Kafkas Univ Vet Fak Derg
26 (1): 143-146, 2020
DOI: 10.9775/kvfd.2019.22196

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

Short Communication

First Molecular Evidence for *Mycoplasma haemocanis* and *Candidatus Mycoplasma haematoparvum* in Asymptomatic Shelter Dogs in Kyrgyzstan

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Article ID: KVFD-2019-22196 Received: 07.03.2019 Accepted: 03.07.2019 Published Online: 03.07.2019

How to Cite This Article

Altay K, Aydin MF, Aytmirzakizi A,Jumakanova Z, Cunusova A, Dumanli N: First molecular evidence for *Mycoplasma haemocanis* and *Candidatus Mycoplasma haematoparvum* in Asymptomatic shelter dogs in Kyrgyzstan. *Kafkas Univ Vet Fak Derg,* 26 (1): 143-146, 2020. DOI: 10.9775/kvfd.2019.22196

Abstract

Mycoplasma haemocanis (Mhc) and *Candidatus Mycoplasma haematoparvum* (CMhp) have been investigated using species specific PCR and sequencing in 170 dogs from Kyrgyzstan. Maximum likelihood estimation (MLE) of the infection rates with 95% confidence intervals (CI) was calculated. The molecular prevalence of hemoplasma infection was 5.29% (CI 2.57-9.34). It was found that, five (2.94%, CI 1.06-6.22) samples were found to be infected with Mhc, one (0.59%, CI 0.03-2.57) sample with CMhp and three (1.76%, CI 0.44-4.52) samples with both species. These results demonstrate that dogs can be exposed to each haemoplasma species and provide first molecular evidence for these species in Kyrgyzstan.

Keywords: *Canine, Haemoplasmas, PCR, Kyrgyzstan*

Kırgızistan'da Asemptomatik Barınak Köpeklerinde *Mycoplasma haemocanis* ve *Candidatus Mycoplasma haematoparvum* İçin İlk Moleküler Kanıt

Öz

Kırgızistan'dan 170 köpekde tür spesifik PZR ve sekanslama ile *Mycoplasma haemocanis* (Mhc) ve *Candidatus Mycoplasma haematoparvum* (CMhp) araştırılmıştır. Enfeksiyon oranlarının en büyük olabilirlik tahmini (MLE) %95 güven aralığında (CI) hesaplandı. Hemoplasma enfeksiyonunun moleküler prevalansı %5.29 idi (CI 2.57-9.34). Beş (%2.94, CI 1.06-6.22) numunenin Mhc ile, bir (%0.59, CI 0.03-2.57) numunenin CMhp ile ve üç (%1.76, CI 0.44-4.52) numunenin de her iki tür ile enfekte olduğu bulundu. Bu sonuçlar, Kırgızistan'daki köpeklerin hemoplasma türlerinin her biri ile maruz kalabileceğini göstermekte ve bu türler için Kırgızistan'daki ilk moleküler kanıtı sağlamaktadır.

Anahtar sözcükler: *Köpek, Hemoplasma, PZR, Kırgızistan*

INTRODUCTION

Vectors and vector-borne diseases have a considerable impact for domestic and wild animals in tropical and subtropical climatic regions worldwide. Haemotropic mycoplasmas or hemoplasmas are bacteria infect in a wide range of vertebrate erythrocytes and recently renamed from *Haemobartonella* and *Eperythrozoon* [1]. Well known canine haemoplasma species, *Mycoplasma haemocanis* (Mhc) and *Candidatus Mycoplasma haematoparvum* (CMhp), cause subclinical or chronic disease in immunocompetent dogs and acute disease with hemolytic anemia in susceptible animals related

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to splenectomy, immunosuppression and concurrent infections. Lethargy, weight loss, fever and anorexia are the other symptoms for acute disease [2,3].

In several studies worldwide, *Mycoplasma* infections have been found in stray, wild and pet dogs in Turkey [4], Nigeria [5], United States [3], Brazil [6], Thailand [7], Iran [8,9], Italy [10], Spain, Portugal, Switzerland and France [2].

Recently, *Hepatozoon canis* infections with high prevalence in dogs from Kyrgyzstan were disclosured [11]. We aimed to investigate frequency of infection with Mhc and CMhp in dogs from Kyrgyzstan using polymerase chain reaction (PCR) and sequence analysis.

MATERIAL and METHODS

Ethic Statement

The Ethic statement was obtained from the Animal Experimentation Ethics Committee of Kyrgyz-Turkish Manas University (Document No: 29.06.2017/2017-06/01).

Study Area and Samples

Bishkek, largest city and capital of Kyrgyzstan, is located at 42.87 latitude and 74.59 longitude, 800 meters above sea level and has a surface area of 169.9 km^2 for city center. Bishkek can show both temperate and continental climate characteristics. Province has an average annual rainfall of 427 mm *(Fig. 1)*. The study was conducted on 170 apparently asymptomatic dogs from May 2016-October 2017. Five mL of blood sample were taken from the *vena cephalica antebrachii* into tubes containing K3EDTA-anticoagulant from shelter dogs with cooperation Kyrgyz-Turkish Manas University Veterinary Teaching Hospital.

