

Improvement of DNA Extraction Method for Dried Blood Spots and Comparison of Four PCR Methods for Detection of *Babesia gibsoni* (Asian Genotype) Infection in Canine Blood Samples

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ABSTRACT. To eradicate canine babesiosis in epidemic areas, mass-screening of the infection situation of *Babesia gibsoni* including occult infection is necessary. The development of cost-effective method for storage and transport of blood samples is required. A highly efficient DNA extraction procedure from dried blood spots (DBS) onto Whatman 3MM filter paper was developed for the diagnosis of *B. gibsoni* infection in dog by PCR. In 3 extraction methods, Chelex-based method in combination with saponin washing and phenol-chloroform-isoamyl alcohol extraction (Saponin-PCI method) provided the best results. Sensitivity of the 4 previously described PCR methods for detection of *B. gibsoni* infection was also compared using serially diluted blood samples of *B. gibsoni*-infected dogs. The PCR method using Gib599F/Gib1270R primer pair provided the best performance. To evaluate the stability of DNA in DBS, DBS of *B. gibsoni*-infected dogs stored at room temperature for 2 months. The stability was superior to whole blood samples stored at -20°C for 2 months. This highly efficient DNA extraction method on DBS using Whatman 3MM filter paper has potential to be cost-effective and high performance tool for storage, and molecular diagnosis of clinical blood sample from dog. This procedure in combination with the PCR method using Gib599F/Gib1270R primer pair may greatly assist in diagnosis of *B. gibsoni* infection in dog populations that are geographically distant.

KEY WORDS: *Babesia gibsoni*, canine, dried blood spot, filter paper.

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Canine babesiosis is a critical disease in dogs caused by infection of *Babesia* species in blood. Most cases of canine babesiosis reported in Japan have been identified as *Babesia gibsoni* (*B. gibsoni*) and *Babesia canis* (*B. canis*). Babesiosis caused by *B. gibsoni* is typically characterized by hemolytic anemia, thrombocytopenia, fever, and splenomegaly [8], and the clinical manifestations is generally more severe than *B. canis* infection [12]. Diagnosis of *B. gibsoni* infection is dependent on microscopic observation of the organisms. However, mild or occult infection of *B. gibsoni* in dogs is often subclinical, and low infection of the organisms is usually undetectable microscopically. Therefore, *B. gibsoni* infection with low parasitemia is easily misdiagnosed as immune mediated hemolytic anemia (IMHA). Diagnosis and treatment of occult infection of *B. gibsoni* may be needed to eradicate canine babesiosis in all epidemic areas. Mass-screening of babesia infection in epidemic areas may be required to find the occult infection of *B. gibsoni* in dogs as the carrier of babesia parasite.

Recently, several PCR methods have been reported to diagnose *B. gibsoni* infection with high sensitivity and specificity [2, 4, 14, 19]. These methods could easily diagnose infection of *B. gibsoni* even if the babesia organisms could not be detected microscopically. However, blood samples of suspicious *B. gibsoni* infection must be transported to

special laboratories that provide diagnostic services based on the PCR technique. Thus, the cost of storage and transport of blood samples under chilled conditions may result in higher clinical test fees. The application of blood samples to commercial available filter paper-based technologies, such as FTA cards (Whatman Bioscience, Cambridge, UK) and IsoCode Stix (Schleicher and Schuell Bioscience, Dassel, Germany), has been reported as a convenient way to keep DNA for epidemiological investigations and diagnosis of infectious disease [9, 15, 18, 20, 28]. Jefferies *et al.* have been demonstrated the nested-PCR methods using the samples stored FTA cards and IsoCode stix provided highly sensitive detection of canine piroplasm [15]. However, the blood sample applied to FTA cards and IsoCode Stix must be used as a small paper pieces for PCR template. This special protocol may restrict the sensitivity of PCR-based detection. Further molecular techniques for greater sensitivity, such as nested-PCR method, may result in higher clinical test fees. Clinical blood samples blotted onto general filter papers have also been reported to have high stability under ambient temperature [3, 13]. However, the presence of PCR inhibitors caused by inefficient DNA extraction procedures lead to limitation of blood volume spotted onto filter paper [16, 24, 26]. The development of a highly sensitive DNA extraction method from whole blood sample blotted onto general filter paper may lead to high cost-efficiency and flexible application of the extracted DNA, and consequently develop the highly sensitive PCR-based detection method.

