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# First Molecular Detection of *Dirofilaria immitis* and *D. repens* in Dogs from Kyrgyzstan

**Mehmet Fatih Aydın[1] · Kürşat Altay[2] · Ayperi Aytmirzakizi[3] · Nazir Dumanlı[3,4]**

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## Abstract

**Background** *Dirofilaria immitis* and *Dirofilaria repens* are the causative agents of cardiopulmonary and subcutaneous dirofilariosis, respectively. This neglected disease mainly seen in dogs, cats and wild carnivores is re-emerging recent years. No study was conducted on dirofilariosis in dogs in Kyrgyzstan.

**Purpose** The goal of this study was to investigate *Dirofilaria* species using PCR and sequencing in dogs from Kyrgyzstan.

**Method** *Dirofilaria* spp. infection in dogs was screened via convential PCR and sequencing in 337 dogs from Kyrgyzstan.

**Result** The overall prevalence of *Dirofilaria* spp. was 0.59% (2/337): DNA of *D. immitis* was detected in one sample and DNA of *D. repens* in second positive sample. In second sample, parallel co-infection of *D. repens* with *Wolbachia* was also found. While *D. immitis* sequence showed 98.70–100% similarity with previously reported sequences of *D. immitis* from dog blood, *D. repens* shared 100% identity with other sequences of *D. repens*.

**Conclusion** These results provided first evidence for *Dirofilaria* spp. in Kyrgyzstan and emphasized the veterinary and medical importance.

**Keywords** *Dirofilaria immitis* · *Dirofilaria repens* · Kyrgyzstan · Dog · Vector-borne diseases · PCR · Sequencing

## Introduction

Dirofilariosis, a significant neglected, re-emerging and globally mosquito-borne diseases, is caused by *D. immitis* and *D. repens*. Canines, felines and sometimes humans are the main hosts for the species [17].

*Dirofilaria repens* can be responsible from subcutaneus granulom, panniculitis and local pruritis in carnivores [17, 23]. Also, it can lead to nodules on hypodermis, conjunctiva and lungs in humans. Being asymptomatic is one of the characteristics of *D. repens* infection. Since *D. repens* has zoonotic potential and the pathogenicity in dogs is fully unexplained [23] treatment of dogs poses a great importance.

*Dirofilaria immitis*, a pathogenic filarial nematode in dogs, other canids and humans [19], cause cardiopulmonary problems (heartworm disease) in many geographic regions of Asia [14, 22, 27, 37, 41, 43], Europe [9, 10, 12, 30], Africa [29, 39], Australia [8] and America [40].

*Dirofilaria* species are transmitted by mosquitos of the genera *Culex*, *Aedes* and *Anopheles* [36] and are prevalent especially in temperate climatic regions [33].

Adult male and female *D. immitis* and *D. repens* live in the cardiopulmonary system [21, 30] and subcutaneous tissues [11], respectively, and microfilariae are transferred into the bloodstream by female worms. Therefore, diagnosis of dirofilariosis in hosts is based on identification of microfilariae in blood samples [18]. Morphological identification of microfilariae by modified knott technique, serological methods [32] or molecular detection of parasite DNA [13, 37] are the most common techniques for the diagnosis.

Recently, our team reported first molecular data for canine hepatozoonosis [1] and canine hemotropic mycoplasma species [2] from Kyrgyzstan. Also, an ongoing research aimed to determine canine *Babesia* species is conducting by the

✉ Mehmet Fatih Aydın
veterinermfa@gmail.com

[1] Department of Public Health, Faculty of Health Sciences, University of Karamanoglu Mehmetbey, 70100 Karaman, Turkey

[2] Department of Parasitology, College of Veterinary Medicine, Sivas Cumhuriyet University, 58140 Sivas, Turkey

[3] College of Veterinary Medicine, Kırgızistan-Türkiye Manas University, 720044 Bishkek, Kyrgyzstan

[4] Department of Parasitology, College of Veterinary Medicine, Firat University, 23119 Elazig, Turkey

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same team. First molecular data obtained with these studies presented that canine vector-borne diseases have veterinary and medical importance for Kyrgyzstan.

The goal of this study was to investigate *Dirofilaria* species using PCR and sequencing in dog bloods from Kyrgyzstan.

## Materials and Methods

### Study Area and Sampling

The study was performed in Bishkek province of Kyrgyzstan (Fig. 1). The geographic location of Bishkek is 42°54′ N latitude and 74°46′ E longitude and it is 800 m above from sea level. Both temperate and continental climate characteristics can be seen and average annual rainfall is 427 mm in the province. Venous blood samples were collected from 337 shelter dogs into tubes containing $K_3$-EDTA anticoagulant with cooperation Kyrgyz-Turkish Manas University Veterinary Teaching Hospital between 2017 and 2019 and stored at − 20 °C until use for molecular analysis. No clinical signs were observed in dogs according to first inspection and all dogs were recorded as healthy or asymptomatic.

