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Detection of *Ehrlichia canis* by PCR in different tissues obtained during necropsy from dogs surveyed for naturally occurring canine monocytic ehrlichiosis

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Abstract

A molecular study for the detection of *Ehrlichia canis* was carried out on tissues obtained at necropsy from randomly selected dogs with the intention of investigating naturally-occurring canine ehrlichiosis. The tissues evaluated for the presence of *E. canis* included lymph nodes, spleen, liver, bone marrow, and blood. Eight of the 18 dogs included were found to be positive for *E. canis* by polymerase chain reaction (PCR) and sequencing of the 16S rRNA gene. Two dogs were positive for *Anaplasma platys* of which one dog was co-infected with *E. canis* and *A. platys*. Blood (5/8) and lymph nodes (5/8) were the tissues found to yield the highest number of positive *E. canis* PCR results with 7/8 dogs positive in the blood or lymph node. *E. canis* and *A. platys* DNA could be amplified by PCR when tissue samples were obtained 72 h after the time of death.

Keywords: *Ehrlichia canis*; *Anaplasma platys*; PCR; Tissue; Necropsy

1. Introduction

Ehrlichia canis is a tick-borne obligate intracellular Gram negative bacterium that infects canine monocytes. The pathogen is transmitted to canines by the brown dog tick, *Rhipicephalus sanguineus*. Experimental inoculations have demonstrated an incubation period of 8–20 days in which the bacteria spread throughout the body in the mononuclear-phagocyte system (Neer and Harrus, 2006). Following incubation, *E. canis* infection may progress into three consecutive clinical stages: acute, sub-clinical and chronic. However, in naturally occurring infections, accurate staging of the disease could be difficult. Because the sub-clinical phase may last several years without any apparent clinical signs, infection may have an illusive nature (Harrus et al., 1998). Experimental infections have indicated that the spleen was most likely to harbour *E. canis* organisms during the sub-clinical phase of canine monocytic ehrlichiosis (CME) (Harrus et al., 1998). Currently, there are few data from dogs naturally infected with *E. canis* regarding the pathogen's pattern of distribution in different tissues.

The presence of *E. canis* DNA in infected dogs has been demonstrated by polymerase chain reaction (PCR) from the blood, bone marrow, spleen, liver, kidney and lymph node in several studies (Iqbal and Rikihisa, 1994; Harrus et al., 1998; Harrus et al., 2004; Mylonakis et al., 2004). However, comparative information on the spread and presence of *E. canis* by PCR analysis in multiple different organs is limited. Likewise, information regarding the post mortem tissue preference for *E. canis* diagnosis by PCR is unavailable. Previous studies on naturally or experimentally infected dogs included samples obtained ante mortem or taken immediately after death (Harrus et al., 1998; Harrus et al., 2004; Mylonakis et al., 2004).

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The integrity of the DNA during molecular analysis of post mortem tissue samples could be influenced by suitable handling of the cadaver. It is important to maintain proper refrigeration of cadavers until the specimen is harvested. Nevertheless, in spite of appropriate temperature conditions, the natural process of cadaver autolysis takes place. Therefore, it is considered necessary to reduce the time frame from death to sample collection and specimen processing as much as possible. Studies documenting the detection of *E. canis* DNA in dogs tissues taken at necropsy are lacking and it was not known whether it is possible to demonstrate this pathogen in dogs by PCR hours to days after death.

Israel is considered an endemic region for canine ehrlichiosis caused by *E. canis*. A previous epidemiological survey from Israel indicated that the seroprevalence of *E. canis* in apparently healthy dogs was 30% (Baneth et al., 1996). The summer months and older animals were found to be associated with a higher incidence of *E. canis* seropositivity. The actual prevalence of *E. canis* infection may differ from the seroprevalence because the presence of serum anti-*E. canis* antibodies indicate previous exposure to infection and not necessarily active infection. However, dogs with clinical ehrlichiosis may not have demonstrable antibodies in the first days after the initial infection prior to the development of a detectable antibody titre (Harrus et al., 1996; Neer and Harrus, 2006).

