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Diagnosis of canine *Hepatozoon* spp. infection by quantitative PCR

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Abstract

Hepatozoon (*H.*) *americanum* and *H. canis* are the etiological agents of canine hepatozoonosis, a disease that is found worldwide and is also prevalent in the southeastern United States. Current laboratory diagnosis of canine hepatozoonosis caused by *H. americanum* is usually dependent on visual identification of *Hepatozoon* "onion skin cysts" in muscle biopsies, an approach that requires invasive sampling and can result in false negatives. We have developed a diagnostic method for detection of *Hepatozoon* spp. DNA that integrates nucleic acid extraction with extensive agitation to maximize DNA extraction efficiency. The DNA extracted from canine EDTA-whole blood is subjected to real-time PCR, and fluorescence resonance energy transfer (FRET) probes detect a signature polymorphism in the amplified DNA. This PCR method amplifies a fragment of the *Hepatozoon* 18S rDNA gene, detects as few as 7 genomic copies of *Hepatozoon* spp. per ml of blood with high specificity, and differentiates between *H. americanum* and *H. canis* amplicons. A surprising 300-fold increase of *H. americanum* 18S rDNA targets occurred during 3–0 days of storage of positive blood specimens. Examination of 614 EDTA-blood samples submitted mostly from the southeastern Unites States from dogs with suspected hepatozoonosis identified *H. americanum* in 167 samples (27.2%). An additional 14 samples (2.3%) were positive for *H. canis*, and 14 samples (2.3%) were positive for both *H. americanum* and *H. canis*. These results suggest that the *Hepatozoon* spp. 18S rDNA quantitative PCR may be a valuable tool that can improve diagnosis and therapy of canine hepatozoonosis.

Keywords: American canine Hepatozoonosis; *Hepatozoon americanum*; *H. canis*; Real-time PCR; Blood; Detection; Diagnosis

1. Introduction

Hepatozoon americanum has been established as the etiological agent of American canine hepatozoonosis (Vincent-Johnson et al., 1997). It is an apicomplexan parasite transmitted by the Gulf Coast tick *Amblyomma maculatum*. Dogs are infected by ingestion of the tick, and are thought to be accidental hosts (Ewing and Panciera, 2003). *H. americanum* infection causes high fever, cachexia, depression, muscle atrophy, anemia, etc., and can be debilitating, or even fatal. After entering the digestive tract of the dogs, *H. americanum* releases sporozoites. The sporozoites migrate via blood or lymphatic vessels to the skeletal muscle, and form the

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typical ‘‘onion skin cysts’’. *H. americanum* then undergoes merogony, and the merozoites released after the matured meronts rupture can induce a pyogranulomatous inflammatory response (Greene, 2006).

H. canis is the primary cause of canine hepatozoonosis (Baneth et al., 2007), and has been found in Asia, Europe, Africa and South America (Eiras et al., 2007). It is different from *H. americanum* and has distinct morphologic features, definitive vector, target organs, and causes less severe disease (Ewing and Panciera, 2003). Recently, Allen et al. (2008) have described the detection of *H. canis* or *H. canis*-like apicomplexan parasites in dogs in the southeastern United States.

The standard diagnosis of canine hepatozoonosis is accomplished by the identification of gamonts in blood smears or of ‘‘onion skin’’ cysts in muscle biopsies (Ewing and Panciera, 2003; Greene, 2006; Karagenc et al., 2006). Mathew et al. (2001) developed an indirect ELISA for diagnosis of American canine hepatozoonosis but this assay is not currently available. Some of these methods do not give highly sensitive and specific results, hence are not capable of providing reliable diagnosis, while others, such as muscle biopsy, require invasive sampling. The fact that *Hepatozoon* spp. gamonts circulate in blood of infected dogs prompted us to attempt PCR diagnosis of canine *Hepatozoon* spp. infection. Several PCR tests for detection of the 18S rDNA gene of *Hepatozoon* spp. have been described (Inokuma et al., 2002; Criado-Fornelio et al., 2007). However, precise data on the detection limits, sensitivity, and specificity of these methods are not available. An important consideration is also that *Hepatozoon* spp. organisms at certain stages in their development may be mechanically resistant (Panciera et al., 2001) which may prevent complete disruption of, and DNA release from *Hepatozoon* cells, thus decreasing detection sensitivity. A possible solution to this problem has been pointed out by Kuske et al. (1998) in their use of a bead mill homogenization step to maximize release of DNA from soil samples.

