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► **To cite this version:**

Mathios E. Mylonakis, Victoria I. Siarkou, Leonidas Leontides, Eleftheria Bourtzi-Hatzopoulou, Vassilios I. Kontos, et al.. Evaluation of a serum-based PCR assay for the diagnosis of canine monocytic ehrlichiosis. *Veterinary Microbiology*, 2009, 138 (3-4), pp.390. 10.1016/j.vetmic.2009.04.015 . hal-00514610

HAL Id: hal-00514610

<https://hal.science/hal-00514610>

Submitted on 3 Sep 2010

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Accepted Manuscript

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PII: S0378-1135(09)00206-5
DOI: doi:10.1016/j.vetmic.2009.04.015
Reference: VETMIC 4418

To appear in:

Received date: 28-11-2008
Revised date: 6-4-2009
Accepted date: 14-4-2009

Please cite this article as: Mylonakis, M.E., Siarkou, V.I., Leontides, L., Bourtzi-Hatzopoulou, E., Kontos, V.I., Koutinas, A.F., Evaluation of a serum-based PCR assay for the diagnosis of canine monocytic ehrlichiosis, *Veterinary Microbiology* (2008), doi:10.1016/j.vetmic.2009.04.015

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

3

4 **Mathios E. Mylonakis**^{a,*}, **Victoria I. Siarkou**^b, **Leonidas Leontides**^c, **Eleftheria**
5 **Bourtzi-Hatzopoulou**^b, **Vassilios I. Kontos**^d, **Alexander F. Koutinas**^a

6

7 ^a*Companion Animal Clinic (Medicine), Veterinary Faculty, Aristotle University of*
8 *Thessaloniki, 11 Stavrou Voutyra str., GR-54627, Greece*

9 ^b*Laboratory of Microbiology and Infectious Diseases, Faculty of Veterinary*
10 *Medicine, Aristotle University of Thessaloniki, University Campus, GR-54124,*
11 *Thessaloniki, Greece*

12 ^c*Laboratory of Epidemiology, Biostatistics and Economics of Animal Production,*
13 *School of Veterinary Medicine, University of Thessaly, 224 Trikalon Str., GR-43100,*
14 *Karditsa, Greece*

15 ^d*Department of Veterinary Public Health, National School of Public Health, 196*
16 *Alexandras Str., GR-11521, Athens, Greece*

17

18 ☆Mathios E. Mylonakis and Victoria I. Siarkou contributed equally in this study.

19 *Corresponding author. Address: 18 Kyprou str., 55133, Thessaloniki, Greece. Tel:
20 +30 2310 994495; E-mail address: mmylonak@vet.auth.gr (M. E. Mylonakis).

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1 **Abstract**

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

17

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

19

1 **1. Introduction**

2 Canine monocytic ehrlichiosis (CME) is an important tick-borne disease of
3 dogs worldwide that is caused primarily by the obligatory intracellular organism
4 *Ehrlichia canis* (Neer et al., 2002). Diagnosis is based on clinical and hematological
5 findings along with laboratory confirmation of infection (Neer et al., 2002). Several
6 sensitive polymerase chain reaction (PCR) assays have been developed for the
7 diagnosis of *E. canis* infection in dogs (Iqbal et al., 1994; Wen et al., 1997; Stich et
8 al., 2002). Successful PCR amplification of *E. canis* DNA in experimentally and
9 naturally infected dogs, especially those with non-myelosuppressive CME, has been
10 reported using whole blood, bone marrow (BM), spleen, liver, kidney, lung and lymph
11 nodes (Iqbal and Rikihisa, 1994; Harrus et al., 1998; Harrus et al., 2004; Gal et al.,
12 2007). In humans, serum has been used occasionally as an alternative substrate to
13 whole blood for the PCR detection of *E. chaffeensis* and *Anaplasma phagocytophilum*
14 DNA in acutely infected individuals, based on the hypothesis that serum contains
15 residual *Ehrlichia*-infected leukocytes, free ehrlichiae organisms, or ehrlichial DNA
16 (Dumler and Bakken, 1996; Massung et al., 1998; Comer et al., 1999; Olano et al.,
17 2003).

