Kafkas Univ Vet Fak Derg
25 (1): 77-81, 2019
DOI: 10.9775/kvfd.2018.20352

Kafkas Universitesi Veteriner Fakultesi Dergisi
ISSN: 1300-6045 e-ISSN: 1309-2251
Journal Home-Page: http://vetdergikafkas.org
Online Submission: http://submit.vetdergikafkas.org

Research Article

Molecular Survey of Hepatozoonosis in Natural Infected Dogs: First Detection and Molecular Characterisation of *Hepatozoon canis* in Kyrgyzstan

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Article ID: KVFD-2018-20352 Received: 13.06.2018 Accepted: 26.09.2018 Published Online: 27.09.2018

How to Cite This Article

Altay K, Aydin MF, Aytmirzakizi A, Jumakanova Z, Cunusova A, Dumanli N: Molecular survey of hepatozoonosis in natural infected dogs: First detection and molecular characterisation of *Hepatozoon canis* in Kyrgyzstan. *Kafkas Univ Vet Fak Derg,* 25 (1): 77-81, 2019. DOI: 10.9775/kvfd.2018.20352

Abstract

Canine hepatozoonosis is a tick-borne protozoan disease and widespread in Europe, Africa, Asia and America. There is not any available data about the presence of *Hepatozoon* infections in dogs in Kyrgyzstan. In the study we aimed that investigate the presence of *Hepatozoon canis* and the prevalance of *Hepatozoon* infections in dogs from Kyrgyzstan using polymerase chain reaction (PCR) and sequence analysis. To determine the prevelance of hepatozoonosis in dogs, a total of 170 blood samples were applied to PCR to amplify a fragment of 666 bp found in 18S SSU rRNA gene of *Hepatozoon* spp. The PCR results shown that *Hepatozoon* infection rate was 28.8% (49/170) in dogs. Eleven representative positive samples were sequenced to classification of the species. The nucleotide sequences were compared to the *H. canis* sequences which registered in GenBank using the basic local alignment search tool. Results of sequence analyse of 11 amplicons indicated that 8 were 100% identical and the other 3 sequences shared 99% similarity with *H. canis*. The sequences were deposited in Genbank with accession numbers from MG917709 to MG917719. It was the first record of *H. canis* in dogs in Kyrgyzstan.

Keywords: *Hepatozoonosis, Hepatozoon canis, Dog, PCR, Sequencing, Kyrgyzstan*

Doğal Enfekte Köpeklerde *Hepatozoon* Enfeksiyonlarının Moleküler Yöntemlerle Araştırılması: *Hepatozoon canis*'in Kırgızistan'da İlk Tespiti

Öz

Köpeklerde hepatozoonozis kenelerle nakledilen ve Afrika, Avrupa, Asya ile Amerika'da yaygın olarak görülen protozoan bir hastalıktır. Kırgızistan'da köpeklerde *Hepatozoon* enfeksiyonlarının varlığına dair bir bilgiye ulaşılamamıştır. Bu çalışmada, polimeraz zincir reaksiyonu (PZR) ve sekans analizi ile Kırgızistan'da köpeklerde *Hepatozoon* enfeksiyonlarının yaygınlığının ve *Hepatozoon canis*'in varlığının belirlenmesi amaçlanmıştır. *Hepatozoon* enfeksiyonlarının prevalansını belirlemek için 170 kan örneğine, *Hepatozoon* spp. 18S SSU rRNA geninin 666 bp'lik kısımını amplifiye etmek üzere PZR uygulanmıştır. PZR sonucunda *Hepatozoon* spp. enfeksiyon oranı %28.8 (49/170) olarak ortaya çıkmıştır. On bir PZR pozitif DNA örneğinin DNA dizilimi belirlenmiş ve elde edilen DNA dizilimleri BLAST programı kullanılarak GenBank'ta kayıtlı diğer dizilimlerle karşılaştırılmıştır. Bunlardan 8'inin GenBankasında kayıtlı *H. canis* DNA dizilimleri ile %100 oranında eşleştiği, diğer 3 dizilimin ise %99 oranında bir benzerliğe sahip olduğu belirlenmiştir. DNA dizilimleri GenBankasına MG917709-MG917719 numaraları altında kaydedilmiştir. Bu çalışma Kırgızistan'da köpeklerde *H. canis*'in belirlendiği ilk çalışma niteliğindedir.

Anahtar sözcükler: *Hepatozoonozis, Hepatozoon canis, köpek, PZR, Sekans analizi, Kırgızistan*

INTRODUCTION

Canine hepatozoonosis is a tick-borne protozoan disease and widespread in Europe, Africa, Asia and America. The first hepatozoonosis case was determined in India, 1905, named as *Leukocytozoon canis*. After the parasite was

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detected in neutrophils, it transferred to the genus of *Hepatozoon* [1,2]. There are more than 340 species in the genus of *Hepatozoon* and two of them infect dogs, *Hepatozoon canis* and *Hepatozoon americanum* [1,3]. On the other hand, some new genotypes have been found in dogs with the molecular-genetic based studies in recent years [4,5]. The disease is transmitted by the ingestion of the vector ticks. *Rhipicephalus sanguineus* in Asia, Europe, Africa and Brazil, *Amblyomma maculatum* in South America are known as vector of canine hepatozoonosis [6-8].

