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Frequency and therapy monitoring of canine *Babesia* spp. infection by high-resolution melting curve quantitative FRET-PCR

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ABSTRACT

Babesia gibsoni and *Babesia canis* are the etiological agents of canine babesiosis, a protozoal hemolytic disease with global significance. Canine babesiosis has been diagnosed by microscopic identification of intra-erythrocytic trophozoites in blood smear, and by serological testing. Here we developed a quantitative fluorescence resonance energy transfer (FRET)-PCR that amplifies a fragment of the *Babesia* spp. 18S rRNA gene with high sensitivity and specificity. Melting curve analysis differentiates *B. gibsoni*, *B. canis canis*/*B. canis vogeli*, and *B. canis rossi* by the disassociation temperature of the fluorescent probes. *Babesia gibsoni* infection was detected in 8 of 48 canine breeds (17%) and 24 of a total of 235 specimens (10.2%) submitted from 22 states of the continental United States of America. A potential blood donor was positive for *B. canis vogeli* infection. In Hong Kong (China), *B. gibsoni* infection was detected in 30 of 64 specimens (46.9%) from 15 of the 24 breeds (63%). While the frequency of canine babesiosis did not associate with seasonal change in Hong Kong, positivity in the USA for *Babesia* spp. infection was higher in Spring and Summer than in Autumn and Winter. The data suggest that environmental factors associated with tick vector exposure rather than genetic susceptibility determine the incidence of canine babesiosis. *Babesia* spp. burdens in blood declined significantly with increasing age of the infected dogs, and therapy with atovaquone and tilmicosin eliminated *B. gibsoni* while doxcycline and berenil did not. This demonstrates that high-resolution real-time PCR analysis may advance diagnosis and therapy monitoring of canine babesiosis.

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1. Introduction

Canine babesiosis is a worldwide, tick-borne, protozoal hemoparasitic disease caused by hemoprotozoan parasites of the genus *Babesia* (Homer et al., 2000). The two predominant species capable of naturally infecting dogs are *Babesia gibsoni* and *Babesia canis* (Boozer and Macintire, 2003). *Babesia gibsoni* is a small and pleomorphic organism, and *B. canis* is a large, piriform-shape organism that exists singly or paired within erythrocytes (Taboada and Lobetti, 2006). A trinomial nomenclature system for *B. canis* has been proposed as *B. canis canis*, *B. canis vogeli*, and *B. canis rossi* based on genetic, serologic, and immunological cross-reactivity studies (Uilenberg et al., 1989; Taboada and Lobetti, 2006; Irwin, 2009). Following the bite of an infected tick, *Babesia* spp. trophozoites are released into host circulation and infect erythrocytes (Homer et al., 2000). Naïve ticks attach to parasitemic dogs and become infected with *Babesia* spp. when they ingest a blood meal. Cases of canine babesiosis may present with a wide variety of clinical signs, ranging from a hyperacute, shock-associated, hemolytic crisis to inapparent and subclinical infection. The acute form of babesiosis is characterized by clinical signs such as pyrexia, weakness, mucous membrane pallor, depression, lymphadenopathy, splenomegaly, and general malaise (Homer et al., 2000; Boozer and Macintire, 2003).

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In the United States of America, *Babesia* spp. infections occur most often in American Pit Bull and American Staffordshire Terriers (Macintire et al., 2002; Taboada and Lobetti, 2006). Canine babesiosis is more prevalent in seasons and geographic regions with high prevalence of ticks and other arthropod vectors (Homer et al., 2000; Macintire et al., 2002). *Babesia* spp. infection is typically diagnosed by identifying the organisms in red blood cells on blood smears. Indirect fluorescent antibody and ELISA tests retrospectively diagnose babesiosis by detecting antibodies against *Babesia* spp. (Taboada and Lobetti, 2006). Organism numbers vary or are low in blood smears, and the host antibody response may require up to 10 days in order to exceed the detection limit. Serological testing usually does not differentiate the *Babesia* species. Therefore, nucleic acid detection and differentiation offer the potential to be the most sensitive and specific approach for detection and differentiation of *Babesia* spp. infections (Altay et al., 2008; Cardoso et al., 2008; Eiras et al., 2008; Solano-Gallego et al., 2008). Here, we describe a quantitative FRET-PCR for genus-specific detection of *Babesia* spp. with high sensitivity and specificity. This PCR detects as few as seven copies of *Babesia* spp. 18S rRNA gene per milliliter of whole blood, but does not amplify or detect any other closely related protozoal parasites or the canine host. Melting curve analysis after PCR conclusively differentiates infections by *B. gibsoni*, *B. canis canis*/*B. canis vogeli*, and *B. canis rossi*.

2. Materials and methods

2.1. Design of primers and probes

Due to the limited availability of other nucleotide sequences of *Babesia* spp., the 18S rRNA was chosen as the target for design of a diagnostic real-time PCR in this study. The *B. gibsoni* sequence (AB118032) was aligned against the canine genome (NW_878652) and 18S rRNAs of the following related protozoan species: *Hepatozoon americanum* (GenBank #AF176836), *Neospora caninum* (U17346), *Theileria parva* (L02366), *Eimeria arnyi* (AY613853), *Toxoplasma gondii* (L37415), *Cryptosporidium parvum* (AF115377), *Trypanosoma cruzi* (AF303659), and *Leishmania infantum* (AJ634364) (Fig. 1).

All oligonucleotides were designed by use of the Vector NTI software (Invitrogen Corporation, Carlsbad, CA, USA). The fluorescein probe was 3′ labeled with carboxyfluorescein (6-FAM) and used unpurified as FRET energy donor probe excited by 488 nm light. The LCRed 640 probe was 5′-labeled, 3′-phosphorylated, HPLC-purified and used as acceptor probe (Integrated DNA Technologies, Coralville, IA, USA).

