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Evaluation of a serum-based PCR assay for the diagnosis of canine monocytic ehrlichiosis☆

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Abstract

The aim of this study was to estimate the relative diagnostic sensitivity and specificity of a polymerase chain reaction (PCR) assay in the serum of dogs with naturally occurring non-myelosuppressive canine monocytic ehrlichiosis (CME), and to investigate the association between PCR positivity and immunofluorescence antibody (IFA) titres for *Ehrlichia canis*. Serum samples obtained from 38 dogs with non-myelosuppressive CME and 12 healthy dogs were analyzed retrospectively. Each serum sample was analyzed in triplicate using an *E. canis*-specific nested PCR assay targeting a 389 bp sequence of the 16S rRNA gene. *Ehrlichia canis* DNA was amplified in 24 of 38 (63.1%) affected dogs; all samples from healthy dogs were negative. A high level of agreement was found among the PCR replicates ($P<0.0001$). Median IFA titre of the 24 PCR-positive dogs was significantly lower than that of the PCR-negative infected dogs ($P=0.0029$), indicating that *E. canis* DNA may circulate prior to the development of a high antibody titre. Serum-based PCR analysis is suggested for the early diagnosis of CME when whole blood samples are not available.

Keywords: Serum; Dog; *Ehrlichia canis*; PCR; 16S rRNA

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1. Introduction

Canine monocytic ehrlichiosis (CME) is an important tick-borne disease of dogs worldwide that is caused primarily by the obligatory intracellular organism *Ehrlichia canis* (Neer et al., 2002). Diagnosis is based on clinical and hematological findings along with laboratory confirmation of infection (Neer et al., 2002). Several sensitive polymerase chain reaction (PCR) assays have been developed for the diagnosis of *E. canis* infection in dogs (Iqbal et al., 1994; Wen et al., 1997; Stich et al., 2002). Successful PCR amplification of *E. canis* DNA in experimentally and naturally infected dogs, especially those with non-myelosuppressive CME, has been reported using whole blood, bone marrow (BM), spleen, liver, kidney, lung and lymph nodes (Iqbal and Rikihisa, 1994; Harrus et al., 1998; Harrus et al., 2004; Gal et al., 2007). In humans, serum has been used occasionally as an alternative substrate to whole blood for the PCR detection of *E. chaffeensis* and *Anaplasma phagocytophilum* DNA in acutely infected individuals, based on the hypothesis that serum contains residual *Ehrlichia*-infected leukocytes, free ehrlichiae organisms, or ehrlichial DNA (Dumler and Bakken, 1996; Massung et al., 1998; Comer et al., 1999; Olano et al., 2003).

To the best of our knowledge, the performance of a serum-based PCR assay in the diagnosis of CME has not been evaluated previously. Therefore, a retrospective study was designed to estimate the relative diagnostic sensitivity, specificity and precision of a PCR assay based on *E. canis* 16S rDNA detection in serum samples from dogs with naturally-occurring non-myelosuppressive CME, and to determine whether PCR results are influenced by sample volume, serum hemolysis or antibody titres against *E. canis* as determined by immunofluorescence assay (IFA).

2. Materials and methods

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2.1. Inclusion criteria for study dogs

Fifty dogs were included in the study. Thirty-eight dogs admitted to the Companion Animal Clinic, between January 1999 and March 2001 were diagnosed with non-myelosuppressive CME as they fulfilled the following five criteria: 1) clinical and clinicopathologic findings compatible with CME (Siarkou et al., 2007); 2) seropositivity to *E. canis* antigens by IFA (reciprocal IgG titre: $\geq$ 100); 3) previous PCR-based confirmation of *E. canis* infection in BM aspirates (Mylonakis et al., 2003; Siarkou et al., 2007); 4) complete recovery following doxycycline treatment and 5) normocellular or hypercellular BM on aspiration cytology. Availability of frozen (-30^{0}C) deposited serum aliquots was also considered. Dogs were excluded from the study if any antiehrlichial medications had been given prior to blood sampling.

Twelve clinically healthy dogs that were seronegative and PCR-negative (in BM samples) for *E. canis* served as controls. The dogs were housed in the Companion Animal Clinic as part of another research project. The management of these animals was in compliance with the guidelines set forth by the European Committee for Care of Animals for Scientific Purposes.

2.2. DNA extraction

Each serum sample was measured (volume in microliters), inspected for hemolysis, and centrifuged at 12,000 x *g* for 20 min at 4° C to concentrate *Ehrlichia*-infected cells and free ehrlichiae organisms. The supernatant was removed and total DNA was extracted from the sediment by using a modification of the QIAamp DNA Mini kit (QIAGEN) protocol, as previously described (Comer et al., 1999). The total concentration and purity of the DNA were measured by spectrophotometry (A_{260} & A_{280}).

