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Unraveling *Dirofilaria* spp. infection: Species diversity, molecular characterization, new diagnostic approaches, and zoonotic outcome
Sónia Vilar Gomes de Sá

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INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR

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“Between animal and human medicine there are no dividing lines - nor should there be. The object is different, but the experience obtained constitutes the basis of all medicine.”

Rudolf Virchow

1858

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Abstract

Dirofilariosis is a vector-borne disease frequent in many countries. Not only infected dogs, but also cats and wild canids (including wolves and foxes), represent important sources of infection for mosquitoes, which are the pathogen vectors. The disease is endemic in Mediterranean countries with increasing incidence in Italy, France, Greece and Spain. Nonetheless, limited epidemiological data is available from Portugal regarding its distribution and impact. In order to add knowledge concerning the presence of *Dirofilaria* spp. in Portugal, 244 dogs in the North of the country were tested through combined analysis of antigens by rapid immunomigration (RIM) test and DNA (Deoxyribonucleic acid) by PCR (Polymerase chain reaction). The latter enabled the differentiation of *D. immitis* from seven other filarial species. Based on RIM, 118 dogs (48.4%) tested positive for *D. immitis* adult worms, and 36 (14,8 %) tested positive to microfilaremia by using PCR, being *D. immitis* the detected species involved. The modified Knott test was only used to detect microfilaremia in samples which presented antigenemia; as a result, from the 118 positive cases identified through RIM, 24 (20.3%) dogs showed microfilariae with characteristic morphology of *D. immitis*. Results indicate that the risk of exposure to *D. immitis* in dogs is high in this region of Portugal, and that prophylaxis against the parasite is advisable to decrease the occurrence of canine infection and disease.

Later on, the presence of *Dirofilaria* spp. in wild canids was assessed using blood samples of 61 animals through RIM and optimized PCR for detection and differentiation of *D. immitis* from seven other filarioids. Three foxes presented *D. immitis* antigen circulation in blood and two wolves tested positive for *D. immitis* microfilaremia through PCR.

To acknoledge new diagnostic approaches, by expanding the use of long-read nanopore-based sequencing technology on nematodes, genomic de novo assembly of a *D. immitis* specimen retrieved from a cardiopulmonary dirofilariosis case was performed using the ONT MinION platform, followed by the study of macrocyclic lactone resistance. The genome size was 87,899,012 base pairs (bp) with a total of 9741 proteins and the subsequent analysis of six loci previously showed that four had a genotype associated with either some loss of efficacy or with resistance phenotype.

Finally, a letter was written to the editor of the prestigious journal Pulmonology (the official journal of the Portuguese Society of Pneumology), regarding the publication of a scientific article in the same journal, which documented the presence of *D. immitis* fragments through

histopathological diagnosis in a lung nodule of a man. The purpose of our letter was to inform that histological characteristics only allow for the determination of the genus *Dirofilaria* and that the identification of the species is much more complex, requiring diagnosis by PCR. The letter also mentioned that there are confirmed cases of *Dirofilaria* spp. circulating in Portugal, namely *D. repens*, which is reported to cause human pulmonary dirofilariosis nodules that can be misdiagnosed as malignant.

Resumo

A dirofilariose é uma doença transmitida por vectores, frequente em muitos países não afectando apenas cães mas também gatos e canídeos selvagens (incluindo lobos e raposas), que representam importantes fontes de infeção para os mosquitos, que são os vetores do agente patogénico. A doença é endémica nos países mediterrânicos, com uma incidência crescente em Itália, França, Grécia e Espanha. No entanto, existem poucos dados epidemiológicos disponíveis em Portugal relativamente à sua distribuição e impacto. Com o objetivo de aumentar o conhecimento sobre a presença de *Dirofilaria* spp. em Portugal, 244 cães do Norte do país foram testados através da análise combinada de antigénios por teste RIM (rapid immunomigration) e DNA (Deoxyribonucleic acid) por PCR (Polymerase chain reaction). Este último permitiu a diferenciação de *D. immitis* de outras sete espécies filariais. Com base no RIM, 118 cães (48,4%) testaram positivo para vermes adultos de *D. immitis* e 36 (14,8%) testaram positivo para microfilaremia através de PCR, sendo *D. immitis* a única espécie detetada. O teste de Knott modificado só foi utilizado para detetar microfilaremia em amostras que apresentavam antigenemia; como resultado, dos 118 casos positivos identificados através do RIM, 24 (20,3%) cães apresentaram microfilárias com morfologia caraterística de *D. immitis*. Os resultados indicaram que o risco de exposição a *D. immitis* em cães é elevado nesta região de Portugal, e que a profilaxia contra o parasita é aconselhável para diminuir a ocorrência de infeção e doença em cães.

Posteriormente avaliou-se a presença de *Dirofilaria* spp. em canídeos selvagens, e para tal 61 amostras de sangue foram testadas através de teste RIM e PCR, este último otimizado para a deteção e diferenciação de *D. immitis* de outras sete espécies filariais. Três raposas apresentaram circulação de antigénio de *D. immitis* no sangue e dois lobos testaram positivo para microfilaremia de *D. immitis* através de PCR.

No sentido de estabelecer novas abordagens de diagnóstico, expandindo a utilização da tecnologia de sequenciação baseada em nanoporos de leitura longa em nemátodos, foi realizada a montagem genómica *de novo* de uma amostra de *D. immitis* retirada de um caso de dirofilariose cardiopulmonar utilizando a plataforma ONT MinION, seguida do estudo da resistência à lactona macrocíclica. O tamanho do genoma foi de 87.899.012 pares de bases com um total de 9741 proteínas e a análise subsequente de seis loci mostrou anteriormente que quatro tinham um genótipo associado a alguma perda de eficácia ou ao fenótipo de resistência.

Por fim, foi elaborada uma carta ao Editor da revista prestigiada Pulmonology (revista oficial da Sociedade Portuguesa de Pneumologia), relativamente à publicação de um artigo científico na mesma revista, que documentava a presença de fragmentos de *D. immitis* através de diagnóstico histopatológico num nódulo pulmonar de um homem. O objetivo da nossa carta foi no sentido de informar que as características histológicas apenas permitem a determinação do género *Dirofilaria* e que a identificação da espécie é bastante mais complexa, sendo necessário o diagnóstico por PCR. Foi referido também na carta que existem casos confirmados de *Dirofilaria* spp. a circular em Portugal, nomeadamente *D. repens*, reportada como causadora de dirofilariose pulmonar humana podendo provocar nódulos e estes muitas vezes serem erradamente diagnosticados como malignos.

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Declaração de Honra

Declaro que a presente tese é de minha autoria e não foi utilizada previamente noutro curso ou unidade curricular, desta ou de outra instituição. As referências a outros autores (afirmações, ideias, pensamentos) respeitam escrupulosamente as regras da atribuição, e encontram-se devidamente indicadas no texto e nas referências bibliográficas, de acordo com as normas de referenciação. Tenho consciência de que a prática de plágio e auto-plágio constitui um ilícito académico.

List of Abbreviations

%	Percentage
AAHA	American Animal Hospital Association
AHS	American Heartworm Society
AP	Acid phosphatase
aPTT	Activated partial thromboplastin time
bp	Base pairs
BUN	Urea nitrogen
CD	Cardiopulmonary dirofilariosis
CHD	Cardiopulmonary heartworm disease
cTnI	Cardiac troponin I
CanD	Canine Dirofilariosis
DIC	Disseminated intravascular coagulation
DNA	Deoxyribonucleic acid
dpi	Days post-infection
EDTA	Ethylenediaminetetraacetic acid
e.g.	For example
ELISA	Enzyme-linked immunosorbent assay~
ESCCAP	European Scientific Counsel Companion Animal Parasites
ESDA	European Society of Dirofilariosis and Angiostrongylosis
FECAVA	Federation of European Companion Animal Veterinary Associations
g	gravitational force
h	Hours
i.e.	That is
IHGS	International Helminth Genomes Consortium
L1	First-stage larvae
L2	Second-stage larvae
L3	Third-stage larvae

L4	Fourth-stage larvae
Kda	Kilodalton
Kg	Kilogram
ITS1	Internal transcribed spacer1
ITS2	Internal transcribed spacer 2
mg	Milligram
ML	Maximum likelihood
ml	Milliliter
MLs	Macrocyclic lactones
ºC	Celsius
per os	Oral administration
PH	Pulmonary hypertension
PCR	Polymerase chain reaction
PT	Prothrombin time
RIM	Rapid immunomigration
SD	Subcutaneous dirofilariosis
SDMA	Symmetric dimethylarginine
SNPs	Single nucleotide polymorphism
TPP	Total plasma protein
UPC	Urine protein/creatinine
w/v	Weight per volume.
V	Volt
VCS	Vena cava syndrome
µL	Microliter
µm	Micrometre

CHAPTER I

State of the Art

1. Introduction and historical perspective of the parasite *Dirofilaria* spp.

Originally from the Latin *dīrus* (“fearful” or “ominous”) + *fīlum* (“thread”), *Dirofilaria* is a genus of nematodes in the superfamily Filarioidea (Railliet and Henry 1911; Simón et al., 2012; Henry, AHS 2020). Francesco Birago described, in 1626, in his Treatise on Hunting: “The dog generates two worms, which are half an arm’s length long and thicker than a finger and red like fire” (Birago, 1626; reviewed by Railliet and Henry 1911). Birago erroneously identified the worms as a larval stage of another parasite, *Dioctophyme renale* (Railliet and Henry 1911; Simón et al., 2012). The dog heartworm was named as *Filaria* in 1856 by American parasitologist Joseph Leidy, whose research identified several adult specimens of *Filaria immitis*, both males and females, which were labelled as coming from a "dog", probably obtained from the abdominal cavity; the genus was later renamed *Dirofilaria* by French parasitologists Railliet and Henry (Railliet and Henry 1911). In South America, the earliest cases of heartworms in dogs arose in 1847, nonetheless the official report was not published until 1875, and in 1974 the American Heartworm Society was founded (AAHA, 2018; AHS, 2020).

Currently, dirofilariosis is understood as a group of parasitosis caused by species of the genus *Dirofilaria* transmitted by mosquitoes belonging to different genera of the family Culicidae (i.e. *Aedes*, *Anopheles*, *Culex* and *Ochlerotatus*) (Alho et al., 2014; Dantas-Torres et al., 2020; Edgerton et al., 2020; Macchioni et al., 2020).

From all species of the genus *Dirofilaria*, the ones considered to be the most relevant are *D. immitis* and *Dirofilaria repens*, once they are zoonotic agents adapted to canine, feline and human hosts, with distinct biological and clinical implications (Dantas-Torres et al., 2013; Capelli et al., 2018; Diakou et al., 2018; Bozidis et al., 2020). *Dirofilaria repens* is accountable for subcutaneous dirofilariosis (SD) in dogs and cats, and for subcutaneous and ocular dirofilariosis in humans (Simón et al., 2012). *Dirofilaria immitis* worms cause canine cardiopulmonary dirofilariosis (CD), also known as cardiopulmonary heartworm disease (CHD), a widespread disease that can have a fatal outcome if animals are not treated (Mendoza-Roldan et al., 2021; Alho et al., 2018).

In fact, *D. immitis* is considered an extremely important worm in veterinary medicine, considering its virulence and increasing incidence (Napoli et al., 2023). Moreover, *D. immitis* in dogs represents a risk for the human population, who may suffer from pulmonary dirofilariosis and, in many cases, pulmonary nodules that can be misdiagnosed as malignant tumours (Fontes-Sousa et al., 2019; Gabrielli et al., 2021). Because of all these issues, the epidemiology, biology, pathogeny, diagnosis, prevention, treatment, drug

resistance, public health, human and animal dirofilariosis are reviewed here.

2. *Dirofilaria immitis* in canids

2.1 Epidemiology of *Dirofilaria immitis*

The epidemiology and distribution dynamics of elements of the genus *Dirofilaria* has suffered modifications due to several factors, including the influence of climate change, human modification of environment, such as irrigating lands for farming, transboundary movements of animals and humans, and changes in the parasite-host relationship such as the outburst of new species of mosquitoes (Alho et al., 2014; Capelli et al., 2018; Fontes-Sousa et al., 2019; Esteban-Mendoza et al., 2020; Farkas et al., 2020).

Several cases of pulmonary heartworm and ectopic infections have been reported in humans, despite the fact that these are considered as accidental hosts for *D.immitis* (Capelli et al., 2018; Djaković et al., 2019; Bozidis et al., 2020; Esteban-Mendoza et al., 2020). The presence of *D. immitis* in dogs represents a risk for the human population (Alberigi et al., 2023).

Although CD has a worldwide distribution, with considerable prevalence in the American continent, Australia and Japan (Dantas-Torres and Otranto, 2013; Esteban-Mendoza et al., 2020), the disease is also endemic in Mediterranean countries with increasing occurrence in Italy, France, Greece and Spain, where the causative agent is considered one of the most widespread animal parasites (Diakou et al., 2018; Laidoud et al., 2019; Macchioni et al., 2020; Mendoza-Roldan et al., 2021). The first study that investigated the overall prevalence of *D. immitis* infection in dogs all over the world, based on published articles, revealed a 10.9% global prevalence, namely of 22.7% in Australia, 12.1% in Asia, 11.6% in America, 10.5% in Europe and 7.6% in Africa (Anvari et al., 2020). The highest prevalence of *D. immitis* in Australia can be explained by the high prevalence of the *Culex* mosquitoes in that region as well as the humidity and seasonal changes which contribute to the survival and development of vector mosquitoes (Dearsley et al., 2019). Based on the results of this study, the countries with the highest and lowest prevalences of *D. immitis* in dogs are Spain with 19.3%, and Canada with 0.2%, respectively (Anvari et al., 2020).

Considering that the climate in Portugal is compatible with the development, reproduction and survival of the vectors, it is appropriate to define Portugal as a country in which canine dirofilariosis by *D. immitis* is endemic (Alho et al 2018). Epidemiological data available in Portugal show that *D. immitis* is present in the country, but little information is known regarding its distribution impact and potential acquisition of new ecosystems (Alho et al.,

2014, 2018; Maia et al., 2015; Ferreira et al 2017). The first documented study on CanD (Canine Dirofilariosis) in Portugal was carried in Águas de Moura (municipality of Palmela) and Herdade do Pinheiro (municipality of Alcácer do Sal), both in the district of Setúbal, in which through research of microfilarie in the blood of dogs, prevalence values of 63.8% and 52.6%, respectively were found (Cambournac and Simões 1943). In 1991, an epidemiologic study on CanD detected in the many regional Directorates of Agriculture in Portugal, a global prevalence of microfilaremiae of 14% using the Knott test. The highest prevalence was verified in Madeira (30%), followed by Ribatejo (16.7%), Alentejo (16.5%) and, finally, the Algarve (12%) (Pereira Da Fonseca et al., 1991). In São Miguel, Azores, in 1995, blood samples were collected and submitted to the modified Knott test with no detection of microfilaremia; however, this same study detected the existence of vectors such as mosquitoes of the genus *Culex* (Medeiros, 1995). After this research, a retrospective study performed in Madeira showed that a total of 22% of dogs were microfilaremic, out of which 89% of the microfilariae were microscopically identified as *D. immitis* and the remaining as *Acantocheilonema reconditum* and *Acanthocheilonema dracunculoides* (Clemente, 1996). Subsequently, in the North of Portugal, in the county of Alijó, 4.4% of the tested dogs were microfilaremic, of which 80% of the microfilariae were identified by acid phosphatase histochemical staining as *A. dracunculoides*, 14% as *D. immitis* and 6% as *A. reconditum*. In the municipality of Sabrosa, 11.8% of the analysed dogs were microfilaremic, all of the larvae identified as *A. dracunculoides* (Santos et al., 2000). Later on, another retrospective study revealed that 1050 cases of canine dirofilariosis were diagnosed in 13 municipalities in Coimbra district, using the diagnostic methods of thick drop techniques and immunochromatography (Sousa et al., 2008a). Additionally, blood samples were collected from 40 dogs and 10 cats in Coimbra district for the identification of *D. immitis* microfilariae according to Knott's test, resulting in eight positive cases in dogs and no positive cases in cats (Sousa et al., 2008b). Soon after, a study conducted in three estuarine areas of Portugal, i.e. Coimbra, Santarém and Setúbal districts, showed through antigen detection 9.7 % positive results for *D. immitis*, whereas the modified Knott's test and the acid phosphatase test detected 12.3% and 10.6% blood microfilariae respectively (Alho et al., 2012). In 2012, a national study found prevalences of *D. immitis* antigen which ranged from 3.6% in apparently healthy dogs to 8.9% in clinically suspected dogs (Cardoso et al., 2012). Afterwards, blood samples taken from dogs, resident in Figueira da Foz (a central region of Portugal), were tested using direct microscopic evaluation of a fresh blood sample, the modified Knott's test and the ELISA antigen detection test and a total prevalence of 27.3% was found; furthermore, using a histochemical technique, *D. immitis* was identified in 73.5% of the microfilaremic samples (Vieira et al., 2014a). Meanwhile, a study performed in three coastal regions in central Portugal showed a global prevalence of canine heartworm of

15.1%, being the highest one in Setúbal, with 24.8%, followed by Coimbra, 13.8%, and Santarém, with 13.2%, using a rapid immunomigration (RIM) technique to detect the presence of *D. immitis* circulating antigens and the modified Knott's test together with acid phosphatase histochemical staining to detect and identify circulating microfilariae (Alho et al., 2014). The same samples of canine blood were analyzed by molecular screening and three animals were identified to be co-infected with *D. immitis* and *A. dracunculoides*; with one dog infected only with *A. dracunculoides*, all confirmed by PCR (Polymerase chain reaction) (Alho et al., 2014). After that, the prevalence of *D. immitis* in cats was detected as 15% by means of testing for circulating anti-*D. immitis* and anti-*Wolbachia* antibodies in central and northern Portugal; furthermore, dogs were also tested for circulating *D. immitis* antigens with a prevalence of 2.1%. The highest feline seroprevalences were 18.7% in Aveiro and 17.6% in Viseu, while the highest canine prevalences were 8.8% and 6.8% in Coimbra and Aveiro, respectively (Vieira et al., 2014b). In another region of Portugal, i.e. the Algarve, antigens of *D. immitis* were detected in 9.4% of the serum samples of dogs by an immunochromatography test (Maia et al., 2015). More recently, from canine blood samples collected in three districts of Portugal (Coimbra, Santarém and Setúbal), 8.8% were positive for *D. immitis* circulating antigen, 13.1% positive for microfilariae by the modified Knott's test and 13.7% were by PCR, being this last one considered the most adequate method for diagnosis and prevalence estimation, as well as being capable of detecting mixed *D. immitis* and *A. reconditum* infections (Ferreira et al., 2017). In Funchal (Madeira), serum samples of cats were tested for *D. immitis* antigen, with an ELISA kit, with a prevalence of 3.5 % (Neves et al., 2020). The latest study carried out in northern Portugal revealed that 48.4% of dogs tested positive for adult forms of *D. immitis* by an immunochromatography test and 14.8% tested positive for microfilaremia by PCR (Gomes-de-Sá et al., 2022a).