2.2. Sample source and extraction of nucleic acids

Between 2006 and 2009, a total of 299 sterile EDTA-whole blood samples were submitted for *Babesia* spp.

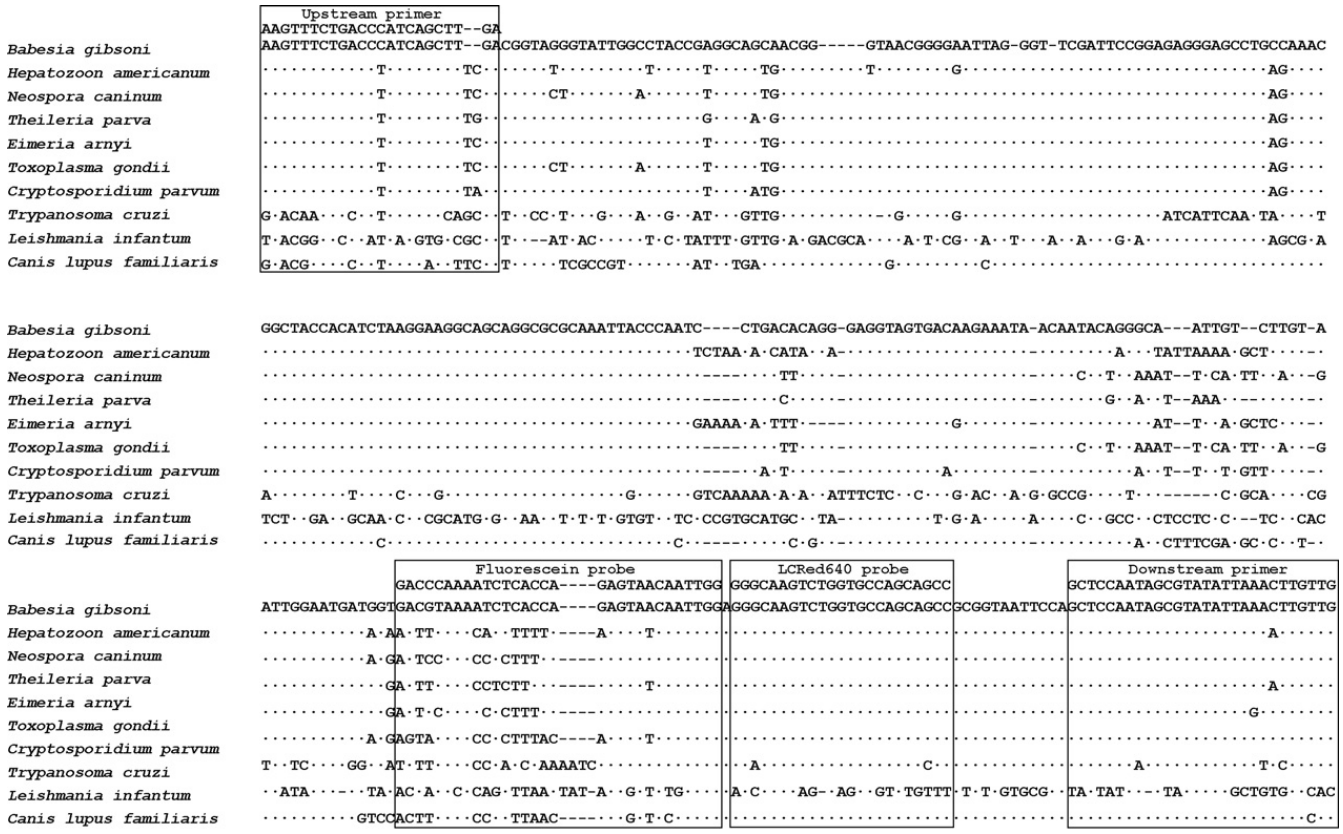

Fig. 1. Alignment of the 18S rRNA amplification target of *Babesia gibsoni* with homologous sequences of related protozoa and the canine host. Dots indicate nucleotides identical to *B. gibsoni*, and dashes represent nucleotide deletions. Primers and probes are shown by boxes. The upstream primer and probes are used as shown, while the downstream primer is used as antisense oligonucleotide. The upstream primer and the fluorescein probe were designed for maximum differences to the homologous sequences.

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Table 1
Summary of *Babesia* spp. 18S rRNA FRET-PCR assays.

Source	Total specimens	*Babesia* spp.positive	
		Number	Percent
USA			
Alabama	62	8	12.9
Missouri	62	1	1.6
Georgia	16	6	37.5
Virginia	14	0	0.0
Louisiana	12	3	25.0
Ohio	11	5	45.5
California	11	0	0.0
Others[a]	47	2	4.3
Total	235	25	10.6
Hong Kong	64	30	46.9
Total	299	55	18.4

[a] Specimens from the remaining 15 states (Arizona, Florida, Illinois, Indiana, Kentucky, Nebraska, New Mexico, New Hampshire, New York, Mississippi, Oregon, Pennsylvania, Tennessee, Texas, Washington) with less than 10 specimens or from unspecified location.

detection from 61 Veterinary Clinics/Hospitals of 22 states in the continental USA and from 2 Veterinary Hospitals in Hong Kong (China) to the Auburn University Molecular Diagnostics Laboratory (Table 1). These samples were shipped at room temperature without prior freezing or refrigeration.

Total nucleic acid extraction was performed as described before (DeGraves et al., 2003) by glass fiber matrix binding and elution with the High-Pure PCR Template Preparation Kit (Roche Molecular Biochemicals, Indianapolis, IN). For each specimen, 700 μl EDTA-whole blood was mixed with an equal volume of binding buffer [6 M guanidine-HCl, 10 mM urea, 20% (v/v) Triton X-100, 10 mM Tris–HCl, pH 4.4], and eluted in 40 μl elution buffer.