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

25 **2. Materials and methods**

1 2.1. *Inclusion criteria for study dogs*

2 Fifty dogs were included in the study. Thirty-eight dogs admitted to the
3 Companion Animal Clinic, between January 1999 and March 2001 were diagnosed
4 with non-myelosuppressive CME as they fulfilled the following five criteria: 1)
5 clinical and clinicopathologic findings compatible with CME (Siarkou et al., 2007); 2)
6 seropositivity to *E. canis* antigens by IFA (reciprocal IgG titre: ≥ 100); 3) previous
7 PCR-based confirmation of *E. canis* infection in BM aspirates (Mylonakis et al.,
8 2003; Siarkou et al., 2007); 4) complete recovery following doxycycline treatment
9 and 5) normocellular or hypercellular BM on aspiration cytology. Availability of
10 frozen (-30°C) deposited serum aliquots was also considered. Dogs were excluded
11 from the study if any antiehrlichial medications had been given prior to blood
12 sampling.

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

18 2.2. *DNA extraction*

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

1 2.3. PCR amplification and analysis

2 A 16S rDNA-based nested PCR assay specific for *E. canis* was used to
3 analyze serum DNA extracts with slight modifications of previously described
4 protocols (Wen et al., 1997; Harrus et al., 1998; Siarkou et al., 2007). Five μl of serum
5 DNA extract was used for the primary amplification in a total reaction mixture of 50
6 μl containing final concentrations of 5 mM MgCl_2 , 200 μM of each deoxynucleoside
7 triphosphate (dNTP), 1.5 U of *Taq* polymerase and 0.4 μM each of primers ECC and
8 ECB. After initial denaturation at 94°C for 5 min, primary amplification consisted of
9 40 cycles of 1 min each at temperatures of 94°C, 60°C, and 72°C for denaturation,
10 annealing, and extension, respectively. Secondary (nested) amplification was
11 performed by using 2 μl of the primary amplification product with the primers “canis”
12 and HE3. Conditions for the nested amplification were the same as those for the
13 primary amplification except for the annealing temperature, which was set at 55°C to
14 achieve optimum amplification. To test the specificity of the serum-based PCR assay
15 the identity of the PCR amplicons (389 bp) as *E. canis* was established in all positive
16 dogs by sequencing with the “canis” and HE3 primers.

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

25 2.4. Statistical analysis

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

9 **3. Results and Discussion**

10 *Ehrlichia canis* DNA was amplified in at least one of three PCR replicates in
11 24/38 dogs with CME, but in none of the control dogs (Table 1). A relative diagnostic
12 sensitivity of 63.1% (confidence interval [CI]: 46-78%) and a specificity of 100% (CI:
13 73-100%) was calculated for the 16S rDNA-based nested PCR assay. *Ehrlichia canis*
14 sequences for all positive dogs were identical to those previously characterized for
15 Greek strains (GenBank accession numbers EF011110 and EF011111) (Siarkou et al.,
16 2007). In *E. canis* infected dogs, complete agreement among replicate PCR results
17 (positive or negative result for all three replicates) was found in 34/38 (89.5%)
18 samples; discordant results were found in 4/38 (10.5%) samples (3 dogs with 2
19 positive and 1 negative result and 1 dog with 2 negative and 1 positive result) (Table
20 1). Overall, a high level of agreement was found among the three PCR replicates
21 ($k=0.85$; $P<0.0001$). The discordant results in 4/38 samples may have been due to the
22 very low levels of template DNA, since all standard precautions were taken for the
23 prevention of contamination. The moderate to high relative sensitivity (when
24 performing 3 repetitions), high relative specificity, and precision suggest serum may

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

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

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

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

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

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

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

4 **Conflict of interest statement**

5 All authors declare no conflict of interest.

6 **Acknowledgements**

7 This study was financially supported by the Companion Animal Clinic and the
8 Laboratory of Microbiology and Infectious Diseases, Veterinary Faculty, Aristotle
9 University of Thessaloniki, Greece. We thank Dr H. Billinis for his valuable help with
10 PCR amplification of *E. canis* DNA in bone marrow samples from a subset of dogs
11 with monocytic ehrlichiosis. We are also grateful to Professor M. Christopher, School
12 of Veterinary Medicine, University of California, Davis, for editing the manuscript.

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- 1 **Table 1.** PCR results (in triplicate), reciprocal IFA titres, serum volume used for
 2 DNA extraction and presence of hemolysis in serum samples from 38 dogs with non-
 3 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	-

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	-

1