The goals of this study were to evaluate *E. canis* infection in a limited number of dogs brought to necropsy in Israel, to assess the tissues in which *E. canis* can be demonstrated by PCR, and to investigate the presence of the pathogen DNA in dogs at post mortem.

2. Materials and methods

2.1. Sample collection

Eighteen dogs that were brought for necropsy at the pathology department of the Kimron institute from June 2005 to November 2005 to investigate their cause of death were chosen randomly to be included in the study. The cadavers were kept under refrigeration at 4 °C until necropsy.

The first dog necropsied on the day of sampling when the pathologist author (EL) was on duty was selected for the study. The presence or absence of tick infestation on the dog cadaver at the time of necropsy was noted. Five tissues were sampled for *E. canis* PCR from each dog: a blood clot from the right atrium of the heart, the right submandibular lymph node, spleen, liver and bone marrow from the right femur. Each tissue section was cut with sterile equipment in order to prevent cross contamination. Special care was taken to avoid sampling of blood with the tissues by taking section blocks from the core of the organs not exposed to free fluid from the surrounding tissues. Samples were then marked and kept in −80 °C for DNA extraction and PCR analysis.

2.2. DNA extraction

The tissues for DNA extraction were harvested in a sterile manner from the centre of each frozen tissue block, in order to prevent cross-contamination. The samples were allowed to thaw and then mechanically homogenized and the DNA was extracted from 200 μL of the lysate by adding 500 μL of lysis buffer (50 mM NaCl, 50 mM Tris, 10 mM EDTA [pH = 8.0]), proteinase K to a final concentration of 250 μg/mL and Triton X-100 (20%) to a final concentration of 1%. Following 2 h incubation at 56 °C and inactivation of proteinase K at 90 °C for 10 min, 500 μL of a mixture of phenol (75%), chloroform (24%) and isoamylalcohol (1%) were added, mixed and centrifuged (12,000 *g*) for 3 min. The supernatant was collected and 500 μL of a mixture of phenol (50%), chloroform (48%) and isoamylalcohol (2%) were added, mixed and centrifuged (12,000 *g*) for 3 min.

The supernatant was collected and 500 μL of a mixture of chloroform (96%) and isoamylalcohol (4%) were added, mixed and centrifuged (12,000 *g*) for 3 min. The supernatant was collected, and 1:10 volume of Na-acetate (3 M) and one volume of ice cold 100% isopropanol (−20 °C) were added and incubated over night at −20 °C. Following centrifugation (14,000 *g*) at 4 °C for 30 min, the supernatant was discarded and the pellet was washed with 150 μL of ethanol (75%, −20 °C) and centrifuged (13,000 *g*) for 15 min. The supernatant was discarded and the pellet was left to dry. The DNA was eluted with 30 μL of ddH_2O overnight at 4 °C.

2.3. PCR amplification and analysis

Two pairs of primers: ECA (5′-AACACATGCAAGTCGAACGGA-3′) combined with the reverse primer HE3 (5′-TATAGGTAC-CGTCATTATCTTCCCTAT-3′) (Wen et al., 1997) and EHR16SD (5′- GGTACCYACAGAAGAAGTCC-3′) combined with the reverse primer EHR16SR (5′-TAGCACTCATCGTTTACAGC-3′) (Brown et al., 2001) were used to amplify DNA products of approximately 400 bp and 345 bp, of the *E. canis* 16SrRNA gene and the *Ehrlichia* genus 16SrRNA gene, respectively. While the EHR primers were designed to amplify a broader spectrum of organisms related to the *Ehrlichia* and *Anaplasma* genera, the ECA/HE3 primers are specific for *E. canis* and are not reported to detect *A. platys* (Wen et al., 1997).

Amplification by PCR was preformed with a 25 μL reaction mixture containing 50 ng of template DNA; 200 μM (each) dATP, dTTP, dCTP and dGTP; 12.5 pmol of each primer; and 0.625 U of Taq DNA polymerase (Bio-Rad Laboratories) in a 1X reaction buffer (50 mM KCl; 10 mM Tris HCl [pH 8.3]; 1.5 mM MgCl).