2.3. Real-time PCR and melting curve analysis

Nucleotide fragments representing partial 18S rRNA of *B. gibsoni*, *B. canis canis*/*B. canis vogeli*, and *B. canis rossi* were synthesized and inserted in the pIDTSMART cloning Vector (Integrated DNA Technologies, Coralville, IA, USA). The plasmid was linearized with *Hin*dIII (Promega, Madison, WI, USA), followed by inactivation of the restriction enzyme at 65 °C for 20 min. DNA was quantified by PicoGreen® DNA fluorescence assay (Molecular Probes, Eugene, OR, USA) for preparation of quantitative standards in a background of purified pGEM® plasmid DNA (Promega, Madison, WI, USA) in TE buffer.

The copy number of *Babesia* spp. genomes was determined by 18S rRNA FRET-PCR performed in a LightCycler® real-time platform with Software version 3.5 (Roche Molecular Biochemicals, Indianapolis, IN) as previously described (DeGraves et al., 2003; Wang et al., 2004). The amplification of single target molecules was verified by limiting dilution of the targets. At nominal single copies per PCR, a Poisson sampling error was observed because only about 70% of the PCRs became positive. In the positive PCRs, the linear portion of the amplification curve showed the same slope as PCRs with high template input. These data established that the PCR was equally effective at single copy input and verified the robust ability of the PCR to detect single target copies. The melting curve for the annealing of the PCR product was determined by monitoring the fluorescence from 50 to 80 °C with a temperature transition rate of 0.2 °C/s (Li et al., 2008). Data were analyzed as

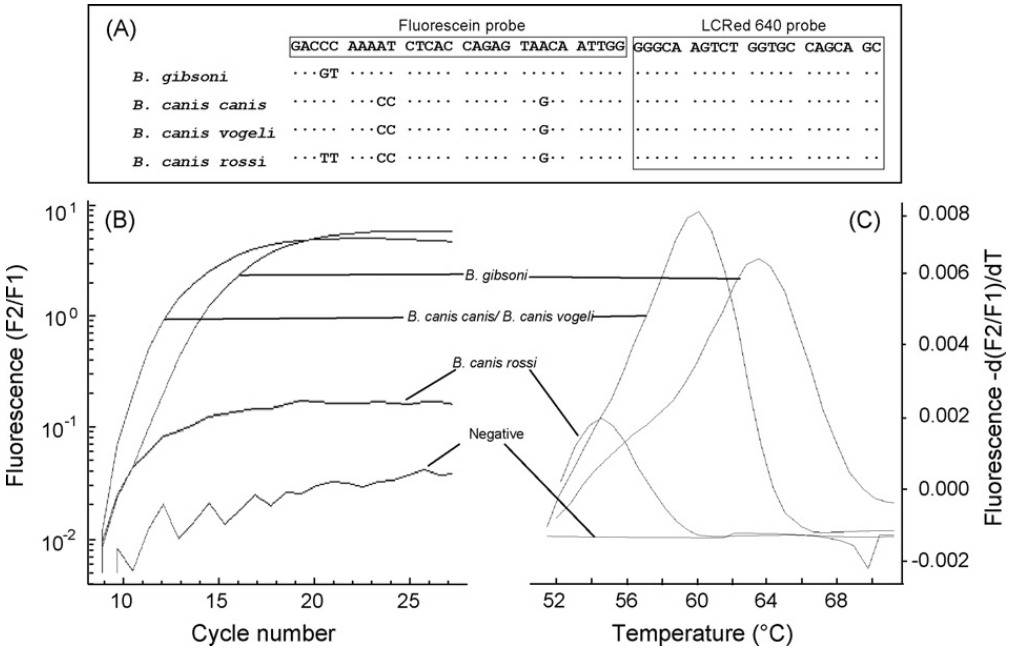

Fig. 2. Amplification and melting curves of the *Babesia* spp. FRET-PCR. (A) The LCRed 640 anchor probe and both primers (shown in Fig. 1) are identical to the sequences of all *Babesia* spp. strains. The fluorescein probe has two mismatches to *B. gibsoni*, three mismatches to *B. canis canis*/*B. canis vogeli*, and five mismatches to *B. canis rossi*. These mismatches are designed to maximize discrimination between sequences in the melting curve analysis. (B) Amplification curves of 1000 copies of *B. gibsoni*, *B. canis canis*/*B. canis vogeli*, and *B. canis rossi* are shown together with the negative control. (C) Melting curves show distinct T_m differences between *B. gibsoni* (~64 °C), *B. canis canis*/*B. canis vogeli* (~60 °C), and *B. canis rossi* (~54 °C).

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640 nm:530 nm (F2/F1) fluorescence ratios, and the first derivative of $F2/F1$ ($-d(F2/F1)/dt$) was evaluated for determination of probe melting temperature (T_m) (Fig. 2).

Specificity of the PCRs was confirmed by amplification and correct melting curves of the synthesized target genes of *B. gibsoni*, *B. canis canis*/*B. canis vogeli*, *B. canis rossi*, and a lack of amplification from extracted DNAs of the whole blood of healthy dogs, or from specimens positive for other related protozoan species such *H. americanum* and *T. gondii* (Fig. 1). PCR products were verified and isolated by 4% MetaPhor agarose gel electrophoresis, and purified for automated DNA sequencing with a QIAquick PCR Purification Kit (Qiagen, Valencia, CA). Nucleotide sequence determination was performed using an ABI automatic DNA sequencer (Model 377; Perkin-Elmer) at the Genomic Sequencing Laboratory (Auburn University, Auburn, AL, USA) using the described forward and antisense primers in Fig. 1.

