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Case Report

Detection of *Dirofilaria immitis* in a brown bear (*Ursus arctos*) in Greece

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Summary

Dirofilaria immitis (canine heartworm) is a filarial nematode found in the pulmonary circulation and the heart of susceptible hosts. It represents an important zoonotic vector-borne disease of domestic dogs and several wildlife species. Herein we report for the first time, the finding of *Dirofilaria immitis* worms in a brown bear killed in a vehicle collision in Northern Greece. The worms were morphologically identified; molecular examination, based on the analysis of the mitochondrial genes 12S (433 bp) and CO1 (610 bp), verified the identification by demonstrating 100% similarity to *D. immitis* specimens deposited in GenBank. Brown bears in Greece occupy habitats that are shared with the potential wild and domestic hosts and the vectors of *D. immitis* and thus may be particularly susceptible to this parasite. This report contributes to the knowledge of dirofilariosis spread in Europe and on the epidemiological threats that may affect the survival of the endangered brown bear in Greece.
Keywords: *Dirofilaria immitis;* brown bear; conservation threats; epidemiology; heartworm

Introduction

Dirofilaria immitis (canine heartworm) is a filarial nematode found in the pulmonary arterial system and in the heart; canine heartworm infections represent an important, potentially life-threatening, zoonotic vector-borne (mosquito) disease (Morchón *et al*., 2012; Taylor *et al*., 2007). The domestic dog (*Canis familiaris*) is the most common host and biological reservoir, although infections have been reported in other carnivores, such as wolves, jackals, foxes and occasionally in humans. Furthermore, in the literature the other rare hosts such as the domestic ferrets, the sea lions and the black bears have been reported (Johson, 1975; McCall, 1998; Taylor *et al*., 2007; McCall *et al*., 2008).
Among the wildlife species, the brown bear (*Ursus arctos*) is one of the most endangered large mammals in Greece. The species is found in two disjunct populations in the north-eastern and western part of Greece and it is estimated that approximately 450 individuals live in the country (Karamanlidis *et al*., 2015). In recent years a significant population recovery has occurred (Karamanlidis *et al*., 2015), but with it also, negative interactions with humans have increased (Karamanlidis *et al*., 2011). Effective management and conservation measures are now, more than ever, necessary for safeguarding the future of the brown bear in Greece; monitoring and understanding the epidemiological effects of parasites is an essential part of the monitoring of wild bear populations globally (Čobádiová *et al*., 2013; Gau *et al*., 1999).
This study reports the finding for the first time of the worm *Dirofilaria immitis* in a wild brown bear in Greece.

Materials and Methods

On the 29th of March 2014, at 04:30 am, a 4 year old brown bear, was heavily injured in a vehicle collision on a highway in Northern Greece (N40.173503, E21.508776). The bear after a light seda-

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tion was transferred to the School of Veterinary Medicine, Aristotle University of Thessaloniki for intensive care, whereas despite the efforts, it died due to its severe injuries.

The necropsy was carried out according to standard procedures. A total of 4 nematode worms were found in the right ventricle of the heart and pulmonary arteries; they were collected into a vial containing ethanol and morphologically identified under a stereoscope. A blood sample was tested for the presence of any circulating microfilariae according to the modified Knott method (Magnis *et al*., 2013). Two of the worms were sent to the Finnish Food Safety Authority Evira (Oulu, Finland) for molecular identification. Genomic DNA was extracted from the parasites using a commercial QiaAmp DNA mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. A 0.5 cm piece of the middle part of the worm was cut off and the remaining ethanol was evaporated. In the final step of extraction the DNA was eluted in a total of 100 µl. Identification was based on the analysis of the mitochondrial genes 12S (433 bp) and CO1 (610 bp). The primer pair Fil12SF-Fil12SR (Casiraghi *et al*., 2004) and Co1intF-Co1intR (Casiraghi *et al*., 2001) was used for amplification.

PCR was performed in 25 µl reaction volumes using 2 µl of template, 0.4 µM of each primer, 200 µM of each dNTP and 1 U DyNAzyme II DNA Polymerase (Thermo Scientific, Finland) under the following conditions: for the 12S gene initial denaturation at 94 °C for 3 min, 40 cycles at 94 °C for 45 s, 50 °C for 45 s and 72 °C for 90 s, followed by a final extension of 5 min at 72 °C and for the COI-gene, initial denaturation at 94 °C for 3 min, 31 cycles at 94 °C for 30 s, 50 °C for 30 s and 72 °C for 1 min, followed by a final extension of 5 min at 72 °C. All PCR reactions were carried out in a XP Cycler (Bioer, Portsmouth, USA). PCR products were visualized on 1.5 % agarose gel electrophoresis, gel-purified by E.Z.N.A.® Gel Extraction Kit (Omega Bio-tek, Norcross, GA, USA) and sequenced using ABI technology. Sequencing was performed using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems Co, CA, USA.) The quality of individual electropherograms was verified visually. Finally, an amplification of the partial 5S rDNA sequence (353 bp) was performed, using the method of (Xie *et al*., 1994) in order to confirm the identification.