Wild animals can be sylvatic reservoirs for *D. immitis* by supporting the transmission of these parasites to domestic animals (Gomes-de-Sá et al., 2022c). At the country level, a study revealed a prevalence of 3.2% of *D. immitis* detected by necropsy in red foxes (*Vulpes vulpes*) (Eira et al., 2006); moreover, in a national serological survey conducted in red foxes in Portugal, 8.5% were positive for *D. immitis* (Alho et al., 2016). *Dirofilaria immitis* was also found in three Eurasian otters (*Lutra lutra*) in Portuguese natural habitats (Torres et al., 2004; Saraiva et al., 2012), afterwards in a collection of pinnipeds: common seals (*Phoca vitulina*), California sea lions (*Zalophus californianus*) and South African fur seals (*Arctocephalus pusillus pusillus*) in a Portuguese oceanographic park (Alho et al., 2017) and more recently antigenemia was detected with an occurrence of 15.8% in red foxes and also, for the first time, microfilaremia in Iberian wolves (*Canis lupus signatus*), in the National Park of Peneda Gerês which was revealed by PCR (Gomes-de-Sá et al., 2022c).

2.2. Biology of *Dirofilaria immitis*

2.2.1. Life cycle

The life cycle of *D. immitis* is moderately long (between 7 and 9 months). This protracted life cycle needs a reservoir of infection, a vectorcapable of transmitting infection, and a receptive host.

Mosquitos, the necessary biologic vectors for transmission of *D. immitis*, become infected as they feed from a microfilaraemic host. It is fundamental to bear in mind that microfilariae cannot become adult heartworms without first evolving into larval stage 1 (L1) in the Malpighian tubules of the mosquito, after that moulting into second-stage larvae (L2), and finally moulting into third-stage larvae (L3) (Taylor, 1960). Once the larvae have reached stage 3, considered the infective stage, they migrate through the body cavity to the head and mouthparts of the mosquito.

Temperature has a vital role in the development of microfilariae to the infective stage. While at a temperature of 27ºC and 80% relative humidity the development takes between 10 and 14 days, with lower temperatures the larvae's maturation takes longer (SIocombe et al., 1989).

Transmission of the infective L3 is achieved when an infected female mosquito feeds on the animal. When the mosquito's stylet enters the animal's skin, there is a rupture in the mosquito's labium and a droplet of haemolymph with infective larvae gets onto its surface (McGreevy et al., 1974) Immediately after the bite, the sexually differentiated larvae enter the animal's body through the wound caused by the mosquito. After 3 days the L3 moults into L4 (fourth-stage larvae). The last moult into immature adults takes place between the 50th and the 70th days. During this time these larvae migrate through the body, ultimately get into the circulatory system, and are taken to the heart and lungs (Kume and Itagaki, 1955; Kotani and Powers, 1982; Lichtenfels et al., 1985). These immature adults arrive at the pulmonary vasculature on the 67th day and by days 90 to 120 all of them have reached this part of the host's body. When they reach the pulmonary arteries, these heartworms have not yet fully matured. The mature female travels between subcutaneous tissues and muscle. About 120 days after infection sexual maturity is attained. At this stage, hosts have circulating microfilariae, which occur 6 months after infection, although it is more common by 7 to 9 months (Kotani and Powers, 1982; Orihel, 1961). As soon as immature heartworms arrive in the lungs, the blood takes them into the small pulmonary arteries (Rawlings, 1980). As the worms get bigger, they successively occupy larger arteries until they become completely mature. If the worm burden becomes heavier, worms can also be placed in the right ventricle (Jackson, 1974; Ishihara et al., 1978; Atwell and Buoro, 1988;). The adults

potentially living more than 7 years and microfilariae up to 2 years (Simón et al., 2012). (Figure 1)

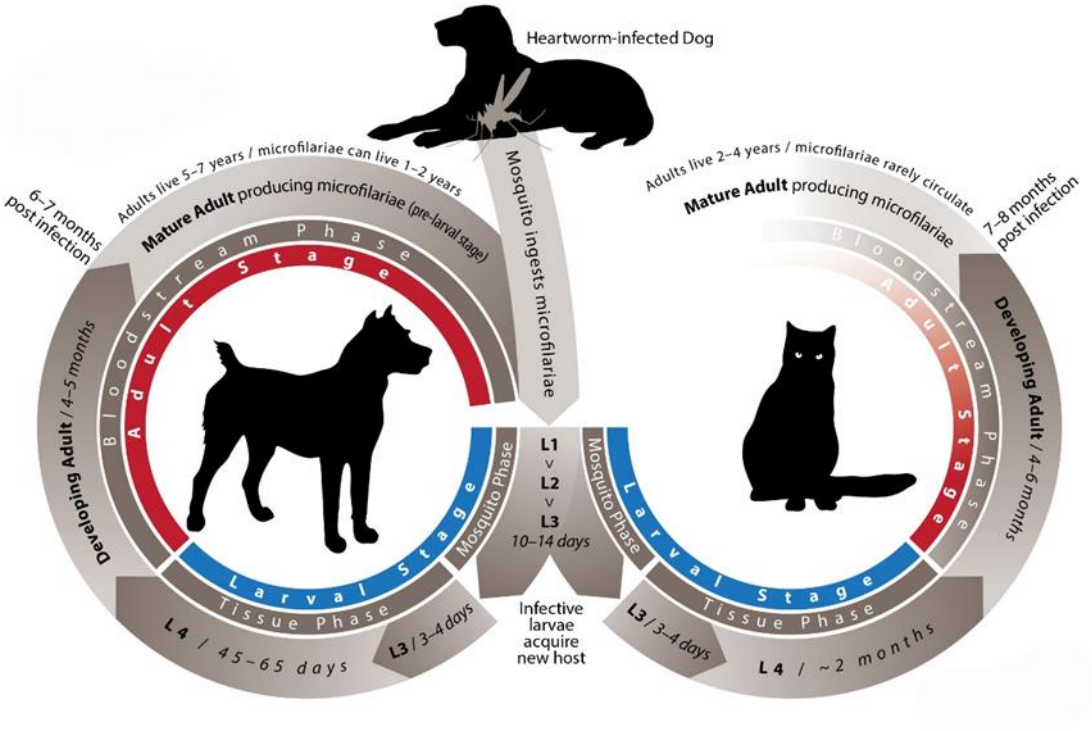

Figure 1 – Life cycle of Dirofilaria immitis (American Heartworm Society [AHS], 2020).

2.2.2. Role of symbiotic bacteria *Wolbachia* spp.

The Endosymbiosis is a close form of symbiotic association in which one organism resides inside the body of another, creating a scope of connections from parasitism to obligatory mutualism (Sullivan, 2017). Many obligate mutual symbiotic associations are based on metabolic enrichment, invigorating the pathways of one or both hosts and increasing the biochemical dynamic (Hosokawa et al., 2010; Nikoh et al., 2014).

In terms of *Wolbachia* spp. and nematode symbiosis, *Wolbachia*, a gram-negative intracellular bacterium, are obligate mutualists for filariae worms and fundamental for the biologic development, reproduction, and survival of nematodes, as well as for supplying critical metabolites to the filarial nematodes (Werren et al., 2008; Ichimori et al., 2014). This bacterium is detected in all filarial developmental stages, i.e. located on the lateral cords of both males and females and in the genital organs of females, and also in microfilariae and the larvae in the vector; however, there is a rapid increase in number as the nematode transitions from its insect vectors to mammalian hosts (Bandi et al., 2001).

Wolbachia spp. have several roles in filariosis, namely fomenting a proinflammatory immune response to onchocercid nematodes by interacting with the host monocytes, macrophages, dendritic cells and neutrophils (Genchi et al., 2011). This manipulation of the host immune system contributes to the extension in the longevity of onchocercid nematodes (Hansen et al., 2010; Sulaiman et al., 2019). In addition, a more powerful immune reaction in response to the release of L3 than to the dead worm proves the predominant role of these bacteria at the development of the early stage of the worm (Genchi et al., 2011).

Molecular approaches play an important role in confirming and characterizing the species of *Dirofilaria* and its *Wolbachia* endosymbionts in blood samples from dogs (Satjawongvanit et al., 2019; Laidoudi et al., 2019; Bawm et al., 2023). Recent studies have explored the connection between molecular detection and the diagnosis of canine filariosis (Satjawongvanit et al., 2019; Laidoudi et al., 2019, Bawm et al., 2023). One of these revealed the detection of *Wolbachia* within filaria-infected dogs and the detected species consisted of *D. immitis* (57.9%), *Brugia pahangi* (19.3%), and *Brugia malayi* (5.3%) by PCR (Satjawongvanit et al., 2019). Another study molecularly detected *D. immitis*, *A. reconditum, Cercopithifilaria bainae* and *Brugia* spp. and the associated *Wolbachia* endosymbionts from canine blood (Laidoudi et al., 2019). Furthermore, by using PCR, 28% and 18% of the samples were found to be positive for *D. immitis* and *Wolbachia* endosymbionts, respectively (Bawm et al., 2023). These data not only contribute to our understanding of the coexistence of *D. immitis* and *Wolbachia* spp. endosymbiosis in dogs, but the findings may also benefit the future prevention and control of dirofilariosis in dogs.

2.2.3. Role of wildlife hosts

The Wildlife carnivores have long been overlooked when considering their possible role in transmitting zoonotic nematodes (Otranto et al., 2019). However, increasing human activities have continuously promoted wild environment invasion, ultimately redefining domestic and wild interface boundaries and consequently increasing the contact between humans and wild animals (Otranto et al., 2019). Moreover, wild animals are frequently exposed to vector-borne pathogens to such an extent that wild carnivores like the grey wolf (*Canis lupus*), red fox (*Vulpes vulpes*) and golden jackal (*Canis aureus*) are now recognized hosts of *D. immitis* (Moroni et al., 2020). The wolves' and foxes' role as *D. immitis* reservoir hosts and their contribution to disease transmission are acknowledged throughout Europe (Morchón et al., 2012).

Variable occurrences have been reported, namely, 32.3% in Spain (Gortazar et al., 1994), 1.6% in Serbia (Penezić et al., 2014), 3.7% in Hungary (Tolnai et al., 2014), 25.2% in Bulgaria (Panayotova-Pencheva et al., 2016), 0.3% in Romania (Ionică et al., 2017) and

2% in France (Medkour et al., 2020).

In Portugal, the prevalence of *D. immitis* detected by necropsy of red foxes ranged from 3.2% in northern-central locations, such as Coimbra district (Eira et al., 2006), to 11.8% in southern and central districts, such as Santarém and Setúbal (Carvalho-Varela et al., 1993). In a serological survey conducted in red foxes in Portugal, 8.5% were positive for *D. immitis* circulating antigens, with positive animals found in northern and southern areas (Alho et al 2018). A study assessed the presence of *D. immitis* in foxes and wolves in Portugal, 15.8% of the foxes were positive for *D. immitis* antigenemia, while 4.8% of the wolves were positive for *D. immitis* microfilaremia by PCR, these can act as a reservoir for further infection if the intermediate mosquito host is present (Gomes-de-Sá et al., 2022c). Another study reported that wolves were suitable *Dirofilaria* hosts and appeared exposed to infection similarly to sympatric unprotected dogs (Moroni et al., 2020). All studies mentioned support the potential epidemiological role in wild carnivore species.

Understanding infection and disease prevalence in wild canids is especially important because these may act as reservoirs, increasing the risk of infection for domestic pets, including urban canids (Pluemer et al., 2019). While in endemic areas frequent chemoprophylactic treatments of domestic dogs reduce the overall prevalence of the infection, wild canids might play a crucial role in the maintenance of infection (Pluemer et al., 2019). Although *Dirofilaria* microfilaremia does not necessarily correlate to an endangerment of the infected animals' health, the individual can act as a reservoir for further infection if the intermediate mosquito host is present (Rishniw et al., 2006).

2.3. Pathogeny of dirofilariosis

Canine Dirofilariosis (CanD) is a cardiopulmonary disease, mainly caused by the presence of the adult parasites and the preferred location of the adult worms is the pulmonary arteries, followed by the right ventricle, which provokes proliferative endarteritis, inducing vascular damage, pulmonary hypertension and congestive heart failure; this last condition develops, ascites, peripheral oedema, hydrothorax, and hydropericardium (McCall et al., 2008; Simón et al., 2012, Carretón et al., 2017; Lemos et al., 2022; Philp et al., 2023; Mõttus et al., 2024) (Figure 2). Vena cava syndrome (VCS) is a serious condition most commonly detected, which happens through the displacement of a mass of worms from the pulmonary arteries to the right ventricle, where they interfere with the function of the tricuspid valve, leading to increased pressure in the right ventricle and the obstruction of the valve lumen, resulting in tricuspid insufficiency (Lemos et al., 2022; Philp et al., 2023). These factors produce volumetric and pressure overloads in the right atrium and the caudal vena cava, with a substantial elevation in venous pressure and difficulty in return circulation, all this frequently

results in the death of the animal because of haemolysis, haemoglobinuria, and disseminated intravascular coagulation (DIC) (Philp et al., 2023). Eosinophilic pneumonia, diagnosed in some dogs with dirofilariosis, produces severe respiratory distress which is provoked by an eosinophilic inflammatory reaction to microfilarial antigens, leading to alveolar dysfunction and impaired gas exchange, causing hypoxemia, hypoxia, and serious respiratory insufficiency (Simón et al., 2012).

Clinical signs develop progressively and the typically reported clinical signs include weakness, exercise intolerance, lethargy, depression, decreased appetite; dehydration, cough, dyspnoea, cachexia, ascites, pale mucous membranes and exertional syncope (Oi et al., 2015; Lemos et al., 2022; Bawm et al., 2023; Mõttus et al., 2024).

During the physical examination weight loss, prostration, abnormal lung sounds; murmur at the tricuspid focus, lameness, hard laboured breathing pattern at rest and pyrexia, may be observed (Oi et al., 2015; Lemos et al., 2022; Mõttus et al., 2024).

Due to the presence of the large adult stages in the pulmonary vasculature and the heart, the infection can result in severe pathology or even death, causing cardiorespiratory insufficiency or severe thromboembolism, reason why exercise restriction is advised in an attempt to minimise these risks (Simón et al., 2012; Mõttus et al., 2024).

Although the degree of disease is normally connected with the worm burden (number of adult parasites) present, the L1 stages released from fertilized adult females necessary for the transmission by mosquitos appear to cause rather little pathology on their own (Simón et al., 2012). Even though canine hosts evidently recognize the presence of heartworms, as demonstrated by the abundant antibody response directed at the parasite, this response is not enough to prevent infection or eliminate the parasite before reproduction takes place (Geary, 2023). Every so often several infected dogs do not present any clinical signs, or, when they do, they are similar to other etiologies, which can lead to misdiagnosis and mistaken for other cardiorespiratory diseases that, if not properly treated, can develop nonspecific and varied cardiorespiratory complications (Keene et al., 2019; American Heartworm Society, 2020; Lemos 2022 et al).

Dirofilaria spp. can cause lesions in other organs owing to erratic localizations, including the brain, liver, eyes, and peritoneal cavity (Venco et al., 2011; Simon et al., 2012). At the hepatic level, hepatomegaly is caused by venous congestion resulting in liver failure, which may be accompanied by jaundice, increased levels of transferases, and coagulation disorders (Venco et al., 2011). Glomerulonephritis is associated with the formation of immune-complexes stimulated by antigens from microfilariae, larvae, and adult worms, with the presence of microfilaria intensifying the condition, likewise, renal lesions can evolve to severe nephrosis induced by proteinuria with renal insufficiency and azotaemia (Paes-de-Almeida et al., 2003; Carretón et al., 2020). A case report documents the detection of an

unusual microfilaria in the urine sediment, focusing attention on the occurrence of microfilariae in unusual locations, such as the bladder (Perles et al., 2024). A study confirmed by PCR the existence of ectopic dirofilariosis caused by *D. immitis* in the subconjunctival and subcutaneous tissues of dogs (Goh et al., 2023). A further study on the presence of microfilariae of *D. immitis* detected nodular pyogranulomatous dermatitis in a dog (Silva et al., 2023).

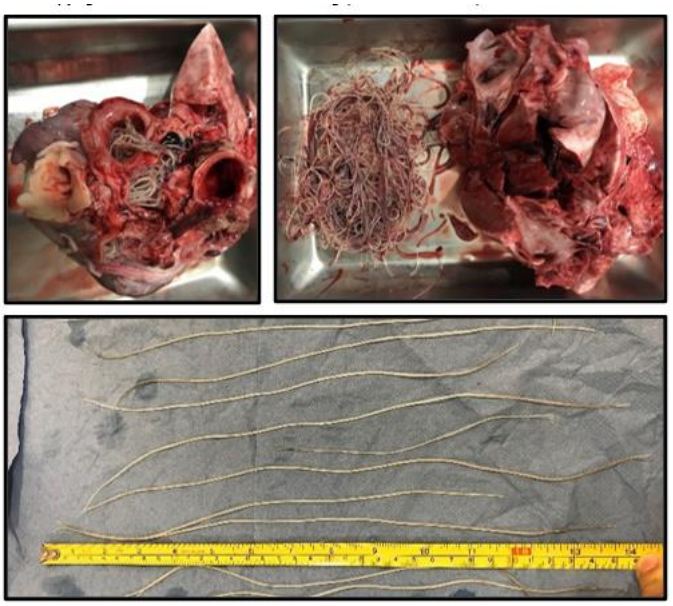

Figure 2 - Adult nematodes of *Dirofilaria immitis (original)*

3. Diagnosis of dirofilariosis

The diagnosis of HWD is possible through the combination of various tests, including: the microscopic detection and morphological identification of circulating blood microfilariae; the detection of circulating adult worm antigens; the detection of DNA using molecular methods; and additional methods such as radiography; echocardiography, and hematology, biochemistry, and coagulation profiles (Gomes-de- Sá et al., 2022a; Fhilp et al., 2023; Mõttus et al., 2024; Murillo et al., 2024).