2.4. Therapeutic intervention of B. gibsoni-infected dogs

Several *B. gibsoni*-infected dogs from Hong Kong received different therapeutic treatments. FRET-PCR was performed to quantify *B. gibsoni* 18S rRNA copies in the whole blood samples, and dynamically monitor the efficacy of therapeutic intervention.

2.5. Statistical analysis

All statistical analyses were performed with the Statistica 7.1 software package (StatSoft, Inc., Tulsa, OK, USA). *Babesia* spp. target numbers were logarithmically transformed. Categorical variables were analyzed by two-tailed Fisher's exact test and odds ratio (OR) analysis (van Belle, 2002). The correlation between animal age and the copy number of *Babesia* spp. targets was analyzed by a linear regression, and *Babesia* 18S rRNA copy numbers of dogs below or above 3 years of age were compared by Mann–Whitney *U*-test. Differences at *P* values ≤ 0.05 were considered significant.

3. Results

3.1. Establishment of Babesia spp. PCR

An optimized design of primers and probes is key to ensure the specificity and sensitivity of quantitative PCR (Kaltenboeck and Wang, 2005). While 18S rRNA is the only available and realistic target for the design of primers and probes in a *Babesia* spp. PCR, there are high similarities of the 18S rRNA sequences between *Babesia* spp. and other protozoan parasites (Fig. 1). Even the 18S rRNA sequences of *B. gibsoni* and the dog show approximately 72% similarity, and the differences result mainly from long indels.

We obtained all available 18S rRNA sequences for *Babesia* spp. in GenBank, and chose one representative of each allele for alignment within the species, and with the 18S rRNAs of other protozoan species and the canine host. This approach identified a target region that is highly conserved among *Babesia* spp. but substantially differs from homologous protozoan and canine sequences. The upstream primer was designed for identity to all *Babesia* spp., but for mismatches and/or deletions to other closely related sequences (Fig. 1). The LCRed 640 probe is identical to all *Babesia* spp., and the 30-base pair fluorescein probe shows 2-bp mismatches to *B. gibsoni*, 3-bp mismatches to *B. canis canis* (DQ297390, AY072926), 3-bp mismatches to *B. canis vogeli* (AY1500615, AY371196), and 5-bp mismatches to *B. canis rossi* (AB303075) (Figs. 1 and 2A). BLAST searches confirmed the specificity of the oligonucleotides (Altschul et al., 1990).

The PCR amplifies the synthesized targets corresponding to the *B. gibsoni*, *B. canis canis* or *B. canis vogeli*, and *B. canis rossi* sequences (Fig. 2B). The amplification curves for *B. gibsoni* and *B. canis canis*/*B. canis vogeli* are robust. *B. canis rossi* exhibits a relatively weak amplification signal due to five mismatches of the fluorescence probe to the *B. canis rossi* amplicon (Fig. 2A and B). Serial dilution of positive specimens showed positive and negative amplifications at multiple amplifications of the limiting dilution, and thus indicated a Poisson sampling error and the ability of the PCR to detect single target copies. The numbers of mismatches between the fluorescein probe and PCR amplicon result in distinct T_m differences, and melting curves, for *B. gibsoni* (~64 °C), *B. canis canis*/*B. canis vogeli* (~60 °C), and *B. canis rossi* (~54 °C) (Fig. 2A and C).

3.2. PCR diagnosis of canine babesiosis

Two hundred and ninety-nine canine EDTA-whole blood samples were submitted to the Auburn University Molecular Diagnostics Laboratory for the diagnosis of *Babesia* spp. infection between 2006 and 2009 (Table 1). All thirty positives of 64 samples (46.9%) submitted from Hong Kong were diagnosed as *B. gibsoni* (Table 1). A total of 235 samples were submitted from 22 states of the continental USA, and 25 of them (10.6%) were positive for *Babesia* spp. (Table 1). Out of the 22 states from which specimens were submitted, *B. gibsoni* positive specimens originated from 6 states including Alabama, Georgia, Louisiana, Ohio, Nebraska, and Tennessee (Table 1, Fig. 3). *B. canis vogeli* was detected in only one specimen of a potential Greyhound dog blood donor from Missouri. *Babesia canis rossi* was not detected in any specimen. Positivity for *Babesia* spp. infection in Hong Kong specimens (46.9%) was significantly higher than in the continental USA (10.6%) (OR = 6.732; $P < 10^{-4}$).

Amplification products from 31 *Babesia* spp. positive specimens were sequenced for confirmation of the complete target and of probe regions, and all showed nucleotide sequences identical to the reported *B. gibsoni* (AB118032) and *B. canis vogeli* (DQ297390, EF052633) sequences, respectively. The sequencing results confirmed the differentiation of *Babesia* spp. by melting curve analysis.

3.3. Association of Babesia spp. infection with breed, age, and season

Twenty-one of 52 canine breeds among the 299 submitted specimens showed *Babesia* spp. infection