A touchdown PCR technique was used. Amplification was performed under the following conditions: 94 °C for 3 min, two cycles of 94 °C for 30 s, 62 °C for 30 s, 72 °C for 30 s, two cycles of 94 °C for 30 s, 60 °C for 30 s, 72 °C for 30 s, two cycles of 94 °C for 30 s, 58 °C for 30 s, 72 °C for 30 s, two cycles of 94 °C for 30 s, 56 °C for 30 s 72 °C for 30 s, two cycles of 94 °C for 30 s, 54 °C for 30 s 72 °C for 30 s, thirty-nine cycles of 94 °C for 30 s, 52 °C for 30 s, 72 °C for 30 s, and final extension at 72 °C for 3 min.

For additional characterization of the *A. platys* sequence obtained, two pairs of primers: Platys (5′–GATTTTTGTCGTAGCTTGCTATG–3′) combined with the reverse primer EHR16SR (Motoi et al., 2001) and MSP465f (5′-TGATGTTGTTACTGGACAGA-3′) combined with the reverse primer MSP980r (5′-CACCTAACCTTCATAAGAA-3′) (Caspersen et al., 2002) were used for the amplification of a 679 bp product of the *A. platys* 16SrRNA gene and a 550 bp product of the *A. phagocytophilum msp2* gene, respectively. Amplification by PCR was preformed with a 25 μL reaction mixture containing 50 ng of template DNA; 200 μM (each) dATP, dTTP, dCTP and dGTP; 12.5 pmol of each of the primers EHR16SR and Platys or 10 pmol of each of the primers MSP980r and MSP465f; and 0.625 U of Taq DNA polymerase (Bio-Rad Laboratories) in a 1X reaction buffer (50 mM KCl; 10 mM Tris HCl [pH 8.3]; 1.5 mM MgCl). PCR amplification was performed by using the following conditions: 95 °C for 5 min, forty cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 90 s, and final extension at 72 °C for 5 min.

All PCR products were electrophoresed through 2% agarose gels in Tris–boric acid–EDTA buffer, and the DNA fragments were visualized by ethidium bromide under UV fluorescence. Selected PCR products were then purified with the Wizard SV gel and PCR clean up system (Promega) according to the manufacturer recommendations. The PCR DNA product was sequenced using BigDye Terminator v 3.1 Cycle Sequencing Kit (Perkin–Elmer, Applied Biosystems Divisions) and ABI PRISM 3100 Genetic Analyzer (Applied Biosystems Divisions) at the Hylabs laboratories (Rehovot, Israel) according to the recommendations

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of the manufacturer. Obtained sequences were evaluated with ChromasPro software version 1.33 and compared to sequence data available from GenBank using the BLAST 2.2.9 program (http://www.ncbi.nlm.nih.gov/BLAST/).

2.4. Pathological and histopathological examinations of dogs

All dogs underwent a gross pathology examination at the Kimron Veterinary Institute in Bet Dagan, Israel. For histological examination, tissue specimens were fixed in 10% buffered formaldehyde, embedded in paraffin, and processed by routine methods for microscopic examination using a haematoxylin and eosin (H&E) stain. Necropsy reports of all dogs were retrieved and utilized for comparison with the PCR results. The reports were retrieved at the end of the study so that the authors were blinded to them while performing the PCR assays and sequence analysis.

3. Results

3.1. Dogs sampled

The signalment, presence of ticks, *Ehrlichia* PCR result and cause of death as described in the pathology report for the 18 dogs included in the study are detailed in Table 1. Nine dogs were positive by PCR for *E. canis* ($n = 8$) or *A. platys* ($n = 2$) with co-infection found in one dog. The dogs were of different breeds with 11 being of mixed breed and no particular breed over-represented in the *E. canis* positive group. The male to female ratio among the 18 dogs was 1, with five males and three females positive for *E. canis* or *A. platys*. An age description was available for 13 dogs with ages ranging from 6 months to 14 years and a median of 7 years. The median age of *E. canis* positive dogs was 5.8 years and the median age of the *E. canis* negative dogs was 8.5 years.