3.1 Parasitological diagnosis

The modified Knott's test and the acid phosphatase histochemical staining test are commonly the most used for microscopic detection, allowing the differentiations from other filarial species (Ferreira et al., 2017; Hays et al., 2020; Gruntmeir et al., 2023). Since treatments may be distinct

according to the filarial species, it is vital to perform morphometric analysis that will differentiate the etiological agents (Gruntmeir et al., 2023). The most important ones are *D. immitis*, *D. repens*, *A. dracunculoides* and *A. reconditum* (Genchi et al., 2011; Hays et al., 2020; Murillo et al., 2024).

The modified Knott's test is the most frequently applied parasitological method, consisting of a concentration method in which 1 ml of EDTA (Ethylenediaminetetraacetic Acid) blood is mixed with 9 ml of 2% formalin and centrifuged for 5 minutes at 500×g. Thenceforth, the supernatant is removed, one drop of blue methylene is added and the sediment is observed under the light microscope (Hays et al., 2020). Through microscopic observation, microfilariae of the four above-mentioned filaria species show (Figure 3) an average length and width as follows: *D. immitis*, 302 µm and 6 µm; *D. repens*, 369 µm and 9 µm; *A. dracunculoides*, 259 µm and 5 µm; and *A. reconditum*, 265 µm and 5 µm, respectively. In terms of front end and caudal end, *D. immitis* shows a conical front end and a straight rear end, *D. repens* has a conical front end and curved caudal end, *A. dracunculoides* presents a round front and straight caudal end, and *A. reconditum* shows a blunt front end and a small hook in the rear end (Venco et al., 2011; Magnis et al., 2013).

Table 1- Morphological characteristics of microfilariae

Species	Length	Width	Characteristics
D. immitis	302 µm	6 µm	Conical front end and a straight rear end
D. repens	369 µm	9 µm	Conical front end and curved caudal end
A. dracunculoides	259 µm	5 µm	Round front and straight caudal end
A. reconditum	265 µm	5 µm	Blunt front end and a small hook in the rear end

Acid phosphatase histochemical staining, also available as a commercial kit, enables to differentiate microfilariae in accordance with the enzymatic activity of acid phosphatases in the anatomical regions (anal pore, excretory pore and internal body) (Yildirim et al., 2007). *Dirofilaria immitis* microfilariae show (Figure 4) two bright red dots of acid phosphatase activity on the excretory and the anal pores, while *D. repens* shows only one dot, near the anal pore and eventually some inner body complex; *A. dracunculoides* reveals three areas of enzymatic activity and *A. reconditum* reveals a diffuse staining (Yildirim et al 2007; Little et al., 2018; Gruntmeir et al., 2023).

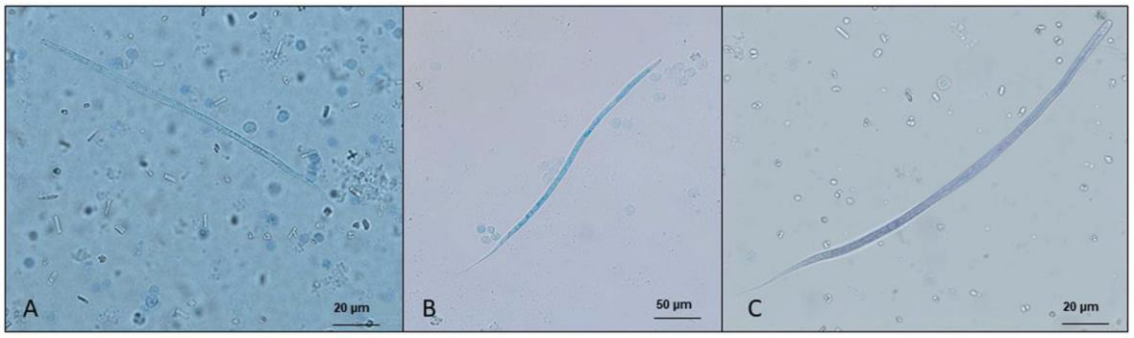

Figure 3- Microfilariae detected in blood samples by a modified Knott's test and stained with methylene blue. (A) *A. reconditum*; scale bar: 20 µm. (B) *D. immitis*; scale bar: 50 µm. (C) *D. repens*; scale bar: 20 µm (Gruntmeir et al., 2023).

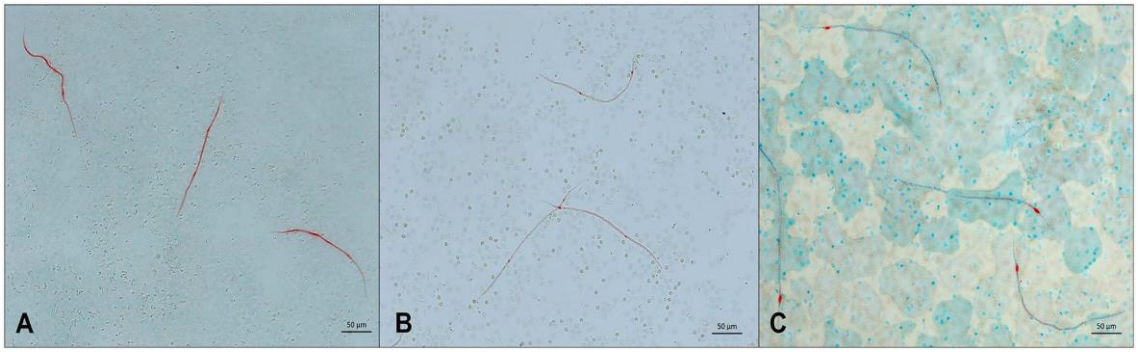

Figure 4 - Microfilariae detected in blood samples stained by acid phosphatase (AP) histochemical method. (A) *A. reconditum*: AP activity through the entire body (red); scale bar: 50 µm. (B) *D. immitis:* AP activity in excretory and anal pores (red); scale bar: 50 µm. (C) *D. repens*: AP activity in anal pores (red); scale bar: 50 µm (Gruntmeir et al., 2023).

3.2. Immunological diagnosis

Serological methods provide a convenient, sensitive and specific way to detect *D. immitis* infection in routine veterinary patients (Panarese et al., 2020; Brianti et al., 2023). One of these tests consists of an immunochromatographic technique aiming at the qualitative detection of *D. immitis* antigen in blood, based on a 14 kDa antigen detection not exclusively related to the feminine genital apparatus. The above-mentioned test successfully identifies infections with a load of only one adult parasite of any type (males, adult females or immature females), with sensitivity and specificity of 94% and 100 %, respectively, when compared to necropsy data. This method is known to be sensitive for the screening of apparently healthy dogs or for confirming clinically suspected *D. immitis* infections, presenting a sensitivity greater than 90% in dogs infected with one adult female worm and 100% in dogs infected with more than one adult female (Henry et al., 2018; Laidoud et al., 2019; Panarese et al., 2020; Brianti et al., 2023). Although these are highly sensitive for *D.*

immitis, recent work has demonstrated limitations in specificity due to cross-reactions with *D. repens*, *Angiostrongylus vasorum*, *Spirocerca lupi* (Venco et al., 2017; Alho et al., 2018; Panarese et al., 2020). Furthermore, another study compared the prevalence of *D. immitis* in serum with and without heat treatment, with results showing a higher prevalence in sera submitted to heat treatment, once the technique allows immune complex dissociation to enhance antigen detection (Murillo et al., 2024). In these situations, diagnosis depends on examining the microfilariae, usually applying the modified Knott's test and PCR (Gomes-de-Sá et al., 2022a; Brianti et al., 2023; Mõttus et al., 2024).

In several studies, the RIM test detected amicrofilaremic infections, because not all animals which were antigen-positive had microfilaremia detected by PCR, proving that RIM also makes it possible to identify amicrofilaremic infections (Gomes-de-Sá et al., 2022a; Lemos et al., 2022). The absence of circulating microfilariae depends on the prepatent period of the parasite (about 7 months) and on the low concentration of microfilariae in the samples (Panarese et al., 2020). Twelve months after infection, due to the development of an immune response or the aging of adult females in the absence of reinfections, amicrofilaremia can also occur (Panarese et al., 2020). This circumstance can lead to false negative results and undiagnosed occult *D. immitis* infections (Miterpáková et al., 2022; Gomes-de-Sá et al., 2022a; Mõttus et al., 2024).

A survey showed that 10 animals, despite being negative in the RIM test, were positive in the PCR and this can be found when the parasite load of adult *D. immitis* females is low, if antigen-antibody complexes are formed or even if microfilariae persist after the death of the adult forms (Little et al., 2018; Panarese et al., 2020; Gomes-de-Sá et al., 2022a). Antigenemia can be suppressed up to about 9 months post-infection in infected dogs receiving chemoprophylaxis based on macrocyclic lactones. If the test is performed before the end of the prepatent period (7 months), the probability of false negatives also increases (Little et al., 2018; AHS 2020).

3.3. Molecular diagnosis

On account of limitations presented by microscopy-based techniques, molecular tools have been broadly utilized for the diagnosis of filariae infections in dogs (Smith et al., 2022; Bawm et al., 2023; Roblejo-Arias et al., 2023; Perles et al., 2024). The use of molecular analyses directed to filarial genomic DNA in blood samples has proven to be a highly sensitive and specific tool to the detection and simultaneous characterization of canine filarial infections (Satjawongvani et al., 2019; Smith et al., 2022; Bawm et al., 2023; Roblejo-Arias et al., 2023; Perles et al., 2024).

Among molecular methods, PCR of the ITS2 (internal transcribed spacer 2) region is

considered the most adequate for the screening and diagnosis of filarial infections in dogs, given the facts it is fast, accurate, and allows for specificity detection and differentiation of *Dirofilaria* spp. from other concurrent blood microfilariae (Ferreira et al 2017). Compared to ITS1 (internal transcribed spacer1) region, it has high variability, and the likelihood of sequencing errors is much higher, and the fact that the ITS2 region is shorter than the ITS1 region makes it easier to amplify using a single pair of primers (Wang et al., 2014). A survey proved that the ITS2 PCR was the most sensitive and specific method to detect mixed infectionsonly if all amplicons are of diferente sizes, by *D. immitis* and *A. reconditum*, particularly in samples with low microfilaremia for which ITS1 amplification failed or gave non-specific results. In addition, even in single or mixed infection cases, species identification of filariae in infected dogs was also more consistent for ITS2. Making use of the same technique, Laidoudi et al. (2019) highlighted the presence of four filarial species *D. immitis*, *A. reconditum*, *C. bainae* and *Brugia* sp. and the associated *Wolbachia* endosymbionts, while Satjawongvani et al. (2019) identified *D. immitis, B. pahangi* e *B. malayi*. Even though the only detected species was *D. immitis*, a study analysed DNA samples in order to detect the presence of microfilarie, by using a conventional PCR targeting the ITS2 region, aiming to differentiate nine filarial species, namely *D. immitis*, *D. repens*, *A. dracunculoides*, *A. reconditum*, *B. malayi*, *B. pahangi*, *Brugia timori*, *Mansonella ozzardi* and *Onchocerca volvulus* (Rishniw et al., 2006). Briefly, pan-filarial primer pair – DIDR-F1 and DIDR-R1 – amplifying products with distinct molecular weights were used. Several studies have revealed that the molecular methods can be a highly sensitive and specific analytic tool used for the diagnosis and simultaneous characterization of infections, providing trustworthy data when compared to parasitological and serological methods (Smith et al., 2022; Bawm et al., 2023; Roblejo-Arias et al., 2023; Perles et al., 2024).

3.4. Clinico-pathological diagnosis

Alterations in the haematological, biochemical and coagulation profiles are common in infected dogs with *D. immitis*. Studies have revealed variations in blood cell count such has non-regenerative normocytic, normochromic anaemia, neutrophilia (segmented neutrophils), eosinophilia and thrombocytopenia (Philp et al., 2023; Lemos et al., 2022; Bawm et al., 2023; Perles et al., 2024).

Regarding serum biochemical analysis, the most common changes are hypoalbuminemia, augmentation in total plasma protein (TPP) concentration, ferritin, and C reactive-protein, being this last one useful for staging the disease and monitoring recovery after treatment (Perles et al., 2024; Philp et al., 2023). The systemic effects of CHD specifically impact cardiac and renal function, which result in an increase of serum concentrations of renal

biomarkers, including serum urea nitrogen (BUN), creatinine and symmetric dimethylarginine (SDMA) (Carretón et al., 2020; Vetter et al., 2023).

It is important to mention that coagulopathy has been described in caval syndrome associated with heartworm disease and coagulation panels revealed elevations in PT (prothrombin time), aPTT (activated partial thromboplastin time), D-dimers and a marked decrease in fibrinogen (Philp et al., 2023). The presence of myocardial injury and heart failure in both acute and chronic CHD has been indicated by evaluating infections through specific cardiac biomarkers, specifically cardiac troponin I (cTnl), myoglobin and D-dimer (Carretón et al., 2017).

Likewise, urine analysis in infected dogs revealed the presence of blood (hematuria), epithelial cells (squamous and transition cells in small aggregates), high protein concentration, high urine protein/creatinine (UPC) ratio and even the existence of microfilariae in the sediment (Carretón et al., 2020; Vetter et al., 2023; Perles et al., 2024). Adding to this, proteinuria may be present both in dogs with high parasite burden and in dogs with low parasite burden, reinforcing the idea that due to non-specific signs of infection, it is mandatory to carry out parasitological, immunological and molecular assessments in dogs (Carretón et al., 2020).

3.5. Imaging findings

Thoracic radiograph and ultrasound provide awareness concerning the clinical status, severity and prognosis of cardiopulmonary disease secondary to heartworm infection, also being recommended for the re-examination following adulticide treatment, as this will be helpful in identifying dogs with persistent pulmonary hypertension (PH), which may benefit from additional management (Philp et al., 2023; Mõttus et al., 2024). Pulmonary hypertension (PH), a hemodynamic and pathophysiologic state present in a wide variety of cardiovascular, respiratory, and systemic diseases, is defined by increased pressure within the pulmonary vasculature (Reinero et al., 2020).

It has been proved that there is no correlation when radiographic and echocardiographic dilated pulmonary arteries are compared, since thoracic radiographies assess more peripheral pulmonary vasculature, while echocardiography can only assess the pulmonary arteries at the level of the heart base (Lemos et al., 2022). Heartworm disease has been described as a predominantly pulmonary arterial disease, which in advanced stages may affect the right heart because of acute or chronic PH (Philp et al., 2023; Mõttus et al., 2024). Pulmonary alterations due to adult filariae result in proliferative endarteritis and thromboembolism, causing different degrees of PH, which can be detected by echocardiography, a non-invasive diagnostic tool that can be performed without sedation

(Lemos et al., 2022; Mõttus et al., 2024).

In echocardiography left ventricular function as well as the morphology and function of the heart valves should be evaluated to identify changes that can affect the function of the right ventricle, namely mild tricuspid valve regurgitation, pulmonary regurgitation, right ventricular systolic dysfunction, right ventricular concentric hypertrophy, dilation of the pulmonary arteries as well as flattening of the interventricular septum (Philp et al., 2023; Perles et al., 2024; Mõttus et al., 2024). Moreover, using echocardiography enables the visualization of adult parasites in the pulmonary arteries and right heart, described as hyperechoic parallel linear bands or double lines, separated by a very thin hypoechoic area (Brianti et al., 2023; Perles et al., 2024; Mõttus et al., 2024). Although echocardiography allows the diagnosis of morphofunctional cardiac abnormalities and can identify the presence of *D. immitis* in the pulmonary artery or right heart chambers, it has low sensitivity for the diagnosis of infection, especially in dogs with low infection load, as the worms are frequently limited to the peripheral branches of the pulmonary arteries, therefore beyond the echocardiographic field of view (Venco et al., 2003).

Thoracic radiography is a useful diagnosis method to evaluate pulmonary lesions and detect heart silhouette changes, and studies have presented as characteristic radiographic features the bronchial pulmonary pattern, interstitial pattern, dilation of the pulmonary arteries, right heart cardiomegaly and pleural effusion following right heart congestive failure (Lemos et al., 2022; Philp et al., 2023; Mõttus et al, 2024). It is important to mention that despite the valences of radiography, abnormalities depend on the severity, chronicity and individual reaction of pulmonary vascularity of the affected animal, resulting in a limitation when compared to other means of diagnosis (Philp et al., 2023; Mõttus et al., 2024; Perles et al., 2024). It was also referred by Lemos at al. (2022) that radiographically examined dogs presented similar clinical findings, regardless of whether they were infected with *D. immitis* therefore, thorough clinical examination and specific parasitological tests must be performed to more accurately confirm or exclude infection.

4. Control of the disease

The death (spontaneous or drug-induced) of dirofilariae may lead to pulmonary artery embolism, and, therefore, as a rule, after diagnosis a combined microfilaricidal and adulticidal treatment under close monitoring must be administered in order to eliminate all stages of heartworms as quickly and completely as possible. Elimination of dirofilariae is important to prevent long-term damage to the pulmonary vessels and is also a prerequisite

for being able to intervene in due time in the event of acute embolism (Glaus et al., 2019; Diakou and Prichard, 2021; Philp et al., 2023).

4.1. Prevention

Heartworm prevention in animals is crucial due to its impact on their health, the complexity, the risk, the cost of treatment (which demands a prolonged period of drug treatment, exercise restriction and occasionally surgery) and zoonotic implications (Carretón et al., 2017; Mõttus et al., 2024).

Since the adult stage of the infection causes morbidity, and a significant infection of adult worms can be fatal, the primary focus of maintaining animal health and welfare is to prevent adult infections from establishing (Brianti et al., 2023; Philp et al., 2023).