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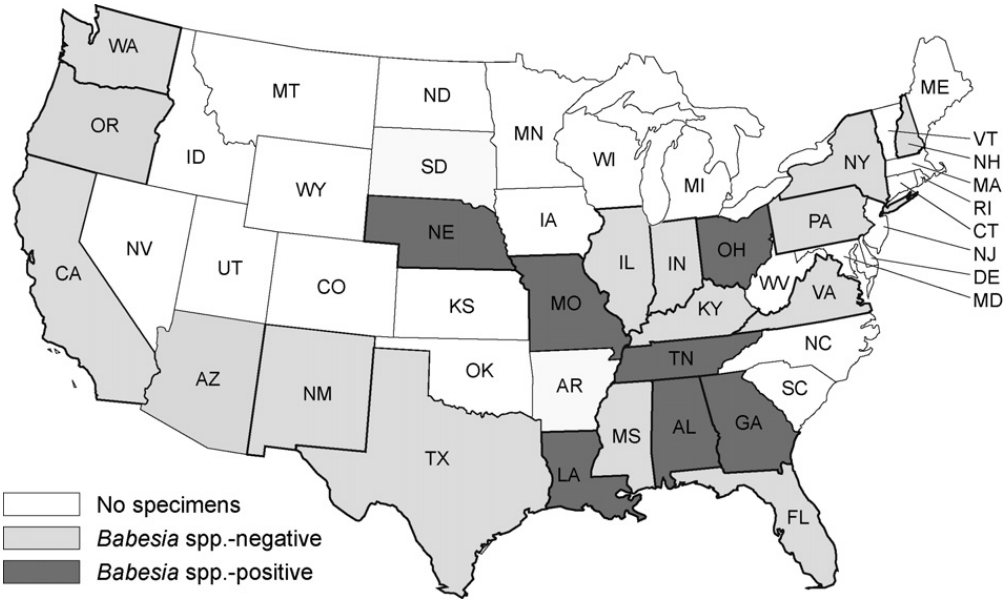

Fig. 3. *Babesia* spp. detection in the specimens from the continental United States of America. EDTA-whole blood samples from dogs were submitted from 22 states of the continental USA and from Hong Kong (China). *Babesia gibsoni* infections were detected in Alabama, Georgia, Louisiana, Nebraska, Ohio, and Tennessee, as well as in Hong Kong. *Babesia canis vogeli* infection was detected in one potential blood donor from Missouri.

(Tables 2A and 2B). Specimens submitted from the USA showed *Babesia* spp. infection in 9 of 48 canine breeds (Table 2A), and from Hong Kong in 15 of 24 breeds (Table 2B). In the USA, approximately 2/3 of all positive results (16/25; 64%) were found in just 16.2% of all submissions (38/235) derived from 8% of all 48 breeds submitted (Bull Terrier, Bulldog, American Staffordshire Terrier, Doberman Pinscher). In contrast, in Hong Kong the 64 percentile of positive specimens was derived from 63% of all 24 breeds submitted and represented 73% of all specimens (47/64). This suggests a breed predilection for *Babesia* spp. infection in the USA, but not in Hong Kong.

The percentage of *Babesia* spp. positive dogs was significantly lower in the USA than in Hong Kong (Table 1 and Fig. 4; $P < 10^{-4}$). The frequency of *Babesia* spp. infection in the USA was significantly lower in Autumn (0/28; 0%; $P = 0.01$ and 0.03) and Winter (9/124; 7.3%; $P = 0.03$ and 0.05) than in Spring (10/53; 18.9%) and Summer (6/37; 16.2%) (Fig. 4). In contrast, the frequency in Hong Kong did not change seasonally (Spring: 11/23 [47.8%]; Summer: 6/14 [42.9%]; Autumn: 9/13 [69.2%]; Winter: 8/16 [50.0%]).

Copy numbers of the target *Babesia* spp. 18S rRNA detected per milliliter whole blood varied from 14 to 4.3×10^7. For 19 specimens from the USA and 28 specimens

Table 2A
Breed distribution of *Babesia* spp. infection in the USA.

Canine breed[a,b]	Specimens from each breed	*Babesia* spp. positive	
		Number	Percent
Greyhound[c]	70	1	1
Mixed breed	18	2	11
Bull Terrier	13	7	54
Bulldog	11	5	45
Labrador Retriever	11	1	9
American Staffordshire Terrier	8	2	25
Doberman Pinscher	6	2	33
Beagle, Chihuahua, Golden Retriever, Old English Shepherd, Poodle, Rottweiler, Jack Russell Terrier	5–3	0	0

[a] Breeds are indicated as designated by the referring veterinarians.
[b] *Babesia* spp. was detected in 1 of 2 specimens from breed Boxer and 1 specimen from breed Cairn Terrier. One or 2 specimens with undetectable *Babesia* spp. were received from the following 32 canine breeds: Airedale Terrier, American Cocker Spaniel, Australia Terrier, Blue Tick Coonhound, Border Collie, Boston Terrier, Cavalier King Charles Spaniels, Chow Chow, Collie, Dachshund, Dalmatian, English Shepherd, English Spring, Fox Terrier, German Shepherd, German Shorthaired Pointer, Italian Greyhound, Japanese Chin, Maltese, Mastiff, Neapolitan Mastiff, Papillon, Pekingese, Pointer, Pomeranian, Pug, Saluki, Schipperke, Shetland Sheepdog, Shih Tzu, Siberian Husky, Weimaraner.
[c] Sixty-two of 70 Greyhound submissions represented potential blood donors, and 1 submission was positive for *B. canis vogeli*.

Table 2B
Breed distribution of *Babesia* spp. infection in Hong Kong.

Canine breed	Specimens from each breed	*Babesia* spp. positive	
		Number	Percent
Golden Retriever	10	6	60
German Shepherd	7	5	71
Pekingese	5	2	40
Hungarian Sheepdog	4	4	100
Schnauzer	4	2	50
Mixed Breed, Pomeranian	3	1	33
English Cocker Spaniel, Labrador Retriever	2	2	100
Siberian Husky	2	1	50
Beagle, Collie, Poodle, Shih Tzu, Welsh Corgi	1	1	100
Affenpinscher, Bichon Frise, Chow Chow, Dachshund, Keeshond, Maltese, Pug, Shetland Sheepdog, Yorkshire Terrier	1–2	0	0

