Development and Evaluation of a Seminested PCR for Detection and Differentiation of *Babesia gibsoni* (Asian Genotype) and *B. canis* DNA in Canine Blood Samples

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Canine babesiosis has recently been recognized as an emerging infectious disease of dogs in North America. We sought to develop a seminested PCR to detect and differentiate *Babesia gibsoni* (Asian genotype), *B. canis* subsp. *vogeli*, *B. canis* subsp. *canis*, and *B. canis* subsp. *rossi* DNA in canine blood samples. An outer primer pair was designed to amplify an ∼340-bp fragment of the 18S rRNA genes from *B. gibsoni* (Asian genotype), *B. canis* subsp. *vogeli*, *B. canis* subsp. *rossi*, and *B. canis* subsp. *canis* but not mammalian DNA. Forward primers were designed that would specifically amplify a smaller fragment from each organism in a seminested PCR. The practical limit of detection was 50 organisms/ml of mock-infected EDTA anticoagulated whole blood. The primer pair also amplified an ∼370-bp fragment of the *B. gibsoni* (USA/California genotype) 18S rRNA gene from the blood of an experimentally infected dog with a high percentage of parasitemia. Amplicons were not detected when DNA extracted from the blood of a dog that was naturally infected with *Theileria annae* at a low percentage of parasitemia was amplified. Due to limited sensitivity, this test is not recommended for the routine diagnosis of *B. gibsoni* (USA/California genotype) or *T. annae*. The PCR test did not amplify *Toxoplasma gondii*, *Neospora caninum*, *Leishmania infantum*, *Cryptosporidium parvum*, or canine DNA under any of the conditions tested. The seminested PCR test was able to detect and discriminate *B. gibsoni* (Asian genotype), *B. canis* subsp. *vogeli*, *B. canis* subsp. *canis*, and *B. canis* subsp. *rossi* DNA in blood samples from infected dogs.

Babesiosis is an important disease of domestic dogs in the United States caused by intraerythrocytic protozoan parasites of the genus *Babesia*. These hemoprotozoan parasites, along with *Theileria* spp., are often referred to as piroplasms. Babesiosis is typically characterized by hemolytic anemia, thrombocytopenia, fever, and splenomegaly. Historically, canine babesiosis has been attributed to infection with either *Babesia canis* or *B. gibsoni*, based on parasite size and the geographic location in which the infection was acquired. Recently, an increased number of genetically unique piroplasms have been identified, and the recognized geographic ranges of various canine piroplasms appear to be expanding. Since Conrad et al. reported the first outbreak of canine babesiosis caused by a small *Babesia* sp. in the United States in 1991, there have been an increasing number of reports of dogs infected with small *Babesia* sp. (4, 9, 14–17, 24). During the California outbreak in 1991, *B. gibsoni* was presumed to be the only small *Babesia* sp. to infect dogs. However, recent studies demonstrate that there are at least three genetically distinct small *Babesia*-like organisms or piroplasms that can infect dogs (15, 24, 25). Based on evolving data, the nomenclature of these small piroplasms is likely to undergo revision.

It is diagnostically important to determine the species, subspecies, and genotype that causes canine babesiosis, since the virulence, prognosis, and response to antibabesial drugs may be different for each organism. We refer here to the Asian genotype of *B. gibsoni* (GenBank accession numbers AF271081, AF271082, AF205636, AF175300, and AF175301) as *B. gibsoni* (Asian genotype) (15, 16, 25), the North American genotype of *B. gibsoni* (AF158702, AF231350, and L13729) as *B. gibsoni* (USA/California genotype) (11, 15, 25), and the European small canine piroplasm (AF188001) as *Theileria annae*, as has been proposed by Zahler et al. (24). Both *B. gibsoni* (Asian genotype) and *B. gibsoni* (USA/California genotype) have been identified in dogs from North America, whereas *T. annae* has only been reported in Europe (9, 24). *B. gibsoni* (Asian genotype) is considered to be virulent in dogs and, to date, no antibabesial treatment has been able to eliminate the infection (22). *B. gibsoni* (USA/California genotype) is also virulent, but its susceptibility to antibabesial therapy has not been well characterized (21, 23). To our knowledge, comparative pathogenicities or responsiveness to antibabesial therapy for *T. annae* has not been studied.