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The aim of this study was to develop a highly efficient DNA extraction method from canine DBS using general filter paper, and to compare sensitivity of the 4 previously described PCR methods for detection of *B. gibsoni* infection in dog under similar condition.

MATERIALS AND METHODS

Evaluation of DNA extraction methods: Blood samples were collected from 5 clinically normal purebred beagle dogs. Peripheral blood samples were obtained from the cephalic vein using blood collection tubes with anticoagulant (EDTA2Na). Blood samples were spotted onto sterilized Whatman 3MM filter paper (3 cm × 3 cm; Whatman Bioscience), and the filter papers were left to dry for 4 hr at room temperature. Two different volumes, 10 μ l and 250 μ l, of dried blood spots (DBS) were prepared to determine the effect of spotted volume. The DBS were stored in a separate plastic bag at room temperature. The remainder of the blood samples was stored at -20°C. The DNA extraction methods used in this study were described below.

Commercial kit: DNA was extracted from 200 μ l of whole blood samples stored at -20°C using the QIAamp DNA blood mini kit (QIAGEN GmbH, Hilden, Germany), according to the manufacturer's instructions. The extracted DNA was stored at -20°C for later analysis as a control sample.

Lysis method [22]: The DBS was cut into strips and the strips were incubated in 900 μ l of lysis buffer containing 50 mM NaCl, 50 mM Tris-HCl (pH 7.4), 10 mM EDTA, 1% Triton X-100, and 200 μ g/ml proteinase K (New England Bio Labs, MA, U.S.A.) at 60°C for overnight. After incubation, phenol-chloroform-isoamyl alcohol (25:24:1) (PCI) extraction and isopropanol precipitation were carried out. The DNA sample was stored at -20°C until use.

Chelex method [17, 23]: The DBS strips were washed with 12.5 ml of ultra pure water. The strips were transferred, and 1.1 ml of 5% suspension of Chelex-100 (Bio-Rad Laboratories, CA, U.S.A.) in ultra pure water was added. After heating at 95°C for 10 min, the sample was centrifuged at 12,000 × g for 2 min, and the supernatant was centrifuged again. The supernatant was stored at -20°C until use.

Saponin method [6, 11, 27]: The DBS strips were incubated in 25 ml of 0.5% saponin (Nacalai tesque, Kyoto, Japan) in PBS (pH 7.4). After incubation at 4°C for 3 hr, the strips were washed twice with PBS. The strips were transferred, and 1.1 ml of 10% suspension of Chelex-100 (Bio-Rad Laboratories) in ultra pure water was added. After incubation at 95°C for 10 min, the sample was centrifuged at 8,200 × g for 5 min at 4°C, and the supernatant was centrifuged again. The supernatant was transferred, and stored at -20°C.

The DNA extraction yield from each method was evaluated by PCR amplification of the canine glyceraldehyde 3-phosphate dehydrogenase (cGAPDH) gene [4]. The PCR conditions were used with 25 μ l reaction volume containing

1 × ExTaq buffer, 1.5 mM MgCl₂, 200 μ M of dNTPs, 0.5 μ M of each primer, 1.25 U of Ex Taq DNA polymerase (TaKaRa Bio, Shiga, Japan), and 0.5 μ l of sample. The number of cycles was optimized for all of control DNA samples to quantify the PCR product in the exponential phase of PCR reaction. DNA amplifications were performed as follows: initial denaturation at 94°C for 5 min, 30 cycles of 94°C for 1 min, 60°C for 1 min, 72°C for 1 min, and a final extension step at 72°C for 5 min. Ten microliters of PCR product was visualized by ethidium bromide staining through a 1.5% agarose gel, and then quantified with a EDAS120 (Kodak, NY, U.S.A.), and NIH image software. To compare the extraction efficiency, cGAPDH signal intensity from each sample was normalized to the signal intensity from a control DNA sample, which was diluted to the expected DNA amount of each extracted sample. The PCR inhibitors contamination in each sample was evaluated by PCR amplification of pUC18 plasmid vector (TaKaRa Bio) added to each extracted sample. M13 primer pair and 0.1 ng of pUC18 plasmid vector into DNA sample were used with PCR conditions as mentioned above. The concentration of pUC18 plasmid vector into DNA samples was determined using serial dilution of pUC18 plasmid vector for quantification of the PCR product in the exponential phase of PCR reaction at 30 PCR cycles. DNA amplifications were performed as follows: initial denaturation at 94°C for 5 min, 30 cycles of 94°C for 30 sec, 60°C for 30 sec, 72°C for 10 sec, and a final extension step at 72°C for 5 min. Ten microliters of PCR product was visualized through 2.5% agarose gel. The signal intensity of pUC18 plasmid vector within the extracted sample was normalized to the signal intensity of pUC18 plasmid vector alone. The 2 replicate tests for evaluation of DNA extraction methods were performed on each sample.