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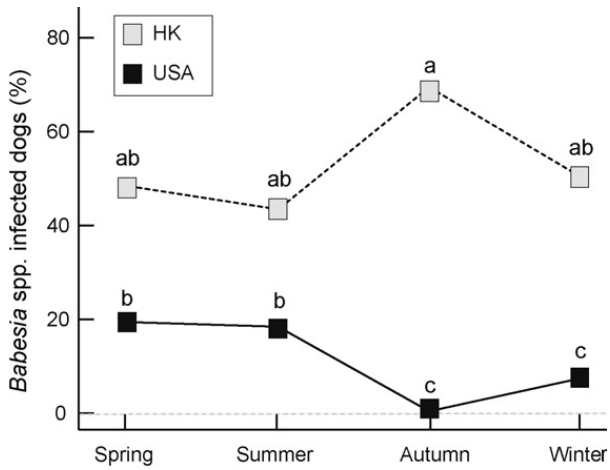

Fig. 4. Association of *Babesia* spp. positivity with season. Percentages of *Babesia* spp. positive animals in Hong Kong were significantly higher than in the USA. The frequency of *Babesia* spp. detection in the USA in Autumn and Winter was significantly lower than in Spring and Summer. Different letters indicate significant differences (two-tailed Fisher's exact test), and data points marked as ab do not differ significantly from data points either marked a or b.

from Hong Kong the age of the animal was known, and significantly correlated negatively with copy number ($r^2 = 0.14$, $P = 0.01$) (Fig. 5). Twenty-two dogs of 3 years of age or younger averaged 1.7×10^5 target copies/ml whole blood, significantly higher than the 1.5×10^4 target copies of the 25 dogs older than 3 years ($P = 0.009$).

From several dogs, multiple specimens were submitted for quantification of *Babesia* spp. infectious burden, to monitor the efficacy of therapeutic intervention. The results showed clearly that efficient therapy eliminated *B. gibsoni*, but only slowly (Fig. 6). For example, dog Nina exhibited a 6651-fold reduction of *B. gibsoni* 18S rRNA copy numbers (199,526 → 30) 3 weeks after treatment with atovaquone, later combined with azithromycin (Fig. 6). The dog Alto, with chronic babesiosis, required 9 weeks for a similar reduction of the *B. gibsoni* burden under therapy with

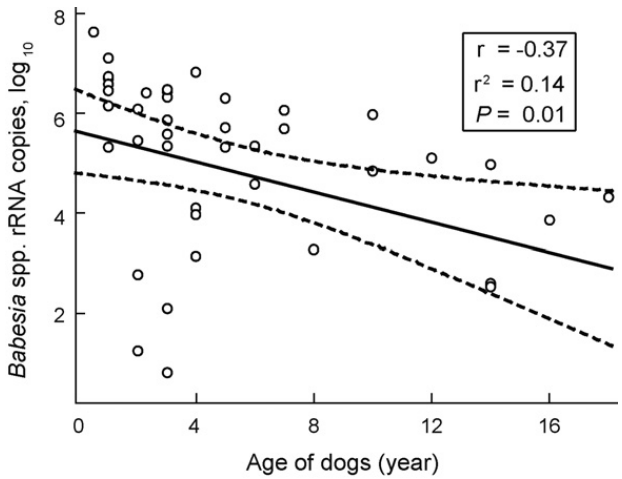

Fig. 5. Animal age correlates negatively to *Babesia* spp. target numbers. In *Babesia* spp. infected dogs, age correlates significantly and negatively with the logarithm of the *Babesia* spp. 18S rRNA copy number per milliliter blood. Each circle represents one dog (19 dogs from the USA and 28 from Hong Kong).

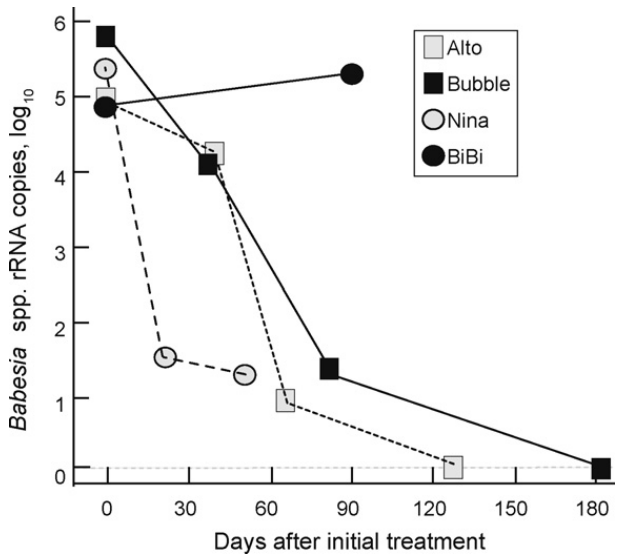

Fig. 6. Change of *B. gibsoni* 18S rRNA copy numbers after therapeutic intervention. Over a period of 21–180 days, therapy with atovaquone (hydroxy-1,4-naphthoquinone) and azithromycin or tilmicosin substantially reduced the target copy numbers of *Babesia* 18S rRNA in the dogs Alto (97,000 → 21,000 → 10 → 0), Bubble (684,420 → 19,800 → 20 → 0), Nina (199,526 → 30 → 20), but administration of doxcycline and berenil (diminazine) increased them in dog BiBi (97,006 → 174,860).

tilmicosin followed by atovaquone and azithromycin (Fig. 6). Another dog, BiBi, showed liver and splenic enlargement and anemia. In this dog, however, the *B. gibsoni* copy number increased from 97,006 to 174,860 after 3 months of therapy attempts by doxcycline and dibenzamidine diaceturate (berenil) parenteral administration (Fig. 6).