There is support for the existence of three subspecies of large canine piroplasms—*B. canis* subsp. *vogeli*, *B. canis* subsp. *canis*, and *B. canis* subsp. *rossi*—based on genetic data, vector specificity, and variations in pathogenicity (10, 20, 26). *B. canis* subsp. *vogeli*, which is found in North America, Europe, and Asia, is considered to be a moderately virulent species, and it is presumed that antibabesial therapy will eliminate the infection. *B. canis* subsp. *canis*, which is mostly found in Europe, has somewhat variable virulence, and the organism is generally considered to respond to antibabesial drugs (10, 20, 26). *B. canis* subsp. *rossi*, which has only been identified in Africa, is considered to be a highly virulent piroplasm that may not be susceptible to the currently available drugs (10, 20, 26).

The definitive diagnosis of canine babesiosis, as well as the

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TABLE 1. Sequences for the oligonucleotide primers used in this study

Primer	Sequence (5′-3′)	Reaction and/or use
5-22F	GTTGATCCTGCCAGTAGT	Full-length 18S rRNA forward primer
1661R	AACCTTGTTACGACTTCTC	Full-length 18S rRNA reverse primer
455-479F	GTCTTGTAATTGGAATGATGGTGAC	Seminested PCR outer forward primer
793-772R	ATGCCCCCAACCGTTCCTATTA	Seminested PCR outer reverse primer
BgibAsia-F	ACTCGGCTACTTGCCTTGTC	Seminested PCR *B. gibsoni* (Asian genotype) specific forward primer
BCV-F	GTTCGAGTTTGCCATTCGTT	Seminested PCR *B. c. vogeli* specific forward primer
BCC-F	TGCGTTGACGGTTTGACC	Seminested PCR *B. c. canis* specific forward primer
BCR-F	GCTTGGCGGTTTGTTGC	Seminested PCR *B. c. rossi* specific forward primer
GAPDH-F	CCTTCATTGACCTCAACTACAT	Detection of PCR inhibitors
GAPDH-R	CCAAAGTTGTCATGGATGACC	Detection of PCR inhibitors

visual differentiation of the species of piroplasms, can be difficult for the clinician. The generally accepted "gold standard(s)" for ruling out babesiosis are splenectomy with or without immune suppression or blood transfusion from the suspect dog into a splenectomized dog (5). For obvious reasons, these procedures are rarely, if ever, performed in the clinical setting to achieve a diagnosis. Historically, light microscopic examination of stained blood smears and serology have provided the primary means of diagnosing babesiosis in dogs. Light microscopic examination cannot be used to establish the genotype of any piroplasm. Antibodies to *Babesia* spp. are often cross-reactive; therefore, serology may not definitively discriminate species or subspecies. In addition, there are reports of canine *Babesia* infections in which piroplasms were not identified by light microscopic examination and/or in which serologic testing yielded false-negative results in dogs that were infected with *Babesia* (4, 6, 17). Since the geographic range of specific piroplasms appears to be expanding, location should not be used as the sole criterion for species or subspecies identification. Although not without limitations, the PCR offers a practical and noninvasive means to detect and differentiate infections with various *Babesia* spp. and also provides a sensitive tool for assessing treatment outcomes. PCR is likely to be more sensitive than light microscopic examination of stained blood smears based on the reported limits of detection for each test (5). Since infected dogs may have antibodies that are unpredictably cross-reactive against other *Babesia* species or subspecies, PCR is more specific than serology. To our knowledge, there are no studies directly comparing all of the available diagnostic tests for babesiosis in a canine population in which the true disease prevalence is known. In the present study we sought to develop a PCR test for canine babesiosis that can detect and differentiate *B. gibsoni* (Asian genotype), *B. canis* subsp. *vogeli*, *B. canis* subsp. *canis*, and *B. canis* subsp. *rossi* and to define the test's limits of detection in canine blood samples.