In this investigation, we developed an integrated nucleic acid isolation and qPCR platform that preserves and efficiently recovers *Hepatozoon* spp. DNA in guanidinium stabilization buffer, enhances the release of *Hepatozoon* spp. DNA by vigorous agitation, binds DNA to a matrix of glass fiber fleece, and elutes sample DNA into a low volume of buffer. A robust real-time PCR format for amplification of the *Hepatozoon* spp. 18S rDNA gene was coupled to detection by fluorescence resonance energy transfer (FRET) oligonucleotide probes. The combined method reveals single target nucleic acid copies in a PCR sample derived from an aliquot of ~140 μl canine blood while maintaining essentially 100% specificity and allowing differentiation of the species found in dogs. Application of this method has shown a surprisingly high prevalence of canine *Hepatozoon* spp. infection, and infection with both *H. americanum* and *H. canis* species of dogs in the United States.

2. Materials and methods

2.1. Design of the real-time PCR

The following 18S rDNA sequences from *Hepatozoon* spp. and related species were obtained from the GenBank: AF176836, AY150067, U17346, L37415, AF115377, AY679105, L19079, AY278443, NR_003286. The sequences were aligned with the ClustalW multiple alignment algorithm (Thompson et al., 1994) as implemented in the Vector NTI 10.1 software package (Invitrogen, Carlsbad, CA). Regions for primers and probes were chosen based on the conserved and variable areas of the alignment. The final sequences are shown in Fig. 1.

2.2. Nucleic acid extraction

Total nucleic acids were extracted from EDTA-whole blood samples by use of the High-Pure PCR Template Preparation Kit (Roche Molecular Biochemicals, Indianapolis, IN) as previously published (DeGraves et al., 2003). For optimization of nucleic acid extraction, blood samples were stored for 0, 3, 6, or 10 days at 4, 25, or 37 °C. After storage, 700 μl aliquots of blood samples were mixed with equal volumes of binding buffer (6 M guanidine–HCl, 10 mM urea, 20% (v/v) Triton X-100, 10 mM Tris–HCl, pH 4.4). Nucleic acid extraction by the Standard protocol followed the procedure described by DeGraves et al. (2003) and was used for all clinical specimens and as control during optimization of the extraction protocol.

For the Bead extraction protocol, samples were mixed with 0.3 g of heparin-washed 0.1 mm zirconia/silica beads (BioSpec Products, Inc., Bartlesville, OK) and shaken for 5 min at maximum setting in the Mini-Beadbeater (BioSpec Products, Inc., Bartlesville, OK). Subsequent extraction followed the protocol described by DeGraves et al. (2003). For washing, beads were suspended in binding buffer containing 500 USP units of heparin sodium/ml (American Pharmaceutical Partners, Inc., Schaumburg, IL). Heparin is negatively charged and is thought to act as blocking agent that

prevents binding of nucleic acids to the zirconia/silica beads (Gadgil and Jarrett, 1999).

For the Optimized extraction protocol, the 1.4 ml combined blood/binding buffer samples were split into two 0.7 ml aliquots and shaken for 5 min at maximum setting in the Mini-Beadbeater (BioSpec Products, Inc., Bartlesville, OK). Subsequent extraction followed the protocol described by DeGraves et al. (2003).

2.3. Real-time PCR

All qPCRs were performed as previously described for the *Chlamydia* 23S rRNA FRET-PCR (DeGraves et al., 2003). Data were analyzed as 640 nm:530 nm (F2/F1) fluorescence ratios (Fig. 2A). For melting curve analyses, the first derivative of F2/F1 (-d(F2/F1)/dt) was evaluated (Fig. 2B). For use as quantitative standards, *H. americanum* DNA was extracted from the blood of positive animals by the High Pure method, and amplified with TTP replacing dUTP. The amplification product was gel purified by use of the QIAquick Gel Extraction Kit (Qiagen, Valencia, CA), quantified by the PicoGreen DNA fluorescence assay (Molecular Probes, Eugene, OR), and the DNA sequence verified by dideoxy-fluorescent automated PCR cycle sequencing. The purified amplicon was diluted at 10,000, 1000, 100, or 10, genome copies per PCR in a background of 100 ng purified salmon sperm DNA in $T_{10}E_{0.1}$ buffer, and used as quantitative standard along with negative buffer and extraction controls (Fig. 2A). Absolute sensitivity of the PCR was established by limiting dilution assay of the positive control and of positive specimens. Specificity of the PCR was confirmed by the lack of amplification from specimens positive for *Toxoplasma gondii*, *Cryptosporidium parvum*, *Babesia gibsoni*, or from negative control samples, but amplification from confirmed *Hepatozoon* spp.-positive specimens. Positive clinical specimens with melting curves deviating from the *H. americanum* melting curve were confirmed by DNA sequencing of the amplification product.

