

Molecular Survey of *Hepatozoon canis* in Dogs from Samsun Province of Northern Part of Turkey

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Abstract: We attempt to address new information of haematozoan infections in dogs from Samsun province of Turkey. The diagnostic techniques using genus specific polymerase chain reaction designed to amplify a fragment of ~666 bp located in 18S ribosomal RNA (rRNA) gene of *Hepatozoon* spp. include microscopic investigations. *H. canis* identities were confirmed by sequencing the 18S rRNA gene. Two hundred (170 stray and 30 owned dogs) blood samples from asymptomatic dogs treated for endo and ecto parasites were examined. There were no detected ticks on dogs in the clinical examinations. None of 200 thin blood smears were positive for the presence of *Hepatozoon* spp. gametocytes. Only one dog sample from 200 with a prevalence of 0.5% was found to be positive for the presence of *Hepatozoon* DNA demonstrates a chronic infection. Partial sequences of the 18S rRNA gene shared 99-100% similarity with the corresponding *H. canis* isolates. Contrary the other molecular surveys on *H. canis* among Turkey, this research revealed a very low prevalence of *H. canis* in dogs from Samsun province of Turkey, and it indicates that ectoparasite control programs have a great impact on decreasing the vector borne parasitic diseases especially in the stray and owned dogs.

Key Words: 18S rRNA gene, DNA sequencing, *Hepatozoon canis*, dogs, Samsun, Turkey

Türkiye'nin Samsun İlindeki Köpeklerde *Hepatozoon canis*'in Moleküler İncelenmesi

Özet: Bu çalışmada Samsun'da köpeklerde haematozoan enfeksiyonlar hakkında yeni bilgilerin verilmesi amaçlanmıştır. Bu amaçla mikroskopik inceleme ile birlikte *Hepatozoon* spp.'nin ~666 baz uzunluğundaki 18S ribosomal RNA (rRNA) gene bölgesini amplifiye eden cins spesifik polimeraz zincir reaksiyon metodu kullanıldı. 18S rRNA gene bölgesinin dizi analizi ile *H.canis* identifiye edildi. Endo ve ektoparazit tedavisi yapılmış asemptomatik köpeklerden 200 (170 sokak ve 30 sahipli) kan örneği toplandı. Köpeklerin klinik muayenelerinde keneler saptanmadı. Çalışmada 200 kan frotisinin mikroskopik bakışında *Hepatozoon* spp. gametositlerine rastlanmadı. Araştırmada % 0,5 enfeksiyon oranında 200 örnekten sadece 1 köpekte *Hepatozoon* DNA'sı pozitif bulunması kronik enfeksiyonu işaret etmektedir. Araştırmada pozitif bulunan örneğin 18S rRNA geninin kısmi DNA dizisi dünyadaki diğer *H. canis* izolatları ile % 99-100 oranında benzerlik gösterdi. Bu çalışmada Türkiye genelindeki *H. canis*'in moleküler incelemelerinin aksine Samsun yöresindeki köpeklerde çok düşük enfeksiyon oranı ile karşılaşıldı. Bu durum sokak ve sahipli köpeklerde vektör kaynaklı parazitler hastalıklarının azaltılması noktasında özellikle ektoparazit kontrol programlarının önemini ortaya koymaktadır.

Anahtar Kelimeler: 18S rRNA gen, DNA dizi analizi, *Hepatozoon canis*, köpek, Samsun, Türkiye

Introduction

Hepatozoon is a tick borne protozoan parasite, classified in the Phylum Apicomplexa and is closely related to *Plasmodium* spp. and Piroplasmids. Representatives of the genus infecting dogs occur both in the New and the Old World. The two species *H. canis* and *H. americanum*, with distinct clinical, pathological, genetic, antigenic aspects and vectors of transmission especially have importance on dogs. However, the distribution of *H. americanum* is re-

stricted to the United States; *H. canis* is geographically more widespread - including the America, the tropical and temperate zone of Europe, Africa, southwestern Asia, southern and Eastern Europe [5,7,12].

Chronically reports and surveys on hepatozoonosis in Turkey have been started with a first case report in Turkey in 1933 [15], thereafter another case report of this disease has been followed [16]. A parasitological, molecular and serological survey

of *H. canis* infection in dogs around the Aegean coast of Turkey including Central Aydın, Kusadası, Selçuk, Central Manisa, Bodrum and Marmaris was presented by Karagenc et al. [10]. Based on molecular investigations of *Hepatozoon* species in dogs reported the infection source as Diyarbakir an inner province of Turkey [2]. Later, Düzlü et al. [8] reported *H. canis* infection in dogs from Kayseri province by Real Time PCR. Moreover, Aktas et al. [1] also reported another molecular and serological survey of *H. canis* infection in domestic dogs from five coastal provinces (Sakarya, Kocaeli, Mersin, Giresun, and Izmir) and four inland provinces (Elazığ, Erzurum, Ankara, and Nevşehir) of Turkey. Finally, molecular detection and characterization of *Hepatozoon* spp. in dogs reported from different locations throughout Konya and Karaman provinces located in Central Anatolia Region of Turkey [4].