Improvement of Saponin method (Saponin-PCI method): To prepare the DBS, 250 μ l of peripheral blood samples from 3 clinically normal beagle dogs were used. Incubation time of DBS with saponin in PBS was set at 1 hr to shorten the time for extraction. The supernatant from the final step of the Saponin method was transferred, and PCI extraction and isopropanol precipitation were carried out as mentioned above. The extracted DNA pellet was lysed in 25 μ l of alkaline lysis buffer (25 mM NaOH, 0.2 mM EDTA, pH 12). After incubation, the sample solution was neutralized by addition of 25 μ l of 40 mM Tris-HCl (pH 5). To evaluate the elimination of PCR inhibitors, the sample solution was serially diluted 2-fold, or 5-fold with TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). The DNA extraction yield of the method and PCR inhibition of each diluted sample were evaluated as mentioned above.

Evaluation of PCR methods: To determine the efficiency of the PCR method for amplification of *B. gibsoni* (Asian genotype) 18S rRNA gene, 4 methods were compared. *B. gibsoni*-infected EDTA anticoagulated whole blood sample was obtained from a dog that was diagnosed with canine babesiosis by microscopic examination of blood smear. The number of red blood cell of the infected dog was 5.23×10^6

/ μ l. Percent parasitemia of the dog was 2.27% based on the average number of 1000-cell count of 3 clinicians. The 5.2 μ l of infected blood sample was added to 1 ml of noninfected EDTA anticoagulated canine whole blood. Percent parasitemia of the sample was 1×10^{-2} percent. The sample was then serially diluted 10-fold by using the noninfected canine whole blood to the percent parasitemia ranging from 1×10^{-3} to 10^{-8} percent. The number of red blood cells of the noninfected dog was 6.18×10^6 / μ l. Three replications of the dilution series were prepared. DNA was extracted from 200 μ l of whole blood samples using the QIAamp DNA blood mini kit (QIAGEN GmbH), and from 250 μ l spotted DBS using Saponin-PCI method. DNA was also extracted from 200 μ l of noninfected canine whole blood using the QIAamp DNA blood mini kit (QIAGEN GmbH) to use for detection of nonspecific PCR product of canine genomic DNA. These 4 sets of primer pairs included PIRO-F/-R and PIRO2-F/-R for nested PCR [2], 455–479F/793–772R and BgibAsia-F/793–772R for semi-nested PCR [4], BgCal-F/-R [19], and Gib599F/Gib1270R [14]. Primer pairs were used with PCR conditions, except for Ex Taq DNA polymerase (TaKaRa Bio) and the reaction buffer, as previously described [2, 4, 14, 19]. All of the PCR products using each primer pairs were extracted from agarose gel, and were checked for amplification of *B. gibsoni* 18SrRNA gene by direct sequencing using each forward primer. The PCR product amplified using PIRO and PIRO2 primer pairs was sequenced both in the forward strand and in the reverse strand to identify the genotype in the blood sample. The DNA sequence of the fragment was identified to be the Asian genotype. The DNA extraction yield was checked by PCR amplification of the cGAPDH gene. The evaluation of each PCR method was performed 2 times on 3 replicate samples.