2.4. Sample collection

Six hundred and fourteen EDTA-whole blood samples collected from dogs for which there was a clinical suspicion of hepatozoonosis and submitted to the Molecular Diagnostics Laboratory between 2006 and 2008 from two Canadian provinces and 28 states of the United States, predominantly from the southeastern US, were analyzed. These samples were sent at ambient temperature without refrigeration and frozen storage. All samples were extracted by use of the Standard protocol and eluted in 40 μl High Pure elution buffer for real-time PCR.

2.5. Statistical analysis

The statistical significance of all data was assessed by two-sided one-way ANOVA or repeated measures ANOVA with the Statistica 7.1 software (StatSoft, Inc., Tulsa, OK).

3. Results

3.1. Development of the canine Hepatozoon spp. FRET PCR

Information about DNA sequences of *Hepatozoon* spp. is sparse, and essentially the only realistic target that allows reliable detection, and also differentiation,

Fig. 1. Alignment of the amplicons of the partial 18S rDNA. Dots indicate nucleotides identical to the *H. americanum* reference sequence, and dashes indicate nucleotide deletions. The human 18S rDNA is 98.5% similar to the available partial canine 18S rDNA sequence. Primers and probes are indicated by boxes. The upstream primer is used as the indicated sequence without gaps. Probes and downstream primer are used as antisense oligonucleotides. The Bodipy 630/650 fluorescent label is added via a linker to the 5′-end of the Bodipy probe, and FAM-6 is directly attached to the 3′-terminal nucleotide of the Fluorescein probe.

of a wide range of *Hepatozoon* spp. is the 18S rDNA. 18S rDNA sequences are highly conserved (95.6% identity) among the protozoal parasites most closely related to *Hepatozoon* spp. These sequences are on average 90.6% identical with the human 18S rDNA sequence (Fig. 1). While comparison of the protozoal sequences to the canine sequence would have been preferable, it was impossible to retrieve the complete canine 18S rDNA sequence. However, the available partial canine 18S rDNA sequence is 98.5% identical to the human sequence.

For design of the PCR, it was important to avoid amplification of the host 18S rDNA. For that reason, regions of the 18S rDNA alignment with distinct polymorphisms between protozoal and human sequences were further inspected. Fig. 1 shows the optimal region identified for amplification, with differences that safely prevent amplification of mammalian 18S rDNA. The design of the primers also creates specificity for *Hepatozoon* spp. among all related protozoal sequences. The Bodipy 630/650 FRET anchor probe targets a virtually 100% conserved sequence of the eukaryotic 18S rDNA (Fig. 1). In contrast, the adjacent Fluorescein probe targets signature sequences of *Hepatozoon* spp. and is not expected to anneal to any other protozoal or mammalian 18S rDNA sequence because of a 4-base deletion in these sequences. This prevents erroneous detection, even if aberrant amplification would occur, and ensures specificity of the assay for *Hepatozoon* spp. In addition, the Fluorescein probe detects signature sequences of *H. americanum* and has 4 mismatches to *H. canis*.

The 18S rDNA FRET PCR detects *Hepatozoon* spp. with high sensitivity, specificity, and accurately quantifies the input template number. The copy number of the standard was determined by PicoGreen assay, and confirmed by limiting dilution assay, in which the standard or positive specimen DNA was diluted until some input aliquots became positive in the PCR while other remained negative (Poisson sampling error). The 10,000, 1000, 100, and 10 copy *H. americanum* standards and *H. americanum*-positive samples all have similar peak levels of the F2/F1 fluorescence signal. An uninhibited amplification curve was observed in all positive PCRs at the limiting dilution, establishing that the PCR is capable of detecting single target copies. The exponential growth of the signal enables generation of an accurate standard equation, based on the crossing points of a cutoff signal level and the *H. americanum* standards, with a correlation coefficient of $R^2 = 1.000$ (Kaltenboeck and Wang, 2005). The copy number of unknown samples is then

