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Short Communication

A molecular and parasitological survey of *Hepatozoon canis* in domestic dogs in Turkey

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ABSTRACT

In this study, asymptomatic dogs in nine provinces of Turkey were surveyed to investigate the prevalence and intensity of *Hepatozoon canis* infection. DNA obtained from blood samples collected from 694 domestic dogs (243 stray, 288 shelter, and 163 pets) of both genders and varying ages were evaluated by polymerase chain reaction (PCR). In addition, 285 thin blood smears prepared from these blood samples were also evaluated for microscopic examination. Direct microscopy revealed *Hepatozoon* gamonts in the peripheral blood of three of 285 (1.0%; 95% confidence interval (CI): 0.21–3.04) tested. Using PCR, 155 of the 694 (22.3%; 95% CI: 19.28–25.61) were found to be positive for the presence of *H. canis* DNA. The prevalence of infection was higher in adult dogs (26.2%; 95% CI: 22.1–30.7) than young animals (16.4%; 95% CI: 12.2–21.3). Although the prevalence determined by PCR was higher in male dogs (24.5%; 95% CI: 19.6–29.9) than in female dogs (20.8%; 95% CI: 16.9–25.1), gender differences were not significant. Pet dogs had a lower prevalence of infection (10.4%; 95% CI: 6.2–16.2) compared to stray (26.3%; 95% CI: 20.9–32.3) and shelter dogs (25.7%; 95% CI: 20.7–31.1), but no significant association between stray and shelter dogs was found for the presence of the parasite. Partial sequences of the 18S ribosomal RNA (rRNA) gene shared 99–100% similarity with the corresponding *H. canis* isolates. This epidemiological survey revealed a high prevalence of *H. canis* in dogs from several provinces in Turkey, and it suggests that the age and origin are associated with the parasite.

1. Introduction

Hepatozoa are protozoan parasites that infect a wide range of domestic and wild carnivores, birds, reptiles, and amphibians (Little et al., 2009). *Hepatozoon canis* and *Hepatozoon americanum* are found in domestic and wild canids, and *H. canis* has long been recognized as the cause of hepatozoonosis in dogs in Asia, Europe, Africa, and Latin America. Dogs become infected with the parasite by ingesting ticks or tick parts containing mature oocysts with infective sporozoites. *H. canis* is considered less virulent than *H. americanum*, and causes seldom clinical signs. The main vector is the brown dog tick, *Rhipicephalus sanguineus*, although several other species have been suggested as potential vectors for *H. canis* (Aktas, 2014; Giannelli et al., 2013). In Europe, the geographical distribution of *H. canis* is

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restricted to the Mediterranean region, and the Balkan and Iberian peninsulas where the tick *R. sanguineus* is frequent (Estrada-Peña et al., 2013). The disease has been detected in the following southeastern European countries: Croatia (Dezdek et al., 2010), Italy (Gabrielli et al., 2010), Bulgaria (Tsachev et al., 2008), and Greece (Mylonakis et al., 2004).

Tick-borne pathogens such as *Theileria*, *Babesia*, and *Anaplasma* have been documented in domestic ruminants and tick vectors in Turkey, but there is limited information regarding the epidemiology of canine hepatozoonosis. A recent molecular study using polymerase chain reaction (PCR) amplification and DNA sequencing was conducted to identify *Hepatozoon* species and to describe developmental stages of *R. sanguineus* feeding on dogs (Aktas et al., 2013). The objective of this survey was to investigate the frequency and distribution of *H. canis* in asymptomatic domestic dogs from nine provinces of Turkey.

2. Materials and methods

2.1. Area of study and collection of samples

A total of 694 domestic dog blood samples were collected from five coastal provinces (Sakarya, Kocaeli, Mersin, Giresun, and İzmir) and four inland provinces (Elazig, Erzurum, Ankara, and Nevşehir) of Turkey (Fig. 1). The fieldwork was undertaken in collaboration with municipal shelter officers, private veterinary clinics, and the Firat University Veterinary Teaching Hospital (FUVTH). Of the total number of dogs, 288 were from municipal shelters, 243 from the FUVTH, and 163 from several private veterinary clinics where stray dogs are surgically neutered and released back to their original location in the scope of official animal control programs. Blood samples collected from FUVTH were from the Elazig Province, where it is located. The sampling was conducted from June 2010 to October 2012. The dogs were of various breeds and age and of both sexes. Gender, age, and origin and body condition were recorded. Age was estimated based on dentition and body size, and dogs were designated as young (6 months to 1 year old) or adult (1–7 years old). The dogs were classified as asymptomatic based on their behavior at the time of sampling, but a detailed clinical examination was not performed. A 3-mL blood sample was taken from the cephalic vein into a tube coated with ethylenediaminetetraacetic acid (EDTA).

2.2. Microscopic examination

A total of 285 thin blood smears (150 from shelter and 135 from stray) were immediately prepared from the blood samples for microscopic examination. The smears were fixed with methanol for 5 min and stained with 5% May–Grunwald Giemsa for 30 min. The stained slides were examined at 1000× magnification for the presence of *Hepatozoon* gamonts. The parasitemia of the infected dogs was determined by counting 500 leukocytes under oil immersion.

