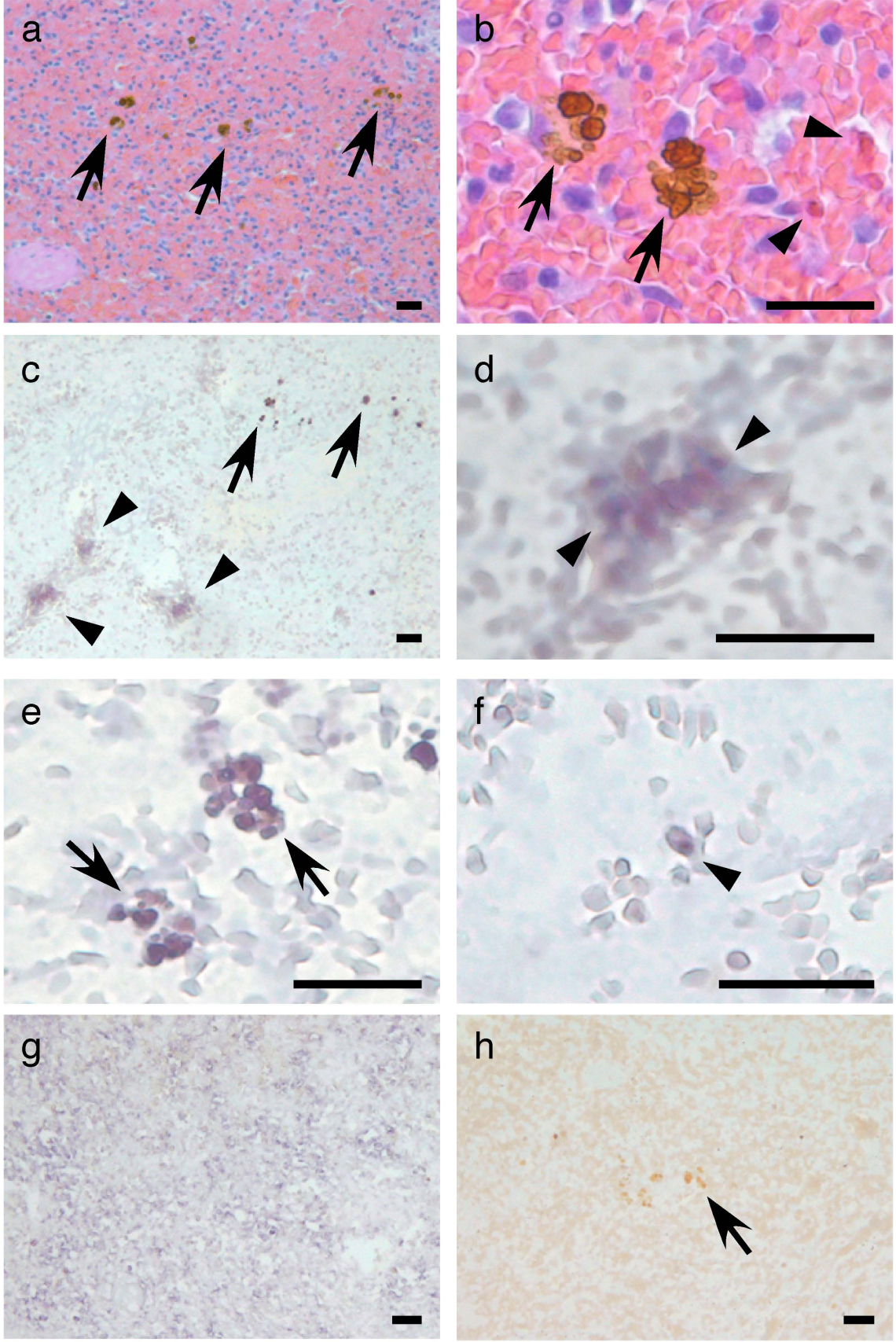
Yamasaki M. et al. Fig. 1



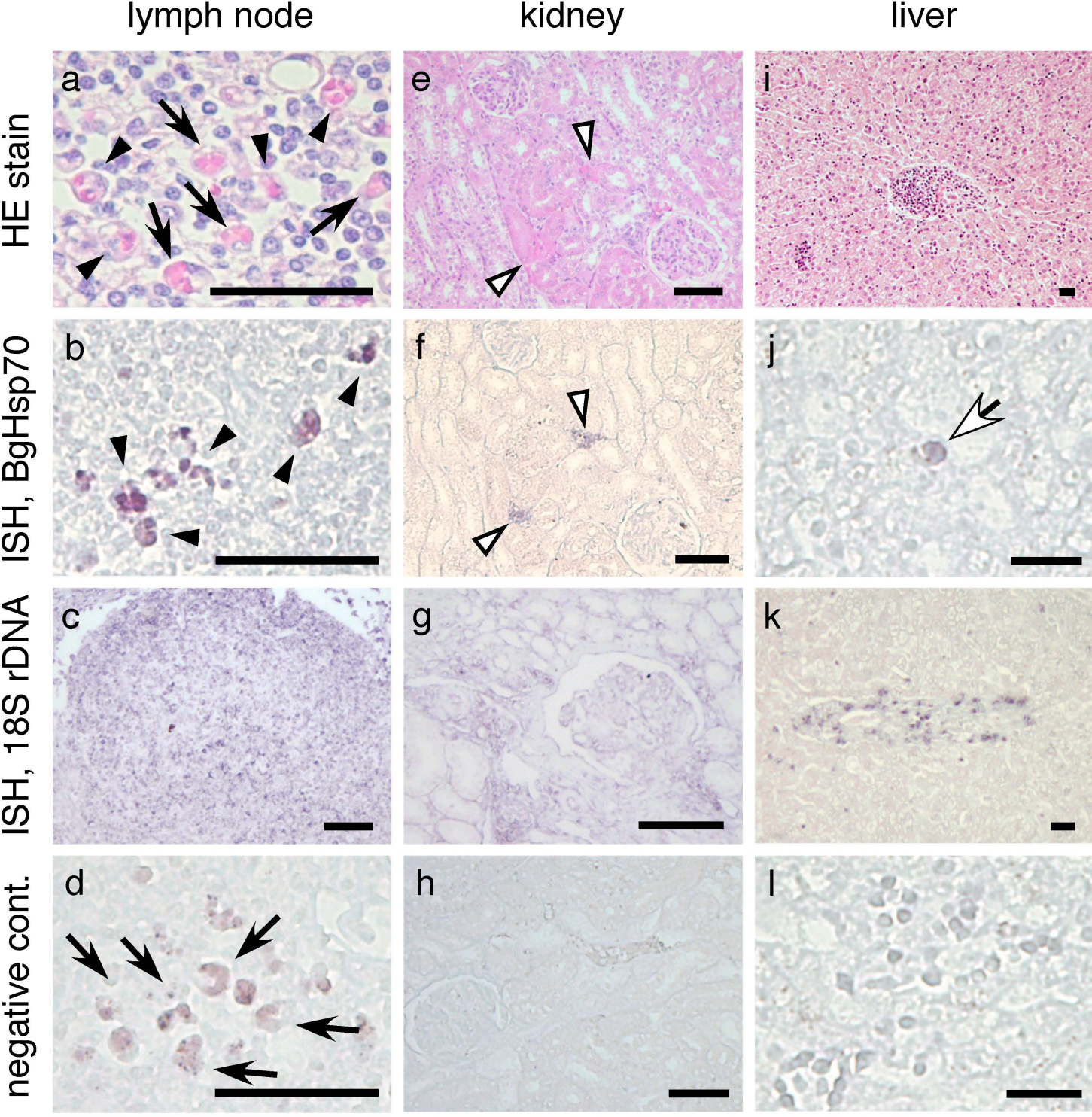
Yamasaki M. et al. Fig. 2



Yamasaki M. et al. Fig. 3



Yamasaki M. et al. Fig. 4



Yamasaki M. et al. Fig. 5

```
canine      :    1'     TCAAGAACGAAAGTCGGAGGTTCGAAGACGATCAGATACCGTCGTAGTTCCGACCA
                        **********************************************************
homo sapiens: 1021" TTAATCAAGAACGAAAGTCGGAGGTTCGAAGACGATCAGATACCGTCGTAGTTCCGACCA

canine      :   57' TAAACGATGCCGACTGGCGATGCGGCGGCGTTATTCCCATGACCCGCCGGGCAGCTTCCG
                    ************** **********************************************
homo sapiens: 1081" TAAACGATGCCGACCGGCGATGCGGCGGCGTTATTCCCATGACCCGCCGGGCAGCTTCCG

canine      :  117' GGAAACCAAAGTCTTTGGGTTCCGGGGGGAGTATGGTTGCAAAGCTGAAACTTAAAGGAA
                    ************************************************************
homo sapiens: 1141" GGAAACCAAAGTCTTTGGGTTCCGGGGGGAGTATGGTTGCAAAGCTGAAACTTAAAGGAA

canine      :  177' TTGACGGAAGGGCACCACCAGGAGTGGAGCCTGCGGCTTAATTTGACCCAACACGGGAAA
                    ********************************************** ************
homo sapiens: 1201" TTGACGGAAGGGCACCACCAGGAGTGGAGCCTGCGGCTTAATTTGACTCAACACGGGAAA

canine      :  237' CCTCACCCGGCCCGGACACGGACAGGATTGACAGATTGATAGCTCTTTCTCGATTCCGTG
                    ************************************************************