Evaluation of storage stability: Whole blood samples and DBS samples, which were prepared as a dilution series of *B. gibsoni*-infected blood for evaluation of PCR methods, were also stored in the following conditions to evaluate stability. DBS was stored in the dark at room temperature. Whole blood sample was stored at -20°C , or $-15.5 \pm 3.5^{\circ}\text{C}$ in a refrigerator with an automatic defroster. After 1 and 2 months' storage periods, DNA was extracted by QIAamp DNA blood mini kit (QIAGEN GmbH) from whole blood samples, and by Saponin-PCI method from DBS. Stability of the blood samples was evaluated by PCR amplification of *B. gibsoni* (Asian genotype) 18S rRNA gene using Gib599F and Gib1270R primer pair. All extracted samples were checked for DNA extraction yield by PCR amplification of cGAPDH gene.

Statistical analyses: Statistical analyses were carried out using the Stat View[®] program (SAS Institute Inc., NC, U.S.A.). Mann-Whitney U test was used to compare DNA amplification yield between the extraction methods. A *P* value of less than 0.05 was considered statistically significant.

Animals: The present study was approved by the NIH guidelines and the regulation of the Local Institutional Ani-

mal Care and Use Committee, and owners signed a consent form before enrolling the dog.

RESULTS

Evaluation of DNA extraction methods: The light brown color contaminations were observed in all DNA samples from DBS. We attempted to quantify the DNA concentration of the all extracted samples from DBS using Optical Density, diphenylamine method [25], DNA Dipstick kit (Invitrogen Japan, Tokyo, Japan), and ethidium bromide staining technique [21], but the accurate DNA concentration was not able to be determined (data not shown). The PCR signal of the cGAPDH was observed in all DNA samples from all extraction methods. The ratios of the signal intensity of the cGAPDH from DBS to that from control samples are shown in Fig. 1. The highest DNA extraction yield was observed in the sample from Saponin method. The PCR inhibition was observed in the sample from Chelex method and Saponin method. The effect of spotted blood volume was observed in the methods without a PCI extraction step (Chelex method and Saponin method). The DNA extraction yield and PCR inhibitors elimination from the sample extracted by Saponin-PCI method was enhanced to be equivalent to or better than that from the control sample (Fig. 2). The PCR inhibition was not observed in all diluted samples.

Evaluation of PCR methods: The semi-nested PCR method using the 455–479F/793–772R and BgibAsia-F/793–772R primer pairs, and the conventional PCR method using Gib599F/Gib1270R primer pair could detect the *B.*

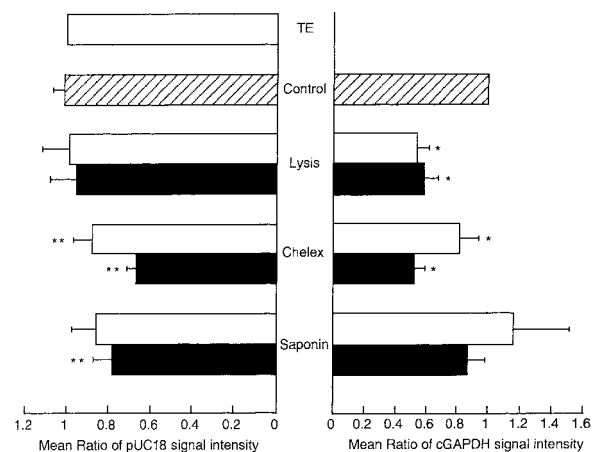


Fig. 1. DNA extraction efficiency from dried blood spot by 3 different extraction methods. The right and left panels show the mean ratio of signal intensity of canine GAPDH and pUC18, respectively. TE, Tris-EDTA buffer alone; Control, control sample extracted by QIAamp DNA blood mini kit; Lysis, Chelex, Saponin, samples extracted by Lysis method, Chelex method, and Saponin method, respectively; Open bar, samples extracted from 10 μ l blood spot; Closed bar, samples extracted from 250 μ l blood spot. Data presented as means \pm SD. * *P*<0.05 vs. control; ** *P*<0.05 vs. TE.