Prevention is achieved by the administration of macrocyclic lactones (MLs), which can be divided into two groups: the avermectins (abamectin, ivermectin, eprinomectin, and selamectin) and the milbemycins (milbemycin oxime and moxidectin). The major structural difference between both classes consists of C13 of the macrocyclic ring: avermectins have sugar residues, while milbemycins are protonated (hydrogen) (Prichard and Geary, 2019; Noack et al., 2021). There are many forms available on the market, such as spot-ons, hard and chewable tablets, and injectable presentations, the majority of which are licensed for heartworm prevention by monthly administration but there are also extended-release injectable forms of moxidectin for administration every 6 or 12 months (Diakou and Prichard, 2021; Noack et al., 2021).

Preventive treatment using MLs should be started in cubs no later than 4 months of age and in the endemic areas a monthly preventative treatment is recommended or a long-acting injectable preventive treatment throughout the year. Additionally, the reduction of exposure to mosquitoes is also highlighted (FECAVA, 2019). Thus, the strategy of the periodic administration is based on the fact that dogs are under continuous exposure to infective mosquito bites throughout the period of transmission and that monthly administration of MLs ensures that no worms will live to reach the pulmonary arteries, even in the case of dosing delayed by a few days (Nolan et al., 2012; FECAVA, 2019). Mosquito control measures are also critical to reducing *D. immitis* infection, including: regular use of mosquito repellent, antifeedant, emptying standing water, installing window screens, and avoiding areas and times of day when mosquitoes are most active (AHS, 2020; ESCCAP, 2021).

4.2. Treatment

In order to fight the disease caused by *D.immitis*, three different treatments can be applied: the first one, heartworm prevention, which stops the establishment of infection by killing L3 and L4; adulticide treatment that kills adult heartworms; and microfilaricidal treatment, which eliminates circulating microfilariae (L1) in infected animals, reducing the number of animals acting as carriers and, therefore, reducing the risk of transmission to mosquitoes. Even though vertebrate hosts acknowledge the presence of heartworms shown by the abundant antibody response directed at the parasite, this response is not enough to prevent infection or remove the parasite before reproduction occurs (Geary et al., 2022; Ketsis et al., 2022; Geary, 2023).

Infection can result in severe disease or death due to the presence of the large adult stages in the pulmonary vasculature and in the heart, being one of the main worries in the treatment of filarial worms the host inflammatory response induced by the death of adult worms within the parasitized tissues (Glaus at al., 2019; Brianti et al., 2023; Philp et al., 2023). Thus, the aims of treatment are to ameliorate the animal's clinical condition and well-being, and remove all forms of the parasite (adult and larval stages) with minimal complications (Glaus et al., 2019; AHS 2020; Mõttus et al., 2024). For this reason, it is fundamental to assess the clinical staging of each animal and the degree of pathology, considering age, size, number of parasites released from fertilized adult females, and severity of pulmonary disease before initiating the therapy (Simón et al., 2012; Glaus et al., 2019; Brianti el al., 2023).

One must not forget that from the day of diagnosis until the end of treatment, restriction of activity is advised for the dogs, this being the most important procedure to minimise the risk of pulmonary thromboembolism and other potentially fatal complications (Diakou and Prichard, 2021; Philp et al., 2023; Mõttus et al., 2024).

In some cases, it is necessary to ensure the stabilization of the dog's health condition before treatment, whilst in other cases, the surgical removal of the worms is the best choice in detriment of pharmacological treatment, although a conjugation between both (pharmacological treatment and surgical removal) may also be an option (Glaus et al., 2019; AHS 2020; Philp et al., 2023; Mõttus et al 2024).

The treatment guidelines of the AHS currently advocate adulticide treatment with melarsomine dihydrochloride, tetracycline-derived antibiotics (doxycycline), macrocyclic lactones and also glucocorticosteroids in some cases. It must be highlighted that pharmacological treatment is recommended to treat dogs with stabilized stages 1, 2 or 3 of the disease. However, in cases of VCS this treatment is only advisable after surgical removal, which remains the safest and most advisable procedure (Alho et al., 2016; Glaus et al., 2019; AHS, 2020; Diakou and Prichard, 2021).

Melarsomine dihydrochloride is an arsenic-containing drug that is approved to kill adult heartworms in dogs; MLs are administered to eliminate the larvae; once melarsomine has incomplete efficacy against young adult worms (less than 4 months old), doxycycline is used (roughly 1 month before adulticide treatment) to remove the symbiotic bacteria *Wolbachia* before killing the adults, which seems to decrease pathological effects associated with the dead of large worms in the host (Prichard and Geary, 2019; AHS 2020; Geary et al., 2022). In cases with high microfilarial counts, antihistamines and corticosteroids are administrated to minimize potential reactions (Brianti et al., 2023; Mõttus et al., 2024). Succinctly, treatment consists in a monthly macrocyclic lactone administration, 28 days of doxycycline given orally (10 mg/kg every 12 h) and three deep intramuscular injections of melarsomine dihydrochloride (1 injection on day 2 of treatment followed 30 days later by 2 injections 24 h apart, all at a dose of 2.5 mg/kg). When doxycycline is not available, minocycline can be utilized as a replacement at a dose of 5 mg/kg twice daily (Philp et al., 2023; Vetter et al., 2023; Mõttus et al., 2024). Whenever the adulticide treatment is contraindicated or unavailable, an alternative therapeutic approach, known as the slow-kill protocol in infected dogs has been used (Brianti et al., 2023; Silva et al; 2023). This protocol was recently recognized by the AHS and the European Society of Dirofilariosis and Angiostrongylosis (ESDA), and consists of doxycycline (10 mg/kg every 12 h) for 4 weeks combined with monthly administration of a topical formulation of 10% (w/v) imidacloprid and 2.5% (w/v) moxidectin for 12 months (AHS, 2020; Brianti et al., 2023; Silva et al., 2023). In any case, there are studies stating that this protocol contributes to the loss of effectiveness against resistant strains, allowing them to be transmitted. It could also promote ML resistance selection in new areas, once the administered ML doses just kill susceptible parasites, leaving unaltered any resistant worms present in the population (Geary et al., 2022; Diakou and Prichard, 2021).

Although both adulticide treatment and the alternative protocol have proved to be globally safe and effective (Vetter et al., 2023; Mõttus et al., 2024), occasionally, several complications may occur during the treatment period, such as congestive heart failure, thromboembolic disease and kidney dysfunction (Brianti et al., 2023; Philp et al., 2023). In a study, thromboembolic-like disorders were detected after treatment had been initiated, as a result doxycycline treatment was temporarily interrupted and fluid therapy was immediately administered together with unfractionated heparin (200 U/kg, subcutaneous every 8 hours for 3 days) and prednisone (1 mg/kg, every 12 hours (h), in gradually decreasing doses for 15 days), resulting in full recovery of that animal after 5 days (Brianti, 2023). The prompt pharmacological treatment in infected dogs is essential to avoid the progression of the disease, impede the provision of microfilariae to vectors and protect dogs from new infections (Mõttus et al., 2024). The VCS caused by *D. immitis* presents itself as

a life-threatening situation, which requires immediate treatment.

In affected animals, adult worms can be can found in the right ventricle and right atrium by echocardiography, and if worm balls disrupt tricuspid valve closure, this favours right-sided congestive heart failure. In these cases, adulticide treatment is unadvised due to the high risk of severe worm embolism (AHS, 2020; Glaus et al., 2019; Kim et al., 2023). Currently, the manual extraction is the preferred method to remove heartworms due to its lower invasiveness, reduction of the vascular endothelium damage and shorter duration of the anaesthesia (Alho et al., 2016; Zlateva-Panayotova et al., 2018; Glaus et al., 2019; Kim et al., 2023). As a rule, the procedure is performed under fluoroscopy, partly combined with fluoroscopic and transoesophageal echocardiographic control, partly under transthoracic echocardiography (Glaus et al., 2019; AHS, 2020). There are several devices used in the heartworm removal, such as forceps, snare, and basket devices, all of these designed to be less invasive than surgical removal through thoracotomy and shorten the procedure time. However, diverse simplified brush-type devices have recently been utilized due to the fact that they are less invasive, less expensive and more effective in heartworm extraction than the above-mentioned conventional devices (Girdan et al.,2018; Zlateva-Panayotova et al., 2018; Kim et al., 2023). Before the procedure, to minimize the complications related to coagulation, drugs such as dalteparin (150 IU/kg, subcutaneous) and clopidogrel (4 mg/kg, *per os*) can be administered, followed by premedication with cefazolin (22 mg/kg intravenous), midazolam and butophanol; cefazolin provides interstitial fluid concentrations effective against the most common commensal organisms on the skin; butorphanol with midazolam provides adequate sedation and analgesia before induction with propofol (Cagnard et al., 2018; Kim et al., 2023; Quandt, 2023). Then anesthesia induction is performed with propofol (3–6 mg/kg, intravenous) and finally maintenance of anaesthesia is done with isoflurane or sevoflurane with 100% oxygen (Alho et al., 2016; Zlateva-Panayotova et al., 2018; Kim et al., 2023). The procedure to remove heartworms using a brush-type device follows the sequence below: the patient is positioned in left lateral recumbency, after dissection, the jugular vein is carefully retracted and a small incision is then made in it to allow the device to enter; then an extraction brush is inserted through the right jugular vein under fluoroscopy into the right side of the heart; the brush device is then rotated to seize the heartworms by the threads and finally the device is extracted with captured heartworms after various rotations. The procedure is concluded after the jugular vein, the subcutaneous tissue and the skin are sutured (Zlateva-Panayotova et al., 2018; Kim et al., 2023).

After treatment, clinical signs relating to the presence of heartworms may be resolved and no murmurs auscultated, haemodynamics improve gradually without any recognisable side effects but pulmonary hypertension may remain (Alho et al., 2016; Glaus et al., 2019; Kim

et al., 2023; Matos et al., 2023). Several studies have demonstrated that the PH after treatment did not present any reduction, because PH in a chronic heartworm infection is not caused by the worms migrating into the heart, but by changes in smaller pulmonary arteries: on the one hand by thromboembolic vascular occlusion, on the other hand by remodelling processes in the arterial walls (Glaus et al., 2019, Maerz et al., 2020; Matos et al., 2023). Within a month of the manual removal of the heartworms a routine adulticidal treatment following the AHS guidelines should be conducted (ivermectin, doxycycline and melarsomine); moreover, to avoid new infections, a monthly routine heartworm prevention is highly recommended (Girdan et al., 2018; AHS, 2020; Kim et al., 2023).

5. Drug resistance of *Dirofilaria immitis*

It is assumed that ML resistance first appeared circa 1998. At that time, MLs for prevention of heartworm had been recommended as the first line of prophylactic treatment for over 10 years, having been registered as 100% effective (McTier et al., 2017; IHGS, 2019). Hence, it is generally assumed that the basis for genetic changes causing ML resistance only arose and was selected in that decade. As such, resistant lines of *D. immitis* are suggested to have been circulating prior the use of ML preventives (McTier et al., 2017; IHGS, 2019). True drug resistance has a genetic basis; thus, continuing efforts to analyze *D. immitis* whole genomes from resistant and susceptible lines could help detect genetic markers for resistance (Bourguinat et al., 2015).The resistance of *D. immitis* to ML was confirmed genetically in vitro and clinically in infected dogs from the Lower Mississippi region of the United States of America (Bourguinat et al., 2011, 2015; Pulaski et al., 2014) and the first case of ML-resistant *D. immitis* infection in Europe was confirmed by Traversa et al. (2024), through genetic analysis showing that the microfilariae had an ML-resistant genotype compatible with a resistant strain. Notwithstanding, due to the fact that ML resistance does not establish easily nor spreads quickly, it may spread from an initial geographical point, via animal and vector mobility, to other regions, while it can also appear as an independent evolutionary process in a new geographical area.

It is important to mention that some biological characteristics of the parasite favour the development of resistance: despite the relatively long lifecycle, the high reproductive rate (female heartworms produce millions of microfilariae), the longevity of both adults and microfilariae, and the somewhat small quantity of parasites in the short-lived intermediate hosts may promote resistance development (Churcher et al., 2008; Panarese et al., 2020; 2021; Geary, 2023). Additionally, there is the likelihood of inbreeding in *D. immitis*, since L3

transmitted by a mosquito have a fair probability of being siblings or half-siblings, presuming that the mosquito became infected by a blood meal from a single infected dog, enhancing as well the resistance development and selection in a parasite such as *D. immitis* (Churcher et al., 2008; Kryda et al., 2019; Panarese et al., 2020; Geary, 2023). Resistance to MLs in *D. immitis* can be identified at different stages of the parasites, namely in L3/L4 (the target of MLs as preventatives), in microfilariae (L1) and in adult parasites (due to the effects of MLs on their reproductive capacity), when MLs are used in the presence of microfilariae and adult parasites (Bowman et al., 2015, 2017).

As previously referred, the resistance to drugs has a genetic basis; therefore, the whole-genome analysis of *D. immitis*, resistant and susceptible strains could help detect genetic markers associated with ML resistance (Diakou and Prichard, 2021; Gomes-de-Sá et al., 2022b). A study investigated the existence of resistant strains through the detection of specific SNPs (single nucleotide polymorphism) that emerge in higher prevalence in known resistant populations, and a whole-genome analysis allowed the comparison between known susceptible and phenotypically resistant populations. According to the results, there was a significant genotype-phenotype association between SNPs detected in ML-resistant strains, i.e. those which did not respond with microfilaremia reduction after MLs administration (Ballesteros et al., 2018). Moreover, these results suggest that ML resistance may be a polygenic trait and, importantly, that there is probably a spectrum of resistant phenotypes (Ballesteros et al., 2018). In yet another study, subsequent analysis of six loci previously characterised as being associated with selection pressure for resistance to MLs showed that four had a genotype associated with either some loss of efficacy or the resistance phenotype, suggesting the circulation of *D. immitis* strains resistant to MLs (Gomes-de-Sá et al., 2022b).

In areas where MLs resistance is established and emerging infections are confirmed, it is advisable the administration of high-dose formulations of moxidectin, as it has been demonstrated that this drug in all forms of products has higher efficacy against resistant strains (Bowman et al., 2015, 2017; Kryda et al., 2019; Prichard and Geary, 2019; McTier et al., 2019). In general, moxidectin shows higher potency against filarial nematodes, probably because of its pharmacokinetic character, which allows better distribution in lipid tissues, lower elimination by the ATP-binding cassette efflux transporters, and a longer half-life (Prichard and Geary, 2019).

Considering the zoonotic potential and life-threatening effects of *D.immitis* in animals, identifying resistant parasites and understanding the factors that promote them is important for maintaining control of the disease.

6. Public health relevance of *Dirofilaria immitis*

Dirofilaria immitis in dogs and also in other animals represents a risk for the human population. The zoonotic implications of these infections are important because, not long after the inoculation by the mosquitoes, there is the possibility of larval migration along human tissues. The parasites do not mature in humans, but, usually, after reaching the pulmonary artery, die, provoking pneumonitis and, subsequently, the formation of a spherical granuloma (Alho et al., 2018; Fontes-Sousa et al., 2019; Gabrielli et al., 2021). The difficulty in diagnosing the aetiology of those lesions represents one of the biggest challenges of human dirofilariosis. In fact, diagnosis in humans is often only reached through unnecessary, invasive procedures, such as surgery for latter histopathological examination of the exerted granuloma, which may have severe side implications (Miterpáková et al., 2022).

Several studies have revealed that human patients can remain asymptomatic or manifest clinically with cough, chest pain, blood-tinged sputum, low-grade fever, eosinophilia, development of ocular, skin and pulmonary nodular lesions misleading to differential diagnosis such as neoplasias (primary or metastatic), hamartomas, tuberculosis, and fungal infections (Alho et al., 2018; Fontes-Sousa et al., 2019; Gabrielli et al., 2021).

Information about the prevalence and distribution of cardiopulmonary parasites is essential for controlling animal diseases and, in the case of *D. immitis*, also for the control of potentially associated illnesses in humans. The presence of higher seroprevalence of *D. immitis* might be due to an hiperendemic status of canine dirofilariosis (Fontes-Sousa et al., 2019; Esteban-Mendoza et al., 2020; Mendoza-Roldan et al., 2021).

Molecular confirmation in human samples demonstrates that the same parasites are currently present in dogs, people and vector insects in the study area (Fontes-Sousa et al., 2019; Mendoza- Roldan et al., 2021; Miterpáková et al., 2022). Nevertheless, the absence of circulating microfilariae disables the use of parasite DNA detection in the patients' peripheral blood samples as a feasible tool for diagnosis, otherwise basing its identification on histopathological analysis of extracted nodules (Gabrieli et al., 2021; Miterpáková et al., 2022).

Due to the lack of sensitive and specific serologic tests, and given the importance of diagnosis, the combination of ultrasound and colour doppler charting could be used as a means to give a presurgical diagnosis, because, according to the ESDA, the visualization of clear-cut findings and characteristics *in D. immitis* nodules is possible, such as: absence of signs of polar vascularity, regular oval shape and hypoechoic inner content. For these reasons, understanding the distribution and occurrence of human cases of dirofilariosis is

quite relevant in the differential diagnosis of subcutaneous and/or pulmonary nodules or other suspected clinical manifestations (Fontes-Sousa et al., 2019; Mendoza-Roldan et al., 2021).

7. Aims and Thesis Outline

This doctoral thesis aimed to investigate the diversity of *Dirofilaria* species, as well as carry out their molecular characterization, assess the potential of different diagnostic approaches and evaluate putative zoonotic implications associated. In order to perform this, specific aims were addressed:

1 – To assess *Dirofilaria* spp. in the northwestern part of Portugal in domestic dogs through combined analysis of antigens, DNA and parasite forms in blood, in order to appraise the diagnostic performance in this context.

2 – To evaluate the presence of *D. immitis* in wolves and foxes in Portugal, given the proximity between dogs and wild canines, through determination of both antigens and microfilaremia of *D. immitis*, using respectively RIM or optimized specific PCR assay, for the simultaneous detection and differentiation of *D. immitis* and seven other concurrent filarioids.

3 – To explore new diagnostic approaches by expanding the use of long-read nanopore-based sequencing technology in nematodes by performing the first *de novo* genomic assembly of a *D. immitis* cardiopulmonary canine heartworm specimen using the ONT MinION platform, followed by the study of macrocyclic resistance to lactones.

4 – To provide insights about the zoonotic potential of dirofilariosis, adopting the One Health perspective, through the dissemination and publicity of evidences with impact on human health.