Results

The subadult male bear was in a good nutritional state. At necropsy, there were multiple skin cuts and abrasions on the head, front legs and right thoracic area. Multifocally diffuse subcutaneous and muscle haemorrhages and fractions were found throughout the right side of the animal. Four adult *Dirofilaria*-like worms were found in the right heart ventricle and pulmonary arteries. No other worms were detected.

The four adult female worms (Figs. 1 – 2) were collected and morphologically identified as *Dirofilaria immitis* (Taylor *et al*., 2007). The adult females size was 10.2 ± 0.5 cm and they contained no microfilariae. Also no microfilariae were found in the blood. The partial sequences of the 12S and CO1 genes were compared to GenBank data and both regions were identical to previous *D. immitis* sequences in GenBank (AM779770.1 and AM749229.1). However, there are no *D. ursi* 12S and COI sequences in GenBank available. Nucleotide blasts of the partial 5S rDNA sequence showed only 85 % identity to *Dirofilaria ursi* sequences in Genbank, thus verifying the previous identification as *D. immitis*.

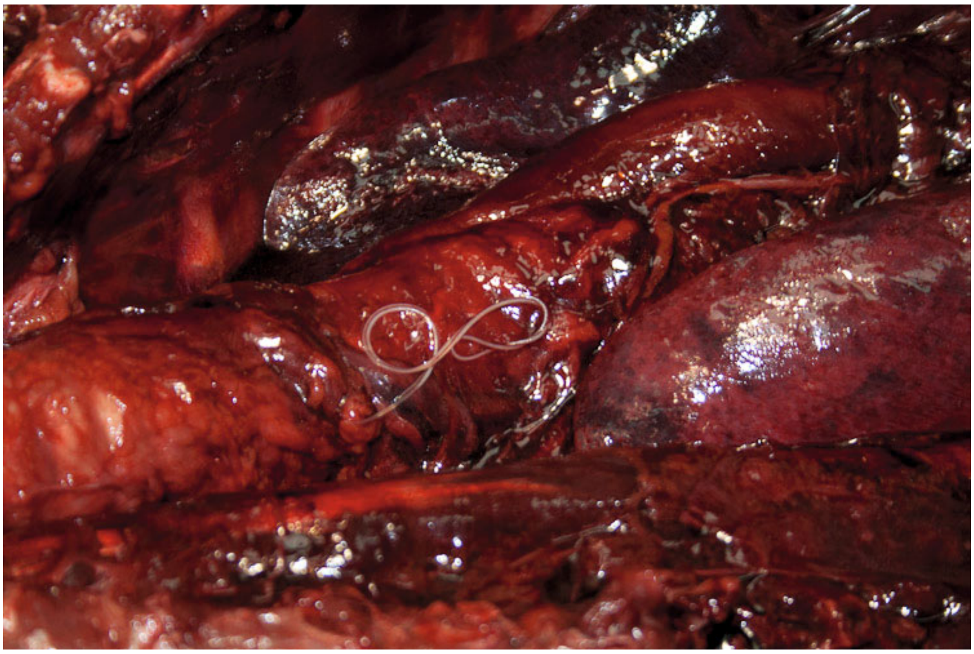

Fig. 1. Female *Dirofilaria* spp worm found in the pulmory artery of a brown bear.