Clinical signs of hepatozoonosis in dogs depend on caused species, the nutritional and the others individual factors. *H. canis* infections can change from subclinical to severe and fatal disease. Subclinical and mild disease is common and generally have low parasitemia but 100% of neutrophils can be infected with *H. canis* in the severe disease which characterised by lethargy, fever and extreme weight loss. *H. americanum* infections causes fever, generalised pain or hyperaesthesia, myositis, muscle atrophy, weakness, depression, reluctance to rise and mucopurulent ocular discharge [2,9,10].

Diagnosis of canine hepatozoonosis may be done with seen of intracytoplasmatic gamonts in neutrophils and monocytes by microscopic examination of the thin blood smears. Secondly the histopathologically meront and monosoic cysts can be investigated. The biopsy of skeletal muscle is the gold standard for the diagnosis of *H. americanum* infections in dogs because gamonts of the parasite are rarely seen in thin blood smears. Serological methods such as indirect immunofluorescence and enzyme-linked immunosorbent assay have been used diagnosis of *Hepatozoon* infections in dogs. But serological methods are generally preferred in epidemiological studies to detection of chronic infections [2,9,10].

In the recent years, molecular-genetic based diagnostic methods such as polymerase chain reaction (PCR) and DNA sequencing have been used for survey of the presence, the characterisation and detection of prevalence of *Hepatozoon* species in dogs [11-14]. These methods have been accepted to be more sensitive and specific than microscopic and serological methods for the diagnosis of hepatozoonosis and the other blood parasites [15-22]. We have not found available data about canine hepatozoonosis in dogs in Kyrgyzstan. In this study we aimed that investigate the presence of *H. canis* and to determine the prevalence of *Hepatozoon* infections in dogs from Kyrgyzstan using polymerase chain reaction (PCR) and sequence analysis.

MATERIAL and METHODS

Study Area and Collection of Blood Samples

Kyrgyzstan Republic is a Central Asia Union country and it borders Kazakhstan to the north, Uzbekistan to the west, Tajikistan to the southwest and China to the southeast. The country is landlocked and mountainous, located in the Northern Hemisphere in the center of the Eurasian continent, as well as it is far away from large water bodies (the seas and oceans) and close of the desert. Bishkek, formerly Pishpek or Frunze is the largest city and the capital of the country [23] *(Fig. 1)*. The blood samples were collected from dogs living in a shelter in Bishkek with cooperation with the shelter's and Kyrgyz-Turkish Manas University Veterinary Teaching Hospital's staffs.

The sampling was carried out from May 2016-October 2017. The dogs have been accepted without clinical signs with behavioral inspection of the dogs, but particular clinical examination was not conducted. A total of 170 blood samples were obtained. The blood samples were collected in tubes containing ethylendiamine tetraacetic acid (EDTA) and stored -20°C until use for DNA extraction.

Fig 1. Map of Kyrgyzstan and location of Bishkek

Total DNA Extraction from Blood Samples and Polymerase Chain Reaction (PCR)

Total DNA extraction from blood samples was performed using a PureLink Genomic DNA mini kit (Invitrogen, Carlsbad, USA) following the manufacturer's instructions. The DNA samples were stored at -20°C until use for PCR. To determine the presence *Hepatozoon* spp. in the DNA samples, a PCR analysis was carried out using Hep-F (5'-ATACATGAGCAAAATCTCAAC-3') and Hep-R (5'-CTTATT ATTCCATGCTGCAG-3') primers which amplify the partial 18S small subunite ribosomal RNA (18S SSU rRNA) gene of *Hepatozoon* species [24]. Sterile water (DNase, RNase free) and *H. canis* positive control DNA were used as negative and positive control in PCR, respectively. *H. canis* positive control DNA was provided from Department of Parasitology, Faculty of Veterinary Medicine, Selcuk University. The PCR was practiced in a touchdown termo-cycler in a total reaction volume of 25 µL according to Aydin et al.[4].

Eleven PCR positive sample products were purified using QIAquick PCR purification kit (Qiagen, Hilden, Germany). The purified PCR samples were sequenced in a commercial company (Iontek, Istanbul, Turkey). The sequences of the partial 18S SSU rRNA gene of *Hepatozoon* spp. obtained in the study were submitted to basic local alignment search tool (BLAST) similarity search. After the sequences were identified they have been deposited in GenBank.

RESULTS

A total of 170 blood samples were analyzed with PCR to amplify a fragment of 666 bp located on 18S SSU rRNA gene of *Hepatozoon* spp. using Hep-F and Hep-R primer pairs *(Fig. 2)*.

Hepatozoon spp. was detected in 49 blood samples of investigated 170 dogs with PCR. Out of 170 field samples, 121 were found as negative in terms *Hepatozoon* spp.

To identify and confirm the PCR positive results, randomly selected representative 11 PCR positive samples were purified and sequenced. A BLAST search performed to compare the sequences alignments with the other *Hepatozoon* spp. sequences registered in GenBank under the accession numbers from MG917709 to MG917719.

