Further data on the distribution of *Dirofilaria* spp. in the Czech Republic in dogs

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Abstract: Based on previously published data, the Czech Republic is regarded an endemic country of the onchocercid nematodes *Dirofilaria immitis* (Leidy, 1856) and *Dirofilaria repens* Railliet et Henry, 1911. Nevertheless, while cases of *D. repens* are commonly reported from dogs in South Moravia, no recent records of *D. immitis* are available. Therefore, the present study was performed to clarify the occurrence of both species of *Dirofilaria* Railliet et Henry, 1910. Blood samples of 551 dogs sampled during 2015 and 2016 were analysed microscopically for presence of microfilaria and blood sera were examined by IDEXX SNAP® 4Dx® test (IDEXX, USA). DNA from blood of microscopically positive dogs was extracted and PCR protocol amplifying fragment of cytochrome c oxidase I (COI) gene was performed; PCR products were then sequenced. All dogs from the Bohemian part of the Czech Republic were negative. The prevalence of *D. repens* in the Moravian region was 5.7% (27/476). BLAST analyses of obtained sequences confirmed the presence of *D. repens* (99–100% identical to KX265049). All sampled animals showed a negative result for *D. immitis* antigen in IDEXX SNAP® 4Dx® test. Our study confirmed the previously reported occurrence of *D. repens* in South Moravia and revealed its spreading from the epicentre to the north and west. PCR with subsequent sequencing together with negative results for *D. immitis* antigen in IDEXX SNAP® 4Dx® test revealed only *D. repens* infection. A previously published autochthonous infection of dogs with *D. immitis* in South Moravia was not confirmed.

Keywords: dirofilariosis, Central Europe, autochthonous infection, COI

Two filaroid nematodes are causative agents of canine dirofilariosis in Europe. The subcutaneous *Dirofilaria repens* Railliet et Henry, 1911 infects predominantly dogs, but can be found also in cats and other carnivores as well as in humans (Otranto et al. 2013). Adults of *Dirofilaria immitis* (Leidy, 1856) reside in the vascular system mostly in canine, sometimes feline hosts and occasionally can also be found in humans (Simón et al. 2012). *Dirofilaria repens* and *D. immitis* exhibit specific developmental patterns with distinct biological and clinical implications.

The nature of *D. repens* infection is frequently asymptomatic. Clinically manifesting cases are usually diagnosed as ezcematous dermatitis and nodular lesions (Sassnau and Genchi 2012). On the contrary, *D. immitis* causes a severe and life-threatening disease which affects pulmonary vasculature, lungs and right chambers of the heart (Furlanello et al. 1998). Both species are zoonotic, especially *D. repens* is gaining attention in Europe as a rising zoonosis with increasing number of clinical cases in humans (Simón et al. 2005, Baptista-Fernandez et al. 2015). Autochthonous human dirofilariosis caused by *D. repens* was confirmed also in the studied area of the Czech Republic (Matějů et al. 2016, Gebauer et al. 2021). The involvement of mosquito vectors in the life cycle of species of *Dirofilaria* makes their transmission and distribution linked to global climatic changes. In spite of the
similarity of temperatures required for extrinsic incubation of larvae of *Dirofilaria* spp. in mosquitoes, *D. repens* is spreading more rapidly than *D. immitis* throughout northern and eastern Europe (Genchi et al. 2011a, Capelli et al. 2018). The highest prevalence of *D. repens* is recorded in the Mediterranean region (Simón et al. 2012), but its occurrence has nowadays been more frequently reported also in central and northern Europe (Genchi et al. 2005, Medlock et al. 2012). Only sporadic *D. immitis* cases were reported in central Europe (e.g., Germany and Austria) in imported dogs, or dogs previously living in endemic areas (Hinaidy et al. 1987, Zahller et al. 1997).

*Dirofilaria immitis* shows growing prevalence in dogs in Slovakia, commonly in co-infections with *D. repens* (Miterpáková et al. 2010, 2021). In the Czech Republic, *D. immitis* was reported by Svobodová et al. (2006) and Dobešová et al. (2007). Since these reports, the Czech Republic is regarded an endemic country, which contrasts with the absence of any diagnosed autochthonous cases of *D. immitis* infection in the past 15 years. Being inspired by a recent publication by Miterpáková et al. (2021) on the distribution of *D. repens* and *D. immitis* in Slovakia and the Czech Republic, we analysed data on the distribution of *Dirofilaria* spp. in the Czech Republic based on samples collected in 2015 and 2016.