Ticks were reported at necropsy on three of the PCR-positive dogs and on three of the PCR-negative animals. The causes of death determined by pathology were variable with four dogs suffering from heat stroke of which one was *E. canis*-positive. Two other dogs had severe anemia and tick infestation of which both were *E. canis*-positive and another dog that died from a snake bite was positive by PCR for *E. canis*. Only one dog was diagnosed by pathology as having died from canine ehrlichiosis and was confirmed as positive by PCR for *E. canis*.

3.2. PCR and DNA analysis

All tissue specimens had undergone PCR with the EHR16SR/EHR16SD and the ECA/HE3 primers. Every sample identified as positive for *Ehrlichia* or *Anaplasma* spp. produced a band corresponding to a PCR product of the expected size. Of the nine positive dogs, seven were positive with the ECA/HE3 primers and four were positive with the EHR16SR/EHR16SD primers. The PCR products were sequenced and found by BLAST analysis to be 98–100% homologous to either a partial sequence of the 16SrRNA gene of the Jake strain of *E. canis* (GenBank accession No. CP000107) or to the partial 16SrRNA gene of *A. platys* (GenBank accession No. AF399917.1).

Among the eight dogs that were *E. canis* PCR positive, seven were positive with the ECA/HE3 primers, and three were positive with the EHR16SR/EHR16SD primers of which two were positive with both sets of primers. The two dogs that had a sequence identical to *A. platys* with the *Ehrlichia* genus specific primers were positive also by PCR with specific primers for *A. platys* (Motoi et al.,

Table 1
Signalment, presence of ticks and necropsy findings from eighteen dogs included in the study

Dog number	Breed	Gender	Age in years	Presence of ticks	PCR for *Ehrlichia*	Cause of death as determined by pathology report
1	Mixed	M	N/A	–	+	Snake bite
2	Mixed	M	N/A	–	+	Undetermined
3	Chow-Chow	F	6	–	+	Chronic hepatitis
4	Mixed	M	N/A	+	+	Blood loss anemia with severe tick infestation
5	Mixed	M	N/A	+	+	Blood loss anemia with jaundice and severe tick infestation
6	Pyrenees	F	5.5	–	+	Chronic hepatitis
7	Dog de-Bordeaux	M	1	–	+	Heat stroke, subaortic stenosis
8	Mixed	F	13	+	+	Chronic ehrlichiosis
9	Mixed	F	0.5	–	+	Undetermined
10	Mixed	M	14	+	–	Leishmaniasis
11	Great Dane	M	N/A	+	–	Heat stroke
12	American Staffordshire Terrier	F	9	–	–	Heat stroke
13	German Shepherd	F	13	–	–	Cholangiocarcinoma
14	Mixed	F	8	+	–	Heat Stroke
15	Mixed	F	4	–	–	Uterine stump haemorrhage
16	Mixed	M	3	–	–	Bacterial pyothorax
17	mixed	M	9	–	–	Malignant mesothelioma
18	Boxer	F	7	–	–	Renal failure due to membranoproliferative glomerulopathy

N/A – not available; M – male; F – female.

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Table 2
Distribution of positive PCR results in the tissues of dogs infected with *Ehrlichia canis* or *Anaplasma platys*

	Blood	Lymph node	Spleen	Liver	Bone marrow
Dog 1	–	–	–	EC	–
Dog 2	EC	–	–	–	–
Dog 3	AP	–	–	–	–
Dog 4	EC	EC	–	–	–
Dog 5	–	EC	–	–	–
Dog 6	EC	EC	–	–	–
Dog 7	N/A	EC	–	–	–
Dog 8	EC	EC	EC	–	–
Dog 9	EC, AP	AP	AP	–	–

EC – *Ehrlichia canis*, AP – *Anaplasma platys*, (–) – negative result, N/A – not available.

2001) and negative with primers for *A. phagocytophilum* (Caspersen et al., 2002).