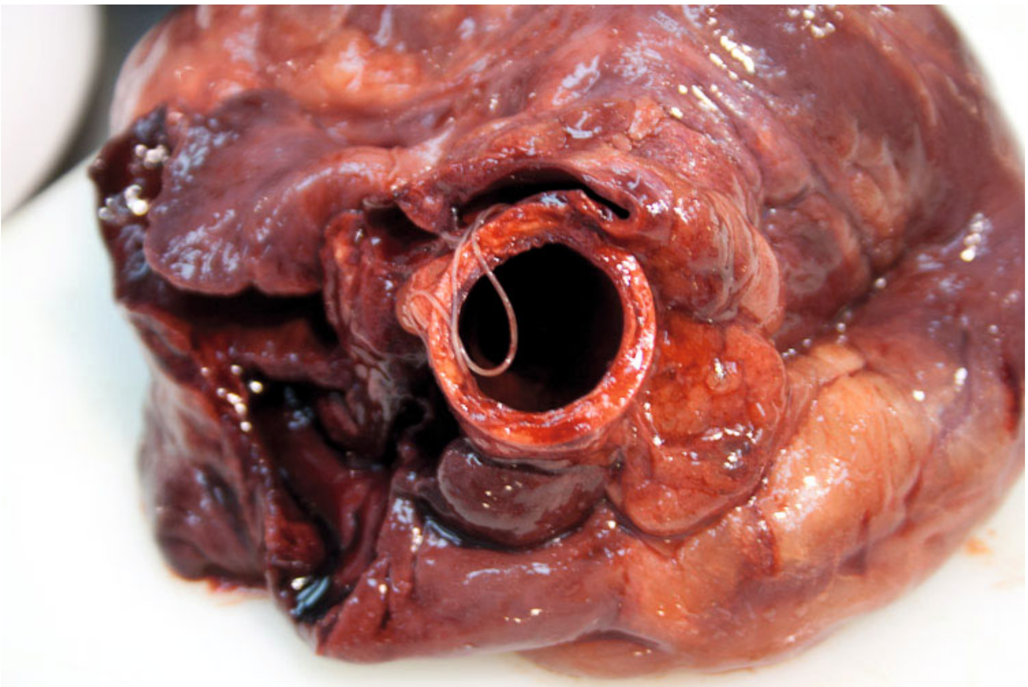

Fig. 2. Adult nematode parasites, morphologically consistent with adult *Dirofilaria spp.* (heartworm) were found in the right ventricle and pulmonary arteries.

Discussion

In Europe dirofilariosis is common in the Mediterranean Basin, although some studies have indicated that the disease is spreading towards areas previously considered canine heartworm-free (i.e. to the Central and North of Europe). This spread is probably facilitated by an increasing number of infected animals moving out of endemic areas, by the introduction of new species of vector mosquitoes, by environmental changes and by the increase of human activities in the new areas (Alho *et al*., 2014; Genchi *et al*., 2009; Morchón *et al*., 2012; Otranto *et al*., 2015). In Greece, dirofilariosis is widespread in domestic dogs, especially in areas of Central and Northern Europe, where this disease is considered endemic (Polizopoulou *et al*., 2000). More specifically, in the region of Macedonia in Northern Greece the dog infection rate may be as high as 34.13 % (Founta *et al*., 1999). Dirofilariosis is particularly common in hunting dogs, as they are at higher risk of contracting the disease during hunting in forest areas with dense mosquito populations (Papazahariadou *et al*., 1994). Furthermore, hunting and military dogs are more often infected than other dogs and it is suggested that the stay outside overnight has the major effect on the exposure of dogs to mosquitoes (Fuehrer *et al*., 2016).

In Greece, even though dirofilariosis is enzootic, it has been recorded so far only in domestic animals and there have been no reports related to wildlife infections. Among Greek wildlife, the brown bear is one of the most charismatic and endangered species, yet little is known about the epidemiological threats to its survival. In fact, information on the epidemiological threats for bears from filarial infections is generally limited and the available information regarding the parasitism of this host with *Dirofilaria immitis* is missing. Johnson (1975) records the infection of the black bear (*Ursus americanus*) with *D. ursi*, and mentions the possibility of the black bear harbouring *D. immitis,* as well. Black bears may become infected with *D. ursi,* which is found in the abdominal cavity and subcutaneous tissues and in the submucosa of the oesophagus. Microfilariae of this nematode can be found also, as in *D. immitis*, in the blood. However, it is considered to be non-pathogenic, contrary to *D. immitis* (Ramsey, 2003).

The role of wildlife species, such as the golden jackal (Ionica *et al*., 2016), coyote in California (Sacks, 1998), the red fox in Australia (Marks and Bloomfield, 1998) and the fox or wolf in Europe (Morchón *et al*., 2012) as reservoir hosts for *Dirofilaria* spp. may be of significant importance in maintaining or spreading the parasite. The presence of *D. immitis* in a brown bear in Greece might have been eventually expected, as throughout its range in Greece, this animal is sympatric with the grey wolf (*Canis lupus*) which may serve as the final host of *D. immitis*. Furthermore, there are several other factors influencing the spreading of the disease, particularly the presence and movement of microfilaremic reservoirs, i.e. hunting dogs, being the most important ones (Morchón *et al*., 2012; Otranto *et al*., 2015). Another fundamental factor in the epidemiology of this disease, is the widespread presence of mosquitoes able to act as vectors (i.e. *Culex, Aedes* and *Anopheles*), as well as the existence of adequate climate conditions for its successful development. Furthermore, it is important to take into account the recent introduction to Europe of a new species of vector mosquito, i.e., *Aedes albopictus*, which besides from being highly adaptable in temperate areas, can also survive in egg-stage throughout the winter (Morchón *et al*., 2012).

The findings of our study have implications for the conservation of bears in Greece and for understanding and managing *D. immitis.* From a wildlife conservation perspective, our study indicates the potential negative effect of vehicle collisions and poaching (i.e., through the finding of a shotgun pellet) on the survival of bears in Greece and highlights the necessity to closely monitor these conservation threats. More importantly however, our findings add dirofilariosis as a new, potential conservation threat to the survival of brown bears in Greece that also needs to be closely monitored. From an epidemiological perspective, the findings of the study contribute to the epidemiological map of dirofilariosis in Europe and to the addition of *Ursus arctos* as a new host for the parasite *D. immitis*. At the same time, it also highlights the potential zoonotic risk for humans living in rural areas, as *D. immitis* is the causative agent of pulmonary dirofilariasis and can produce benign pulmonary nodules (Simon *et al*., 2012).

Conflict of interest statement

Authors do not have any conflict of interest

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