Nucleic Acid Extractions and PCR Assay

For genomic DNA isolation, 200 µL blood was processed with a commercial kit [PureLink Genomic DNA mini kit (Invitrogen, Carlsbad, USA)]. Target DNA's were kept at -20°C until analysis. To determine each species, a single PCR analysis were made in a final 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. Sequence, specificity, target gene and product sizes for primers were demonstrated in *Table 1*. PCR was performed with an initial denaturation step of 94°C for 5 min was followed by 32 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min. A final extension step at 72°C for 5 min was also applied [9]. Positive control DNA for Mhc (GenBank accession no: MG594502) and CMhp (GenBank accession no: MG594500) species and negative controls (nuclease-free water) were also used in the PCR

Fig 1. Map of Kyrgyzstan and location of Bishkek

Table 1. *Primers used in this study*

Primer	Sequence (5'-3')	Specificity	Target Gene	Product Length (bp)	Reference
Forward	GAAACTAAGGCCATAAATGACGC	Mhc	16S rRNA	309	[9]
Reverse	ACCTGTCACCTCGATAACCTCTAC				
Forward	ACGAAAGTCTGATGGAGCAATAC	CMhp	16S rRNA	328	[9]
Reverse	TATCTACGCATTCCACCGCTAC				

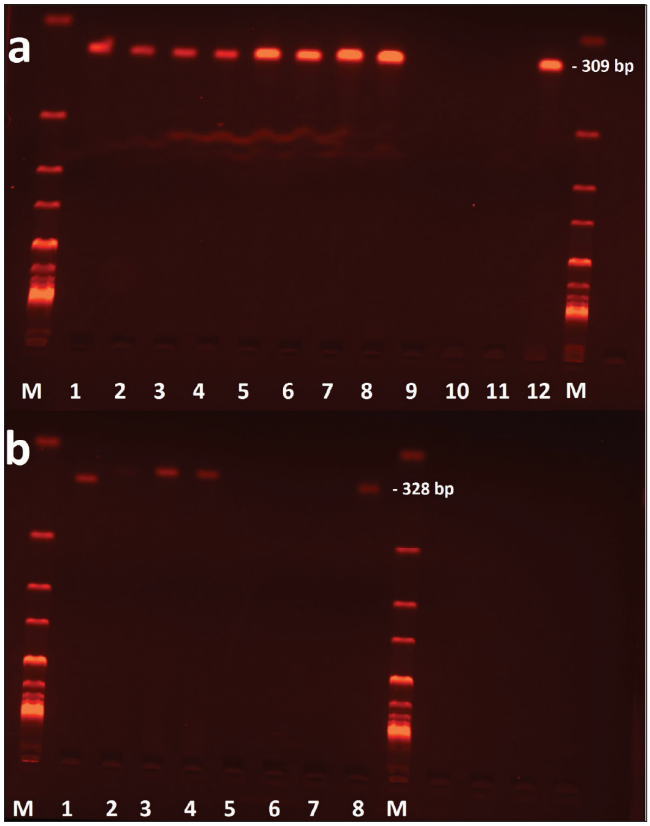

Fig 2. Agarose-gel electrophoresis of *Mycoplasma haemocanis* (a) and *Candidatus Mycoplasma haematoparvum* (b) specific polymerase chain reaction. M: 1 kb DNA ladder-marker, lane a1-a8: *Mycoplasma haemocanis* positive dog blood samples; lane a9, a10: negative dog blood samples, lane a11: negative control distilled water; lane a12: positive control DNA from dog, lane b1-b4: *Candidatus Mycoplasma haematoparvum* positive dog blood samples; lane b5, b6: negative dog blood samples, lane b7: negative control distilled water; lane b8: positive control DNA from dog

Table 2. *Molecular prevalence of canine hemoplasma species detected by PCR in Kyrgyzstan (n=170)*

Overall Prevalence (n = 9)		*Mycoplasma haemocanis*	*Candidatus Mycoplasma haematoparvum*	*Mhc+CMhp*
5		5	-	-
3		-	-	3
1		-	1	-
Total	9 (5.29%) (CI; 2.57-9.34)	5 (2.94%) (CI; 1.06-6.22)	1 (0.59%) (CI; 0.03-2.57)	3 (1.76%) (CI; 0.44-4.52)

reaction. Five microliters of PCR product was separated using electrophoresis (100 V, 60 min) in a 1.5% agarose gel stained with ethidium bromide and visualized using Gel Doc (Bio-Rad, Hercules, CA, USA) *(Fig. 2)*.