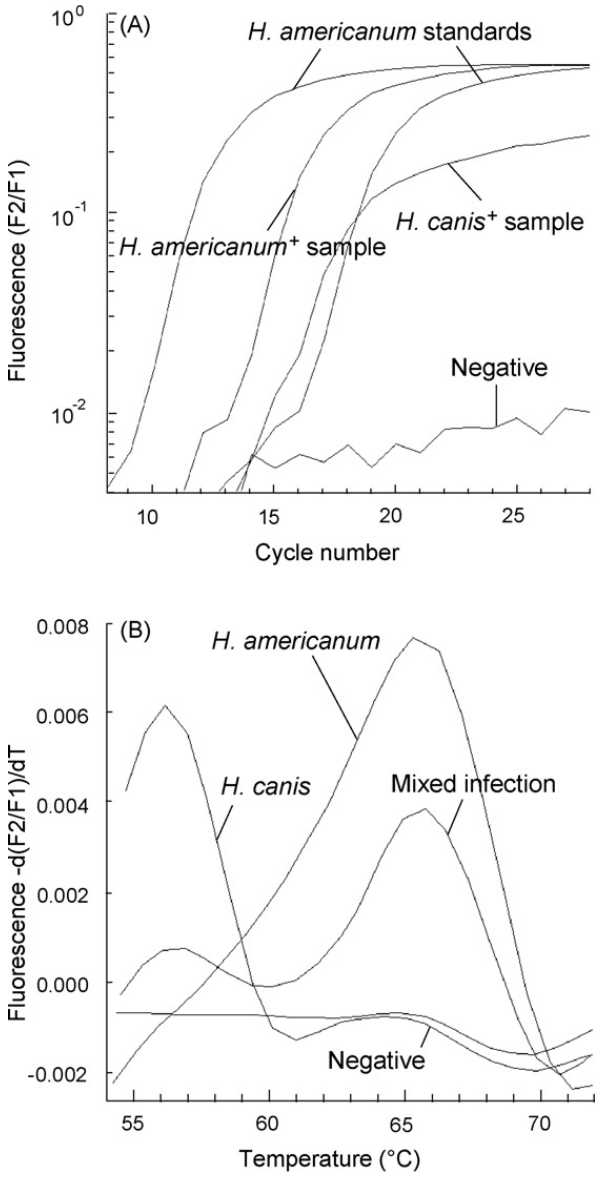

Fig. 2. Validation of the *Hepatozoon* spp. FRET-PCR. Comparison to amplification of standard containing 1000 or 10 *H. americanum* amplicons quantifies the sample copy number, and melting curve analysis speciates positive samples. (A) Amplification curves of *H. americanum* standards with 1000 and 10 amplicon copies, an *H. americanum*-positive sample, an *H. canis*-positive sample, and the negative control are shown. (B) Distinct melting peaks differentiate *H. americanum* and *H. canis*. *H. americanum* has a T_m of 65.5 °C and *H. canis* of 56.3 °C. A sample with dual infections shows melting peaks at both temperatures.

calculated by use of this equation and the crossing point of the sample (Fig. 2A). *H. canis*-positive samples do not reach as high a level of the F2/F1 fluorescence signal as *H. americanum*-positive samples do, which can be used for preliminary discrimination between the two *Hepatozoon* species. Analysis of the product melting curves after amplification allows reliable differentiation between *H. americanum* and *H. canis*, and also

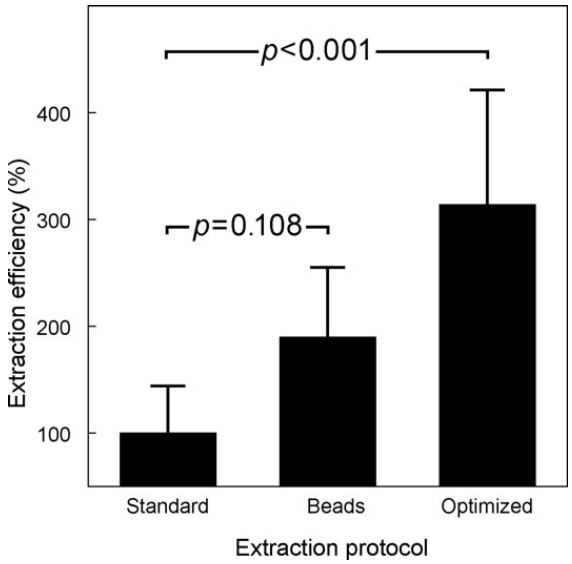

Fig. 3. Comparison of relative extraction efficiencies of *Hepatozoon americanum* DNA by use of three different protocols. EDTA-whole blood was obtained from a *Hepatozoon americanum*-positive dog. In the standard protocol, 0.7 ml EDTA-whole blood was mixed with 0.7 ml binding buffer, digested with Proteinase K at 65 °C for 20 min, and then extracted by use of the High Pure Extraction kit. In the Beads protocol, samples were similarly extracted after shaking with zirconia/silica beads in the Mini-Beadbeater for 5 min. More *H. americanum*/PCR is detected in the Beads protocol than without beads, but the difference is not statistically significant ($p = 0.108$; two-tailed one-way ANOVA). In the Optimized extraction protocol, EDTA-whole blood samples were also extracted after shaking in the Mini-Beadbeater without use of zirconia/silica beads. The 1.4 ml volume of the sample-binding buffer mixture was divided into 2 × 0.7 ml volumes, shaken with the Mini-Beadbeater for 5 min, digested with Proteinase K, and combined again for extraction. This Optimized protocol achieved an approximately 3-fold higher extraction efficiency than the standard protocol ($p < 0.001$). $n = 42$, 28, and 35 for Standard, Beads, and Optimized extraction protocols, respectively.