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CHAPTER II

Assessment of the circulation of *Dirofilaria immitis* in dogs from northern Portugal through combined analysis of antigens, DNA and parasite forms in blood

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Assessment of the circulation of *Dirofilaria immitis* in dogs from northern Portugal through combined analysis of antigens, DNA and parasite forms in blood

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Abstract

Dirofilariosis is a vector-borne disease frequent in many countries. Not only infected dogs, but also cats and wild canids (including wolves and foxes), represent important sources of infection for mosquitoes, which are the pathogen vectors. The disease is endemic in Mediterranean countries with increasing incidence in Italy, France, Greece and Spain, but limited epidemiological data is available from Portugal regarding its distribution and impact. Aiming to clarify this, canine whole blood samples (n = 244) from the north of Portugal were tested for *Dirofilaria* spp. antigens by use of a commercial rapid immunomigration test. Polymerase chain reaction (PCR) and the modified Knott test were also used to assess the presence of microfilariae. Results were also compared to assess the performance of each test used. Of the 244 animals tested, 118 (48.4%) were positive for *Dirofilaria immitis* (heartworm) in the serological adult worm rapid antigen detection test, and 36 (14.8%) had circulating microfilariae, identified as *D. immitis.* A combined positivity of 51.6% (126/244) was found. Results indicate that the risk of exposure to *D. immitis* in dogs is high in this region of Portugal, and that prophylaxis against the parasite is advisable to decrease the occurrence of canine infection and disease. The present study highlights the diagnostic value of serological and molecular tests in determining the prevalence of *D. immitis*.

Keywords

Diagnosis, *Dirofilaria immitis*, Dogs, Portugal

1. Introduction

Cardiopulmonary dirofilariosis caused by heartworm *Dirofilaria immitis*, also known in dogs as canine heartworm disease, and subcutaneous dirofilariosis caused by *D. repens* are zoonoses whose agents have adapted to canine, feline and human hosts, with distinct biological and clinical implications (Dantas-Torres and Otranto, 2020; Capelli et al., 2018; Diakou et al., 2019; Bozidis et al., 2020). *Dirofilaria* spp. are transmitted by mosquitoes belonging to different genera of the family Culicidae (i.e. *Aedes*, *Anopheles*, *Culex* and *Ochlerotatus*) (Alho et al., 2014; Dantas-Torres and Otranto, 2020; Edgerton et al., 2020; Macchioni et al., 2020). *Dirofilaria immitis* is a parasitic nematode that infects domestic and wild canids as well as other mammals, including humans. Cardiopulmonary dirofiloriosis, the associated disease, typically occurs in temperate, tropical, and subtropical areas of the world, with the agent being transmitted by several mosquito species especially from the genera *Culex, Aedes*, and *Anopheles* (Barriga, 1982). These mosquitoes deposit infective

larvae (stage L3) at the biting site on the host's skin. These larvae moult into the next stage (L4) 3–12 days post-infection (dpi), later moulting into preadult worms at 50–70 dpi, which migrate to the pulmonary artery and right ventricle (70–85 dpi), then reaching sexual maturity at 120 dpi. Female worms initiate the production of microfilariae (L1) 6-9 months post-infection, with adults potentially living more than 7 years and microfilariae up to 2 years (Simón et al., 2012). Recently, the epidemiology and distribution dynamics of elements of the genus *Dirofilaria* has suffered modifications due to several factors, including the influence of climate change, human modification of environment, such as irrigating lands for farming, transboundary movements of animals and humans, and changes in the parasite-host relationship such as the outburst of new species of vector mosquitoes (Alho et al., 2014; Capelli et al., 2018; Fontes-Sousa et al., 2019; Esteban-Mendoza et al., 2020; Farkas et al., 2020). Several cases of pulmonary heartworm and ectopic infections have been reported in humans, despite the fact that these are considered as accidental hosts for *Dirofilaria* spp. (Capelli et al., 2018; Djakovic´ et al., 2019; Bozidis et al., 2020; Esteban-Mendoza et al., 2020). The presence of *D. immitis* in dogs represents a risk for the human population (Morchón et al., 2012). Although cardiopulmonary dirofilariosis has a worldwide distribution, with considerable prevalence in the American continent, Australia and Japan (Dantas-Torres and Otranto, 2013; Esteban-Mendoza et al., 2020), the disease is also endemic in Mediterranean countries with increasing incidence in Italy, France, Greece and Spain, where the causative agent is considered one of the most widespread animal parasites (Diakou et al., 2019; Laidoudi et al., 2020; Macchioni et al., 2020; Mendoza-Roldan et al., 2021). Worms live live in the pulmonary arteries and right cardiac ventricle of domestic and wild canids. Infection may cause a wide variety of clinical signs, which go from cough to dyspnoea and exercise intolerance to severe vascular and pulmonary disease with hearth failure that may cause death (Alho et al., 2018). The zoonotic implication of these infections is important because, not long after the inoculation by the mosquitoes, there is the possibility of larval migration along human tissues (Gabrielli et al., 2021). This may provoke ocular, skin and also pulmonary nodular lesions which are frequently and wrongly diagnosed as pulmonary carcinomas (Alho et al., 2018; Fontes-Sousa et al., 2019; Gabrielli et al., 2021). Information about the prevalence and distribution of cardiopulmonary parasites is essential for the control of animal diseases and, in the case of *D. immitis*, for the control of potentially associated illnesses in humans. Some studies refer that the presence of higher seroprevalence of *D. immitis* might be due to the hiperendemic status of canine dirofilariosis (Fontes-Sousa et al., 2019; Esteban-Mendoza et al., 2020; Mendoza-Roldan et al., 2021). There are cases of lesions which were initially incorrectly identified has malignant neoplasm and therefore required invasive procedures prior to reaching the correct diagnosis (Gabrielli et al., 2021). Also, the absence of circulating microfilariae disables the use of parasite DNA

detection in peripheral blood samples as a feasible tool for diagnosis, chiefly basing its identification in histopathological findings of extracted nodules (Gabrielli et al., 2021). For these reasons, understanding the distribution and occurrence of human cases of dirofilarioses is quite relevant in the differentiated diagnosis of subcutaneous and/or pulmonary nodules or other suspected clinical manifestations (Fontes-Sousa et al., 2019; Mendoza-Roldan et al., 2021). Epidemiological data available in Portugal show that *D. immitis* is present in the country, but little information is known regarding its distribution impact and potential acquisition of new ecosystems (Alho et al., 2014, 2018; Maia et al., 2015; Ferreira et al 2017). Tests for the detection of antigens provide a convenient, sensitive and specific way to identify *D. immitis* infection in routine veterinary patients (Genchi et al., 2018; Henry et al., 2018; Laidoudi et al., 2020; Panarese et al., 2020), while the polymerase chain reaction (PCR) is a valuable tool for the screening and diagnosis of filarial infections in dogs, due to the rapid and accurate detection and differentiation of *Dirofilaria* spp. from other concomitant blood microfilariae, allowing species confirmation by specific primers or sequencing (Hou et al., 2011; Ferreira et al., 2017; OH et al., 2017; Little et al., 2018; Gomes-de-Sá et al., 2022a). Considering that the climate in Portugal is compatible with the development, reproduction and survival of the vectors, it is appropriate to define Portugal as a country in which canine dirofilariosis by *D. immitis* is endemic (Alho et al 2018). Caminha, located in the North of Portugal, with a population of about 16,000 human inhabitants, shares the same propitious climate as the rest of the country. Up to this moment there has been no study of dirofilariosis conducted in this municipality. Wild animals can be sylvatic reservoirs for *D. immitis* by supporting the transmission of these parasites to domestic animals (Gomes-de-Sá et al., 2022c). At the country level, a study revealed a prevalence of 3.2% of *D. immitis* detected by necropsy in red foxes (*Vulpes vulpes*) (Eira et al., 2006); moreover, in a national serological survey conducted in red foxes in Portugal, 8.5% were positive for *D. immitis* (Alho et al., 2016). *Dirofilaria immitis* was also found in three Eurasian otters (*Lutra lutra*) in Portuguese natural habitats (Torres et al., 2004; Saraiva et al., 2013), afterwards in a collection of pinnipeds: common seals (*Phoca vitulina*), California sea lions (*Zalophus californianus*) and South African fur seals (*Arctocephalus pusillus pusillus*) in a Portuguese oceanographic park (Alho et al., 2017) and more recently antigenemia was detected with an occurrence of 15.8% in red foxes and also, for the first time, microfilaremia in Iberian wolves (*Canis lupus signatus*), which was revealed by PCR, in the National Park of Peneda Gerês (Gomes-de-Sá et al., 2022c). Epidemiological studies are important to follow the spread of canine heartworm disease and the availability of diagnostic tools options contributes greatly to the reduction of the number of severe clinical cases through timely drug treatment. To add knowledge in Portugal, we assessed the presence of *Dirofilaria* spp. In dogs from the municipality of Caminha, northwest part of the

country, through combined analysis of antigens, DNA and parasite forms in blood.

2. Material and Methods

Blood samples were collected from a total of 244 dogs from the municipality Caminha, located in the northern border of Portugal with Spain. Blood was taken at the request of a public kennel as part of the surveillance and control scheme for vector-borne diseases. Canine profiles, including sampling date, age, gender, breed and use of anti-parasitic drugs, were recorded. In terms of age, the dogs were divided into three groups: [1–2], [3–6] and [7–11] years **(Table 1)**. The sample consisted of 140 neutered females and 104 neutered males, all tested dogs were of mixed breed and none of the dogs had received anti-parasitic drugs.

Table1 Prevalence of *Dirofilaria immitis* infection in dogs from the municipality of Caminha (northwestern Portugal) as determined by adult worm RIM, PCR and combined results.

Variable/category	Dogs sampled (n)	Relative distribution (%)	% (n) antigen positivity	% (n) PCR positivity	% (n) combined positivity
Sex			*p* = 0.642p	*p* = 0.431	*p* = 0.784
Female	140	57.4	50.0 (70)	12.9 (18)	53.6 (75)
Male	104	42.6	46.2 (48)	17.3 (18)	51.0 (53)
Age group (years)			*p* = 0.026	*p* = 0.528	*p* = 0.014
[1 – 2]	34	13,9	32.4 (11)	8.8 (3)	35.3 (12)[a]
[3 – 6]	83	34.0	42.7 (35)	14.5 (12)	47.0 (39)
[7 – 11]	127	52.0	55.9 (71)	16.5 (21)	60.6 (77)[a]
Total	244	100	48.4 (118)	14.8 (36)	51.6 (126)

PCR: polymerase chain reaction

[a] *p* = 0.042. Bonferroni's correction has been incorporated by multiplying a previously significant pairwise p value (0.014) by 3. Only statistically significant differences are shown for pairwise comparisons of age group categories (i.e. [1–2] and [7–11]).

Two millilitre of blood was taken from the cephalic vein and immediately transferred to a tube containing EDTA. For the initial screening of *D. immitis* infection, RIM kits (Uranotest *Dirofilaria* ®, Barcelona, Spain) were used, according to the manufacturer's instructions. To determine if the antigen-positive dogs were a source of *D. immitis* for mosquitoes, the dogs were tested by PCR and the modified Knott test. For DNA detection, nucleic acids were

extracted from blood using the QIAamp® DNA Mini Kit (Qiagen, Valencia, CA, USA), using an automated QIAcube nucleic acid extractor (Qiagen GmbH, Germany). DNA was stored at -20 ◦C until further analysis. A negative extraction control was processed along with each batch of 12 samples. DNA specimens were initially screened for the presence of microfilariae by using a conventional PCR targeting the ITS2 region. Briefly, pan-filarial primer pair – DIDR-F1 and DIDR-R1 – amplifying products with distinct molecular weights were used to differentiate nine filarial species (Rishniw et al., 2006). For all reactions, a total of 5 µl of genomic DNA was added to 12.5 µl Xpert Fast Hotstart Mastermix (2X) with dye (KAPA Biosystems, Woburn, MA, USA; Grisp, Porto, Portugal), 5.5 µl of deionized sterile water and 1 µl (10 µM) of each of the primers in a 25 µl final volume of the reaction mixture. The reactions were carried out in an automatic DNA thermal cycler 100 (Bio-Rad Laboratories, Hercules, CA, USA), including negative and positive controls (extracted from an adult *D. immitis* female). The PCR amplification products were visualized by Xpert green (Grisp) fluorescence after electrophoresis in a 1.5% agarose gel at 100 V for 40 min. To confirm species identification, all amplicons of expected size were sequenced bidirectionally for genetic characterization. Briefly, amplicons were purified with GRS PCR & Gel Band Purification Kit (Grisp), and bidirectional sequencing was performed by Sanger method, using the respective primers. Sequences were manually corrected using the BioEdit Sequence Alignment Editor v 7.1.9 software package, version 2.1 (Ibis Biosciences, Carlsbad, CA, USA) and further analysis were performed by comparison with the sequences available in the NCBI (GenBank) nucleotide database (http://blast.ncbi.nlm.nih.gov/Blast). Phylogenetic analysis was performed using MEGA version 6.0 software (Tamura et al., 2013). The obtained consensus sequences in this study and representative sequences for *O. volvulus, D. immitis* and *D. repens* obtained from GenBank were used for the phylogenetic analysis. Maximum likelihood (ML) method was applied. The ML bootstrap values were estimated using 1000 replicates with Tamura 3-parameter as the correction model, estimated as the best substitution model by MEGA version 6.0 software. We deposited the 5.8S-ITS2-28S of *D. immitis* sequences recovered in this study in GenBank. The modified Knott test was also performed on blood samples from the 118 dogs that were positive for *D. immitis* antigen, according to described methods (Magnis et al., 2013; Soares et al., 2022). The chi-square test was used to compare seropositivity values between different categories of the same independent variables (i.e. sex and age group). Cohen's kappa coefficient (κ) measured agreement beyond chance between diagnostic test results from paired samples (i.e. from the same animal). A probability (p) value < 0.05 was regarded as statistically significant. Statistical analyzes were done with IBM® SPSS® Statistics 26.0® software.

3. Results

Of the 244 shelter dogs tested, 118 (48.4%) were positive for *D. immitis* based on the RIM for adult worms, and 36 (14.8%) were found to be positive by PCR to detect microfilaremia (Fig. 1). Moreover, the use of this latter test optimized the differentiation of *D. immitis* from eight other filarioids. When comparing results positive by RIM and/or PCR, combined positivity showed to be significantly higher in dogs of the [7–11] year group (60.6%) when compared with those in the group of [1–2] years (35.3%) ((p = 0.042; Table 1). The difference between the number of female (53.6%) and male (51.0%) dogs positive for *D. immitis* was not considerable. Eighty-two of the 118 antigen-positive animals were not detected for *D. immitis* DNA, a circumstance which reveals 69.5% occult infections.

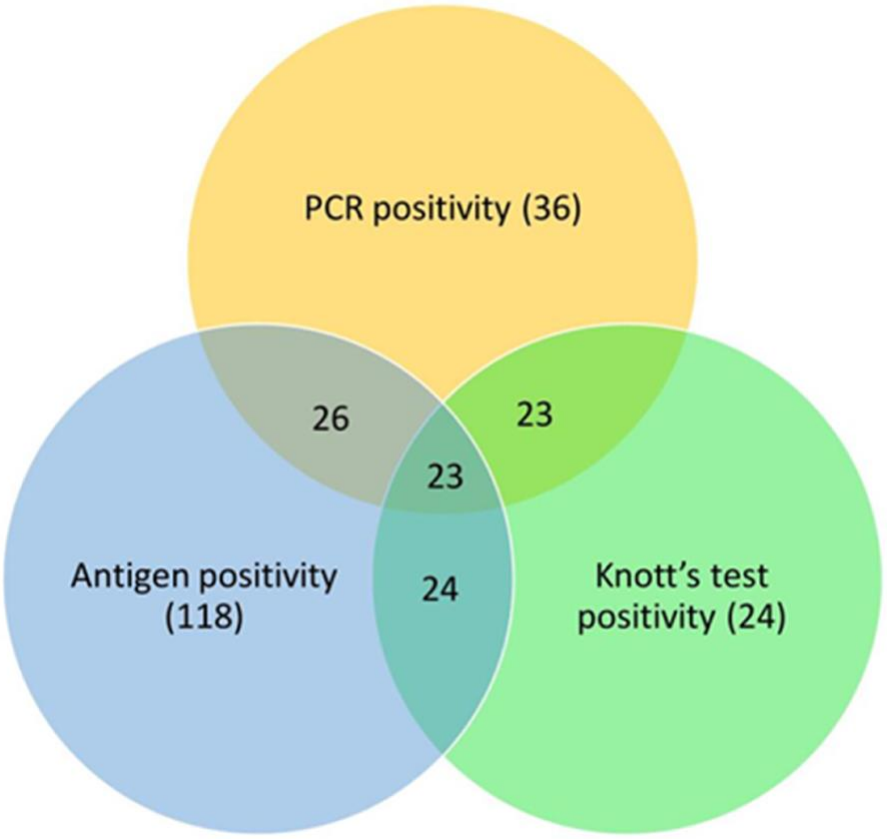

Fig. 1. Venn diagram on dirofilariosis diagnosis by RIM, PCR and Knott tests. Numbers in parentheses indicate the intersection positivity of tests.

On the other hand, 10 (7.9%) of the 126 antigen-negative samples yielded positive results for the presence of *D. immitis* microfilariae DNA. Amplicon size assessment suggested that all detected microfilariae were from *D. immitis*. Bidirectional sequencing revealed 98.3% - 100% identity with the *D. immitis* reference sequences (GenBank MN596213, KX932106,

MW019916, KX932113, MN332198, KY863453, MF962487). The following accession numbers were assigned to the sequences obtained in this work: OK632232-OK632250, OK632252-OK632268. Phylogenetic analysis based on the 460 nt partial region of the 5.8S-ITS2-28S regions showed that sequences OK632235, OK632240, OK632243, OK632250, OK632263 shared 99.44–100% identity between them and clustered with a *D. immitis* sequence retrieved from a mosquito in Turkey and a *D. immitis* sequence from a dog in Iran (Fig. 2). On the other hand, sequences OK632232- OK632234, OK632236- OK632239, OK632241-OK632242, OK632243-OK632249, OK632252- OK632262 and OK632264-OK632268 showed to be 90.91%-100% identical between them and clustering with a *D. immitis* sequence from a mosquito in Portugal and a *D. immitis* sequence from a Red Panda in China Fig. 2.

