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Case Report

First report of heartworm (*Dirofilaria immitis*) infection in an imported dog in Lithuania

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Summary

Over the past decade, increasing numbers of autochthonous cases of heartworm infection have been reported in the countries of Eastern Europe where previously only imported cases were described. In this report we have described the first clinical case of *Dirofilaria immitis* infection in an imported dog in Lithuania.

In 2018, a 5-year-old male Spanish greyhound (Spanish galgo) was imported to Lithuania from southern Spain and referred to a small animal veterinary clinic in Vilnius for wellness screening. Circulating microfilariae and female antigens of *D. immitis* were detected using the Knott's test and SNAP 4Dx Plus Test (IDEXX Laboratories, Portland, USA). The diagnosis was confirmed using molecular analysis. Treatment according to the guidelines recommended by the American Heartworm Society was applied. This is the first confirmed report of canine heartworm infection in an imported dog in Lithuania. Heartworm-infected dogs transported to North-Eastern Europe from endemic areas could act as microfilarial reservoirs for the local mosquito population, which could increase the risk of spreading the disease.

Keywords: Dirofilariasis; Heartworm; *Dirofilaria immitis*; Lithuania

Introduction

Among all filarial species the most relevant for dogs in Europe are *Dirofilaria repens* and *Dirofilaria immitis*. Adult nematodes of *D. repens* most often are found in subcutaneous tissues, whereas *D. immitis* is the causal agent of canine and feline cardiopulmonary dirofilariasis (McCall *et al*., 2008). Both species are zoonotic, responsible for human ocular/subcutaneous (*D. repens*) and pulmonary (*D. immitis*) dirofilariasis. Although humans are a less suitable host for these parasitic nematodes due to specific immune response that destroys the worm in most cases (Simón *et al*., 2005), in the past two decades the number of infections in humans has been rising (Pampiglione & Rivasi, 2000; Fuehrer *et al*., 2016).

Dogs infected with *D. immitis* usually develop severe life-threatening symptoms. Progression of the disease is chronic. Firstly, adults develop in the vascular and pulmonary system and eventually in the right chambers of the heart (McCall *et al*., 2008).

The infective larvae (L3) of both *D. repens* and *D. immitis* are transmitted by more than 70 mosquito species, including some species of the Lithuanian mosquito fauna (Cancrini *et al*., 2006; Bernotienė, 2012). Looking at the biological life cycle of parasites, both species require the same time interval and temperature for incubation in same mosquito species (Genchi *et al*., 2009).

Over the past decade, increasing numbers of autochthonous cases of heartworm infection have been reported in the following countries of Eastern Europe where previously only imported cases

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were described: Belarus (Şuleşco *et al*., 2016), Czech Republic (Svobodová *et al*., 2006), Hungary (Jacsó *et al*., 2009; Tolnai *et al*., 2014), Poland (Świątalska & Demiaszkiewicz, 2012), Slovakia (Svobodova *et al*., 2005; Miterpáková *et al*., 2008) and Russia (Kartashev *et al*., 2011). Rapid spread of this parasitic infection in non-endemic regions is caused by several factors. Climate change plays the essential role in the spreading of vector-borne diseases in Europe (Genchi *et al*., 2005). The risk season for the transmission of the disease due to more suitable conditions for vector development is becoming longer (Genchi *et al*., 2009).

Perhaps the most important factor for the spread of dirofilariasis into new areas is the increased movement of infected dogs due to the simplification of human and animal traveling rules and regulations in Europe (Genchi *et al*., 2011).

A growing number of vector-borne pathogens (such as *Babesia canis* and *Anaplasma phagocytophilum*) has been observed in recent years in Lithuania (Radzijevskaja *et al*., 2008, 2017; Paulauskas *et al*., 2014), including *D. repens* (Jankauskaitė *et al*., 2011; Paulauskas *et al.,* unpublished). According to the observations of veterinary practitioners, the highest incidence of canine subcutaneous dirofilariasis (*D. repens*) is registered in the central and western parts of Lithuania.