4. Discussion

Few gene sequences are available for *Babesia* spp. that can serve as a target for PCR detection. Only complete or partial sequences of the 18S rRNA gene of numerous isolates or of amplification products of canine *Babesia* are available. Amplification of 18S rRNA of protozoal parasites requires careful design of primers and probes since the canine host 18S rRNA shares long regions of identical sequences with these parasites, due to the extreme evolutionary conservation of 18S rRNA. We initially identified all *Babesia* spp. 18S rRNA sequences of more than 750 nucleotide length and aligned them among themselves and with related protozoa and canine 18S rRNA. This approach identified regions conserved among *Babesia* spp. but different from other protozoa and the canine sequence (Fig. 1). These regions served as template to search for all, including short, *Babesia* spp. 18S rRNA sequences. Only sequences with 80% or more overall identity to the template were retained while sequences that were less similar were excluded (e.g., *B. gibsoni* GenBank sequence #EU084671 is only 44% identical to *B. gibsoni* #AB118032). Typically, 16S and 18S rRNA genes show less than 5% interspecies heterogeneity (Maden et al., 1987; Clarridge, 2004; Altay et al., 2008; Bhoora et al., 2009), and we therefore considered sequences with less than 80% identity not as derived from *Babesia* spp. The retained *Babesia* spp. sequences were aligned in order to identify

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conserved as well as polymorphic regions of the 18S rRNA gene of *Babesia* spp. that also differed substantially from other protozoa. The conserved regions were used for design of *Babesia* genus-specific primers and the LightCycler Red 640-labeled anchor probe, while the polymorphic regions were used for design of the fluorescein-labeled probe. This approach allowed the amplification and detection of the 18S rRNA of all *Babesia* organisms, but also differentiation due to mismatches of the fluorescein probe that differed for each species/subspecies resulting in different probe melting temperatures (Fig. 2). Furthermore, the substantial differences ($\geq$8 mismatches; Fig. 1) in the fluorescein probe region also prevented detection of any other protozoal genus.

The availability of clinical specimens from multiple dog breeds, continuously submitted over the course of 3 years, from multiple geographic regions within the USA and Hong Kong, permitted the analysis of the influence of breed and geographic origin on the frequency of canine babesiosis. In all but one of the positive specimens from both the USA and Hong Kong, the identified species was *B. gibsoni*. In one blood sample from a healthy dog in Missouri that was screened as a blood donor, a copy number of *B. canis vogeli* targets was identified (33,600/ml blood) that is typically associated with acute infection (Table 2A). Transfusion-associated canine *B. gibsoni* infection has been reported (Stegeman et al., 2003), and we report here for the first time the detection of *B. canis vogeli* from a potential blood donor. Since most *B. canis vogeli*-infected dogs are subclinical carriers (Taboada et al., 1992), the finding of *Babesia* infection in a potential blood donor dog highlights the importance of screening for this infection in blood donors regardless of the dogs' physical appearance, complete blood count results, or their geographic origin. The overwhelming preponderance of *B. gibsoni*, particularly in diseased dogs, and the lack of clinical disease in the *B. canis vogeli*-infected dog confirm that *Babesia* disease in dogs is largely caused by *B. gisoni* in the USA and in Hong Kong.

In the continental USA, *Babesia* spp. infection occurred mostly in Southeastern states (Table 1 and Fig. 3), and more often in Spring and Summer than in Autumn and Winter (Fig. 4). This regional and seasonal distribution is consistent with the concept that the tick vector population has a pivotal importance in the transmission of canine *Babesia* spp. infection. The recognized tick vectors for canine babesiosis include *Ixodes hexagonus*, *Rhipicephalus sanguineus*, *Dermacentor reticulates*, and *Haemaphysalis* (Taboada and Lobetti, 2006). No seasonal variation of the frequency of *Babesia* spp. infection was observed in the samples from Hong Kong (Fig. 4), a geographic region with subtropical climate, little annual temperature fluctuation, and no freezing temperatures in Winter. Again, these results are consistent with the understanding that a consistently high vector population favors the transmission of *Babesia* spp. infection.

In the USA, 64.0% of all *Babesia* spp. positive specimens were from just 4 canine breeds that represented 16.2% of all specimens submitted from a total of 48 breeds (Table 2A). In contrast, in samples from Hong Kong, no single breed dominated in positive submissions, and 63% of all breeds from which samples were received represented 73.4% of all submitted specimens and 64% of *Babesia* spp. positive specimens in Hong Kong (Table 2B). These vastly different breed distribution patterns of *Babesia* spp. positivity strongly argue against a dominant host genetic component in susceptibility to canine babesiosis, and favor a strong environmental influence. The dominant positive breeds in the USA, such as Bull Terrier, Bulldog, American Staffordshire Terrier, Doberman Pinscher, are typical outdoor dogs and may experience a higher tick exposure than other breeds in the USA. In Hong Kong, canine crowding in the few available green spaces, combined with year-round high tick populations, may favor uniform exposure for all dogs and result in the very high and uniform frequency of canine babesiosis in Hong Kong (Gubler et al., 2001; Taboada and Lobetti, 2006; Zákovská et al., 2007).

Babesia spp. burdens in blood declined significantly with increasing age of the infected dogs (Fig. 5). While only one of 10 submissions from dogs below 1 year of age old was positive for *B. gibsoni* infection, this sample exhibited the highest target copy numbers (42,657,951 per 1 ml whole blood) among all specimens (Fig. 5). Compared to dogs more than 3 years of age, dogs below 3 years of age showed 10-fold higher target copies. It is unclear if this lower intensity of infection is the result of an age-related differential susceptibility or of partial immune protection built up during previous exposure.

Determination of infectious burden proved particularly helpful for the evaluation of therapeutic approaches against clinically inapparent chronic infections with *Babesia* spp. While obtained from clinical samples rather than a specifically designed investigation, data suggested that administration of atovaquone combined with azithromycin is an effective, albeit relatively slow-acting, therapy to eliminate *B. gibsoni* (Fig. 6). In contrast, therapy with doxcycline and berenil failed to reduce the parasite burden and may be ineffective. The reasons for these differences in apparent therapeutic efficacy, if they exist, are unclear and need to be investigated further in controlled experiments.