identifies mixed infections (Fig. 2B). The four mismatches between the Fluorescein probe and the *H. canis* 18S rDNA sequence result in an almost 10 °C reduction in melting temperature from 65.5 to 56.3 °C, and allow unambiguous discrimination of the amplification product of both *Hepatozoon* species found in dogs (Fig. 2B).

3.2. Optimization of DNA extraction

Hepatozoon spp. organisms, just as many other protozoal parasites, have developmental stages with presumably high mechanical resistance (Panciera et al., 2001). This raises the issue, if lysis of membranes by the chaotropic guanidinium buffer, followed by proteinase K digestion, as used in our Standard DNA extraction protocol, is adequate to release DNA sufficient for sensitive detection, or if mechanical disruption of the membranous structures of *Hepatozoon* spp. is required. To address this question, we used blood from dogs persistently infected with *H. americanum*, and extracted DNA by glass fiber binding and elution after different approaches for mechanical disruption or without disruption. The Standard protocol followed the procedure described earlier (DeGraves et al., 2003) that incorporates gentle agitation during 20 min of proteinase K digestion at 72 °C, while the Beads and Optimized protocols included vigorous shaking with or without zirconia/silica beads for 5 min prior to proteinase K digestion.

Use of zirconia/silica beads in the Beads protocol does not significantly increase extraction efficiency (Fig. 3). During these experiments the question appeared if increased DNA release by the use of beads was counterbalanced by binding of the released DNA to the beads. We addressed this question by washing the beads prior to extraction in binding buffer containing 500 USP units of heparin sodium/ml to block subsequent DNA binding (Gadgil and Jarrett, 1999). While this method improves DNA recovery, the improvement is not statistically significant. To examine if the shaking volume (full microcentrifuge tube vs. half-full tube) influenced results, we split samples and combined them after shaking. Again, this approach improves *H. americanum* DNA recovery, but not to the level of significance. Finally, we asked if shaking without beads released more DNA, and again found improvement, albeit non-significant. In the final Optimized protocol, we combined these non-significant improvements to a convenient method of shaking half-full sample tubes without beads, and obtained significantly improved, over 3-fold increased DNA recovery as compared to the Standard protocol (Fig. 3). This approach is now in routine use for DNA extraction in *Hepatozoon* spp. PCR diagnosis in our laboratory.

3.3. Effect of storage of the EDTA-blood samples on H. americanum detection

To ensure that submission of untreated EDTA-blood samples at ambient temperatures did not negatively affect *Hepatozoon* spp. detection rates, we examined the influence of sample storage on target recovery and PCR results. Interestingly, extended incubation of the EDTA-whole blood samples at refrigerator temperature or above dramatically increases the number of *H. americanum* genomes detected by PCR (Fig. 4). Storage at 37 °C in particular, significantly increases detection over storage at 4 or 25 °C after 3, 6 or 10 days (Fig. 4A). The average number of *H. americanum* genomes