### Molecular Analysis

200 µl were used for isolation of genomic DNA by commercial kit [PureLink Genomic DNA mini kit (Invitrogen, Carlsbad, USA)] as described Atas et al. [3]. Genomic DNA's were stored at − 20 °C until analysis.

All samples were investigated by conventional PCR analysis using three pairs primers specified *D. immitis*/*D. repens*, *D. repens* [28] and *D. immitis* [42]. Sequences, specificity, target gene, product length and thermal conditions for the primers were demonstrated in Table 1. Final reaction volume of PCR was 25 µl and it contained sterile water—13 µl (DNase, RNase free), PCR buffer—2.5 µl [750 mM Tris–HCl (pH 8.8), 200 mM $(NH_4)_2SO_4$, 0.1% Tween 20], $MgCl_2$—2.5 µl (5 mM), deoxynucleotide triphosphates—2 µl (1.25 mM), Taq DNA polymerase—0.1 µl (1.25 U) [Solis BioDyne, Tartu, Estonia], primers—1.25 µl × 2 (20 pmol/µl) and template DNA—2.5 µl. *D. immitis* and *D. repens* positive DNA's and nuclease-free water were used as positive and negative controls in the PCR, respectively. One DNA sample of *D. repens* infected dog were also investigated in terms of the bacterial endosymbiont *Wolbachia* with generic primers which amplify a 492–498 bp fragment of the 16S rRNA gene [5, 35].

Five microliters of amplicons separated in a 1% agarose gel (100 V, 60 min), stained with ethidium bromide thereafter and viewed with gel documentation system (Bio-Rad, Hercules, CA, USA).

Sequencing were performed to validate PCR results and to compare with the other sequences available in GenBank. Sanger sequencing were performed at one direction using forward primer by a commercial company (BMLabosis, Ankara, Türkiye). After editing the sequences by Chromas version 2.6.5 (https://technelysium.com.au/wp/) they were compared by BLAST (Basic Local Alignment Search Tool) with other sequences available in GenBank (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The sequences of *D. immitis*


**Fig. 1** Map of the Kyrgyzstan, showing the location of Bishkek

**Table 1** Primers used in this study

| Primer | Sequence (5′–3′) | Specificity | Target gene | Product length (bp) | Thermal conditions | References |
|---|---|---|---|---|---|---|
| DIDR-F1 | AGTGCGAATTGCAGACGCATTGAG | *D. immitis* *D. repens* | 5.8S-ITS2-28S | 542 | – 94 °C to 2 min<br>– 94 °C to 30 s, 57 °C to 30 s, 72 °C to 30 s (35 cycles)<br>– 72 °C to 7 min | Rishniw et al. [28] |
| DIDR-R1 | AGCGGGTAATCACGACTGAGTTGA | | | 484 | | |
| DR-COI-F1 | AGTGTTGATGGTCAACCTGAATTA | *D. repens* | COI | 209 | | |
| DR-COI-R1 | GCCAAAACAGGAACAGATAAAACT | | | | | |
| Di-16S-rRNA-F | AGCTCGTAGTTGGATCTGCAT | *D. immitis* | 16S SSU rRNA | 453 | – 95 °C to 10 min<br>– 95 °C to 1 min, 55 °C to 1 min, 72 °C to 1 min (35 cycles)<br>– 72 °C to 10 min | Vezzani et al. [42] |
| Di-16SrRNA-R | CGTCAAGGCGTATTTACCG | | | | | |

and *D. repens* were deposited in GenBank databases under accession nos.: MK026169 and MK457363, respectively.

## Result

According to result of 337 dog blood samples investigated using the genus specific PCR the overall prevalence of *Dirofilaria* spp. was 0.59% (2/337). The species-specific PCR confirmed DNA of *D. immitis* in one sample (0.29%) and DNA of *D. repens* in second sample (0.29%).

For the genus-specific identification a fragment of 542 and 484 bp located on 5.8S-ITS2-28S genes of *Dirofilaria* spp. was amplified using DIDR-F1 and DIDR-R1 primer pairs. Also, 209 and 453 bp of COI and 16S SSU rRNA genes of *D. repens* and *D. immitis,* respectively, was amplified for the species identification. Co-infection of *D. repens* and *Wolbachia* was detected in one positive dog blood sample (Accession number: MK452249).