MATERIALS AND METHODS

Samples. *B. gibsoni* (Asian genotype)-infected whole-blood samples were either obtained from a specific-pathogen-free splenectomized dog that was infected intravenously with blood from a dog that was confirmed to be infected with *B. gibsoni* (Asian genotype) or from dogs (n = 5) from North America that were confirmed to be infected with a small piroplasm. *B. canis* subsp. *vogeli*-infected canine whole-blood samples were obtained from dogs (n = 3) from North America that were confirmed via light microscopy to be infected with large piroplasms. A *B. canis* subsp. *canis*-infected canine whole-blood sample was obtained from a specific-pathogen-free splenectomized dog that was infected intravenously with blood from a dog that was confirmed to be infected with

B. canis subsp. *canis*. *B. canis* subsp. *rossi*-infected canine whole-blood samples, kindly provided by George Moore (U.S. Army Medical Department Center and School, Fort Sam Houston, Tex.), were obtained from dogs (n = 2) from South Africa that were confirmed via light microscopy to be infected with large piroplasms. *B. gibsoni* (California/USA genotype)-infected canine whole-blood samples were obtained from a specific-pathogen-free splenectomized dog that was infected intravenously with blood from a dog that was confirmed to be infected with *B. gibsoni* (California/USA genotype). The original *B. gibsoni* (California/USA genotype) isolate was kindly provided by Patricia Conrad (University of California, Davis). A *T. annae*-infected canine whole-blood sample from Spain, kindly provided by Sam Telford III (Harvard University, Boston, Mass.), was obtained from a dog confirmed via light microscopy to be infected with small piroplasms. *Toxoplasma gondii* and *Neospora caninum* DNAs were kindly provided by Nick Sharp (Animal Critical Care Group of Vancouver, Burnaby, British Columbia, Canada). DNA was also extracted from the anticoagulated whole blood of a dog that was naturally infected with *Leishmania infantum* (13). *Cryptosporidium parvum* DNA was kindly provided by Lance Perryman (Colorado State University, Fort Collins).

Primer design. Oligonucleotide primers were designed based on the canine *Babesia* 18S rRNA genes reported in GenBank (3). For the amplification of the nearly full-length *Babesia* 18S rRNA genes, primers (5-22F and 1661R) were designed to amplify *Babesia* 18S rRNA genes but not mammalian 18S rRNA genes.

For the seminested PCR, an outer primer pair (455-479F and 793-772R) was designed that would amplify an approximately ~340-bp fragment from *B. gibsoni* (Asian genotype) (AF271081, AF271082, AF205636, AF175300, and AF175301), *B. canis* subsp. *vogeli* (AJ009796 and AY072925), *B. canis* subsp. *canis* (AJ009795 and AY072926), and *B. canis* subsp. *rossi* (L19079) that spanned a hypervariable region of the 18S rRNA gene. Then, specific internal primers were designed for *B. gibsoni* (Asian genotype) (BgibAsia-F), *B. canis* subsp. *vogeli* (BCV-F), *B. canis* subsp. *canis* (BCC-F), and *B. canis* subsp. *rossi* (BCR-F) that were paired with the outer reverse primer in the seminested secondary reaction to amplify 185-, 192-, 198-, and 197-bp amplicons, respectively. The sequences of the oligonucleotide primers used in the present study are listed in Table 1. All primers were synthesized by Integrated DNA Technologies, Coralville, Iowa.

Preparation of DNA. DNA was isolated from canine whole-blood samples by using the QIAamp DNA Blood Mini-Kit according to the manufacturer's instructions. Plasmid DNA was isolated with the QIAprep Spin Miniprep according to the manufacturer's instructions.

PCR. The nearly full-length *B. gibsoni* (Asian genotype) and *B. canis* subsp. *canis* 18S rRNA genes were amplified by PCR. Amplification of the nearly full-length 18S rRNA genes was performed by using 25-μl reactions. Each 25-μl reaction contained a 1× concentration of PCR buffer II (Perkin-Elmer), 0.625 U of *Taq* polymerase, 0.5 μl of DNA template, 1.5 mM MgCl$_2$, 12.5 pmol of each primer, and a 200 μM concentration of each deoxynucleoside triphosphate. The cycling conditions were 95°C for 5 min, followed by 35 amplification cycles (95°C for 1 min, 56°C for 1 min, and 72°C for 1 min), and a final extension step at 72°C for 5 min (PCR ExPress; Thermo Hybaid, Middlesex, United Kingdom).