The aim of this report is to describe the clinical case of *D. immitis* in the dog imported from Spain and therefore to draw attention of veterinary practitioners and owners that the presence of *D. immitis* infected dogs could influence the spread of canine heartworm disease in Lithuania and other areas in North-Eastern Europe.

Material and Methods

Case history and observations

In January 2017, a 5-year-old male Spanish greyhound (Spanish galgo) was found free ranging in Cádiz (province of southern Spain) by a Lithuanian family. The dog was owned by local hunters, but after a leg trauma it was not suitable for hunting. The dog had lost significant amount of its body fat and muscle mass, weighed 13 kg (normal weight of a male Spanish greyhound dog is 27 – 29 kg) and was lethargic and exercise-intolerant. In April 2018, the dog arrived in Lithuania. In May 2018, the dog was referred to a small animal veterinary clinic in Vilnius for wellness screening.

Serology

Serology for circulating female (*D. immitis*) antigens and tick-transmitted pathogens (*Borrelia burgdorferi*, *Ehrlichia canis*, *Ehrlichia ewingii*, *Anaplasma phagocytophilum* and *Anaplasma platys*) was performed using SNAP 4Dx Plus Test (IDEXX Laboratories, Portland, USA).

Microfilaria

The Knott's test was used to detect circulating microfilariae. One ml of EDTA blood was mixed with 9 ml of 2 % formalin in a 12 ml tube and centrifuged for 5 minutes. The supernatant was poured off and two drops of the sediment was transferred to a slide covered with a coverslip and examined with a microscope using low power magnification.

PCR

A molecular analysis was performed to confirm the diagnosis. Genomic DNA was extracted from 200-µl aliquots of EDTA blood (taken from *Vena cephalica* of the examined dog) using the GeneJet Whole Blood Genomic DNA Purification kit (Thermo Fisher Scientific, Lithuania) according to the manufacturer's instructions. Species identification was based on amplification of partial internal transcribed spacer 2 (ITS-2) region of the ribosomal DNA using the panfilarial primer set DIDR-F1 and DIDR-R1 which allow to differentiate between *D. immitis* and five other filariae found in dogs (Rishniw *et al*., 2006). PCR amplification was carried out in a total volume of 20 µl containing 1X PCR buffer, 1.5 mM MgCl2, 250 µM dNTPs, 0.5 µM of each primer, 1 U of Taq polymerase (Thermo Fisher Scientific, Lithuania) and 1 µl of DNA template. Amplification was performed as follows: denaturation at 94 °C for 2 min and 35 cycles of denaturation (30 s at 94 °C), annealing (30 s at 60 °C), and extension (30 s at 72 °C) and a final extension (7 min at 72 °C). The amplification product was detected after electrophoresis in 1.5 % of agarose gel, revealed by staining with ethidium bromide and visualized under UV light. The PCR product was purified using a commercial kit (GeneJet Gel Extraction Kit, Thermo Fisher Scientific, Lithuania) and analysed by sequencing (performed at Macrogen Europe, Amsterdam). The obtained sequence was compared to those available in the GenBank database by using the Basic Local Alignment Tool (BLAST) analysis and Mega 6.0 software. Sequence obtained in this study was aligned with sequences derived from GenBank using CLUSTAL W algorithm. The phylogenetic tree was generated using maximum likelihood method (ML) with 1000 bootstraps replications.

Following the confirmation of the diagnosis, echocardiography and chest radiography were carried out to evaluate the patient prognosis.

Treatment

Treatment using the guidelines recommended by the American Heartworm Society (Nelson *et al*., 2014) was applied.

Adjunct therapy

The routine dose of prednisone at 0.5 mg/kg twice daily for the first week and 0.5 mg/kg once daily for the second week, 0.5 mg/kg every other day for the third and fourth weeks and doxycycline at 10 mg/kg twice daily for 4 weeks was applied. To kill susceptible larval stages milbemycin oxime (0.5 mg/kg) at day 30 of treatment was administered orally and continued monthly (Nelson *et al*., 2014).

