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Original article

Molecular detection and characterization of *Hepatozoon* spp. in dogs from the Central part of Turkey☆

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ABSTRACT

Canine hepatozoonosis is a tick-borne protozoal disease caused by *Hepatozoon* spp. Two species of *Hepatozoon* are currently known to infect dogs as *Hepatozoon canis* and *H. americanum*. Although *H. canis* generally causes a chronic infection with relatively mild clinical alterations compared to *H. americanum*, infection by *H. canis* can be life-threatening. The disease is widespread in USA, Africa, Europe, South America, and Asia. To determine the frequency of infection with *Hepatozoon* spp. in stray dogs from Central Anatolia Region of Turkey, a total of 221 blood samples collected over a three-year period were evaluated by using genus specific Polymerase Chain Reaction (PCR) designed to amplify a fragment of 666 bp located in 18 S rRNA gene of *Hepatozoon* spp. Eight (3.61%) blood samples were positive for *Hepatozoon* spp. For the classification of species, all positive PCR products were purified with a PCR purification kit and sequenced. Sequencing results of eight representative amplicons indicated that 6 were 98–99% identical to the sequence of *H. canis* and the other 2 sequences were 95–97% identical to the sequence of *Hepatozoon* spp. So it was named *Hepatozoon* sp. MF. A phylogenetic tree was constructed from the sequences of the tick-borne agents identified previously and in this study using the neighbor-joining method. The nucleotide sequences were compared to the *H. canis* sequences reported in Turkey using the nucleotide Basic Local Alignment Search Tool (BLAST) program. The results of this study are significant in terms of the presence of a novel canine *Hepatozoon* genotype.

Introduction

Hepatozoon species are apicomplexan parasites from the family Hepatozoidae. There are more than 300 species in the genus *Hepatozoon* and about 46 of them infect mammals (Smith, 1996). Canine hepatozoonosis is a tick-borne disease caused by the protozoal agents *Hepatozoon canis* and *Hepatozoon americanum* transmitted by the brown dog tick, *Rhipicephalus sanguineus* and the Gulf Coast tick, *Amblyomma maculatum* respectively (Baneth et al., 2003; Little et al., 2009). *H. canis* is the most common species associated with canine hepatozoonosis in Europe, Asia, Africa and Latin America (Baneth and Vincent-Johnson, 2005; Karagenc et al., 2006; Little et al., 2009; Aktas, 2014; Kaewkong et al., 2014). *H. canis* affects the reticuloendothelial system organs such as spleen, lymph nodes and bone marrow, and it causes the symptoms such as elevated white blood cell counts, stiffness, pain, weight loss, lethargy, anemia and fever; although generally chronic or mild disease, the infection can be life-threatening in severe clinical manifestations associated with high parasite load (Baneth, 2011). In life cycle of canine *Hepatozoon* spp., dogs serve as intermediate host that merogony and gametogony stages of development take place in, and ticks act as definitive host that sexual development and sporogony phases occur (Baneth et al., 2007).

H. canis infections in dogs have been described in worldwide, including the USA, South America, Africa, Europe and Asia (Vojta et al., 2009), only a small number of cases and epidemiological surveys have been reported in Turkey to date (Tüzdil, 1933; Voyvada et al., 2004; Karagenc et al., 2006; Aktas et al., 2013). Turkey is located between the 36–42° northern parallels and the 26–45° eastern meridians forming a land bridge between Europe and Asia with her land area of 770,760 km^2 consisting of seven geographical regions and showing different ecological and climate conditions. Although there have been a number of studies of the prevalence of tick-borne protozoan parasites (*Theileria* and *Babesia*) in tick

☆ Nucleotide sequence data reported in this paper is available in GenBank, EMBL 21 and DDBJ databases under accession numbers from KF439864 to KF439867.

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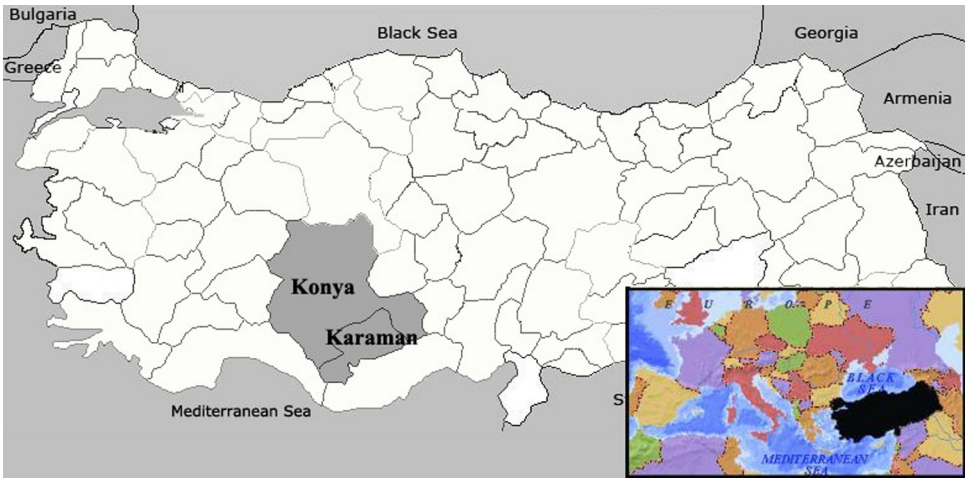

Fig. 1. Turkey map showing the study area.

vectors and domestic animals in Turkey (Altay et al., 2008; Aydin et al., 2012, 2013, 2015). There is paucity on canine tick-borne diseases that represents whole country. This study was planned to determine the prevalence of *Hepatozoon* spp. infections in dogs from the Central Anatolia Region of Turkey by using PCR.