The PCR conditions were optimized for the annealing temperature (55 to 62°C by increments of ~1.4°C) and MgCl$_2$ concentration (1.0 to 5.0 mM by increments of 0.5 mM) by using the experimentally infected *B. gibsoni* (Asian genotype) and noninfected canine DNA as positive and negative controls, respectively. The optimal conditions were used with a 50-μl reaction volume containing 1.25 U of AmpliTaq Gold (Perkin-Elmer)/reaction, 25 pmol of each primer, 200 μM concentrations of each deoxynucleoside triphosphate, 1.5 mM MgCl, and a 1× concentration of PCR buffer II. DNA amplification with the

outer primer pair was performed in a thermal cycler (PCR ExPress) at the following temperatures: initial denaturation at 95°C for 5 min, followed by 50 amplification cycles (95°C for 45 s, 58°C for 45 s, and 72°C for 45 s), and a final extension step at 72°C for 5 min.

Seminested PCRs (i.e., specific forward primers paired with the outer reverse primer) were each carried out in separate tubes under the same conditions as the outer primer pair, except for the following: 0.5 μl from the initial reaction was used as a DNA template, and the reactions were amplified for 30 cycles. In order to prevent PCR amplicon contamination, sample preparation, reaction setup, PCR amplification, and amplicon detection were all performed in separate areas. Positive and negative controls were used in all processing steps, including the DNA extraction. The presence of PCR inhibitors in DNA samples that tested negative in our PCR test was excluded by the amplification of a fragment of the GAPDH (glyceraldehyde-3-phosphate dehydrogenase) gene. The presence of *T. annae* DNA in the sample from Spain was confirmed by amplification of the nearly full-length 18S rRNA gene as described above.

Amplicon detection. All PCR products were visualized after electrophoresis in a 2% agarose gel containing 0.2 μg of ethidium bromide/ml by transillumination with an UV light.

Cloning and sequencing of PCR products. The PCR products were cloned into a plasmid vector (PCR 2.1; Invitrogen, Carlsbad, Calif.), and *Escherichia coli* (TOP10′; Invitrogen) strain was transformed according to the protocol of the supplier. Recombinants were selected by the blue-white color of colonies, and plasmid DNA from at least three clones, for each isolate, were sequenced. Recombinant plasmid DNA was sequenced bidirectionally with the infrared fluorescence-labeled primers M13R-700 (5′-CAGGAAACAGCTATGACC ATG) and T7-800 (5′-TAATACGACTCACTATAGGGCGA) synthesized by LI-COR, Inc., Lincoln, Nebr. The previously described internal sequencing primers 515F and 1391R were also used for full-length 18S ribosomal DNA sequencing (18). The sequencing reaction conditions were as follows: 2 min at 92°C, followed by 30 amplification cycles (30 s at 92°C, 15 s at 55°C, and 30 s at 72°C) (Hybaid PCR ExPress). The sequencing reactions were analyzed by polyacrylamide gel electrophoresis (3.75%) on an automated DNA sequencer (i.e., the LI-COR 4200 DNA sequencer).

Limit of detection. The DNA concentrations of the purified plasmids containing nearly full-length *B. gibsoni* (Asian genotype) (sequence identical to AF271081) and *B. canis* subsp. *canis* (sequence identical to AY072926) 18S rRNA genes were determined by spectrophotometry (Biospec-Mini; Shimadzu Corp., Columbia, Md.). These plasmid clones were individually serially diluted 10-fold in Tris-EDTA buffer to concentrations ranging from 1,000,000 to 10 plasmids/ml. One microliter of each dilution was then used as a template for the primary PCR.