3.3. Tissue distribution

Blood (5/8) and lymph nodes (5/8) were the tissues found to yield the highest number of positive *E. canis* PCR results (Table 2). Seven of eight dogs were positive in the blood or lymph node. The spleen and liver yielded only one positive *E. canis* result, respectively. Interestingly, the liver was the only positive tissue in dog No. 1. Surprisingly, the bone marrow was negative for *E. canis*in all dogs. *Anaplasma platys* was found in multiple organs including the spleen, lymph node and blood in dog No. 8 and only in the blood of dog No. 3.

3.4. Relationship between the time of necropsy and identification of infection

Of the 18 dogs included in the study, eight had necropsy performed within 24 h of the time of death, eight dogs 24–48 h post mortem, and the last two dogs 48–72 h post mortem. Four of the eight dogs from the first group, 4/8 dogs from the second group and 1/2 dogs from the last group were positive by PCR for either *E. canis* or *A. platys*. Blood and lymph node were positive by PCR in dogs necropsied within 72 h post mortem whereas the spleen was only positive in dogs necropsied within 48 h post mortem, the liver was positive 48 h post mortem and the bone marrow was negative at all time points (Table 3).

Table 3
The relationship between the times of death and necropsy, and the distribution of positive PCR results in the tissues of dogs infected with *Ehrlichia canis* or *Anaplasma platys*

Number of hours between time of death and time of necropsy (h)	Blood	Lymph node	Spleen	Liver	Bone marrow
Up to 24	3/4	3/4	1/4	0/4	0/4
Up to 48	2/4	2/4	1/4	1/4	0/4
Up to 72	1/1	1/1	0/1	0/1	0/1

The number of dogs positive in each tissue is compared to the total number of positive dogs necropsied at that time.

4. Discussion

In this molecular study, PCR detected *Ehrlichia* or *Anaplasma* DNA in half of the dog cadavers included in the investigation and sampled at necropsy during the summer and autumn months. Eight of the 18 dogs were infected with *E. canis* and two were positive for *A. platys*. This was an unexpectedly high rate of infection considering that the dogs were not selected for this study by criteria that favoured animals with a higher tendency to be infected with these two pathogens. Moreover, the necropsy pathology reports considered ehrlichiosis as the cause of death in only one of these dogs.

Canine ehrlichiosis in Israel is an endemic disease (Baneth et al., 1996; Harrus et al., 1997b; Baneth et al., 1998). A previous study from Israel indicated that 30% of apparently healthy dogs sampled were seropositive to *E. canis* and had been exposed to infection (Baneth et al., 1996). In addition to this serological evidence, the molecular data presented in the current study from a limited number of dead dogs brought to necropsy further suggested that infection is present in a high proportion of dogs with ehrlichial DNA found in the blood or haemolymphatic tissues. Canine ehrlichiosis in these PCR-positive dogs was probably either a sub-clinical infection, or an exacerbating factor that contributed to other disease conditions present in the dogs and masked by the presenting condition.

Although the finding of *Ehrlichia* DNA in tissues does not necessarily correlate with the presence of viable organisms, a study evaluating the presence of *E. canis* DNA during treatment with doxycyline in experimentally infected dogs has shown that it could not be detected from the blood or spleen within 9 and 60 days from treatment initiation, respectively (Harrus et al., 2004). Based on these findings it is presumed that DNA from dead *E. canis* organisms is probably cleared up in living dogs and does not persist in the tissues for a long time after the elimination of infection by successful treatment or by an effective host immune response. *Anaplasma platys* has also been reported from Israel before in conjunction with *E. canis* or independently from it (Harrus et al., 1997a). Its role as a canine pathogen has been suggested but this remains to be further elucidated (Breitschwerdt, 2005).

Blood clots and lymph nodes were the specimens found to yield the highest number of positive PCR results from the dog cadavers. The spleen and liver were positive in only a small proportion of dogs and the bone marrow was negative in all animals. This is in contrast to results from ante mortem PCR studies on natural and experimental *E. canis* infection (Harrus et al., 1998; Harrus et al., 2004; Mylonakis et al., 2004). The spleen was superior to blood and bone marrow for PCR detection of *E. canis* when preformed on fine needle aspirates of experimentally-infected dogs in the sub-clinical stage of ehrlichiosis (Harrus et al., 1998). In an

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experimental study on acutely infected dogs, the spleen and blood were equally efficacious in yielding positive PCR results, however, *E. canis* was amplified from the spleen after it was eliminated from the blood following doxycyline therapy (Harrus et al., 2004). In a study on the chronic form of canine ehrlichiosis in naturally-infected dogs, *E. canis* DNA was amplified from bone marrow aspirates of 13/19 dogs. PCR was not performed on other tissues in this study (Mylonakis et al., 2004).