2.3. DNA extraction and PCR amplification

DNA was extracted using the QIAamp DNA Blood Mini kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. For the detection of *H. canis*, a PCR assay was performed using primers Hep-F (5′-ATACATGAGCAAAATCTCAAC-3′) and Hep-R (5′-CTTATTATTCCATGCTGCAG-3′), which amplify a fragment of 666 bp of the 18S ribosomal RNA (rRNA) gene of *Hepatozoon* species (Inokuma et al., 2002). The PCR was performed in a total reaction volume of 25 μL containing 2.5 μL of 10 × PCR buffer (100 mM Tris–HCl (pH 9), 500 mM KCl, and 1% Triton X-100), 2.5 μL of $MgCl_2$, 250 μM of each of the four deoxynucleotide triphosphates, 2 U Taq DNA polymerase (Promega, Madison, WI, USA), and 20 pmol of each primer. The cycling conditions were 94 °C for 5 min followed by 30 cycles at 94 °C for 30 s, annealing at 55 °C for 30 s, extension at 72 °C for 60 s, and a final extension step at 72 °C for 5 min. Positive control DNA previously isolated from a dog naturally infected with *H. canis* (Gen-Bank accession no. JQ867390) and negative control DNA (uninfected canine blood DNA and distilled water) were included in each PCR assay. The amplification products were visualized on 1% agarose gels stained with ethidium bromide and observed under UV illumination. DNA amplicons were purified with a PCR purification kit (Qiagen, Hilden, Germany). DNA sequences obtained were evaluated with Chromas Lite software, version 2.01 (Technelysium Pty, Ltd), and compared for similarity to sequences deposited in GenBank.

2.4. Data analysis

The prevalence (%) and 95% binomial exact confidence intervals (CIs) were calculated for the microscopy and PCR results for *H. canis* using Sourceforge. net® (http://sampsize.sourceforge.net/iface/index.html). The association between the prevalence of *H. canis* by PCR and host factors (gender, age, and origin) was compared. Pearson's chi-squared test was used, and p-values of ≤ 0.05 were considered statistically significant.

2.5. Ethical approval

This study was approved by Firat University's experimental animal ethic committee (approved protocol no. 16.02.2010-15).

3. Results

3.1. Thin blood smears

Thin blood smears revealed the number of parasites in infected dogs ranging from one to seven. *H. canis* gamonts were observed to be ellipsoidal, surrounded by a capsule within the leukocyte cytoplasm. The standard deviation of gamonts was determined as 9.28–11.56 μm × 4.22–6.70 μm.

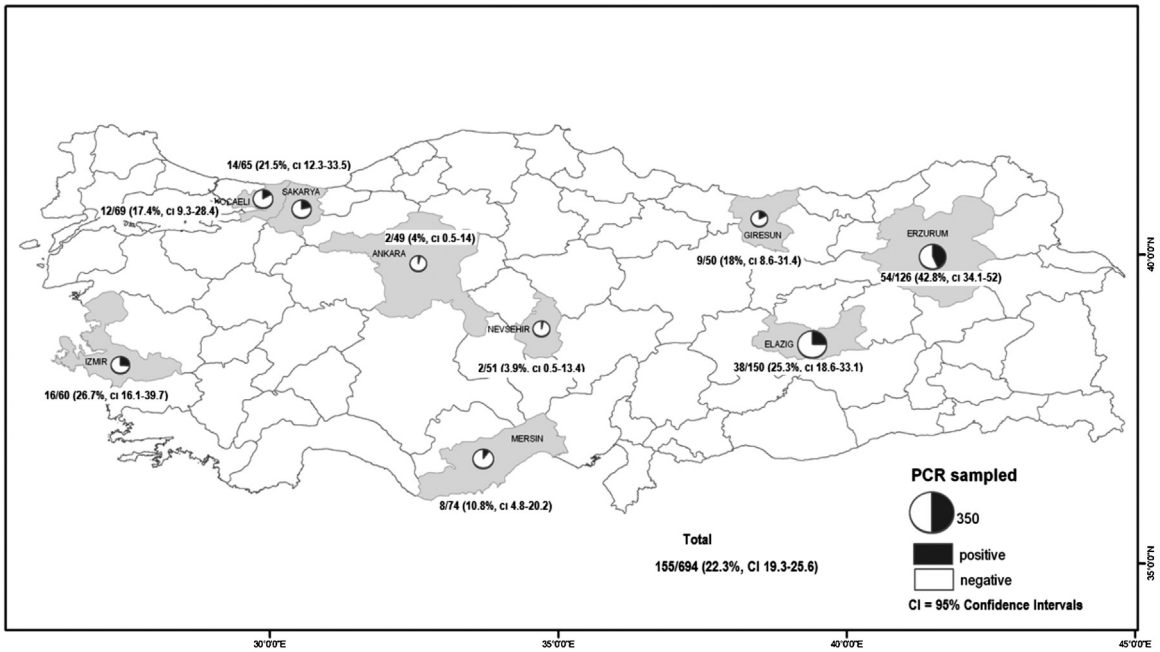

Fig. 1. Shows the province boundaries and the frequency of *Hepatozoon* infection in naturally infected dogs using PCR by location in nine provinces of Turkey.