homo sapiens: 1261" CCTCACCCGGCCCGGACACGGACAGGATTGACAGATTGATAGCTCTTTCTCGATTCCGTG

canine      :  297' GGTGGTGGTGCATGGCCGTTCTTAGTTGGTGGAGCGATTTGTCTGGTTAATTCCGATAAC
                    ************************************************************
homo sapiens: 1321" GGTGGTGGTGCATGGCCGTTCTTAGTTGGTGGAGCGATTTGTCTGGTTAATTCCGATAAC

canine      :  357' GAACGAGACTCTGGCATGCTAACTAGTTACGCGACCCCCGAGCGGTCGGCGTCCCCCAAC
                    ************************************************************
homo sapiens: 1381" GAACGAGACTCTGGCATGCTAACTAGTTACGCGACCCCCGAGCGGTCGGCGTCCCCCAAC

canine      :  417' TTCTTAGAGGGACAAGTGGCGTTCAGCCACCCGAGATTGAGCAATAACAGGTCTGTGATG
                    ************************************************************
homo sapiens: 1441" TTCTTAGAGGGACAAGTGGCGTTCAGCCACCCGAGATTGAGCAATAACAGGTCTGTGATG

canine      :  477' CCCTTAGATGTCC
                    *************
homo sapiens: 1501" CCCTTAGATGTCCGGGGCTGCACGCGCGCTACACTGACTGGCTCAGCGTGTGCCTACCCT
```

Fig. A. Aligment of the nucleotide sequence of PCR products, which amplified with 18S PCR Primer Pair (QuantumRNA™ Classic 18S Internal Standards) from canine cDNA, and Homo sapiens 18S ribosomal 1 (RN18S1)(GenBank Accession No. NR_003286). Nucleotide, which are conserved between dog and homo sapiens, are marked by asterisks (*).