A report on PCR performed on tissue specimens taken immediately after euthanasia from three dogs experimentally infected with *E. canis* that remained carriers after treatment with doxycycline revealed that *E. canis* DNA could be demonstrated in the blood, liver, spleen, lymph nodes and kidneys (Iqbal and Rikihisa, 1994). The comparison of these studies is difficult due to the differences in natural vs. experimental infection, the different stages of disease, sampling methodologies, the nature of the tissues evaluated, and ante mortem vs. post mortem obtainment of specimens. However, the fact that the spleen was positive for *E. canis* in only 1/8 dogs and that the bone marrow was negative in all dogs in the current study differ considerably from the results of other studies. Since no other study evaluated cadavers long after death and in the process of autolysis, the low yield of *E. canis* DNA in the spleen and bone marrow may be explained by more progressive nucleic acid degradation in these organs, as compared with the blood and external lymph nodes.

Another possible explanation for the negative PCR obtained in our study may be a false negative result due to a small size of aliquot which did not contain detectable pathogen DNA. The finding of *E. canis* DNA in the liver of dog No. 1 without evidence of *E. canis* in other organs is interesting as the liver is readily accessible for biopsies ante mortem and has not been previously included as a target for *E. canis* PCR. In agreement with the results of the present study, we suggest that blood and lymph node should be the preferred tissues to be sampled when the diagnosis of canine monocytic ehrlichiosis is pursued at necropsy. However, testing of additional tissues such as the liver or spleen could increase the rate of pathogen detection.

The time that elapsed between death and the performance of necropsy did not seem to affect the overall result of PCR for the dogs. Equal proportions of dogs were positive by PCR for *E. canis* and or *A. platys* at different times after death. Four of eight of the dogs were necropsied within 24 h of death, 4/8 within 48 h and 1/2 within 72 h. Molecular laboratory analysis of post mortem tissue samples could be influenced by proper refrigeration of cadavers, the time frame from death to sample collection and the natural process of carcass autolysis. When performing PCR on samples taken at necropsy, the integrity of the DNA is important, since after death, DNA is degraded by DNAses that are released from lysed cells. The blood and lymph node were the only tissues positive 72 h post mortem in this study. This could possibly be due to better preservation of *Ehrlichia* DNA or alternatively to the higher prevalence of the pathogen in these two tissues. Our results indicate that performing PCR for *E. canis* 72 h from the time of death may still yield positive results.

The EHR16SR/EHR16SD and the ECA/HE3 primer sets used in this study amplified a different DNA fragment of the *E. canis* 16SrRNA gene. The lack of complete overlap between results obtained with the different primer sets may be due to differences in nucleotide sequences among local strains of *E. canis*, differences in reactions sensitivities, or inhibition of the reaction in some samples possibly as a result of post mortem changes.

The presence of ticks and the dogs' age were not found to be related to PCR positivity with *E. canis* in the limited number of animals included in the study. This could be due to the small sample. Alternatively, the dogs may have been treated with acaricidals as a part of their medical management. In addition, ticks could have left the cadavers during their storage prior to necropsy.

5. Conclusions

The presence of *E. canis* and *A. platys* can be studied in dog cadavers using PCR, preferably blood and lymph node specimens. Pathogen DNA can be reliably amplified by PCR when tissue samples are obtained 72 h post mortem.

The rate of *E. canis* infection found among the small number of dogs brought to necropsy in Israel and included in this study suggests that the presence of this pathogen in an endemic area for the disease should be suspected more often. The involvement and contribution of *E. canis* to other pathogenic conditions should be further investigated.

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