Materials and methods

Study area and collection of field samples

The investigation was carried out on 221 apparently asymptomatic dogs from different locations throughout Konya and Karaman provinces located in Central Anatolia Region of Turkey (Fig. 1) during 2010–2013.

All animals were examined for general clinical investigations such as rectal temperature, visual observation of mucous membranes, abnormalities in size of subcutaneous lymph nodes. The information about the age, sex and breed of animals was recorded (data not shown). After clinical inspections, blood samples were taken from the vena cephalica antebrachii into sterile tubes with K_3EDTA-anticoagulant.

DNA extraction

One hundred twenty-five microliters of blood was added to 250 μl of lysis buffer (0.32 M sucrose, 0.01 M Tris, 0.005 M $MgCl_2$, 1% Triton X-100, pH 7.5). The mixture was centrifuged at 11.600 × *g* for 1 min. The pellet was washed three times with 250 μl lysis buffer by centrifugation. The supernatant was discarded, and the final pellet was resuspended in 100 μl of PCR buffer (50 mM KCl, 10 mM Tris–HCl (pH 8), 0.1% TritonX-100, pH 8.3). Proteinase K (50 μg/ml) was added to the pellet suspension, and the mixture was incubated at 56 °C for 1 h. Finally, the samples were heated at 100 °C for 10 min (Aydin et al., 2013). Genomic DNAs were kept at −20 °C until use.

Polymerase chain reaction and agorose jel electrophoresis

Amplification of the 666 bp fragment of 18S ssrRNA gene of *Hepatozoon* spp. was performed using PCR protocol using the forward HepF 5′-ATACATGAGCAAAATCTCAAC-3′ and the reverse HepR 5′-CTTATTATTCCATGCTGCAG-3′ primers (Inokuma et al., 2002). The PCR was performed in a touchdown thermocycler in a total reaction volume of 25 μl containing PCR buffer [750 mM Tris–HCl (pH 8.8), 200 mM $(NH_4)_2SO_4$, 0.1% Tween 20], 5 mM $MgCl_2$, 125 μM deoxynucleotide triphosphates, 1.25 U Taq DNA polymerase (Promega, Madison, WI, USA), primers (20 pmol/μl) and template DNA. Touchdown PCR was performed with 50 cycles of 94 °C for 20 s, 67–59 °C for 45 s, and 72 °C for 45 s, followed by a final extension at 72 °C for 10 min. The annealing temperature was decreased every second cycle with 2 °C to a "touchdown" temperature of 57 °C.

PCR product was visualized using Gel Doc (Bio-Rad, Hercules, CA, USA) in a 1.5% agarose gel stained with ethidium bromide after electrophoresis (100 V, 60 min). PCR products were purified using the Qiaquick purification kit (Qiagen, Hilden, Germany) and sequencing was conducted by a commercial company (Iontek, Istanbul, Turkey). Sequences were determined in both directions (using the forward and reverse primers).

Phylogenetic analysis

The sequences of the partial 18S rRNA gene of *Hepatozoon* identified in this study have been deposited in GenBank under the Accession Nos.: KF439864–KF439867. Each construct was sequenced at least three times and subjected to BLAST similarity searches. A phylogenetic tree was constructed from the sequences of the 18S rRNA genes of some tick-borne agents infect carnivores using the neighbor-joining method by the MAFFT Multiple Sequence Alignment Software Version 7 (Katoh and Standley, 2013). The nucleotide sequences used in this study are available in GenBank under the following accession numbers: DQ111764 for *Babesia canis rossi*; AM183216 for *Babesia canis vogeli*; HM212628 for *Theileria annae*; JQ867388 for *Hepatozoon felis*; DQ439540, DQ060329, KC584777 and FJ497019 for *H. canis*.

Results

Out of 221 field samples, 8 (3.61%) were found positive in terms of *Hepatozoon* spp. by touchdown PCR.

All of the positive samples were confirmed by sequence comparisons. Two *Hepatozoon* sequences were identified. Distribution and frequency of canine *Hepatozoon* species detected by PCR in Central Anatolia in Turkey was summarized in Table 1.

The partial sequences of the 18S rRNA genes for *H. canis* and *Hepatozoon* sp. MF were deposited in the EMBL/GenBank databases under accession numbers from KF439864–KF439867. *H. canis* sequences shared 99% identity with the previously reported sequences for the 18S rRNA gene of *H. canis*, also the other sequence differed clearly from the all known *Hepatozoon* species (Fig. 2).