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2.3. PCR amplification and analysis

A 16S rDNA-based nested PCR assay specific for *E. canis* was used to analyze serum DNA extracts with slight modifications of previously described protocols (Wen et al., 1997; Harrus et al., 1998; Siarkou et al., 2007). Five µl of serum DNA extract was used for the primary amplification in a total reaction mixture of 50 µl containing final concentrations of 5 mM $MgCl_2$, 200 µM of each deoxynucleoside triphosphate (dNTP), 1.5 U of *Taq* polymerase and 0.4 µM each of primers ECC and ECB. After initial denaturation at 94°C for 5 min, primary amplification consisted of 40 cycles of 1 min each at temperatures of 94°C, 60°C, and 72°C for denaturation, annealing, and extension, respectively. Secondary (nested) amplification was performed by using 2 µl of the primary amplification product with the primers "canis" and HE3. Conditions for the nested amplification were the same as those for the primary amplification except for the annealing temperature, which was set at 55°C to achieve optimum amplification. To test the specificity of the serum-based PCR assay the identity of the PCR amplicons (389 bp) as *E. canis* was established in all positive dogs by sequencing with the "canis" and HE3 primers.

PCR amplification was performed in triplicate on all serum DNA extracts. A positive result was defined as the presence of *E. canis* amplicons in at least one of the three PCR replicates. To detect possible PCR inhibitors, all samples were also tested at a 1:10 dilution; moreover, an aliquot of each negative sample was spiked with control DNA (*E. canis* strain GR21) and reassayed (Osborne and Smith, 2005). In separate experiments, bovine serum albumin (BSA) was added to the amplification mixture at 0.01% or 0.4% concentration to eliminate the potential inhibitors (Harrus et al., 1998; Abu Al-Soud and Radstrom, 2001).

2.4. Statistical analysis

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The level of agreement among the three PCR replicates was measured by using a kappa statistic. The mean volume (µl) of serum concentrated in infected dogs, with and without positive PCR results, was compared by Student's t-test. The frequency of sample hemolysis in PCR-positive and -negative serum samples from infected dogs was compared by using a Pearson's chi-squared test. The Wilcoxon Rank Sum Test was used to compare median anti-*E. canis* antibody titres (IFA results) between affected dogs with or without positive serum PCR results. All analyses were evaluated at a 5% level of significance.

3. Results and Discussion

Ehrlichia canis DNA was amplified in at least one of three PCR replicates in 24/38 dogs with CME, but in none of the control dogs (Table 1). A relative diagnostic sensitivity of 63.1% (confidence interval [CI]: 46-78%) and a specificity of 100% (CI: 73-100%) was calculated for the 16S rDNA-based nested PCR assay. *Ehrlichia canis* sequences for all positive dogs were identical to those previously characterized for Greek strains (GenBank accession numbers EF01110 and EF011111) (Siarkou et al., 2007). In *E. canis* infected dogs, complete agreement among replicate PCR results (positive or negative result for all three replicates) was found in 34/38 (89.5%) samples; discordant results were found in 4/38 (10.5%) samples (3 dogs with 2 positive and 1 negative result and 1 dog with 2 negative and 1 positive result) (Table 1). Overall, a high level of agreement was found among the three PCR replicates ($k=0.85$; $P<0.0001$). The discordant results in 4/38 samples may have been due to the very low levels of template DNA, since all standard precautions were taken for the prevention of contamination. The moderate to high relative sensitivity (when performing 3 repetitions), high relative specificity, and precision suggest serum may

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be a valuable substrate in the molecular diagnosis of CME when a whole blood sample is not available.

Because *Ehrlichia* sp. are intracellular bacteria, and a low level of bacteremia is typically observed in infected individuals, whole blood has been used in the majority of relevant medical and veterinary studies (Iqbal et al., 1994; Wen et al., 1997; Childs et al., 1999; Stich et al., 2002). Only a few medical reports have evaluated the sensitivity of 16S rDNA-based PCR assays for the diagnosis of human monocytic and granulocytic ehrlichiosis in serum samples (Massung et al., 1998; Comer et al., 1999; Olano et al., 2003). Sensitivity was substantially lower in humans with monocytic (6% and 7%) (Comer et al., 1999; Olano et al., 2003) and granulocytic (7% and 43%) (Massung et al., 1998; Comer et al., 1999) ehrlichiosis, than in dogs with monocytic ehrlichiosis in the present study. This difference may be partially explained by the fact that sensitivity in our study was determined in a group of dogs with confirmed CME, as opposed to studies in humans that included both confirmed and suspected cases of ehrlichiosis. The different concentration of microorganisms in serum samples and use of different DNA extraction methods also may account for the discrepant results.