To determine the practical limit of detection of the seminested PCR, leukocyte-reduced packed red blood cells from *B. gibsoni* (Asian genotype) or whole blood from a *B. canis* subsp. *canis*-infected dog with parasitemias of known percentages were added to whole blood obtained from a noninfected dog. For the *B. gibsoni* (Asian genotype) mock-infected samples, 500 ml of whole blood was collected by sterile methods, using CPDA-1 as an anticoagulant, from the jugular vein of a splenectomized dog that had been experimentally infected with *B. gibsoni* (Asian genotype) via intravenous injection. The blood was leukoreduced by using a commercially available leukocyte reduction filter. (Purecell; Pall Corp., East Hills, N.Y.). The leukoreduced blood was centrifuged at 4,000 rpm, and the plasma was removed. The red blood cell count was determined in an automated cell counter, and a thin smear was stained with a modified Wright stain to determine the percent parasitemia. After leukoreduction and concentration, the red blood cell count was 12.5 × 10⁶ cell/μl with a parasitemia of 10% based on a 1,000-cell count. Then, 40 μl (5 × 10⁷ organisms) of these leukoreduced *B. gibsoni* (Asian genotype)-infected concentrated red blood cells was added to 960 μl of noninfected EDTA anticoagulated canine whole blood. These samples were then serially diluted 10-fold by using the noninfected canine whole blood. The red blood cell count of the noninfected dog was 6.8 × 10⁶ cells/μl. For the mock-infected *B. canis* subsp. *canis* samples, 3 ml of EDTA anticoagulated whole blood was collected via jugular venipuncture from a splenectomized dog that had been infected intravenously with *B. canis* subsp. *canis*. The *B. canis* subsp. *canis*-infected dog's red blood cell count was 5.7 × 10⁶ cell/μl with a parasitemia of 9% based on a 1,000-cell count. Next, 100 μl (5 × 10⁷ organisms) of these *B. canis* subsp. *canis*-infected red blood cells was added to 900 μl of noninfected EDTA anticoagulated canine whole blood. These samples were then 10-fold serially diluted by using the noninfected canine whole blood. The red blood cell count of the noninfected dog was 6.8 × 10⁶ cells/μl. The final test concentrations of both mock-infected samples ranged from 500,000 to 5 organisms/ml. Total DNA was extracted from 200-μl aliquots of these dilutions by
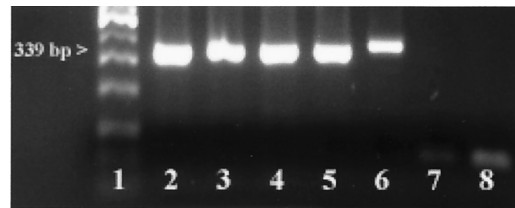

FIG. 1. Analysis of seminested PCR products (outer primer pair only) by 2% agarose gel electrophoresis and ethidium bromide staining. From left to right the lanes contain the following: molecular weight marker (lane 1), *B. gibsoni* (Asian genotype) (lane 2), *B. canis* subsp. *vogeli* (lane 3), *B. canis* subsp. *canis* (lane 4), *B. canis* subsp. *rossi* (lane 5), *B. gibsoni* (California/USA genotype) (lane 6), *T. annae* (lane 7), and negative (i.e., no-DNA) control (lane 8).

using a commercially available DNA extraction kit. A 5-μl portion of each dilution was used as a template for the primary PCR.

Specificity. Portions (5 μl) of DNA extracted from *T. gondii*-, *N. caninum*-, *C. parvum*-, and *L. infantum*-infected canine whole blood and a noninfected dog were used as templates for the PCRs.

DNA sequence analysis. The DNA sequences generated in the present study were aligned and compared to the DNA sequences in GenBank (3) by using a computer program. (MegAlign; DNASTAR, Inc., Madison, Wis.).

Animals. All work with live animals in the present study was conducted under the approval of and in accordance with the guidelines of the North Carolina State University Institute for Animal Care and Use Committee.

RESULTS

PCR. Nearly full-length (~1.7-kb) 18S rRNA genes were amplified from the experimentally infected *B. gibsoni* (Asian genotype), *B. canis* subsp. *canis* samples, and the naturally infected *T. annae* sample from Spain (data not shown).