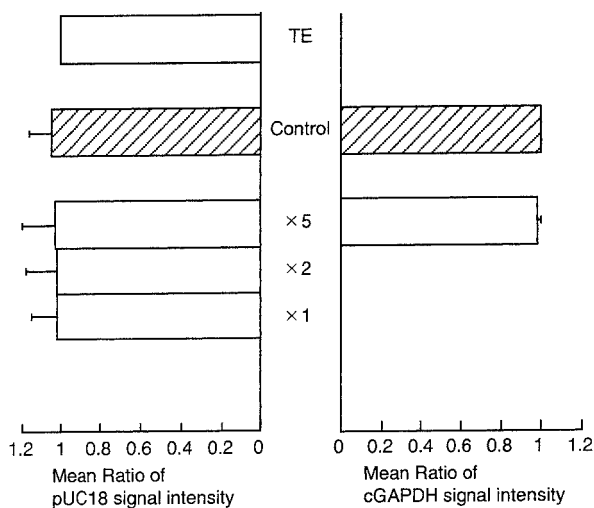


Fig. 2. DNA extraction efficiency from dried blood spot by Saponin-PCI method. Right and left panel show the mean ratio of signal intensity of canine GAPDH and pUC18, respectively. TE, Tris-EDTA buffer alone; Control, control sample extracted by QIAamp DNA blood mini kit; $\times 5$, $\times 2$, $\times 1$, 5-fold (same as expected DNA concentration of control sample), 2-fold, and 1-fold (undiluted) diluted samples, respectively. The mean ratio of signal intensity of canine GAPDH from 2-fold and 1-fold (undiluted) diluted samples were not measured. Data presented as means \pm SD.

gibsoni (Asian genotype) at 10^{-5} percent parasitemia (Fig. 3b). The nested PCR method using the PIRO-F/PIRO-R and PIRO2-F/PIRO2-R primer pairs could detect *B. gibsoni* (Asian genotype) at 10^{-4} percent parasitemia from whole blood sample, and at 10^{-5} percent parasitemia from DBS. The PCR method using the BgCal-F/BgCal-R primer pair could not detect *B. gibsoni* (Asian genotype) in all dilution samples. Nonspecific PCR product of canine genomic DNA was observed in all amplifications (Fig. 3a, b).

Evaluation of storage stability: The signal of *B. gibsoni* (Asian genotype) 18SrRNA gene could be detected at 10^{-5} percent parasitemia in all replicate samples from DBS and whole blood samples prior to storage (Table 1). The highest stability was observed in DBS stored at room temperature. After storage for 2 months, the signal of *B. gibsoni* (Asian genotype) 18SrRNA gene could also be detected at 10^{-5} percent parasitemia in 2 of 3 replicate samples from DBS. In the sample from whole blood stored in a refrigerator with an automatic defroster, the signal of *B. gibsoni* (Asian genotype) 18SrRNA gene could not be detected at 10^{-5} percent parasitemia after storage for 2 months.

DISCUSSION

The detection of hemoprotozoan parasite in blood samples by PCR usually requires a highly sensitive DNA extraction method because of the presence of inhibitors in blood.