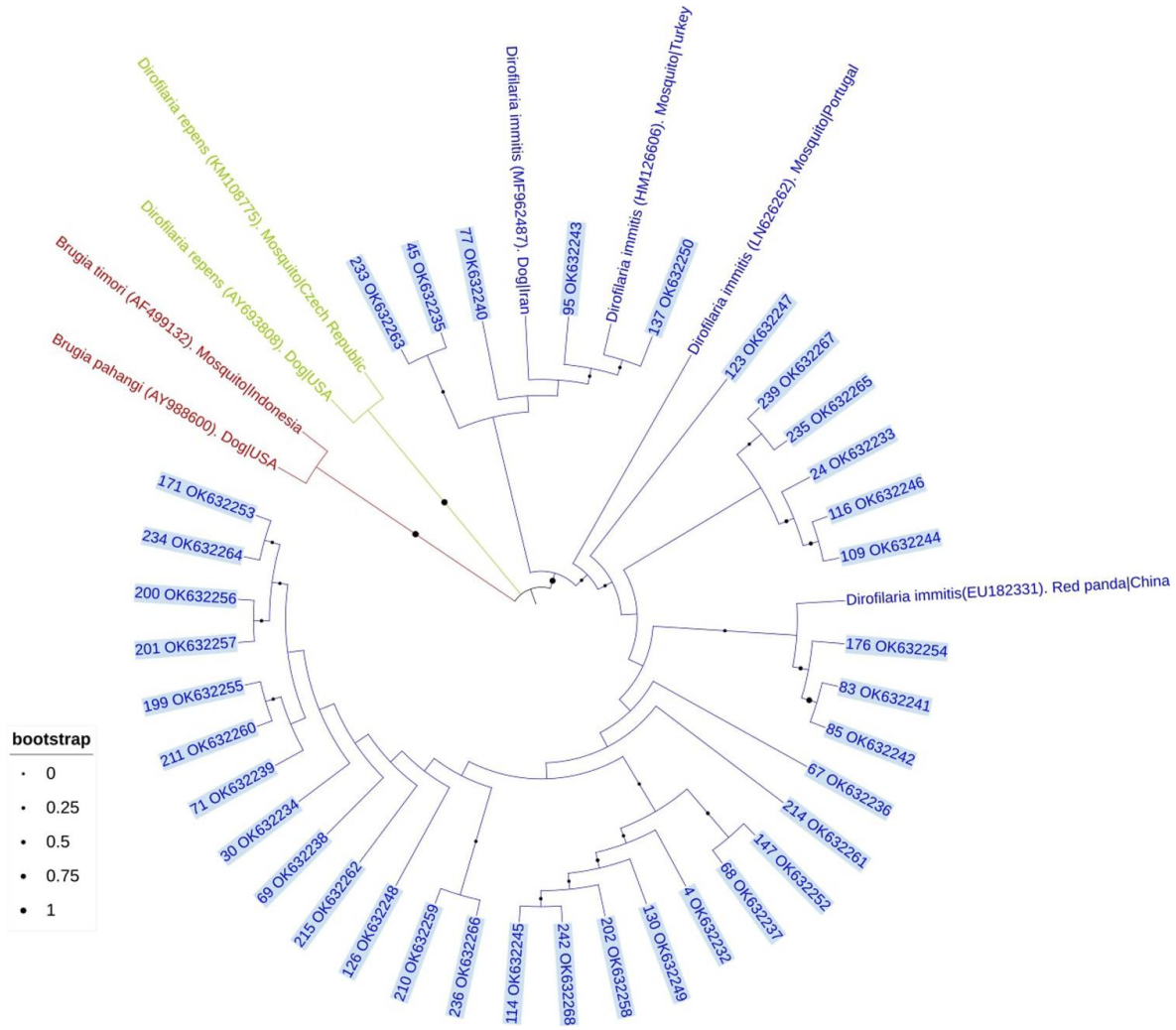

Fig. 2. Phylogenetic analysis of *Dirofilaria immitis* found in dogs in Portugal. The evolutionary history was inferred by using the ML method based on the Tamura et al. (2013) 3-parameter model.

The decision of not using the Knott test in all the tested animals one of the limitations of this study. It was assumed that PCR would be more reliable for the detection of microfilaremia,

because it allows differentiation from other filarial species and Knott test does not. As the RIM is aimed at detecting adult forms, Knott test would be used to detect microfilaremia in samples which presented antigenemia. Microfilariae were detected in blood samples of 20.3% (24/118) dogs by the modified Knott's technique (Fig. 1). These 24 dogs were found to host microfilariae with characteristic morphology of *D. immitis*. The agreement between modified Knott and PCR results for the same 118 blood samples had a κ value of 0.879 ($p < 0.001$), which represents an almost perfect agreement beyond chance.

4. Discussion

From the total amount of 244 dogs, 118 tested positive for adult forms antigens, 36 showed microfilaremia through PCR, and all detected microfilariae were of *D. immitis*. Eighty-two (i.e. 118 minus 36) occult infections were identified and the largest percentage of infections were from the group of [7–11] years. In the diagnosis of human dirofilariosis, indirect methods are not useful because L1, which is the stage mostly responsible for activating an immunological reaction in infections with filarial nematodes in humans, seldom appears in human dirofilariosis, and therefore the detection of antibodies against filariae as well as the utilization of DNA in peripheral blood samples of the majority of patients is not possible (Gabrielli et al., 2021). For that reason, the majority of reports of human dirofilariosis have been based on histopathological findings of extracted nodules, in which biopsy is considered the conclusive diagnostic method, even though its invasive nature could be a limitation. For a biopsy, and only when the parasite extraction is possible, the methods of molecular diagnosis, based on direct sequencing of the parasites' DNA, play a fundamental role in the identification of the etiological agent involved (Fontes-Sousa et al., 2019; Gabrielli et al., 2021). The examination of microscopic morphological features of human dirofilariosis should surely be taken into consideration for solitary nodules of uncertain nature in subcutaneous tissues or mucous membranes, especially when patients live in areas where high infection prevalence in dogs is reported (Gabrielli et al., 2021). In order to avoid the use of invasive diagnostic means, in-house tests – i.e. non-invasive test currently available – would be an adequate option in defining the nature of the pulmonary cysts, so that the presence of antibodies to *D. immitis* and its symbiotic bacteria *Wolbachia* spp. could be detected (Fontes-Sousa et al., 2019). In the present work, a RIM test was used to detect adult worms, and 118 positive animals were found positive from a sample of 244 shelter dogs. According to the manufacturer's information, this test consists of an immunochromatographic technique aiming at the qualitative detection of *D. immitis* antigen in blood, based on a 14 kDa antigen detection not exclusively related to the feminine genital

apparatus, infections with a load of only one adult parasite of any type (males, adult females or immature females), with sensitivity and specificity of 94% and 100%, respectively, when compared to necropsy data. This method is known to be sensitive for screening a population of apparently healthy dogs or for confirming a clinically suspected *D. immitis* infection presenting a sensitivity greater than 90% in dogs infected with one adult female worm and 100% in dogs infected with more than one adult female (Genchi et al., 2018; Henry et al., 2018; Laidoudi et al., 2020; Panarese et al., 2020). Although these are highly specific and sensitive for *D. immitis*, recent work has demonstrated limitations in specificity due to cross-reactions with *D. repens* or *Angiostrongylus vasorum* (Schnyder and Deplazes, 2012; Venco et al., 2017; Alho et al., 2018). In these cases, diagnosis depends on examining the microfilariae, usually applying a modified Knott test and PCR. In the present work, the RIM test detected 89 amicrofilaremic infections (75.4%), because only 29 of the 118 antigen-positive animals had microfilaremia as detected by PCR. These results demonstrate that RIM also make it possible to identify amicrofilaremic infections. Contrarily to our study, heartworm antigens were detected by Borthakur et al. (2016) in 141 dogs, but with only 32 (22.7%) being considered as occult infections, because 109 animals with *D. immitis* microfilariae were detected by PCR. The absence of circulating microfilariae depends on the prepatent period of the parasite (about 7 months) and on the low concentration of microfilariae in the samples (Panarese et al., 2020). Twelve months after infection, due to the development of an immune response or the aging of adult females in the absence of reinfections, amicrofilaremia can also occur (Panarese et al., 2020). This circumstance can lead to false negative results and undiagnosed occult *D. immitis* infections (Genchi et al., 2018; Miterpáková et al., 2022). In the present study, 10 animals despite being negative for the rapid antigen detection test, were PCR positive. This can be found when the parasite load of adult females of *D. immitis* is low, if there is formation of antigen-antibody complexes or even if there is persistence of microfilariae after death of the adult forms (Velasquez et al., 2014; Little et al., 2018; Panarese et al., 2020). Antigenemia can be suppressed up to about 9 months post-infection in infected dogs receiving chemoprophylaxis based on macrocyclic lactones. If the test is performed before the end of the pre-patent period (7 months), the possibility of false negatives also increases (Little et al., 2018; AHS, 2020). Several studies have demonstrated that molecular methods can be a highly sensitive and specific analytical tool for the simultaneous diagnosis and characterization of infections, providing more reliable data when compared to serological and parasitological methods (Hou et al., 2011; Ferreira et al., 2017; Mircean et al., 2017; Little et al., 2018). The modified Knott test allows the detection and differentiation of *D. immitis* from other filarial species (Magnis et al., 2013; Genchi et al., 2018; Moreira et al., 2019). There are also cases in which incorrect identification of microfilariae occurs, namely when there is a high

concentration of *Dirofilaria* spp. or *Acanthocheilonema* spp. in a given area, failure in fixation techniques, variation of reference values and relying on specialist training to accurately differentiate the filariae (Rishniw et al., 2006; Magnis et al., 2013; Little et al., 2018). The PCR test was chosen for the detection of circulating microfilariae and, of the 244 tested, 36 dogs were considered positive for *D. immitis*. Twenty-six samples (22.0%) from 118 *D. immitis* antigen positive dogs were considered positive for *D. immitis* microfilariae DNA. Subsequently, sensitivity of PCR was compared with that of the modified Knott test, revealing 24 animals with microfilaremia. In the present study, there were no significant differences between parasitological and molecular tests. Similar results were obtained in studies in which the modified Knott test was considered effective, sensitive and compatible with PCR (Ferreira et al., 2017; Soares et al., 2022). In order to obtain accurate results, a combination of several diagnostic methods should be used, because although microfilaremia does not necessarily compromise the health of an infected patient, this can still act as a reservoir for further infection of other dogs if the intermediate mosquito host is present. Although filarial species *(*e.g*. D. immitis, D. repens, A. reconditum, A. dracunculoides,* can be distinguished using molecular tools (Rishniw et al., 2006), in the present study species other than *D. immitis* were not detected both in modified Knott or PCR tests. The same happened in studies in which no other filarial species were detected, probably due to the low pressure of suitable vectors in the environment (Ferreira et al., 2017, Panarese et al., 2020). In the present study, the difference between the number of female and male dogs positive for *D. immitis* was not considerable, which is in agreement with other studies that showed that *D. immitis* infection related to the sex of the canids was not statistically significant (Hou et al., 2011; Anvari et al., 2019; Miterp´akov´a et al., 2018; Esteban-Mendoza et al., 2020). The present study showed a significantly higher prevalence (combined positivity) in older dogs than in younger ones. Similar results were obtained by other authors (Hou et al., 2011; Ferreira et al., 2017; Anvari et al., 2019; Miterp´akov´a et al 2018). The gradual increase of infection associated with increasing age of dogs can be explained by the cumulative probability of exposure to mosquito bites in older animals, prolonged proliferation of microfilariae in the blood, and insufficient immunity against adult parasites (Hou et al., 2011; Anvari et al., 2019). These results suggest that the risk of exposure to *D. immitis* in dogs is high in Caminha and this can be explained by the fact that all animals tested had access to outdoors and, therefore, had a greater degree of exposure to vectors. It should also be noted that they lived in areas close to water sources and none of the animals tested were subjected to any prophylactic measure. Prophylaxis against the parasite is advisable to reduce the occurrence of infection and canine disease, so the application of ectoparasiticides and repellents is essential. The role of wolves and foxes as *D. immitis* reservoir hosts and their contribution to disease transmission are acknowledged

throughout Europe and therefore, understanding infection and disease prevalence in wild canids is especially important because these may act as reservoirs, thus increasing the risk of infection for domestic pets, including urban canids (Morchón et al., 2012; Otranto and Deplazes, 2019; Pluemer et al., 2019; Gomes-de-Sá et al., 2022c). While in endemic areas regular chemoprophylactic treatments of domestic dogs reduce the general prevalence of the infection, wild canids are likely to play a decisive role in the maintenance of infection (Otranto and Deplazes, 2019), recently a study assessed *D. immitis* occurrence in a population of Portuguese wolves and foxes by searching for *D. immitis* antigens and microfilaremia and confirmed the existence of antigenemia in these wild animals, with microfilaremia being detected for the first time in wolves (Gomes-de-Sá et al., 2022c).

5. Conclusion

The present study confirms a substantial circulation of filarioid parasites in stray and shelter dogs of Caminha, highlighting the diagnostic value of serological tests and identification of microfilariae of *D. immitis* by PCR. Considering that *D. immitis* is a zoonotic parasite, the application of ectoparasiticides and repellents is highly recommended for dogs in this region. Systematic monitoring studies are required to better understand the environmental risk factors and to identify the competent mosquito vectors in the local epidemiology of dirofilariosis.

Ethics approval and consent to participate

All procedures complied with the Portuguese legislation for the protection of animals used for scientific purposes (i.e. Decree-Law no. 113/2013 of 7 August 2013), which transposes European legislation (i.e. Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010).

CRediT authorship contribution statement

Sónia Gomes-de-Sá: Writing – review & editing, Data curation, Formal analysis, Methodology, Resources, Writing – original draft. Sérgio Santos-Silva: Methodology, Data curation, Formal analysis. Alícia de Sousa Moreira: Methodology, Data curation, Formal analysis. Patrícia Ferreira Barradas: Irina Amorim: Methodology, Data curation, Formal analysis. Luís Cardoso: Methodology, Data curation, Formal analysis. João R. Mesquita: Data curation, Formal analysis, Methodology, Resources, Writing – original draft, Writing –

review & editing.

Data Availability

Data will be made available on request.

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CHAPTER III

Dirofilaria immitis antigenemia and microfilaremia in Iberian wolves and red foxes from Portugal

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***Dirofilaria immitis* antigenemia and microfilaremia in Iberian wolves and red foxes from Portugal**

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Abstract

Background:

Dirofilaria immitis (*Spirurida; Onchocercidae*) is a parasitic nematode, which causes cardiopulmonary dirofilariosis in wild and domestic canines and felines, and also pulmonary dirofilariosis in humans [1]. Mosquitoes (*Diptera; Culicidae*) are vectors to *Dirofilaria* spp., making the parasite distribution susceptible to changes, as well as to rapid and significant variations in defined geographic regions, such as movement of infected animals, the introduction of new mosquito species and anthropogenic activities in ecosystems [2, 3]. Dirofilariosis is endemic in the Mediterranean countries, including Portugal, particularly because of the appropriate geographic and climatic conditions [4]. The climate in Portugal is typically temperate with warm and dry summers. It is considered to be divided in subtypes, namely the first with hot summers and average temperatures > 22 °C in the warmest months and the second with warm summers with average temperatures ≤ 22 °C in the warmest months and with ≥ 4 months with the average temperatures > 10 °C [5]. Wildlife carnivores have long been overlooked when considering their possible role in transmitting zoonotic nematodes [6]. However, increasing human activities have continuously promoted wild environment invasion, ultimately redefining domestic and wild interface boundaries and consequently increasing the contact between humans and wild animals [6]. Moreover, wild animals are frequently exposed to vector-borne pathogens to such an extent that wild carnivores like the gray wolf (*Canis lupus*), red fox (*Vulpes vulpes*) and golden jackal (*Canis aureus*) are now recognized hosts of *D. immitis* [7]. Wolves and foxes' role as *D. immitis* reservoir hosts and their contribution to disease transmission are acknowledged throughout Europe [8]. However, in Portugal, little information is known regarding *D. immitis* circulation, and the studies available are limited to a few surveys and case reports, possibly underestimating the relevance of these nematodes [3]. Moreover, the extent to which wild carnivores remain reservoirs for *D. immitis* is still unknown. As such, the aim of the present investigation was to assess the presence of *D. immitis* in wolves and foxes in Portugal by searching for both *D. immitis* antigens, using a rapid immunomigration test (RIM), and for *D. immitis* microfilaremia, by using a species-specific polymerase chain reaction (PCR) assay optimized for the simultaneous detection and differentiation of *D. immitis* and eight others concurrent filarioids.