During the primary reaction of the seminested PCR, an ~340-bp product was amplified from *B. gibsoni* (Asian genotype), *B. canis* subsp. *vogeli*, *B. canis* subsp. *canis*, or *B. canis* subsp. *rossi*-infected canine whole-blood samples. An ~370-bp product was amplified from the *B. gibsoni* (USA/California genotype)-infected canine whole-blood samples. No amplicons were detected when DNA from the *T. annae*-infected canine whole-blood sample was used as a template for the PCR (Fig. 1). The PCR tests did not produce amplicons when canine DNA or DNA extracted from *T. gondii*, *N. caninum*, or *C. parvum* was used as a template (data not shown).

During the secondary seminested reaction, the test was able to differentiate *B. gibsoni* (Asian genotype), *B. canis* subsp. *vogeli*, *B. canis* subsp. *canis*, and *B. canis* subsp. *rossi* when the specific internal primers were paired with the reverse primer in the secondary reactions (Fig. 2). Occasionally, after the second round of amplification both the ~340-bp and the 185- to 200-bp amplicons were visualized on the gel, but test interpretation was not affected (Fig. 2). An additional ~300-bp amplicon of unknown significance was also detectable after the secondary seminested PCRs. In both the primary and secondary reactions, low-molecular-weight bands (~25 to 50 bp) were often detected in both positive and negative samples, including the no-DNA controls. These low-molecular-weight bands were presumed to be "primer dimers."

Amplicon contamination was not detected in any of the negative control samples at any time. As determined by amplification of GAPDH, no PCR inhibitors were detected in any
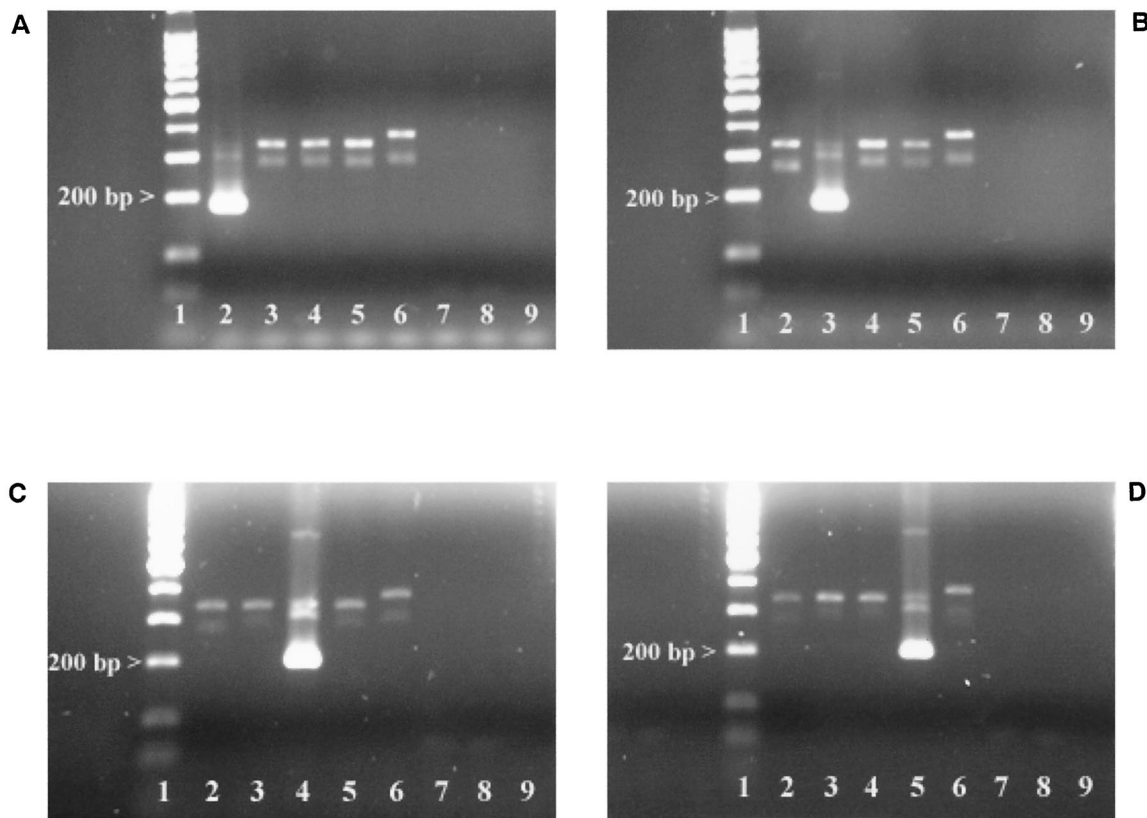
FIG. 2. Analysis of the specificity of the seminested PCR by 2% agarose gel electrophoresis and ethidium bromide staining.(A) Analysis of seminested *B. gibsoni* (Asian genotype)-specific secondary reactions; (B) analysis of seminested *B. canis* subsp. *vogeli*-specific secondary reactions; (C) analysis of seminested *B. canis* subsp. *canis* specific secondary reactions; (D) analysis of seminested *B. canis* subsp. *rossi*-specific secondary reactions. In panels A to D the lanes contain, from left to right, the following: molecular weight marker (lane 1), *B. gibsoni* (Asian genotype) (lane 2), *B. canis* subsp. *vogeli* (lane 3), *B. canis* subsp. *canis* (lane 4), *B. canis* subsp. *rossi* (lane 5), *B. gibsoni* (California/USA genotype) (lane 6), *T. annae* (lane 7), canine genomic DNA (lane 8), negative (i.e., no-DNA) control (lane 9).