Adulticide therapy

Following the adjunct therapy, three-dose regimen of melarsomine (one injection of 2.5 mg/kg body weight followed at least one

month later by two injections of the same dose 24 hours apart) was used (Nelson *et al*., 2014).

Ethical Approval and/or Informed Consent

Animal care and handling were carried out in accordance with institutional guidelines.

Results

During the clinical examination, no symptoms of heartworm disease were detected. Thoracic radiograph and echocardiography did not show any identifiable abnormalities commonly found with heartworm disease and appeared fairly normal, most likely due to an early asymptomatic stage of infestation with *D. immitis*.
In ELISA negative results were reported for *B. burgdorferi*, *E. canis*, *E. ewingii*, *A. phagocytophilum*, *A. platys* antibodies and positive for circulating female (*D. immitis*) antigens. Low numbers of microfilaria were found in the examined blood sample using the Knott's test.
Visualization of the PCR product of the examined sample by gel electrophoresis demonstrated DNA fragment of the expected size for *D. immitis* (542 bp). The sequence analysis of partial ITS-2 region revealed that the sequence had 99 – 100 % identity (with 1- to 3 nucleotides difference) with the *D. immitis* sequences deposited in GenBank, thereby confirming the *D. immitis* diagnosis. The phylogenetic analysis based on partial ITS-2 region sequence (Fig. 1) shows phylogenetic relationship of *D. immitis* obtained in present study and other filarial nematodes. Partial ITS2 region sequence was submitted to the GenBank database under the accession number MH663471.

Using the recommended treatment within 12 hours of the first injection of melarsomine, tenderness at the injection site, fever, lethargy and tremor were noted and after 24 hours the symptoms completely disappeared. Two weeks after the first injection of melarsomine, the dog started to gain weight, became more active and started to tolerate exercises. No side effects were noticed after the second and third melarsomine injection. Knott's test ant circulating female antigen test will be repeated at day 120 and 271 from starting treatment.
Information about this case are summarized in Table 1.

Table 1. Summarized case information.

Species	Domestic dog
Breed	Spanish greyhound
Age	5 year old
Imported from	Spain
Imported in	Lithuania
Weight before treatment	13.0 kg
Weight after treatment	21.0 kg
Symptoms	• Lethargic • Exercise-intolerant
Knott's test	Positive
Serology	Positive
PCR	Positive
Adjunct therapy	• Prednisone (0.5 mg/kg) • Doxycycline (10 mg/kg) • Milbemycin oxime (0.5 mg/kg)
Adulticide therapy	• Melarsomine (2.5 mg/kg)