Keywords: *Dirofilaria immitis*, Foxes, Wolves, Portugal, Wildlife

Methods

Blood samples were collected from a total of 61 wild carnivores, 42 wolves (*C. lupus signatus*) and 19 foxes (*V. vulpes*), during 2010–2012, from a National Park located at the northern border of Portugal (Peneda-Gerês National Park). Samples from these animals were collected as part of a carnivore protection program, an initiative that manages the collection of recently deceased animals suspected to be poisoned. Tissues and other matrices are available for studies considered to be of value for the assessment of animal population morbidities and mortalities. For the initial detection of *D. immitis* infection, rapidantigen detection test kits (Uranotest Dirofilaria ®, Barcelona, Spain) were used, according to the manufacturer's instructions. This test consists of an immunochromatographic technique aiming at the qualitative detection of the *D. immitis* antigen in blood, which resources to 14 kDa antigen detection not related to the parasite's feminine genital apparatus, hence detecting both male and female parasites. The platform detects infections with a load of only one adult parasite of any type (males, adult females, immature females), with sensitivity and specificity of 94% and 100%, respectively, compared to necropsy, according to the manufacturer's information. To detect microfilaremia, DNA was extracted from blood using the QIAamp® DNA Mini Kit (Qiagen, Valencia, CA, USA), using an automated QIAcube nucleic acid extractor (Qiagen GmbH, Hilden, Germany). DNA was stored at − 20 °C until further analysis. A negative extraction control was processed along with each batch of 12 samples. DNA specimens were initially screened for the presence of microfilaria by using a conventional PCR targeting the 5.8S-internal transcribed spacer (ITS) 2–28S regions of the genome. Briefly, pan-filarial primer pair − DIDR-F1 and DIDRR1 − amplifying products with distinct molecular weights were used to differentiate nine filarial species, namely *Acanthocheilonema dracunculoides* (584 base pairs [bp]), *Acanthocheilonema reconditum* (578 bp), *Brugia malayi* (615 bp), *Brugia pahangi* (664 bp), *Brugia timori* (625 bp), *Dirofilaria immitis* (542 bp), *Dirofilaria repens* (484 bp), *Mansonella ozzardi* (430 bp) and *Onchocerca volvulus* (470 bp). For all reactions, a total of 5 µl of genomic DNA was added to 12.5 µl Xpert Fast Hotstart Mastermix (2×) with dye (GRiSP, Porto, Portugal), 5.5 µl of deionized sterile water and 1 µl (10 µM) of each of the DIDR-F1 and DIDR-R1 primers in a 25-µl final volume of the reaction mixture. The reactions were carried out in an automatic DNA thermal cycler 100 (Bio-Rad Laboratories, Hercules, CA, USA), including negative and positive controls (extracted from an adult female *D. immitis*). The PCR amplification products were visualized by Xpert Green DNA Stain direct (GRiSP, Porto, Portugal) fluorescence after electrophoresis in a 1.5% agarose gel at 100 V for 40 min. To confirm species identification, all amplicons of expected size were sequenced

bidirectionally for genetic characterization. Briefly, amplicons were purified with GRS PCR & Gel Band Purification Kit (GRiSP, Porto, Portugal), and bidirectional sequencing was performed by Sanger method, using the respective primers. Sequences were manually corrected using the BioEdit Sequence Alignment Editor v 7.1.9 software package, version 2.1 (Ibis Biosciences, Carlsbad, CA, USA), and further analyses were performed by comparison with the sequences available in the NCBI (GenBank) nucleotide database (http:// blast. ncbi. nlm. nih. gov/ Blast). Phylogenetic analysis was performed using MEGA version 6.0 software [9]. The obtained consensus sequences in this study and representative sequences for *O. volvulus*, *D. immitis* and *D. repens* obtained from GenBank were used for the phylogenetic analysis. Maximum likelihood (ML) method was applied. The ML bootstrap values were estimated using 1000 replicates with Tamura 3-parameter as the correction model, estimated as the best substitution model by MEGA version 6.0 software. We deposited the 5.8S-ITS2-28S of *D. immitis* sequences recovered in this study in GenBank.

Results and discussion

For the initial detection of *D. immitis* infection, out of the 61 wild carnivores screened by rapid antigen detection, three had D*. immitis* antigen circulation in blood (4.9%; 95% confidence interval [CI] 1.0 − 13.7). All three wild carnivores showing antigenemia were foxes, yielding an occurrence of 15.8% (95% CI 3.4−39.6). The same 61 animals were again tested for microfilaremia through conventional PCR, and only two wolves (1.6%; 95% CI 0.0−8.8) tested positive, with a prevalence in the whole sampled lupine population of 4.8% (95% CI 0.6 − 16.2). Both positive samples yielded amplicons with 542 bp, being presumptively positive for *D. immitis*. After bidirectional sequencing, the consensus sequences showed to be identical. Basic Local Alignment Search Tool confirmed the identity (100%) of D. immitis in both lupine samples. The following accession numbers were assigned to the sequences obtained in this work: OK632269 and OK632270. One of the sequences obtained (OK632270) spanned only 77 nucleotides (nt); hence, phylogenetic analysis was performed with the other sequence. Phylogenetic analysis based on the 460 nt partial region of the 5.8S-ITS2-28S regions showed clustering with *D. immitis* (Fig. 1). The present study evaluated *D. immitis* occurrence in a population of Portuguese wolves and foxes by searching for both *D. immitis* antigens and *D. immitis* microfilaremia, using a RIM test and a species-specific PCR assay followed by sequence confirmation, respectively. *Dirofilaria immitis* is usually detected by specific antigen testing and/or identification of microfilariae [1]. Despite the negative heartworm antigen test result, the

infected animals may still show microfilaremia in the blood [1]. In Europe, eight species of filarioids, including zoonotic species, have been reported mainly in domestic dogs, and occasionally in wild carnivores [10]. Species discrimination is of high clinical and epidemiological importance because of zoonotic concerns and therapeutic implications [1]. The application of molecular analysis targeting filarial DNA is a highly sensitive and specific analytical tool for the diagnosis and simultaneous characterization of filarial infections, thus being an extremely valuable approach [1]. In this study, none of the 42 wolf samples presented evidence for *D. immitis* antigenemia. Nevertheless, two wolves were positive for *D. immitis* microfilaremia by PCR (4.8%; 95% CI 0.6–16.2%). Although *Dirofilaria* microfilaremia does not necessarily correlate to an endangerment of the infected animal's health, the individual can act as a reservoir for further infection if the intermediate mosquito host is present [1]. To the best of our knowledge, only one study has reported that wolves were suitable *Dirofilaria* hosts and appeared exposed to infection similarly to sympatric unprotected dogs [7]. Until today, microfilaremia had never been found, thus hampering the assessment of the impact of wolves on infection maintenance. Interestingly, the present study shows that none of the foxes presented microfilaremia, but three were positive for *D. immitis* antigenemia (15.8%; 95% CI 3.4–39.6). Variable occurrences have been reported, namely, 32.3% in Spain [12], 1.6% in Serbia [13], 3.7% in Hungary [14], 25.2% in Bulgaria [15], 0.3% in Romania [10] and 2% in France [16]. In Portugal, the prevalence of *D. immitis* detected by necropsy of red foxes ranged from 3.2% in northern-central locations, such as Coimbra district [17], to 11.8% in southern and central districts, such as Santarém and Setúbal [18]. In a serological survey conducted in red foxes in Portugal, 8.5% (10/118) were positive for *D. immitis* circulating antigens, with positive animals found in northern and southern areas [3]. It should be noted that the lack of microfilaremia can be related to several factors, including unisexual infections, pre-patency or the host's immune response leading to the elimination of microfilariae. Comparisons between studies should be made with caution as different assays with distinct sensitivities/specificities were used.

Conclusions

The present study provides molecular and serological evidence for *D. immitis* infection in wild carnivore species present in Portugal, supporting their potential epidemiological role. While in endemic areas frequente chemoprophylactic treatments of domestic dogs reduce the overall prevalence of the infection, wild canids might play a crucial role in the maintenance of infection. Understanding infection and disease prevalence in wild canids is especially important because these may act as reservoirs, increasing the risk of infection

for domestic pets, including urban canids [19]. Infected microfilaremic carnivores may, in the presence of competent vector species, also act as reservoir hosts. To the best of our knowledge, this is the first detection of *D. immitis* microfilaremia in wolves, supporting that these animals can have a role as *D. immitis* reservoirs.

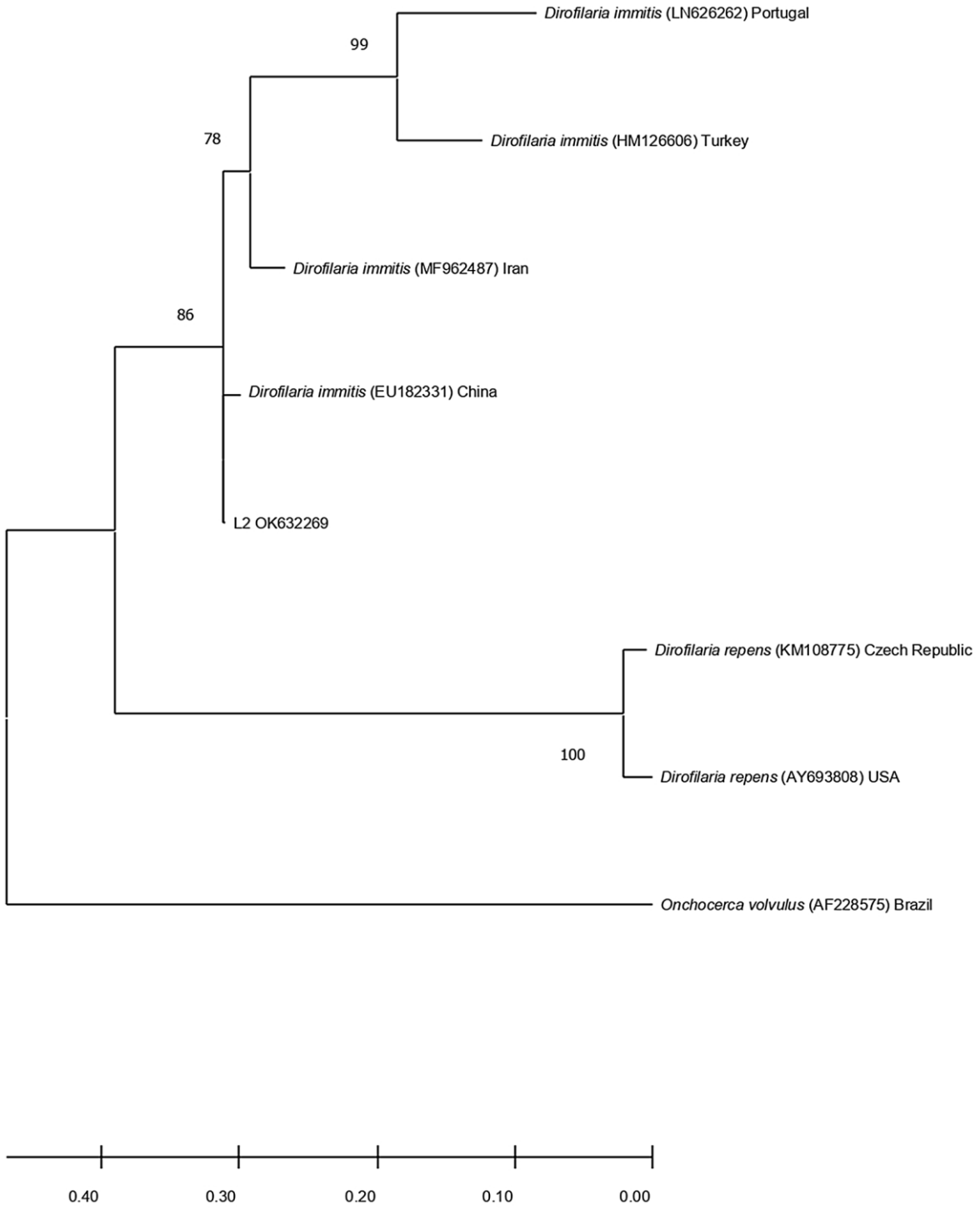

Fig. 1 Phylogenetic analysis of *D. immitis* found in wolves in Portugal. The evolutionary history was inferred by using the ML method based on the Tamura 3-parameter model. The analysis involved

eight sequences. The *D. immitis* sequence characterized in this study is represented by sample code (L2) followed by the GenBank accession number (OK632269). Other sequences are represented with the species name followed by the corresponding accession number and country. Branch lengths are measured as the number of substitutions per site. Reliability of internal branches was assessed using the bootstrapping method (1000 replicates)

Abbreviations

bp: Base pairs; CI: Confidence interval; ITS: Internal transcribed spacer; ML: Maximum likelihood; nt: Nucleotide(s); PCR: Polymerase chain reaction; RIM: Rapid immunomigration.

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Authors' contributions

SGdS: conducted sample collection and testing and drafted the manuscript; SSS: conducted testing and data analysis; AdSM: conducted testing and data analysis; PFB, IA: supervised the study, conducted data analysis and drafted the manuscript; LC: supervised the study and interpreted data; JRM: supervised the study, designed the work and interpreted data. All authors have read and approved the final manuscript.

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Availability of data and materials

Data supporting the conclusions of this article are included within the article.

Declarations

Ethics approval and consent to participate

All procedures complied with the Portuguese legislation for the protection of animals used for scientific purposes (i.e. Decree-Law no. 113/2013 of 7 August 2013), which transposes European legislation (i.e. Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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CHAPTER IV

De Novo Assembly of the *Dirofilaria immitis* Genome by Long-Read Nanopore-Based Sequencing Technology on an Adult Worm from a Canine Cardiopulmonary Dirofilariosis Case

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De Novo Assembly of the *Dirofilaria immitis* Genome by Long-Read Nanopore-Based Sequencing Technology on na Adult Worm from a Canine Cardiopulmonary Dirofilariosis Case

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Simple Summary: *Dirofilaria immitis* is a zoonotic parasite that infects canids and other vertebrates. We expanded the use of long-read nanopore-based sequencing technology by performing genomic de novo assembly of a *D. immitis* specimen retrieved from a canine cardiopulmonary dirofilariosis case by using the ONT MinION platform. We also identified loci previously characterized as being associated to macrocyclic lactone resistance selection pressure. The identification of a resistant zoonotic parasite alerts for the overuse of macrocyclic lactone in the region.

Abstract

Dirofilaria immitis is a zoonotic parasitic nematode that infects domestic and wild canids, among its vertebrate hosts. The genetic analysis of *D. immitis* nowadays transcends the need for genetic taxonomy of nematodes, such as the study of resistance to macrocyclic lactone. We expanded the use of long-read nanopore-based sequencing technology on nematodes by performing genomic de novo assembly of a *D. immitis* specimen retrieved from a canine cardiopulmonary dirofilariosis case using the ONT MinION platform, followed by the study of macrocyclic lactone resistance. The assembled genome of *D. immitis* consists of 110 contigs with an N50 of 3687191. The genome size is 87.899.012 bp and contains a total of 9741 proteins; 6 ribosomal RNAs, with three belonging to the small subunit (18S) and three to the large subunit (28S); and 73 tRNAs. Subsequent analysis of six loci previously characterized as being associated to macrocyclic lactone resistance selection pressure showed that four have a genotype associated with either some loss of efficacy or the resistance phenotype. Considering the zoonotic potential of *D. immitis*, the identification of a resistant parasite alerts for the overuse of macrocyclic lactone in the region, which poses a potential risk to both veterinary and human public health.

Keywords
Dirofilaria immitis, genome, macrocyclic lactone resistance, long-read

1. Introduction

Dirofilaria immitis is a parasitic nematode that infects domestic and wild canids as well as other animals, including humans. The associated disease typically occurs in temperate, tropical, and subtropical areas of the world, with the agent being transmitted by several mosquito species such as those belonging to the *Culex*, *Aedes*, and *Anopheles* genera [1]. These mosquitoes deposit infective stage larvae (L3) at the biting site, which penetrate the

host's skin. The L3 molt into L4 3–12 days post-infection (dpi), later molting into preadult worms 50–70 dpi, which migrate to the pulmonary artery and right ventricle 70–85 dpi, reaching sexual maturity 120 dpi [2]. Females initiate the production of microfilariae (first larval stage) 6–9 months post-infection, with adults living more than 7 years and microfilariae living up to 2 years [2]. *Dirofilaria immitis* worms can cause canine cardiopulmonary dirofilariosis (also known as heartworm disease in dogs), a widespread disease that can have a fatal outcome if animals are not treated [3]. Moreover, *D. immitis* in dogs represents a risk for the human population, who may suffer from pulmonary dirofilariosis and, in many cases, pulmonary nodules that can be misdiagnosed as malignant tumors [4].

Morphological analysis is commonly used for the differentiation of nematode species, but not without its drawbacks such as scarce distinguishable characters, a circumstance that may hamper their classification to the species or even genus level [5]. This is not necessarily the case for *D. immitis*, which can easily be distinguished from other filarioids, taking into account the cardiac location of adults. Nonetheless, genetic analysis nowadays transcends the need for genetic taxonomy of nematodes. An example is the requirement for ascertaining resistance to macrocyclic lactones, known to have a genetic origin [6]. Macrocyclic lactones such as milbemycin oxime, ivermectin, moxidectin, and selamectin are widely available drugs that are used to prevent the establishment of the L3–L4 *D. immitis* stages in dogs and cats. However, loss of efficacy has been described since 2005, and whole-genome analysis has been performed on *D. immitis* isolates to characterize their genetic profile and differences that could potentially be associated with evident loss of efficacy and resistance [6]. Nematode characterization based on markers such as the internal transcribed spacer (ITS) regions of the ribosomal RNA locus, the 28S large subunit ribosomal RNA gene (28S LSU rRNA), the 18S small subunit ribosomal RNA gene (18S SSU rRNA), and the cytochrome oxidase I gene (coi) followed by Sanger sequencing is a widespread, lowcost approach to ascertain genetic profiles [5]. However, the data generated are limited, and novel sequencing strategies such as single-molecule real-time sequencing or third-generation sequencing have reduced the expenses and the necessary hardware for obtaining thorough data on highly contiguous genome assemblies, permitting comprehensive, nearreal-time biomonitoring of samples [7,8]. The aim of the present work was to expand the use of long-read nanopore-based sequencing technology on nematodes by performing genomic de novo assembly of a *D. immitis* specimen retrieved from a canine cardiopulmonary dirofilariosis case using the ONT MinION platform.

2. Material and Methods

2.1. Extraction of Parasite DNA

Adult *D. immitis* worms (n = 32) were observed at routine parasitological investigation in the right heart and pulmonary artery of a dog that died from an unrelated cause in the municipality of Caminha, northern Portugal, November 2020. The city of Caminha is located by the mouth of the Minho River (circa 340 km in length), under a wet Atlantic climate, being the area of the country with the highest precipitation, reaching around 1800 mm on average and peaking at more than 3500 mm [9]. The dog hosting the *D. immitis* worms was kept in a municipal kennel since birth and had not received any macrocyclic lactone treatment. No ethics permission was obtained since the parasites were taken during regular post-mortem evaluation. One nematode was selected, washed three times in phosphate-buffered saline at pH 7.2, frozen at – 80º C, and subjected to mechanical disruption with a disposable pestle. Nucleic acid extraction followed the previously described procedures [7]. Briefly, the homogenate was incubated with 20 µL proteinase K (Qiagen, Hilden, Germany) and 180 µL of Buffer ATL (Qiagen) for 48 h at 56º C, with vortexing (200 rpm) in a thermoblock (Eppendorf Epp Thermomixer; Hamburg, Germany). High-molecular-weight (100–200 kb) DNA was then extracted using a magnetic-bead-based protocol (MagAttract HMW DNA kit; Qiagen) as described by the manufacturer. Eluted DNA (on 100 µL of 10 mM tris-HCL) was evaluated for size distribution on an agarose (0.8%) gel. DNA was assessed on a Nanodrop spectrophotometer (ThermoFisher, Waltham, MA, USA). Genomic DNA size selection was then performed using a 0.4× volume of AmpureXP beads (Beckman Coulter, Brea, CA, USA) in order to remove smaller fragments.