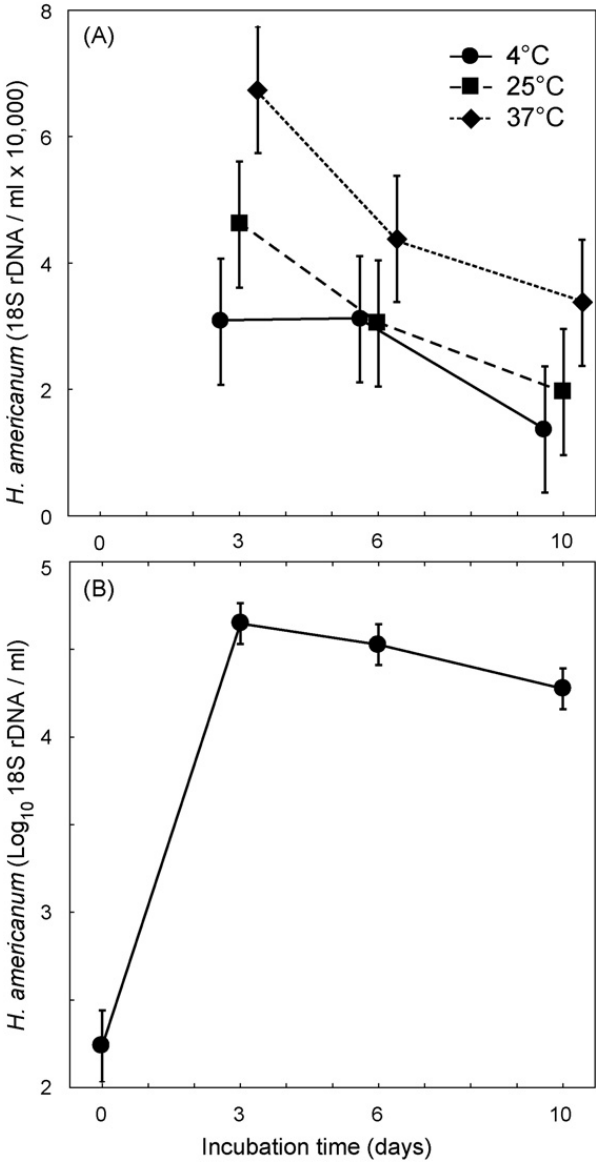

Fig. 4. Storage of the EDTA-blood sample affects *Hepatozoon* detection by real-time PCR. EDTA-whole blood was obtained from a *Hepatozoon americanum*-positive dog, and stored at 4, 25, or 37 °C for 3, 6, or 10 days, respectively. DNA was extracted by use of the Optimized protocol. (A) Evaluation of the combined storage temperature–time effects on detection of *H. americanum* DNA. Storage at 37 °C significantly increases sensitivity over lower temperatures ($p < 0.001$ for 37 °C vs. 4 °C and 25 °C; repeated measures ANOVA), and detected copy numbers are highest on day 3 ($p < 0.001$ for 3 days vs. 6 and 10 days; two-tailed one-way ANOVA). (B) Evaluation of the storage effect. Combined data for each time point were evaluated. Any storage of the EDTA-blood sample significantly increases the amount of *H. americanum* genomes detected by PCR ($p \leq 0.0001$; two-tailed one-way ANOVA). The number of *H. americanum* genomes detected on day 3 is approximately 300-fold higher than on day 0 without storage, and decreases after day 3 (day 3 vs. day 10, $p = 0.0002$; two-tailed one-way ANOVA). $n = 5$ for each temperature/time point.

detected on day 3 after storage of the combined 4, 25, and 37 °C samples is approximately 300-fold higher than on day 0 without storage, and decreases slowly after day 3 (Fig. 4B).

3.4. Laboratory diagnosis of canine hepatozoonosis by the 18S rDNA FRET PCR

A total of 614 EDTA-whole blood samples from dogs with tentative clinical diagnosis of hepatozoonosis, submitted to the Auburn University Molecular Diagnostics Laboratory mostly from the Southeast of the United States, were analyzed in years 2006–2008. Of these samples, 195 (32%) were positive for *Hepatozoon* spp. DNA (Table 1). Of the positive samples, 167 (85.6%) were positive for *H. americanum*, 14 (7.2%) were positive for *H. canis*, and 14 (7.2%) were mixed infections that were positive for both *H. americanum* and *H. canis*. All samples with melting peaks that differed from the T_m of 65.5 °C for *H. americanum* had melting peaks around 56.3 °C, essentially identical to that of *H. canis*. The DNA sequences for all these samples were obtained and were identical to the *H. canis* sequence. *H. canis* gamonts in segmented neutrophil leukocytes of an *H. canis*-positive dog are shown in Fig. 5. The range of detected *Hepatozoon* spp. 18S rDNA target copies per ml blood was 7 (a single copy/PCR) in cases of chronic disease to more than 200,000 copies of *H. americanum* DNA in acutely diseased dogs.