Nucleotide sequences of partial 18S SSU rRNA gene of *Hepatozoon* spp. obtained in the study (MG917709 to MG917719) were aligned with the GenBank registered 18S SSU rRNA gene sequences of *H. canis* (KX712126, KX880505, KY197000). Sequence alignment of the PCR positive samples were identified as *H. canis*. BLAST analyse showed that 8 isolates were shared 100% similarity with *H. canis* isolates while the other 3 isolates were shared 99% similarity from Czeck Republic (accesion number: KX712126), Iran (accesion number: KX880505), Turkey (accesion number: KY197000).

DISCUSSION

Canine hepatozoonosis, a worldwide protozoon parasitic infection, has been reported in many countries of Africa, America, Asia and Europe such as Nigeria [25], Italy [14], Thailand [26], Croatia [27], Brazil [28], Argentina [11], and Turkey [13]. There are not any available data on presence *Hepatozoon* infections in dogs in Kyrgyzstan. The DNA based molecular diagnostic techniques such as PCR, RLB are widely used for detection and identification of blood parasites from animals. These techniques have superiority in specifity and sensitivity, and they permit to identification new genotypes and/or species [11-22]. Otronto et al.[14] showed that PCR the most sensitive assay for the detection of *H. canis* infection in dogs. They suggest that PCR can be used in epidemiological studies as a convenient diagnostic test. Using PCR, this study revealed hepotozoonozis has a high prevalence in dogs in Kyrgyzstan. 49 out of 170 blood samples were found as positive for *Hepatozoon* spp. with PCR. The result showed that hepatozoonosis has a high prevalence in dogs in Kyrgyzstan.

Hepatozoon canis and H. americanum are primary agents of canine hepatozoonosis. *H. canis* is more prevalent species than *H. americanum* in dogs. *H. canis* is seen in Europe,

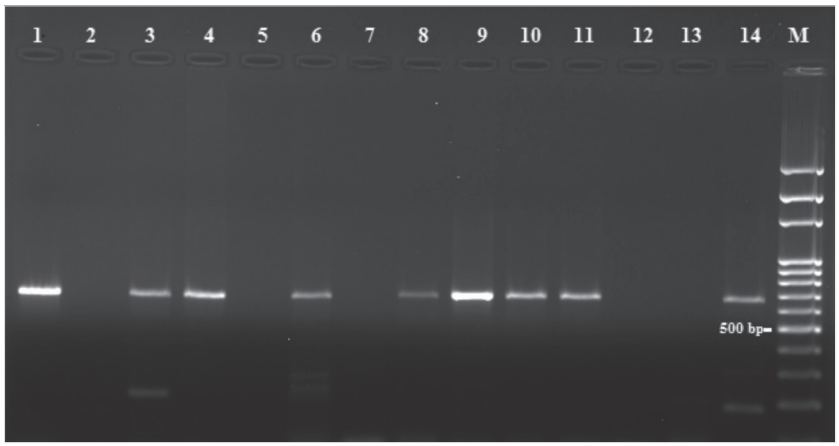

Fig 2. Agarose-gel electrophoresis of *Hepatozoon* spp. specific polymerase chain reaction products using Hep-F and Hep-R primers. Lane 1, *Hepatozoon canis* positive control DNA from dog; lane 2, negative control distilled water; lane 3,4,6,8,9,10,11,14, positive dog blood samples; 5,7,12,13 negative dog blood samples

Asia, Africa and also America while *H. americanum* is limited in America continent [1-3,29]. In this study 49 samples were detected positive with *Hepatozoon*-genus specific PCR. The partial sequences were determined of 11 PCR positive samples. The positive samples were identified as *H. canis* according to sequence alignments. BLAST analysis of the sequences showed that 8 *isolates* were shared 100% similarity with *H. canis* isolates while the other 3 isolates were shared 99% similarity from Czeck Republic (accesion number: KX712126), Iran (accesion number: KX880505), Turkey (accesion number: KY197000). It was the first detection of *H. canis* in dogs from Kyrgyzstan using molecular methods. There is need more comprehensive studies to detection of the infection status in Kyrgyzstan.

Currently, there are no protective vaccines for the prevention of canine hepatozoonosis. Therefore, the only powerful method to prevent the canine hepatozoonosis is the control of vector ticks. On the other hands wild carnivores such as jackals have been found naturally infected with *H. canis* [30]. This situation enhances the importance of epidemiological knowledge of *Hepatozoon* infections in terms of the development and application of appropriate control strategies. This survey reveals the high prevalence of canine hepatozoonosis in dogs in Kyrgyzstan, and *H. canis* is the primary agent of the diseases in the country. There is still need more prevalent epidemiological studies on canine hepatozoonosis in the country.

Acknowledgments

We thank Dr. Ferda Sevinc (Selcuk University) for *Hepatozoon canis* positive control DNA animal shelter staffs and Kyrgyz-Turkish Manas University Veterinary Teaching Hospital staffs for their assistance in sample collection.

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