**Materials and Methods**

**Sampled animals and geographical location**

A total of 551 dogs were sampled in collaboration with private veterinarians in 2015 and 2016. Dogs in veterinary clinics were sampled after owners’ agreement and included healthy dogs visiting veterinarians for prevention as well as dogs with health problems. Dogs in shelters were sampled by the study authors. The majority of sampled dogs (476) originated from the southern and central part of the South Moravian region; remaining animals (75) originated from South Bohemia and from different localities of Central and West Bohemia. Information on breeds of dogs and their age, sex, health status, lifestyle (indoor/outdoor housing) and travelling history were collected. Blood was obtained by venipuncture of *vena cephalica*; 1–3 ml of the blood was preserved in EDTA and 1–3 ml was processed to obtain the blood serum; two blood smears were prepared for each sampled animal.

**Microscopy and serology**

The blood of all dogs sampled was examined microscopically for the presence of microfilariae after transport to the laboratory. One ml of all EDTA samples was used for modified Knott’s test: 1 ml EDTA blood was mixed with 9 ml of 2% formaldehyde solution in a 15 ml centrifuge tube and centrifuged for 5 min at 1,500 U/min. The supernatant was discarded, leaving 1 ml of solution to which a few drops of methylene blue solution was added. The sediment was transferred to glass slides, covered with coverslips.
and examined by light microscopy at magnifications 100× and 400×. The blood smears were stained with Dip-Quick (Jorgensen Laboratories, Inc., Loveland, Colorado, USA) and examined under a light microscope. Collected blood sera from all dogs were examined by IDEXX SNAP® 4Dx® test (IDEXX, Westbrook, Maine, USA) according to manufacturer’s instructions.

DNA isolation, PCR and sequencing

Genomic DNA Mini Kit (Geneaid Biotech, New Taipei, Taiwan) was used to extract DNA from 200 μl of EDTA blood from microscopically positive dogs. PCR amplifying ~600–700 bp of gene for cytochrome oxidase I (COI) of filarial nematodes was performed using the primer set COIntF and COIntR (Casiraghi et al. 2001). PCR was performed in 20 μl volumes under the following conditions: 2.5× of 5 Prime Hot Mastermix Buffer (5 Prime Inc., Gaithersburg, Maryland, USA), 0.2 μM of each forward and reverse primers, 1 unit of HotMasterTaq DNA polymerase (5 Prime Inc., Gaithersburg, MD, USA) and 1 μl of the template DNA. Cycling conditions included 94 °C for 45 s, 52 °C for 45 s, and 72 °C for 90 s, repeated for 40 cycles. PCR products were separated by electrophoresis in 1% agarose gel and visualised by MidoriGreen (NIPPON Genetics, Düren, Germany) and UV transilluminator, purified with Gel/PCR DNA Fragment Extraction Kit (Geneaid Biotech, New Taipei, Taiwan) and commercially Sanger sequenced (Macrogen Europe, Amsterdam, the Netherlands). All obtained sequences were edited using the ChromasPro 1.5 (Technelysium) and compared to the GenBank database using the BLAST algorithm. In two samples with lower BLAST identity due to low sequencing signal quality, further PCR was performed. In this protocol, species-specific primer Drcox1F and universal COIntR (Casiraghi et al. 2001, Latrofa et al. 2012) were used to amplify ~450 bp COI fragment of Dirofilaria repens to confirm the BLAST results.

RESULTS

Out of 551 dogs examined, 27 were microscopically detected as Dirofilaria-positive. Positive dogs included 13 females and 14 males of various breeds and crossbreeds aged 2–15 years. All positive animals originated from the Moravian part of the Czech Republic, with higher frequency in the Břeclav district (Fig. 1); the northernmost record was from Kroměříž. As all dogs from Bohemian part of the Czech Republic were Dirofilaria-negative, they are excluded from prevalence analysis. The prevalence of Dirofilaria repens in the Moravian region was 5.7% (27/476). Out of 27 positive dogs, four had history of traveling abroad (Croatia, Italy, Spain, Serbia and Slovakia) and seven positive animals were dogs from shelters without known traveling history. Five of these positive shelter dogs were from the infection hotspot in South Moravia, two were from the town of Kroměříž, which is the northernmost area of detection. All Knott-positive animals were positive also by the PCR for filarial nematodes. All 27 positive samples were sequenced. Thirteen COI sequences (621–689 bp) were obtained in good quality for analyses, the remaining lower quality sequences were checked with BLAST and showed identity with D. repens. Analysed sequences belong to three unique haplotypes differing in 1–2 single nucleotide polymorphism (SNP) sites (0.1–0.3%). The haplotypes Hap1, Hap2 and Hap3 were represented by ten, two and one sequence, respectively. BLAST analyses of all three haplotypes sequences invariably confirmed the presence of D. repens. Haplotype Hap1 and Hap2 had 99.85% identity with D. repens (GenBank accession no. KX265049) from a human from Croatia and haplotype Hap3 was 100% identical to D. repens (GenBank accession no. KX265048) from a dog from Italy. Final sequences of the COI were deposited in the GenBank database under the accession numbers MW675691 (Hap1), MW675692 (Hap2) and MW675693 (Hap3). All sampled animals showed a negative result for Dirofilaria immitis antigen in IDEXX SNAP® 4Dx® test.