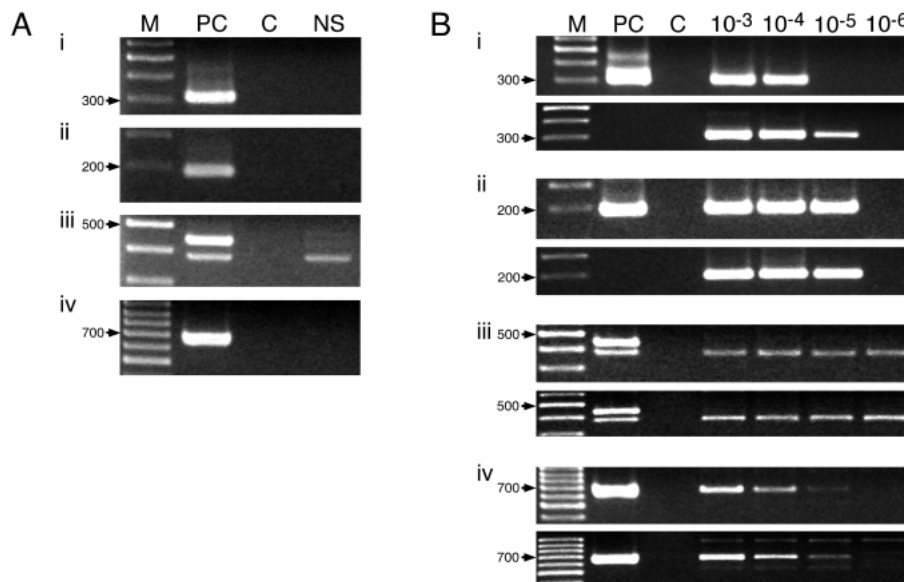


Fig. 3. Sensitivity of 4 different PCR methods for detection of *B. gibsoni* (Asian genotype) 18S rRNA gene using serially diluted *B. gibsoni*-infected canine blood. PCR product of 4 different methods, nested PCR using PIRO-F/-R and PIRO2-F/-R primer pairs (i), semi-nested PCR using 455-479F/793-772R and BgibAsia-F/793-772R primer pairs (ii), PCR using BgCal-F/-R primer pair (iii), and PCR using Gib599F/Gib1270R primer pair (iv), was analyzed by agarose gel electrophoresis and ethidium bromide staining. M, molecular weight marker of 100 bp ladder; PC, sample extracted by QIAamp DNA blood mini kit from *B. gibsoni*-infected canine whole blood; C, distilled water instead of DNA sample. A *B. gibsoni* (Asian genotype) 18S rRNA gene detection from noninfected canine whole blood used for dilution. NS, sample extracted by QIAamp DNA blood mini kit from noninfected canine whole blood. B *B. gibsoni* (Asian genotype) 18S rRNA gene detection from dilution series of *B. gibsoni*-infected canine blood. The upper panel of each pair of panels shows the sample extracted by QIAamp DNA blood mini kit from whole blood. The lower panel of each pair of panels shows the sample extracted by Saponin-PCI method from DBS. 10^{-3} to 10^{-6} , percent parasitemia of the blood samples.

Table 1. The influence of different storage conditions and storage periods on the sensitivity of detection of *B. gibsoni* (Asian genotype) 18S rRNA gene by PCR

Storage period	Storage condition	Percent parasitemia					
		10 ⁻³	10 ⁻⁴	10 ⁻⁵	10 ⁻⁶	10 ⁻⁷	10 ⁻⁸
0	DBS ^{a)}	3	3	3	1	0	0
	Whole blood	3	3	3	0	0	0
1 month	DBS	3	3	3	0	0	ND ^{d)}
	Whole blood						
	–20°C ^{b)}	3	3	3	0	0	ND
2 month	–15 ± 3.5°C with defroster ^{c)}	3	3	1	0	0	ND
	DBS	3	3	2	0	0	ND
	Whole blood						
	–20°C	3	3	1	0	0	ND
	–15 ± 3.5°C with defroster	3	3	0	0	0	ND

Results are reported as the detectable number of 3 replicate samples. a) Dried blood spot stored at room temperature, b) Whole blood samples stored at –20°C, c) Whole blood samples stored at –15 ± 3.5°C in a refrigerator with an automatic defroster, d) Not done.

To develop a sensitive extraction method for DBS onto filter paper, three DNA extraction methods; Lysis method, Chelex method, and Saponin method, were evaluated. The DNA extraction efficiency in Chelex method and Saponin method estimated as the mean ratio of signal intensity of the cGAPDH was not accurately evaluated, because both of methods provided insufficient elimination of PCR inhibitors. Chelex-100 used in the Chelex method and Saponin method is a chelating resin with a high affinity for multivalent metal ions. This has been identified to remove PCR inhibitors by binding metal ions [23]. The blood proteins, such as Immunoglobulin G and lactoferrin, were also considerable as a major inhibitor of PCR in blood [1], because the PCI extraction step added to the Saponin method resulted in higher DNA extraction efficiency and elimination of PCR inhibitors. Our result suggested that the blood proteins, which may contain extraction steps from DBS on the filter paper, should be eliminated by PCI or other extraction procedures for higher efficiency. The additional advantage of Saponin-PCI method is to concentrate DNA from at least 250 μ l of DBS with high quantity and quality. High quantity and quality of DNA sample lead to increase the sensitivity of PCR-based detection, and to apply the other molecular techniques.