Out of the 28 states from which specimens were submitted, positive specimens originated from 13 states, mainly from the Southeast, but some also from Washington, California, Nebraska, Vermont, or Virginia. *H. canis* was detected in samples submitted from

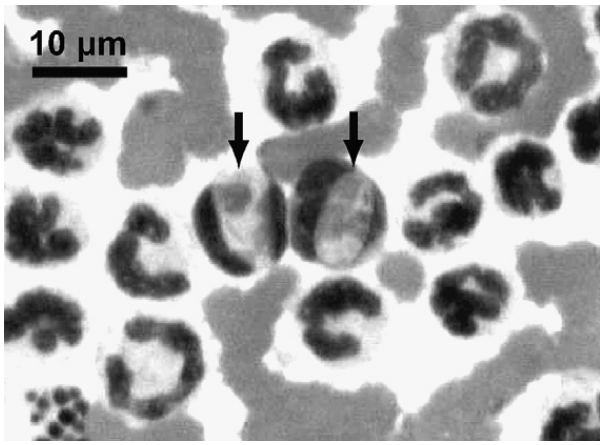

Fig. 5. *Hepatozoon canis* gamonts detected in segmented neutrophil leukocytes in the Wright's Giemsa-stained blood smear of an infected dog. EDTA-blood was obtained from a dog highly positive (>10,000 genome copies/ml blood) for *H. canis* in the PCR test.

Table 1
Summary of *Hepatozoon* spp. 18S rDNA FRET-PCR tests

State	Total specimens	*H. americanum*$^{+}$	*H. canis*$^{+}$	*H. americanum*$^{+}$ and *H. canis*$^{+}$	Total % positive
Alabama	268	83	6	9	36.6%
Georgia	63	18	2	0	31.8%
Mississippi	56	23	3	4	53.6%
Texas	50	12	0	0	24.0%
Louisiana	42	10	0	1	26.2%
Oklahoma	17	9	0	0	52.9%
North Carolina	16	1	0	0	6.3%
Virginia	10	1	1	0	20.0%
Others[a]	92	10	2	0	13.0%
Total	614	167	14	14	31.8%

[a] Includes specimens from the remaining 20 states, from which less than 10 specimens were submitted, and specimens with unidentifiable sources.

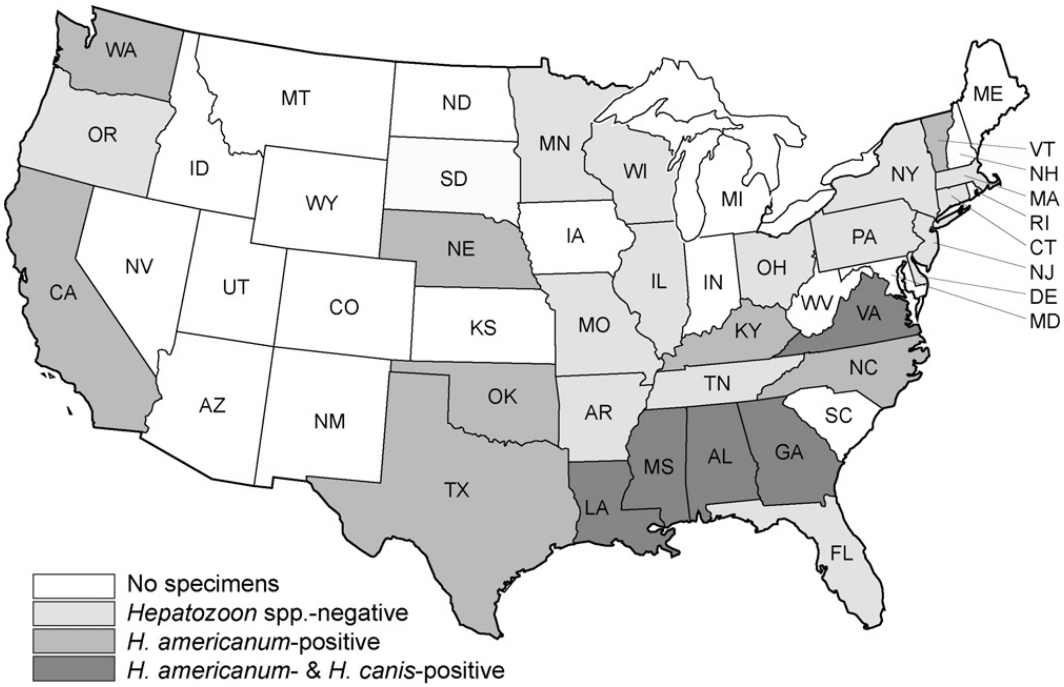

Fig. 6. Geographical distribution of *Hepatozoon* spp.-positive specimens in the continental United States. Blood samples from dogs with suspected hepatozoonosis were submitted from 28 states. *Hepatozoon* spp. was diagnosed in specimens from 13 states (Table 1). In California, Kentucky, Nebraska, North Carolina, Oklahoma, Texas, Vermont, and Washington, *H. americanum*, but not *H. canis*, was detected, whereas in Alabama, Georgia, Louisiana, Mississippi and Virginia, both *H. americanum* and *H. canis* were detected.

Alabama, Georgia, Louisiana, Mississippi, and Virginia (Fig. 6).