Table 1
Comparison of *H. canis* positivity in naturally infected dogs by PCR in relation to gender, age, and origin (stray, shelter, and pets).

	Gender		Age		Origin		
	Female	Male	Young	Adult	Stray	Shelter	Pet
No. samples	408	286	275	419	243	288	163
Positive	85 (20.8%)	70 (24.5%)	45 (16.4%)	110 (26.2%)	64 (26.3%)	74 (25.7%)	17 (10.4%)
95% CI	17.0–25.1	19.6–29.9	12.2–21.3	22.1–30.7	20.9–32.3	20.7–31.1	6.2–16.2
p-value*	>0.05		<0.05		>0.05		<0.05

95% CI, 95% confidence intervals.
* Pearson's chi-squared test.

3.2. Prevalence of hepatozoonosis in dogs

Three of 285 thin blood smears were positive for the presence of *Hepatozoon* gametocytes. All of the samples positive by microscopy were confirmed to be infected with *H. canis* by PCR and sequencing. With PCR, 155 of the 694 dogs were found to be infected with *H. canis*. *H. canis* infections were found in all provinces, with the percentage of positive dogs in each province ranging from 3.9% (95% CI: 0.5–13.4) to 42.8% (95% CI: 34.1–52) (Fig. 1). Comparison of *H. canis* positivity in naturally infected dogs by PCR in relation to gender, age, and origin (stray, shelter, and pets) is presented in Table 1. Although positivity obtained by PCR was higher in male dogs (24.5%; 19.6–29.9) than in female dogs (20.8%; 95% CI: 17.0–25.1), significant gender differences were not found ($p > 0.05$). The frequency of *H. canis* infections was higher in adult dogs than in younger dogs ($p < 0.05$). The prevalence of *H. canis* infection was 26.3% (95% CI: 20.9–32.3) among stray dogs, 25.7% (95% CI: 20.7–31.1) among shelter dogs, and 10.4% (95% CI: 6.2–16.2) among pet dogs. Pet dogs had a lower prevalence compared to stray and shelter dogs ($p < 0.05$); instead, no significant association between stray and shelter dogs was found for the presence of the parasite ($p > 0.05$) (Table 1).

To determine the relationship of tick burden with the intensity of *H. canis* infection, dogs sampled in the Diyarbakır Province in inland Turkey were examined for the presence of ixodid ticks. Visual examination revealed 41.26% of the dogs to be infested with adult and nymphal *R. sanguineus*, and PCR positivity for *H. canis* infection was correlated with the presence of ticks on dogs (Aktas et al., 2013).

To confirm the PCR-positive results, randomly selected representative PCR positive samples were sequenced. A BLAST search performed with the 18S rRNA gene sequences of *H. canis isolates* shared 99–100% similarity to various GenBank sequences.

4. Discussion

Using direct microscopy of stained blood smears, this study observed circulating gamonts of *H. canis* in 3/285 (1.0%; 95% CI: 0.2–3) of dogs sampled. The finding was not surprising, as no dog showed evidence of clinical signs of

infection. Similar positivity rates have been reported in a previous study (Amoli et al., 2012). In the present study covering 9 Turkish provinces, *H. canis* infections were found in all, with the percentage of positive dogs in each sampled province ranging from 3.9 (95% CI: 0.5–13.4) to 42.8% (95% CI: 34.1–52). We suggest that *H. canis* is present throughout Turkey.

The overall prevalence of *Hepatozoon* infection revealed by PCR in the present study was similar to previous reports in domestic dogs from the Aegean coast of Turkey (22.3%) (Karagenc et al., 2006), but lower than that observed in Italy (57.8%) (Otranto et al., 2011). It has been suggested that the prevalence of *H. canis* may be related to the distribution and population density of the vector (Otranto et al., 2011), the sampling methodology, and the characteristics of the targeted dog population (Gomes et al., 2010).

In this study, *H. canis* infection was more frequent in adult dogs than in younger dogs similar to a previous study (Gomes et al., 2010). This may be attributed to cumulative exposure of older animals to the parasite. It is well documented that certain tick-borne pathogens, including *H. canis*, may be associated with animal long-term subclinical infections (Rani et al., 2011). The increased contact with the vector may also be the reason for a higher prevalence in adults compared with younger dogs. However, it has been asserted that the differences in prevalence of *H. canis* infection in adult and young dogs was not significant (Rojas et al., 2014), suggesting that factors such as vector density, geographic distribution, and host immune status may play a role in the prevalence of *H. canis* infection. Stray and shelter dogs showed a significantly higher prevalence of *H. canis* infection in comparison with pet dogs.

Although the prevalence was higher in males than in females, gender differences were not significant in the present study. The finding is consistent with previous reports of no correlation of gender with the presence of infection (Gomes et al., 2010). In conclusion, this epidemiological survey reveals the high prevalence of *H. canis* infection in dogs in Turkey, and it suggests that infection is associated with origin, but not with age.

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