Sequencing and Molecular Classification

One positive sample for each species were selected to validate PCR results. After purification of PCR products by QIAquick PCR purification kit (Qiagen, Hilden, Germany) sequencing were performed by a commercial company (Macrogen, South Korea). Sequences were edited by Chromas versiyon 2.6.5 (http://technelysium.com.au/wp/) and compared with the other sequences available in Genbank *(https://blast.ncbi.nlm.nih.gov/Blast.cgi)*. The sequences of the partial 16S ribosomal RNA gene of Mhc and CMhp have been deposited in GenBank databases under accession no: MK015018 and MK026012 respectively.

RESULTS

Distribution and frequency of hemoplasma species in 170 dogs were determined. The overall prevalence for hemoplasma infection was 5.29% (CI 2.57-9.34). Nine animals were found to be infected by one or more species *(Table 2)*. Mixed infections were determined in 3 of 170 samples with a rate of 1.76% (CI 0.44-4.52) and single infections were in 6 with a rate of 3.53% (CI 1.41-7.03). While five (2.94%, CI 1.06-6.22) samples were found to be infected with Mhc, CMhp was detected in one (0.59%; CI 0.03-2.57) sample.

Obtained sequences comparisons exhibited that while Mhc sequence identified in this study (MK015018) showed 99-100% similarity with the previously reported sequences for the 16S ribosomal RNA gene of Mhc (KY117656, KP715858, EF416567, AB848714) and *Mycoplasma haemofelis* (KM275238, KR905462), CMhp sequence (MK026012) shared 99-100% identity with sequences for the 16S ribosomal RNA gene of CMhp (MG594500, KF366443, HQ918288) and 97-98% identity with *Candidatus Mycoplasma haemominutum* (JQ689947, AY150974, JQ044683).

DISCUSSION

This study exhibits, for the first time, molecular evidence and prevalence of hemoplasma in dogs in Kyrgyzstan. The overall molecular prevalence of canine hemoplasma species was 5.29% (CI 2.57-9.34). Mhc, CMhp and Mhc + CMhp prevalences were 2.94% (CI 1.06-6.22), 0.59% (CI 0.03-2.57) and 1.76% (CI 0.44-4.52) respectively with this study. Low prevalence of hemoplasmas determined with this study was similar to data from Italy 4.5% [10] and Nigeria with 7.7% [5]. While higher prevalences were reported in Portugal 40% [2], Iran 23% [9] and Turkey 15.3% [4]; low prevalence was from USA 1.3% [3]. Low prevalence in the studied area may be correlated to climate conditions and/or lack of vector diversity.

In the present study Mhc has higher molecular prevalence than CMhp and this result is in accordance with previous publications [5,9]. Three dogs (1.76%) were found to be infected for both Mhc and CMhp. Similar to our results, mix infections were determined in several studies [2,4,9].

Diagnosis of hemotropic *Mycoplasma* species is based on microscopic examination of thin blood smears [8] or PCR analysis targeting 16S rRNA gene fragment [1,12]. Microscopic examination of smears may be useful and cheap in acute cases but it is not possible to discriminate species

and also determine carrier animals with this method. Molecular methods have always been found superior to microscopic examination for detection and differentiation of hemotropic *Mycoplasma* spp. and other tick-borne agents [13,14]. In this study a species-specific PCR assay with high sensitivity and specificity were applied to determine carrier animals for hemotropic *Mycoplasma* spp. in dogs. It was determined that overall prevalence of canine hemoplasma species was 5.29% in Kyrgyzstan and Mhc and CMhp circulate there. We recommend species-specific PCR for Mhc and CMhp in routine screening of blood donors.

Transmission of canine hemoplasma species is associated with haematophagous arthropods like fleas and ticks [2]. Also, blood transfusion from carrier dogs to splenectomized dogs induces transmission the organism [12]. Furthermore, CMhp is accepted as a zoonotic microorganism [15]. Since it was determined with this study dog population in Kyrgyzstan is carrier for both Mhc and CMhp, veterinarians and medical doctors should take these species into consideration in suspected cases.

In conclusion canine hemoplasma species were determined in Kyrgyzstan dog population for the first time and the molecular prevalence for hemotropic *Mycoplasma* spp. in Kyrgyzstan dog population is 5.29%. It was also determined that dog population in Kyrgyzstan exposure to either or both hemoplasma species of Mhc and CMhp and Mhc has higher molecular prevalence than CMhp. It is suggested that further studies aimed to determine molecular prevalence of canine hemoplasmas and potential arthropod vectors should be conducted in other provinces of Kyrgyzstan. We also suggest to veterinarians to be conscious for canine hemoplasma among anemic dogs and routine screening of blood donors may be useful to prevent spread of disease.

Acknowledgments

The authors thank Dr. Munir Aktas and Dr. Sezayi Ozubek (Department of Parasitology, College of Veterinary Medicine, Firat University, Turkey) for providing positive control DNA's of Mhc and CMhp and also, we are grateful to animal shelter staffs and Kyrgyz-Turkish Manas University Veterinary Teaching Hospital staffs for their kind help during sample collection. The authors declare that they have no conflicts of interest.

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