4. Discussion

Canine hepatozoonosis is a common disease of dogs in the southeastern United States. The traditional methods of diagnosis, which are based on organism identification or serologic testing, are unsatisfactory because of their low sensitivity. Novel molecular techniques such as PCR have detected *Hepatozoon* spp. with highly improved sensitivity (Jittapalapong et al., 2006). In this investigation, we developed a robust platform for efficient DNA extraction and real-time PCR amplification of a 198 bp signature fragment of the *Hepatozoon* spp. 18S rDNA gene. Our analyses show a very high specificity of detection, with no background signal from even the most closely related protozoal genera, and maximum sensitivity as evidenced by consistent detection of single target DNA copies in a high background of host DNA. Mechanical disruption of EDTA-whole blood specimens resulted in a 3-fold increase in detection of target copy numbers, and has been now incorporated into our DNA extraction

protocol. The detection of the amplification product by FRET probes not only further increases the specificity of the method, but also allows convenient differentiation of the critical *Hepatozoon* species found in dogs. The routine identification of *H. canis* and mixed *H. americanum*/*canis* infections in dogs has proven the value of this approach.

Our investigation into optimal sampling conditions has uncovered a curious and potentially significant phenomenon. We detect up to a 300-fold increase of *Hepatozoon* spp. 18S rDNA copy numbers after storage of EDTA-whole blood specimens. This effect can be observed over a wide temperature and time range, but is most pronounced at 37 °C incubation for 3 days. Our data do not support the notion that this increase is achieved solely by improved DNA extraction efficiency. In the specimens that we tested we did not see a 300-fold increase of gamonts; in fact, we detected neither before nor after incubation *H. americanum* gamonts in the smears of blood that we incubated. Although not demonstrated experimentally, some speculate ''that certain merozoites (released from muscle ''onion cysts'') do not become gamonts but, rather, give rise to a new merogonous cycle'' (Ewing and Panciera, 2003). Our data suggest that, if such a merogonous cycle could occur in circulation rather than muscle, it may account for the increase seen upon incubation, perhaps because the process was accelerated under the microaerophilic conditions in a stored blood sample.

While we have established the absolute sensitivity and specificity of the *Hepatozoon* spp. 18S rDNA PCR in this study, we have not yet determined negative and positive predictive values of the assay by comparison with other diagnostic methods. However, our observations of PCR-positive and -negative patients, their clinical appearance, and the number of detected target copies allow some tentative conclusions. Most patients that we examined were negative for *Hepatozoon* spp. in blood smears, but showed clinical disease consistent with hepatozoonosis. The unsatisfactory diagnostic situation was in fact the main reason for development of this assay. Our data strongly suggest that many cases of canine hepatozoonosis remain undiagnosed by standard methodology, and further suggest that gamonts may be present in circulating leukocytes of most *Hepatozoon*-infected dogs. The number of detected copies of *Hepatozoon* spp. 18S rDNA typically correlated well with the clinical presentation of diseased dogs. Up to 200,000 copies per ml blood were detected in acutely sick animals, while copy numbers as low as 7 per ml blood were detected in chronically diseased dogs, and in particular in animals under continuous anti-*Hepatozoon* therapy. Our results suggest that the *Hepatozoon* spp. 18S rDNA quantitative PCR can also be used for monitoring therapeutic efficacy. A small number of patients (<10) was positive by muscle biopsy, yet *Hepatozoon* spp. DNA was not detected even in repeated PCR assays. This suggests that not every case of canine hepatozoonosis is characterized by circulating gamonts. Comparative diagnosis by PCR and muscle biopsy should provide a conclusive answer.

Hepatozoonosis was thought endemic in the southeastern US only. However, we found *Hepatozoon* spp. DNA in samples from 13 states, including California, Nebraska, Vermont, Virginia, and Washington, as well as the southeastern states, including Alabama, Georgia, Kentucky, Louisiana, Mississippi, North Carolina, Oklahoma, and Texas. The identification of *H. canis* in almost 15% of the positive specimens also was unexpected, but confirmed simultaneous observations by Allen et al. (2008) in Oklahoma, and rebuts the notion that *H. canis* is not present in North America. Interestingly, the vast majority of *H. canis*-positive cases was detected in the two coastal southern states Mississippi and Alabama. Furthermore, *H. americanum* and *H. canis* result in different disease symptoms and require different therapeutic strategies (Greene, 2006). The *Hepatozoon* spp. 18S rDNA quantitative PCR may therefore be a valuable diagnostic tool that can improve diagnosis and therapy of canine hepatozoonosis.

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