Showing different ecological and climate conditions the Middle Black Sea Region of Turkey has a great importance of the distribution of vector borne parasitic diseases. Therefore, a molecular study was attempted to determine the prevalence of *Hepatozoon* spp. infection in a coastal province, Samsun, from the Middle Black Sea Region of Turkey by using blood smear, and PCR amplification and DNA sequencing was conducted to identify *Hepatozoon* species.

Materials and Methods

Animals

A total of 200 domestic dog blood samples were collected from Samsun province of Turkey. The field work was undertaken in collaboration with Samsun Metropolitan Municipality's Incapacitated Orphaned Animal Care Center officers, and colleges in the Ondokuz Mayıs University Faculty of Veterinary Medicine's Teaching Animal Hospital. Of the total number of 200 dogs with different breeds and sexes, 170 were from municipal shelters, 30 from the Ondokuz Mayıs University Faculty of Veterinary Medicine's Teaching Animal Hospital. Because of the shelter management policy all dogs that were enrolled in the study were treated for endo and ecto parasites, same as all owned dogs were also treated for endo and ecto parasites. Based on their detailed clinically examinations dogs were determined as

asymptomatic. Venous blood was taken from the cephalic vein, with 2 ml evacuated into a plain additive tube with K3 EDTA (7.5 per cent 0.040 ml) for blood firm sampling and PCR. Sampling procedures was conducted between 2010 and 2013. The protocol for sampling had been reviewed and approved by the Ethical Review Committee of the Ondokuz Mayıs University (No: HAYDEK/109). Thin blood smears were taken from the cephalic vein, fixed with ethanol, stained with Giemsa, and screened for *Hepatozoon* gametocytes.

DNA isolation, amplification and sequencing

The genomic DNA was extracted from blood samples using a DNeasy Blood and Tissue Kit (Qiagen) according to manufacturer's instruction. Polymerase chain reaction (PCR) targeting the partial 18S rRNA gene sequences of *Hepatozoon* spp. were performed. PCR was carried out in a final volume of 50 µl, containing 10-50 ng of extracted DNA, 1X PCR Buffer with KCl (Thermo Scientific), 1.5 mM of MgCl₂ (Thermo Scientific), 0.2 mM each dNTPs (Thermo Scientific), 20 pmol of each primer and 2 U of Taq DNA Polymerase (Thermo Scientific). Fragments of ~ 666 bp of the partial 18S rRNA gene were amplified using the primers HepF (5'-ATACATGAGCAAATCTCAAC-3') and HepR (5'-CTTATTATTCATGCTGCAG-3') [9]. The PCR was performed in a Thermo PxE 0.2 thermal cycler (Thermo Scientific) and the conditions were as follows: 5 min at 94°C, then 30 cycles of 30 s at 94°C, 30 s at 55°C and 1 min at 72°C followed by a final elongation of 5 min at 72°C. PCR products were electrophoresed in 1.5% agarose gel (Prona) in a TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8.3) (Thermo Scientific), stained with ethidium bromide (Sigma) and visualized by UV transillumination (DNR, Bio-imaging system). The size of the amplified fragments was estimated by comparisons with the 1000 bp DNA Ladder (Thermo Scientific).

Hepatozoon canis identities were confirmed by sequencing the 18S rRNA gene. The 18S rRNA gene amplification product was sent to sequencing company (Macrogen, Korea) for purification and sequencing in both directions using same primers. Sequencing was carried out directly on purified fragments with ABI PRISM 310 genetic analyzer

(Applied Biosystems, Foster City, CA, USA), using the ABI PRISIM® BigDye terminator cycle sequencing ready reaction kit. Sequence quality was assessed using Vector NTI Advance 11.5 (Invitrogen).

Phylogenetic analysis

The obtained sequences were verified by forward and reverse comparisons, assembled and edited with using Contig Express in Vector NTI Advance 11.5 (Invitrogen). The obtained consensus sequences were compared with previously published data for identification by using the Basic Local Alignment Search Tool (BLAST) via GenBank database [3]. Sequences were aligned with previously characterized sequences of other known *H. canis* using ClustalW in Mega 5.0 (<http://www.megasoftware.net/>) multiple sequence alignments [14] and adjusted manually. Genetic distances were calculated using the Kimura two-parameter model with pairwise deletion in Mega 5.0 [13]. The nucleotide sequence was deposited in GenBank database under the accession numbers: KX588232.