of the DNA samples that were negative when our PCR test was used.

Limit of detection. The absolute limit of detection, with plasmid clones of nearly full-length *B. gibsoni* (Asian genotype) and *B. canis* subsp. *canis* 18S rRNA genes diluted in Tris-EDTA buffer, was one molecule/reaction (data not shown). The practical limit of detection of *B. gibsoni* (Asian genotype) and *B. canis* subsp. *canis* organisms diluted in noninfected canine whole blood was 50 organisms/ml. The second round of amplification with a specific internal primer paired with the reverse primer did not improve the limit of detection but did improve ease of interpretation due to the enhanced visualization of bands on the gel.

Sequencing. The sequences of the nearly full-length 18S rRNA gene clones for *B. gibsoni* (Asian genotype) and *B. canis* subsp. *canis* were 100% identical to GenBank accession numbers AF271081 and AY072926, respectively. The sequences of the ~340-bp amplicons from the North American dogs naturally infected with small piroplasms were identical to GenBank accession numbers AF271081, AF271082, AF205636, AF175300, and AF175301 (*B. gibsoni* Asian genotype). The sequences of the ~340-bp amplicons from the North American dogs naturally infected with large piroplasms were ≥99% identical to GenBank accession numbers AJ009795 and AY072926 (*B. ca-

nis* subsp. *vogeli*). The sequences of the ~340-bp amplicons from the dog experimentally infected with *B. canis* subsp. *canis* was identical to GenBank accession number AY072926 (*B. canis* subsp. *canis*). The sequences of the ~340-bp amplicons from the South African dogs naturally infected with large piroplasms were ≥99% identical to GenBank accession number L19079 (*B. canis* subsp. *rossi*). The sequence of the 369-bp amplicon from the dog experimentally infected with *B. gibsoni* (USA/California) was 98% identical to GenBank accession numbers AF158702 and AF231350 (*B. gibsoni* USA/California genotype). The sequence of the nearly full-length *T. annae* 18S rRNA amplicon was ≥99% identical to GenBank accession number AF188001 (*T. annae*).

DISCUSSION

For several reasons, a definitive diagnosis of canine babesiosis can be difficult to achieve in the clinical setting. Light microscopic examination cannot consistently differentiate species or subspecies. The lack of standardized serologic assays, the presence of cross-reactive antibodies, and recent changes in the geographic ranges of several canine piroplasms have also further complicated the diagnosis of babesiosis in dogs. In the present study, we describe a seminested PCR that has a prac-