DISCUSSION

Both species of Dirofilaria infecting European carnivores show remarkably different distribution patterns and pathogenicity (Genchi et al. 2011a). The correct discrimination between the two species is essential in small animal practice, as both species differ also in clinical impact on their hosts. There is no doubt that increased movement of hosts of Dirofilaria spp., together with ongoing climate changes, impact the distribution of filariosids in Central Europe (Genchi et al. 2011b).

Since the initial report by Svobodová et al. (2006), the Czech Republic is considered an area of endemic distribution of both species of Dirofilaria, which contrasts with virtual absence of heart worm disease in the Czech Republic. In contrast, both Dirofilaria repens and Dirofilaria immitis are well established in lowlands of neighbouring Slovakia (Miterpáková et al. 2010, 2021). Dirofilaria repens is well-known among the veterinary clinicians in South Moravia and its presence was confirmed in mosquitoes (Rudolf et al. 2014) as well as in human cases (Matějů et al. 2016, Gebauer et al. 2021). In the most recent study, Miterpáková et al. (2021) provided comparative data on the distribution of D. immitis and D. repens in Slovakia and the Czech Republic, reporting 1.9% prevalence of D. repens in dogs from the Czech Republic.

In our study, we detected microfilaraeic dogs by the Knott’s test and confirmed the identification of microfilariae by PCR targeting part of the COI gene. All microscopically positive animals showed negative result for D. immitis antigen in IDEXX SNAP® 4Dx® test and all were positive in PCR. Sequence analysis confirmed D. repens in all cases. The overall prevalence of D. repens including dogs from both the Bohemian and Moravian territory in our sample set was 4.9%, which is higher than a prevalence recently reported by Miterpáková et al. (2021). However, the sampling in the latter study involved more dogs from the Bohemian territory. Our sampling effort was concentrated to the areas of the most probable occurrence of D. repens and D. immitis, based on climatic data and previously published records (Svobodová et al. 2006, Dobešová et al. 2007, Rudolf et al. 2014, Matějů et al. 2016, Gebauer et al. 2021). For the Moravian region the prevalence of D. repens was calculated to be 5.7%.

Nevertheless, it should be considered that we were unable to confirm the autochthonous nature of the infection in a significant portion of the dogs examined due to their travel history.
el history (four cases) or unknown origin (seven dogs from animal shelters). Collected information on breeds of dogs, their age, sex, health status and indoor/outdoor housing did not reveal any risk factors of infection with *D. repens*.

As seen from Fig. 1, *D. repens* is well-established in the domestic dog population in lowland areas along the Dyje and Morava rivers, extending northwards to the Kroměříž region in middle Moravia. The distribution corresponds well with previously diagnosed cases of human subcutaneous dirofilariosis (Matějů et al. 2016, Gebauer et al. 2021). In contrast, we did not detect any case of *D. immitis*, which contrasts with historical data from the same areas presented by Svobodová et al. (2006). As the *D. immitis* cases in the study in question were detected by ELISA-based approach (PetChek kit, IDEXX Laboratories), it is quite possible that the positive results reflected a cross-reactivity with *Angiostrongylus vasorum* (Railliet, 1866) as previously reported (Schnyder and Deplazes 2012).

In conclusion, presented data combined with results of Mitterpáková et al. (2021) represent a robust evidence of the absence of *Dirofilaria immitis* in the Czech Republic and this should be reflected in publicly available data sources (ESSCAP Guideline Maps 2021). This assumption correlates with the absence of autochthonous heart worm disease in veterinary clinics (personal communication with local veterinarians, data not shown). The area of detected cases represents a region with the warmest climate in the Czech Republic, but the ongoing process of climate warming combined with dog traveling and numerous imports of dogs from *D. immitis* endemic areas stresses further need for monitoring of infections with species of *Dirofilaria*, preferably in close collaboration with veterinary practitioners.

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**Authors’ contributions.** DM, DDB and MN designed the study. JJ, MN, BŠM, BC and LH took samples from dogs, modified Knott’s test and examined samples. MN performed molecular analyses. JJ evaluated the data and wrote the manuscript. All the authors read and approved the final manuscript.

**REFERENCES**


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