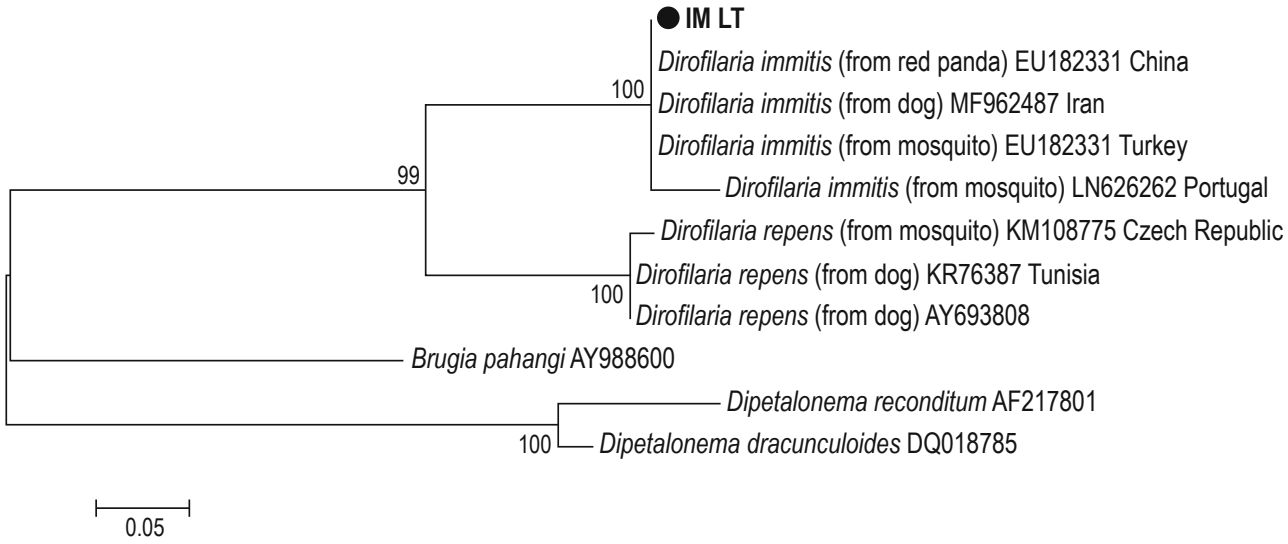

Fig. 1. The phylogenetic tree generated using maximum likelihood (ML) method, based on partial ITS-2 sequences. Maximum Likelihood bootstrap values associated with the branches. Sequence of *D. immitis* obtained in this study are marked with black circle (accession number MH663471).

Discussion

Previous research and published reports suggest that the geographical range of canine cardiopulmonary dirofilariasis has expanded in Europe in the past decade due to increasing movement of dogs and global changes in climate (Genchi *et al*., 2005). Many reports about imported cases of dirofilariasis have been published in different Central and Eastern European countries in the past decade and a few years later those areas became endemic for heartworm disease (Genchi *et al*., 2011).

Despite that both species, *Dirofilaria repens* and *Dirofilaria immitis,* require the same conditions for development, subcutaneous dirofilariasis spreads faster in new non-endemic areas than heartworm disease (Genchi *et al*., 2011). There are several explanations for it. Firstly, heartworm disease most often ends lethally due to adult worm development in the cardiovascular system, for that reason, infected dogs have shorter period of transmitting the disease, than the dogs infected with *D. repens*. Secondly, most cases of the subcutaneous dirofilariasis infection are asymptomatic, therefore dogs infected with *D. repens* could be the source of infection for several years. Dogs infected with *D. immitis* often develop clinical signs and are referred to the veterinary clinic where appropriate treatment is provided and transmission of the disease is prevented (Genchi *et al*., 2011).

Despite the fact that the period suitable for heart worm transmission in Lithuania is short (Genchi *et al*., 2005), previously reported autochthonous cases of subcutaneous dirofilarias and knowledge that both filarial species require the same temperature and the same time interval for incubation in vector suggest that an increase in the autochthonous heartworm cases in this area is possible. Furthermore, numerous suitable vector species (such as *Culex pipiens* s.l.*, Anopheles maculipennis* s.l.*, Aedes vexans*) for parasite development and transmission are found in Lithuania (Bernotienė, 2012).

Due to the complicated parasite life cycle, heartworm disease is chronic and asymptomatic in the primary stage of development. Veterinary clinicians in non-endemic areas lack experience in identifying the disease, therefore most cases are under diagnosed, microfilaremic dogs often do not get appropriate treatment and become the source of infection to the local mosquito population (McCall *et al*., 2008).

This is the first case report of heartworm infection in Lithuania in the dog imported from Southern Europe confirmed by serological, cytological and molecular methods. On the basis of this report it can be stated that heartworm-infected dogs transported to Lithuania from parasite endemic areas could act as donors of microfilariae to local mosquito species. Protocols of periodic heartworm antigen testing, in particular for traveling dogs, enable diagnosing early stages of heartworm disease and preventing transmission of microfilariae. A clear understanding of the biological life cycle of *D. immitis,* importance of asymptomatic dog treatment, and disease prevention in healthy dogs are critical to stop the spread of the disease in previously non-endemic areas.

Conflict of interest statement

Authors state no conflict of interest.

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