To validate PCR results parts of target genes amplified from positive samples were sequenced. A BLAST analysis was performed to compare results with the other *Dirofilaria* spp. sequences available in the GenBank. *D. immitis* and *D. repens* sequences were submitted to GenBank under the accession numbers of MK026169 and MK457363, respectively. Obtained *D. immitis* sequence with this study (MK026169) showed 98.70–100% similarity with previously reported sequences of *D. immitis* from dog blood (FJ765450-South Korea, KJ183078-Turkey, MN696499-France, MN241432-Ecuador, HM124350-Argentina, AB973230-Japan, MN795071- French Guiana). Sequence of *D. repens* in this study (MK457363) shared 100% identity with other sequences of *D. repens* available in GenBank (MN728180-donkey-Egypt, MN728215-dog-Egypt, MN696498-dog-France, MK495735-dog-Cote d'Ivoire, AB973229-human-Japan) and *Onchocerca cervicalis* (DQ094174).

## Discussion

This is the first study aimed to determine *D. immitis* and *D. repens* prevalence with molecular methods in Kyrgyzstan. *Dirofilaria* species have cosmopolitan distribution and a wide range of host. Primarily *Dirofilaria* species cause serious disease in domestic and wild canids worldwide [17] and it was reported that the number of human dirofilariosis (particularly *D. repens* infection) is a serious public health concern [11].

Prevalence of both *D. immitis* and *D. repens* was 0.29% in Kyrgyzstan with this study. This result is of great importance as it is the first evidence for these species in the country. On the other hand, prevalence of both species was found very low. We think this may be due to some important reasons. First, the sampling was made randomize and animals were clinically healthy. Second one is "presence and circulation of microfilaria in blood is influenced from several factors like sampling time" [15]. Third one is mosquito vectors are not common in the sampling region due to the climatic conditions. Prevalence of *D. immitis* was determined as 21.3% in France [38], 14.5% in Tunisia [29], 13.7% in Portugal [10], 8.0% in Mexico [40], 2.3–4.02% in Iran [4, 24], 1.5–3.7% in Turkey [3, 13, 37] and 0.0% in Cape Verde [20] with PCR. Environmental factors particularly climate and ecologic features influence mosquito population and this can be related to prevalence of dirofilariosis. Compared to other molecular prevalence in various parts of the world, a low prevalence (0.29%) was found in asymptomatic dogs in Kyrgyzstan. This may be due to combined several factors. Further investigations should be conducted with postmortem examination and other diagnostic methods. We also suggest focus to investigate parasite prevalence in mosquitoes to specify risk areas in Kyrgyzstan.

*Dirofilaria repens* infection is usually with low or non-pathogenicity in dogs. Undiagnosed dogs and pet traveling

can cause to complete of parasite's life cycle. From all reasons above, it is thought that *D. repens* spreading is faster than *D. immitis* [6, 24]. *D. repens* prevalence in asymptomatic dogs with PCR was 26.0% in Iran [24], 6.0% in Cape Verde [20], 3.0% in Tunisia [29], 2.7% in Lithuania [31], 1.12% in Mexico [26], 0.0–1.8% in Turkey [13, 37], 0.0% in Portugal [10], 0.0% in France [38], 0.0% in Algeria [39]. With this study, *D. repens* was first detected in dogs in Kyrgyzstan and the parasite prevalence was low when compared to some studies. We advise further investigations and treatment protocols on subcutaneous dirofilariosis in dogs under the concept of "One Health".

*Wolbachia*, a gram-negative bacteria included in Anaplasmataceae family, is very common worldwide and present in several arthropod and filarial nematode species [7]. *Wolbachia* is in a mutualist relationship with filarial nematodes and they have a positive effect on nematode reproduction [16]. Additionally, this mutualist relationship increases the severity of dirofilariosis and from this viewpoint use of antibiotics against to *Wolbachia* is a new strategy to fight against dirofilariosis [25]. In several studies, *Wolbachia* DNA was detected in *D. immitis* and/or *D. repens* infected blood samples [34, 37]. Although *Wolbachia* DNA wasn't detected in *D. immitis* infected sample, one sample infected with *D. repens* was also found to be infected with *Wolbachia* with this study. Similar to our results, Sabūnas et al. reported that *Wolbachia* DNA was detected in Lithuanian dogs infected with *D. repens* [31]. Our results also provided first data for *Wolbachia* harbours in Kyrgyzstan. Further investigations are advised concerning this issue.

Dogs are accepted as a reservoir host for *Dirofilaria* spp. for mosquito vectors and there is a strong relationship between infection prevalence in dogs and humans [6]. Although no data for *Dirofilaria* spp. prevalence in mosquito vectors and no human subcutaneous *D. repens* infection was reported in Kyrgyzstan until today, we recommend to medical doctors should take into consideration dirofilariosis in suspected cases.

In conclusion, this study provided first evidence for *D. immitis* and *D. repens* in Kyrgyzstan. It is suggested that detailed epidemiological surveys should be conducted in mosquito vectors and domestic and wild hosts also in humans for dirofilariosis in the country.

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## Compliance with Ethical Standards

**Conflict of interest** The authors declare that they have no conflicts of interest.

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

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