To determine the efficient PCR method for amplification of *B. gibsoni* (Asian genotype) 18S rRNA gene, 4 methods were compared based on the lowest detectable percent parasitemia. The limitation of interpreting differences in sensitivities of PCR methods based on the lowest detectable percent parasitemia has been described [4]. The degree of anemia could result in a difference in the total number of parasite/volume when the samples with identical percent parasitemia were examined. In this study, the same serially diluted *B. gibsoni*-infected whole blood sample, and same PCR reagents were used to evaluate the PCR methods. Thus, the accurate comparable results for interpreting differences in sensitivities of 4 PCR methods could be provided from this study. The PCR method using Gib599F/

Gib1270R primer pair provided the best performance among 4 methods. The semi-nested PCR method using the primer pair 455–479F/793–772R and BgibAsia-F/793–772R provided lowest detection limit and highest signal intensity of the *B. gibsoni* (Asian genotype) 18SrRNA gene. However, the methods could be unsuitable for routine screening in terms of cost, time, and reduction of sequence error for genotypic characterization. The differences of sensitivity of 4 PCR methods compared in this study were 100 to 1000-fold or more. The PCR method using the primer pair BgCal-F/BgCal-R could not detect *B. gibsoni* (Asian genotype) in all dilution samples. Nonspecific PCR product of canine genomic DNA, which has been described in the original paper, was also observed in all amplifications (Fig. 3a, b). The detection limit of the method was lesser than the accepted limit of detection (0.001% parasitemia) for light microscopic examination of stained blood smear in our condition [5]. These results suggested the necessity and importance of preliminary test for the sensitivity of PCR in each laboratory condition.

In the study described here, DBS samples stored at room temperature provided higher stability of *B. gibsoni* (Asian genotype) 18SrRNA genes than other storage conditions for 2 months. This result is consistent with those of other studies showing the detection efficiency of visceral leishmaniasis, *Plasmodium falciparum*, and human immunodeficiency virus in dried blood [7, 13, 22]. Automatically defrosting condition affected the stability of DNA in whole blood sample. Several cycles of freeze-thawing of the blood sample have been reported to cause no appreciable reduction in the strength of PCR signals [13]. The DNA stability of the blood sample may be affected by frequently changing the storage temperature, even if the sample is stored under chilled conditions, and the sensitivity of PCR diagnosis may also be reduced. DBS tested in this study was not stored under controlled humidity. Other studies have reported that dried blood spotted onto filter paper and stored at higher humidity resulted in 10- to -100 fold loss of sensitivity of

PCR [13]. In contrast, rapid drying of filter paper provided a 10-fold higher sensitivity than more lengthy drying condition at room temperature. Further studies of desiccated storage conditions may provide improvement of DNA stability.

The PCR method using Gib599F/Gib1270R primer pair in combination with Saponin-PCI method could detect the *B. gibsoni* (Asian genotype) at 10^{-5} percent parasitemia from DBS onto filter paper using one round of PCR. The detection limit of the method reported in this study was superior to methods previously described for the detection of *B. gibsoni* using nested-PCR with sample stored FTA cards and IsoCode stix [15]. Although previous studies have been described the benefits of FTA cards and IsoCode stix as a blood storage method for detection of pathogens [9, 15, 18, 20, 28], these commercially available filters have few benefits for diagnosis of canine babesiosis except for long-term stability of DNA. The stability of blood DNA onto Whatman 3MM filter paper reported in this study have sufficient potential for practical use in terms of cost, time, and sensitivity. The safety for prevention of spreading pathogens in our protocol was not confirmed in this study. The FTA cards can be embedded with substances that inactivate pathogens such as bacteria and viruses [18, 20]. However all types of filter paper-based samples should be handled under safety procedures for prevention of spreading pathogens, because all of infectious pathogens have not been confirmed inactivation.

The Saponin-PCI DNA extraction method from canine DBS using Whatman 3MM filter paper has potential to be cost-effective and high performance tool for storage, and molecular diagnosis of not only *B. gibsoni* infection but also other infectious pathogens from clinical blood sample, because the saponin and Chelex base extraction method for DBS have also been reported to be usable for detection of human immunodeficiency virus [10]. This procedure may also provide the benefit to diagnosis of *B. gibsoni* infection in dog populations that are geographically distant when combined with the PCR method using Gib599F/Gib1270R primer pair.

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