The sensitivity of PCR analysis in the diagnosis of other bacterial diseases can be improved by simply increasing the volume of serum concentrated (Demaerschalck et al., 1995; Goessens et al., 1995). In the present study, serum volume did not appear to affect PCR results, although volumes as low as 250 µl and as high as 1500 µl were tested (Table 1). Mean serum volumes of affected dogs having positive or negative PCR were 1041.7 µl and 1017.9 µl, respectively, and did not differ significantly. However, when the circulating bacterial load is low (i.e. during the myelosuppressive phase of the disease), serum volume may be more likely to affect the PCR result.

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Similarly, serum hemolysis did not have an impact on the sensitivity of the PCR assay in the present study; slight to moderate hemolysis was observed in 17/38 (44.7%) samples (Table 1). Although hemolyzed serum contains a number of potential PCR inhibitors, such as hemin (Klein et al., 1997), we did not detect the presence of inhibitors in any of the serum samples in this study.

Among the 38 affected dogs, the median reciprocal IFA titre for PCR-positive dogs was significantly lower than for PCR-negative dogs (400 vs 800, respectively) (P=0.0029) (Table 1), similar to what has been reported in humans infected with *E. chaffeensis* and *A. phagocytophilum* (Comer et al., 1999). These data may indicate that PCR-amplifiable *E. canis* DNA circulates early in the course of the disease, prior to the development of a high antibody titre. Although seronegative diseased dogs were not tested in this study, PCR may be a suitable tool for the detection of *E. canis* DNA in serum even before seroconversion, as has also been observed in dogs with experimental *E. canis* infection and in humans with monocytic and granulocytic ehrlichiosis, thus facilitating early diagnosis of the disease (Iqbal et al., 1994; Wen et al., 1997; Massung et al., 1998; Comer et al., 1999; Childs et al., 1999). False negative results due to the putative inhibitory effect of IgG on PCR (Abu Al-Soud et al., 2000) was most likely minimal, as inhibitors were not detected in any of the serum samples, including those with high antibody titres.

The decision to investigate the diagnostic performance of the PCR assay in dogs having the non-myelosuppressive (acute) rather than the myelosuppressive (chronic) form of CME in the present study was based on the short-lasting antigenemia documented in experimental acute *E. canis* infections in dogs, which presumably is attributable to rapid propagation and multiplication of the bacterium in mononuclear cells (Waner et al., 1996). A similar phenomenon has yet to be

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demonstrated in dogs with chronic *E. canis* infection. However, evaluation of the performance of a serum-based PCR assay in dogs with severe chronic CME would be an interesting objective for a future study.

Conflict of interest statement

All authors declare no conflict of interest.

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Table 1. PCR results (in triplicate), reciprocal IFA titres, serum volume used for DNA extraction and presence of hemolysis in serum samples from 38 dogs with non-myelosuppressive monocytic ehrlichiosis (*Ehrlichia canis*)

Dog #	PCR			IFA	Serum (µl)	Hemolysis
	1	2	3			
1	-	-	-	800	1000	-
2	-	+	-	400	1500	+
3	+	+	+	400	1500	+
4	-	-	-	1600	1300	+
5	+	+	+	800	700	-
6	+	+	-	1600	500	+
7	-	+	+	200	300	-
8	-	-	-	1600	1500	+
9	+	+	+	400	1500	-
10	+	+	+	400	900	-
11	+	-	+	400	1300	-
12	+	+	+	400	1500	-
13	+	+	+	200	900	-
14	-	-	-	800	1500	-
15	-	-	-	800	1000	+
16	-	-	-	400	250	+
17	+	+	+	200	1000	+
18	+	+	+	800	500	+
19	-	-	-	1600	1500	+
20	+	+	+	400	800	-
21	-	-	-	800	1100	+
22	-	-	-	1600	500	+
23	-	-	-	200	500	+
24	+	+	+	200	1000	+
25	+	+	+	400	1500	+
26	+	+	+	200	400	-

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27	-	-	-	1600	800	-
28	-	-	-	400	1500	-
29	-	-	-	200	500	-
30	+	+	+	200	1100	+
31	+	+	+	400	900	-
32	+	+	+	100	1500	-
33	+	+	+	100	1400	-
34	+	+	+	100	1000	+
35	+	+	+	800	1400	-
36	+	+	+	200	700	-
37	+	+	+	200	1200	-
38	-	-	-	400	1300	-

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