tical limit of detection of 50 organisms/ml of canine whole blood. The PCR is specific for the diagnosis and differentiation of *B. gibsoni* (Asian genotype), *B. canis* subsp. *vogeli*, *B. canis* subsp. *canis*, and *B. canis* subsp. *rossi*. Based on the lowest number of detectable organisms/ml of whole blood, the limit of detection test is 180-fold lower than the reported limit of detection for a previously described PCR-based test for *B. gibsoni* (Asian genotype) (1). Unfortunately, the majority of reports describing PCR-based tests for the diagnosis of canine babesiosis have not reported a limit of detection or, in the case of one study (12), only reported the limit of detection in terms of the lowest detectable percent parasitemia (7, 10, 14–17, 26). Although the lowest detectable percent parasitemia is often reported for the detection of piroplasmosis, comparisons of tests based on the percent parasitemia can be difficult to interpret. For example, *Babesia*-infected animals can have red blood cell counts ranging from severely anemic to normal (1.5×10^6 to 7.6×10^6 red blood cells per μl); therefore, the degree of anemia could result in as much as a fivefold difference in the total number of parasites/volume when samples from different animals with identical percent parasitemias are examined. The lowest estimated percent parasitemia that was detectable by our test was 0.00000073%, which is about 1,300-fold lower than the accepted limit of detection (0.001% parasitemia) for light microscopic examination of stained blood smears (5). Despite the aforementioned difficulty in interpreting differences in test sensitivities based on the percent parasitemia, it seems unlikely that differences in red blood cell counts would account for a 1,300-fold difference.

The clinical sensitivity of the PCR could not be determined in the present study and to our knowledge has not been described for any other PCR tests for canine babesiosis. In order to determine the clinical sensitivity, a population of animals in which the true prevalence of infection is known (i.e., experimental infection) is required. The detection limit of our test is superior to or comparable to the tests that have been described for the detection of piroplasmosis in other species (2, 8, 19). A blinded study comparing PCR and serologic testing has been performed evaluating the clinical sensitivity of a PCR test in cattle experimentally infected with *B. bovis* (8). The PCR test used in that study had an absolute sensitivity that was similar to our PCR for canine babesiosis. That study demonstrated false-negative PCR tests in 30% of the samples, especially when the samples were obtained from chronically infected cattle with a low percent parasitemia. However, the clinical sensitivity was improved to >90% by retesting the cattle that had tested negative by PCR 10 to 14 days later. A similar clinical sensitivity would be expected for the PCR test described here.

The approach used to develop the outer primer pair of our seminested PCR should permit the detection of previously unrecognized and/or unsequenced piroplasms with genetic variation in this hypervariable region of the 18S rRNA gene. If the ~340-bp amplicon is detected but species-specific amplification is not possible with the internal primers, then the ~340 bp can be sequenced directly for genotype and species identification. Surprisingly, despite a 4-bp difference on the 3′ end of the forward primer compared to the *B. gibsoni* (USA/California genotype) (AF216496 and AF175300) rRNA gene sequences, an 18S rRNA gene amplicon was detected when a sample containing high numbers of parasites (30% para-

sitemia) was used as a template for the outer primer pair reaction. Despite an identical sequence in the primer regions, amplicons were not detected when a *T. annae* (AF188001)-infected sample with a lower percent parasitemia (0.01%) was used as a template. Since there were no detectable amplicons in the noninfected canine whole-blood samples under the testing conditions, we chose not to increase the annealing temperature to eliminate the mispriming of the *B. gibsoni* (USA/California genotype). Although the test might be useful for identifying some *B. gibsoni* (USA/California genotype)- or *T. annae*-infected samples in which there are large numbers of organisms, we do not recommend the use of this test due to the limited sensitivity for the routine diagnosis of *B. gibsoni* (USA/California genotype) or *T. annae*. In these instances, i.e., when a 370-bp amplicon is detected, we recommend that either specific tests for *B. gibsoni* (USA/California genotype) or *T. annae* be performed or that the amplicons be sequenced to confirm the genotype of the organism.

In conclusion, we describe here a PCR test for the diagnosis of canine babesiosis that can detect and differentiate *B. gibsoni* (Asian genotype), *B. canis* subsp. *vogeli*, *B. canis* subsp. *canis*, and *B. canis* subsp. *rossi* with a defined limit of detection. This test should improve the diagnostic capabilities for the detection and differentiation of canine *Babesia* spp. in clinical samples and facilitate future research studies that assess canine infection with these organisms.

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