Results

In the present study, information on ecto parasiticide treatment was available for all dogs. Therefore,

there were no detected ticks on dogs in the clinical examinations. None of 200 thin blood smears were positive for the presence of *Hepatozoon* spp. gametocytes. In contrast, results out of 200 samples, only 1 (0.5%) were found positive in terms of *Hepatozoon* spp. by PCR in the stray dog. The only PCR-positive sample was confirmed by 18S rRNA gene sequence comparisons in Genbank. The percent identities among *H. canis* isolates from Turkey (Samsun, KX588232) showed 99-100 % identity with various geographical isolates of *H. canis* from the Iran (KT736298), Hungary (KJ572976), Italy (GU371447; GU371448; GU371449; GU371450; KP644235), Malaysia (KT267961), Croatia (FJ497019; FJ497022; HM212626), Brazil (AY461375) and Spain (AY150067) from GenBank. Moreover, in Turkey, pairwise comparison between the 18S rRNA gene sequences of the *H. canis* isolates from Samsun (KX588232) and other *H. canis* isolates from different regions (strain H130, KF034776; Konya B172, KF439867; Aydın, DQ060328; Kusadasi, DQ060324; Selcuk, DQ060329; Bodrum, DQ060327; Marmaris, DQ060326; TrKysHcan1, KJ513198; isolate DD11, JQ867390; Konya B167, KF439866; Manisa, DQ060325) showed differences ranging from 0.0 to 0.9 % (Table 1).

Table 1. Pairwise comparison of nucleotide sequence differences (in percent) in the 18S rRNA among *H. canis* isolates in Turkey.

	1	2	3	4	5	6	7	8	9	10	11	12
1. Samsun, KX588232	-											
2. strain H130, KF034776	0.000	-										
3. Konya B172, KF439867	0.002	0.002	-									
4. Aydın, DQ060328	0.005	0.005	0.007	-								
5. Kusadasi, DQ060324	0.005	0.005	0.007	0.000	-							
6. Selcuk, DQ060329	0.007	0.007	0.009	0.002	0.002	-						
7. Bodrum, DQ060327	0.007	0.007	0.009	0.002	0.002	0.000	-					
8. Marmaris, DQ060326	0.007	0.007	0.009	0.002	0.002	0.000	0.000	-				
9. TrKysHcan1, KJ513198	0.007	0.007	0.009	0.002	0.002	0.000	0.000	0.000	-			
10. isolate DD11, JQ867390	0.007	0.007	0.009	0.002	0.002	0.000	0.000	0.000	0.000	-		
11. Konya B167, KF439866	0.007	0.007	0.009	0.002	0.002	0.000	0.000	0.000	0.000	0.000	-	
12. Manisa, DQ060325	0.009	0.009	0.011	0.005	0.005	0.007	0.007	0.007	0.007	0.007	0.007	-

Discussion

Hepatozoonosis as one of the other tick-borne diseases plays a major hazard to domestic and wild canids globally. Therefore, epidemiological studies are essential to describe the trends of infection with a particular pathogen and its vectors locally and regionally. Knowledge on vector-borne infections at the local level allows veterinary practitioners to recognize the pathogens that can affect their patients facilitating a prompt diagnosis and treatment [6]. To date no study about canine hepatozoonosis from Samsun province of Turkey has been reported. Although PCR is considered the most sensitive detection method for canine hepatozoonosis, microscopic examination of blood smears is a simple technique frequently used for the diagnosis of this infection. This technique may be used also as an epidemiological tool for studies in areas where canine hepatozoonosis is endemic or where it is suspected [11]. However, negative results may lead to misdiagnosis of the disease especially in tick free patients.

Previous studies even molecularly presented in different parts of Turkey represented higher infection rates [1,2,4,8,10,16] contrarily to our results. One of the explanations of this very low infection rate of our result may be depended to the successful ecto parasite treatment of the sampled dogs. While, all shelter samples same as all owned dogs were also treated for endo and ecto parasites. The only positive result of the study also demonstrates the chronic infection in the dogs, which only could be detected by PCR. Same as Otranto et al. [11], this study suggests that when no information is available on the date of potential infective tick exposure, PCR on either blood or buffy coat should be preferred to cytology for the diagnosis of *H. canis* infection. This detection of the chronic infection even in one sample shows the distribution of hepatozoonosis in Samsun province, and precautions should be taken for disease protection.

Therefore, we believe that this molecular survey has an importance for clarifying the canine hepatozoonosis, and guide the practitioners for continuing ecto parasite treatment who may suffer patients have with high fever and potential infective tick exposure in the northern part of Turkey.

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