In conclusion, this study has established a FRET-PCR with high sensitivity and specificity for quantitative detection of *Babesia* spp. in dogs. Melting curve analysis following amplification and real-time detection conclusively differentiates canine *Babesia* species and subspecies. The data on canine babesiosis reported by use of this novel diagnostic method conform with previous studies. The rapid reporting of the results within a day combined with quantitative diagnosis of relevant *Babesia* species may advance therapy and prognosis of canine babesiosis.

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References

Altay, K., Aydin, M.F., Dumanli, N., Aktas, M., 2008. Molecular detection of *Theileria* and *Babesia* infections in cattle. Vet. Parasitol. 158, 295–301.

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Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J., 1990. Basic local alignment search tool. J. Mol. Biol. 215, 403–410.

Bhoora, R., Franssen, L., Oosthuizen, M.C., Guthrie, A.J., Zweygarth, E., Penzhorn, B.L., Jongejan, F., Collins, N.E., 2009. Sequence heterogeneity in the 18S rRNA gene within *Theileria equi* and *Babesia caballi* from horses in South Africa. Vet. Parasitol. 159, 112–120.

Boozer, A.L., Macintire, D.K., 2003. Canine babesiosis. Vet. Clin. North Am. Small Anim. Pract. 33, 885–904.

Cardoso, L., Costa, A., Tuna, J., Vieira, L., Eyal, O., Yisaschar-Mekuzas, Y., Baneth, G., 2008. *Babesia canis canis* and *Babesia canis vogeli* infections in dogs from northern Portugal. Vet. Parasitol. 156, 199–204.

Clarridge III, J.E., 2004. Impact of 16S rRNA gene sequence analysis for identification of bacteria on clinical microbiology and infectious diseases. Clin. Microbiol. Rev. 17, 840–862.

DeGraves, F.J., Gao, D., Kaltenboeck, B., 2003. High-sensitivity quantitative PCR platform. Biotechniques 34, 106–110 112–115.

Eiras, D.F., Basabe, J., Mesplet, M., Schnittger, L., 2008. First molecular characterization of *Babesia vogeli* in two naturally infected dogs of Buenos Aires, Argentina. Vet. Parasitol. 157, 294–298.

Gubler, D.J., Reiter, P., Ebi, K.L., Yap, W., Nasci, R., Patz, J.A., 2001. Climate variability and change in the United States: potential impacts on vector- and rodent-borne diseases. Environ. Health Perspect. 109 (Suppl. 2), 223–233.

Homer, M.J., Aguilar-Delfin, I., Telford III, S.R., Krause, P.J., Persing, D.H., 2000. Babesiosis. Clin. Microbiol. Rev. 13, 451–469.

Irwin, P.J., 2009. Canine babesiosis: from molecular taxonomy to control. Parasit. Vectors 2 (S4), 1–9.

Kaltenboeck, B., Wang, C., 2005. Advances in real-time PCR: application to clinical laboratory diagnostics. Adv. Clin. Chem. 40, 219–259.

Li, Y., Wang, C., Allen, K.E., Little, S.E., Ahluwalia, S.K., Gao, D., Macintire, D.K., Blagburn, B.L., Kaltenboeck, B., 2008. Diagnosis of canine *Hepatozoon* spp. infection by quantitative PCR. Vet. Parasitol. 157, 50–58.

Macintire, D.K., Boudreaux, M.K., West, G.D., Bourne, C., Wright, J.C., Conrad, P.A., 2002. *Babesia gibsoni* infection among dogs in the southeastern United States. J. Am. Vet. Med. Assoc. 220, 325–329.

Maden, B.E., Dent, C.L., Farrell, T.E., Garde, J., McCallum, F.S., Wakeman, J.A., 1987. Clones of human ribosomal DNA containing the complete 18 S-rRNA and 28 S-rRNA genes. Characterization, a detailed map of the human ribosomal transcription unit and diversity among clones. Biochem. J. 246, 519–527.

Solano-Gallego, L., Trotta, M., Carli, E., Carcy, B., Caldin, M., Furlanello, T., 2008. *Babesia canis canis* and *Babesia canis vogeli* clinicopathological findings and DNA detection by means of PCR-RFLP in blood from Italian dogs suspected of tick-borne disease. Vet. Parasitol. 157, 211–221.

Stegeman, J.R., Birkenheuer, A.J., Kruger, J.M., Breitschwerdt, E.B., 2003. Transfusion-associated *Babesia gibsoni* infection in a dog. J. Am. Vet. Med. Assoc. 222, 959–963 952.

Taboada, J., Harvey, J.W., Levy, M.G., Breitschwerdt, E.B., 1992. Seroprevalence of babesiosis in greyhounds in Florida. J. Am. Vet. Med. Assoc. 200, 47–50.

Taboada, J., Lobetti, R., 2006. Babesiosis. In: Greene, C.E. (Ed.), Infectious Diseases of the Dog and Cat. Elsevier, St. Louis, Missouri, USA, pp. 722–736.

Uilenberg, G., Franssen, F.F.J., Perie, M., Spanjer, A.M.M., 1989. Three groups of *Babesia canis* distinguished and a proposal for nomenclature. Vet. Q. 11, 33–40.

van Belle, G., 2002. Statistical Rules of Thumb. John-Wiley & Sons, Inc., New York, NY.

Wang, C., Gao, D., Vaglenov, A., Kaltenboeck, B., 2004. One-step real-time duplex reverse transcription PCRs simultaneously quantify analyte and housekeeping gene mRNAs. Biotechniques 36, 508–516 518–519.

Zákovská, A., Netusil, J., Martiníková, H., 2007. Influence of environmental factors on the occurrence of *Ixodes ricinus* ticks in the urban locality of Brno-Pisárky, Czech Republic. J. Vector Ecol. 32, 29–33.