2.2. Library Preparation and Sequencing

The 1D genomic ligation (SQK-LSK109) library preparation kit (ONT, Oxford, UK) was used imputing 1.2 µg of extracted genomic DNA, and libraries were then developed as instructed by the manufacturer with a calculated final library quantity assessment at 467 ng. Then, 79.3 ng was loaded onto the MinION sequencer using an R9.4.1 flow cell managed by the MinKNOW software (version 18.12.9, ONT). The ONT MinION Mk1B platform was used with active channel selection performed at every 1.5 h, resourcing to
no script modifications. Refueling of the flow cell was performed 24 h after initiation by first extracting excess liquid from the waste chamber, followed by the addition of 37.5 µL of SQB and 37.5 µL of H2O on the SpotON sample port. An additional 24-hour run was then

performed.

2.3. Base-Calling, Genome Assembly, and Read Alignments

After completion of the sequencing run, Guppy (version 2.3.5, ONT) was used for basecalling signal data (fast5 files), with the generated fastq files used to generate statistics with NanoPlot (version 1.19.0). Raw reads were processed using Porechop software (version 0.2.4) with the default parameters to trim sequences from all known Oxford Nanopore adapters. After trimming, all reads were used to perform a complete genome assembly using Flye software (version 2.8.3) with the parameters "-nano-raw" to specify the input type data and an expected genome size of 100 Mb. Contigs obtained at the assembly step were processed using Kraken2 to locate potential contaminants in the sample. Some contigs were identified as belonging to *Canis lupus*, *Homo sapiens*, or *Wolbachia* and therefore removed from the dataset for further analyses. Contigs potentially belonging to *D. immitis* were corrected by mapping the raw reads against the assembled contigs through the Pilon software (version 1.24). Confirmation of the species was double-checked by using the small subunit of the ribosomal RNA (18S) against the NR database using NCBI blast (Evalue of 0.0 and 98.91% identity with accession AB973231.1) and blast against the SILVA SSU database, where the best match was again AB973231.1 belonging to *D. immitis*. The corrected contigs were compared against the three available *D. immitis* genomes at NCBI (GCA_009829315.1, GCA_001077395.1, and GCA_013365355.1) to verify the integrity of our assembly. The completeness of the assembly was verified through BUSCO software (version 4) using the nematode database. Final contigs were annotated in order to locate coding regions using Genemark-ES software (version 4.65) with self-training and default parameters. Non-coding regions belonging to rRNAs and tRNAs were also located with RNAmmer software (version 1.2) and tRNAscan-SE (version 2.0), respectively. Functional annotation for the coding regions was performed using Sma3s (version 2), retrieving GO terms and EC numbers for enzymes. Deposition of the Whole Genome Shotgun project was performed at DDBJ/ENA/GenBank under the accession code JAKNDB000000000, with the current version (described in the present paper) being JAKNDB010000000. Single-nucleotide polymorphism (SNP) loci genotypes that differentiate loss of efficacy/ resistant populations from susceptible *D. immitis* populations, previously referred to as markers for macrocyclic lactone resistance in *D. immitis* in the United States [6] and in Australia [10], were identified in the *D. immitis* genome with the Basic Local Alignment Search Tool (BLAST) and Interactive Genome Viewer (IGV) for full characterization of the sample.

3. Results and Discussion

The assembled genome of *D. immitis* consists of 110 contigs with an N50 of 3687191. The majority of the contigs had a very good match with one or more of the compared genomes without any sign of fragmentation. The genome size is 87.899.012 bp and contains a total of 9741 proteins; 6 ribosomal RNAs, with three belonging to the small subunit (18S) and three to the large subunit (28S); and 73 tRNAs. The results obtained for BUSCO show that the genome is complete to a high level, showing the following values (C:93.9% [S:93.5%, D:0.4%], F:1.7%, M:4.4%, n:3131). We therefore present evidence that a single ONT MinION flow cell can produce sufficient data to assemble a contiguous, high-level, near-full-length genome of *D. immitis*. Noteworthily, parasite genome assemblies used as references are still today considered to be vastly fragmented, frequently hampering more in-depth analysis [11]. A total of 42 loci in the *D. immitis* genome, with the corresponding SNP associated with resistance selection pressure or at least loss of efficacy for various macrocyclic lactones [6,10], were studied. Subsequent analysis of the loci associated with macrocyclic lactone resistance selection pressure showed that 27 of the 42 loci have a genotype associated with either some loss of efficacy or the resistance phenotype (Table S1). Interestingly, the dog hosting the *D. immitis* worm collected for this study was kept in a municipal kennel since birth and had not received any macrocyclic lactone treatment, a fact which suggests that resistance-associated SNPs were likely acquired from ancestral *D. immitis* previously circulating in the region. It is assumed that macrocyclic lactone resistance first appeared circa 1998. At that time, macrocyclic lactone *D. immitis* preventives had been recommended for use as the first line of treatment to prevent heartworm for over 10 years, having been registered as 100% effective [12,13]. Hence, it is generally assumed that the basis for genetic changes causing macrocyclic lactone resistance only arose and was selected in that decade. As such, resistant lines of *D. immitis* are suggested to have been circulating prior the use of macrocyclic lactone preventives [12,13]. True drug resistance has a genetic basis; hence, continuing efforts to analyze *D. immitis* whole genomes from resistant and susceptible lines could help detect genetic markers for resistance [6]. Long-read sequencing technologies allow for democratizing access to powerful sequencing options, reducing the cost of de novo genome assemblies of understudied organisms. TheMinION platform was first made available in 2014, presenting a compact, novel, and lightweight sequencing platform that could produce long reads used for real-time base-calling [14]. The sequencer uses a nanopore that holds a biological membrane where DNA is driven while producing differences in electrical current that are measured and translated as different DNA bases [14–16]. The ONT MinION platform can not only provide a low-cost

option for projects that were previously considered costprohibitive but also grants access to this technology in underdeveloped areas that do not have the infrastructural capacity for sequencing. Moreover, the sequence quality seems to allow for the profiling of drug resistance patterns, which allows for more informative treatment options [14].

4. Conclusions

In conclusion, this work provides, for the first time, the de novo assembly of the *D. immitis* genome using long-read nanopore-based sequencing technology. We show that a single ONT MinION flow cell can produce sufficient data to assemble a contiguous, high-quality genome from a complex nematode. The data from this study also provide information suggesting the circulation of macrocyclic-lactone-resistant *D. immitis* in northern Portugal. Considering the zoonotic potential of *D. immitis*, the identification of a resistant parasite alerts for the overuse of macrocyclic lactone in the region, which poses a potential risk to both veterinary and human public health.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/ani12111342/s1, Table S1. Macrocyclic lactone resistance profile in a *Dirofilaria immitis* collected from a dog in northern Portugal, November 2020.

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CHAPTER V

Correspondence: “The One Health concept applied to dirofilariosis—a zoonotic disease”

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Correspondence:"The One Health concept applied to dirofilariosis—a zoonotic disease"

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We have read with interest the paper by Silva et al.1 “human pulmonary dirofilariosis: a pitfall in solitary pulmonary nodule”.

After careful analyses, we would like to comment on certain statements from the article.

In this paper authors report the case of a 38-year-old man who presented to the emergency department with face edema, eosinophilia (2000/uL) and a chest X-ray showing a small peripheral solitary lung nodule on the right lung. Post-operative histopathological diagnosis was consistent with a central zone of necrosis surrounded by granulomatous inflammation and a fibrous wall. Besides that, a filarial worm was found in the lumen of an artery within the area of necrosis containing remnants of *Dirofilaria immitis*.

The zoonotic implication of *Dirofilaria* spp. Infections is important since shortly after the inoculation of stage 3 larvae (L3) by vector mosquitoes, there is the possibility of larval migration along human tissues. [2] This can provoke ocular, skin and pulmonary nodular lesions, which are frequently and erroneously diagnosed as pulmonary carcinomas.[2]
Moreover, the diagnosis of malignant neoplasm requires invasive procedures before reaching the correct diagnosis.[2]

The paper by Silva et al.[1] caught our attention as, upon biopsy, the morphological identification of dirofilariae parasites can be difficult to obtain, due to a loss of parasite integrity after tissue excision with consequent underdiagnosis, and a diagnosis based solely on the histological features only allows the determination of the genus *Dirofilaria*.[3] In addition, there are confirmed *Dirofilaria* spp. Circulating in Portugal, namely *Dirofilaria repens,* [4] which has been reported to cause human pulmonary dirofilariosis with nodules that can be mistakenly diagnosed as malignant.[5] Noteworthy, Ferrari et al. [5] have reached definite diagnosis by multiplex PCR targeting mitochondrial cytochrome oxidase subunit I gene (mtDNAcox1).

Determining *Dirofilaria* spp. based solely on histological diagnosis is yet to be confirmed and, when the parasite’s DNA extraction is possible, the methods of molecular diagnosis based on sequencing can play a fundamental role in the identification of the etiological agent involved.[2] Hence, we would like to highlight the diagnostic accuracy of PCR followed by dideoxy chain termination sequencing as a valuable and affordable method to confirm worm species. Dirofilariosis is not difficult to treat when diagnosed with accuracy; however, it remains an underdiagnosed infection and disease because of the complexity in identifying the parasites involved. The use of molecular biology techniques to detect and identify them

is likely to overcome the complexity associated to the diagnosis.[3]

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CHAPTER VI

General discussion and concluding remarks

General discussion

Factual data on prevalence, distribution, impact and potential acquisition of new ecosystems are crucial to control animal and zoonotic diseases such as dirofilariosis.

In Chapter II, in order to add knowledge concerning the presence of *Dirofilaria* spp. in Portugal, 244 dogs in the North of the country were tested through combined analysis of antigens by RIM and DNA by PCR. The latter enabled the differentiation of *D. immitis* from seven other filarioids species. Based on RIM, 118 dogs (48.4%) tested positive for D. *immitis* adult worms, and 36 (14,8 %) tested positive to microfilaremia by using PCR being *D. immitis* the detected species involved. The modified Knott test was only used to detect microfilaremia in samples which presented antigenemia; as a result, from the 118 positive cases identified by RIM, 24 (20.3%) dogs showed microfilariae with characteristic morphology of *D. immitis*. All this information was possible due to the importance of using a combination of different diagnostic methods, emphasising the diagnostic accuracy and high performance of serological tests to detect antigens of adult worms, and through PCR to detect microfilaremia. Nevertheless, it is important to accentuate that none of these tests should be used separately, for there are cases in which RIM detects amicrofilaremic infections, but there are other cases where results are negative for RIM and positive for PCR (Miterpáková et al., 2022; Lemos et al., 2022; Mõttus et al., 2024. These data play a pivotal role when determining the prevalence of existing filarial species, showing that *D. immitis* is the only species found. Additionally, these results raise legitimate concerns not only because it refers to a zoonosis, but also because, of all the existing filarial species this is one of the species that leads to the development of the most serious clinical signs (Brianti et al., 2023; Philp et al., 2023). This knowledge was only possible due to the combined use of different diagnostic methods, which underline and emphasise the diagnostic accuracy associated with the high performance of serological tests for the detection of adult worm antigens, and of the PCR technique used to detect microfilaremia. The results herein achieved are in accordance with other studies which refer that the molecular methods are not only a highly sensitive and specific analytical tool for the detection and simultaneous characterization of the different species, but also provide more reliable data when compared to other tools (Hou et al., 2011; Ferreira et al., 2017; Mircean et al., 2017; Little et al., 2018). The high risk of exposure to *D. immitis* in the study can be explained by the fact that all tested animals had a greater degree of exposure to vectors, lived in areas close to water sources and none of the animals tested were subjected to any prophylactic measure. In fact, prophylaxis against the parasite is highly recommended to minimize the occurrence of

infection and canine disease, so the application of ectoparasiticides and repellents is essential. It must also be mentioned the role of the climate in the spread and increase of the prevalence, and Portugal has a compatible climate to the development, reproduction and survival of the vectors and it is, therefore, accurate to define Portugal as a country in which canine dirofilariosis by *D. immitis* is endemic (Esteves-Guimarães et al., 2024). A recent study developed in Greece, where the climate resembles ours, confirms that *D. immitis* infection in dogs is spreading since canine seroprevalence rates have increased in all regions previously considered and the likelihood of a dog becoming infected with the parasite has increased 4.1 times (Symeonidou et al., 2024). As such, the implementation of mosquitoe control measures can have a chief role to reduce *D.immitis* infection including: regular use of mosquito repellent, antifeedant, emptying standing water, installing window screens, and avoiding areas and times of day when mosquitoes are most active (AHS, 2020; ESCCAP, 2021; Brianti et al., 2023; Philp et al., 2023). Since the implementation of the above-mentioned measures is not always possible, and in order to avoid cases of infection of adult worms, which can be fatal, the administration of MLs comprise a strategy to make sure that no worm survives (Nolan et al., 2012; FECAVA, 2019). Prevention will also interrupt the spread of canine cardiopulmonary dirofilariosis because microfilariaemic dogs are reservoirs, especially those without clinical signs and treatment (Symeonidou et al., 2024). Heartworm prevention in animals is crucial because of its impact on their health, the complexity, the risk, the cost of treatment and zoonotic implications (Carretón et al., 2020; Mõttus et al., 2024). Since the adult stage of the infection causes morbidity, and a significant infection of adult worms can be fatal, the primary focus of maintaining animal health and welfare is to prevent adult infection from establishing (Brianti et al., 2023; Philp et al., 2023).

Chapter III refers to the high prevalence identified and the geographical area considered, in which dogs frequently have close contact with wild canids and likely share the same arthropod vectors. For these reasons, there was interest in adding knowledge and information regarding the presence of *Dirofilaria* spp. in wolves and foxes and establish measures to assess the presence of *Dirofilaria* spp. To accomplish this, blood samples of 61 wild canids were further screened through RIM and PCR for the detection and differentiation of *D. immitis* from seven other filarioids. Three foxes presented *D. immitis* antigen circulating in blood and two wolves tested positive for *D. immitis* microfilaremia through PCR. Studies reveal that given the permanent exposure of wild canids to vector-borne pathogens, the grey wolf (*Canis lupus*), red fox (*Vulpes vulpes*) and golden jackal (*Canis aureus*) are now recognized hosts of *D. immitis* (Alho et al 2018; Moroni et al., 2020). The results stated in this chapter utterly intensify the concern of new infections, as it is the

first ever detected case of microfilaremia in wolves. Therefore, it is concluded that *D. immitis* can also infect wolves and that these hosts can act as a reservoir for new infections, whenever mosquitoes, as intermediate hosts, are present.

Based on the above, and in an attempt to develop novel or alternative diagnostic approaches and genetic targets, analysis of the complete genome of *D. immitis* was carried out in Chapter IV. In fact, as a clinical veterinarian, throughout my professional career I had the opportunity to accompany several clinical cases in which the failure of prophylactic treatment with MLs was evident. Therefore, it has become more obvious that possible resistance to MLs could be occurring in dogs, similar to what has been reported in other countries where resistance of *D. immitis* to ML was confirmed genetically in infected dogs from the Lower Mississippi region of the United States of America (Bourguinat et al., 2011, 2015; Pulaski et al., 2014). And the first case of ML-resistant *D. immitis* infection in Europe was confirmed by Traversa et al. (2024), through genetic analysis showing that the microfilariae had an ML-resistant genotype compatible with a resistant strain. It must also be pointed out the mediatic attention that ivermectin gained as one of the drugs to combat COVID-19, which led to its inappropriate and even dangerous use and its wide sale and distribution through illegitimate online pharmaceutical markets (Barac et al., 2022). In this sense, it is very likely that this event may have contributed significantly to the emergence of possible resistant strains. On the other hand, the biological characteristics of the parasite such as the longevity of both adults and microfilariae, and the small quantity of parasites in the short-lived intermediate favour the development of resistance (Churcher et al., 2008; Panarese et al., 2020; Diakou and Prichard, 2021; Geary, 2023). Additionally, there is the likelihood of inbreeding in *D. immitis*, since L3 transmitted by a mosquito have a equitable probability of being siblings or half-siblings, assuming that the mosquito became infected by a blood meal from a single infected dog, intensifying also the resistance development (Churcher et al., 2008; Kryda et al., 2019; Panarese et al., 2020; Geary, 2023)

Thus, de novo genomic assembly of a *D. immitis* specimen retrieved from a case of canine cardiopulmonary dirofilariosis was performed using the ONT MinION platform followed by the study of macrocyclic resistance to lactones. The genome size is 87,899,012 bp with a total of 9741 proteins and the subsequent analysis of six loci previously showed that four presented a genotype associated with either some loss of efficacy or resistance phenotype. Genetic markers, previously described for resistance to MLs, were recently reassessed in a study, but now in a chromosomic context, thereby mapping single nucleotide polymorphisms previously associated with macrocyclic lactone resistance in the new genome assembly, revealing the physical linkage of high-priority variants on chromosome

3 (Gandasegui et al., 2024). Thus, it was possible for the first time the de novo assembly of the *D. immitis* genome from a complex nematode using long-read nanopore-based sequencing technology; simultaneously, information was provided which suggested the resistance of macrocyclic-lactone in northern Portugal, alerting to the possibility of misuse of MLs in this region which poses a potential risk to both veterinary and human public health.

Finally, in chapter IV, through the constant reading of the newly published literature on the topic, I have identified a scientific article in the prestigious journal Pulmonology (the official journal of the Portuguese Society of Pulmonology), which documented the presence of remnants of *D. immitis* by means of histopathological diagnosis in a lung nodule of a man. Subsequent to this publication, a letter to the editor was written to the aforementioned journal advising that histological features only allow the determination of *Dirofilaria* genus and that the species identification is quite more complex, requiring a PCR approach (Gabrielli et al., 2021). Moreover, there are confirmed cases of *Dirofilaria* spp. circulating in Portugal, namely *D. repens*, which has been reported to cause human pulmonary dirofilariosis nodules that can be mistakenly diagnosed as malignant (Maia et al., 2015).

Molecular confirmation in human samples demonstrates that the same parasites are currently present in dogs, people and vector insects in the study area proving that in a certain way they are in close contact (Fontes-Sousa et al., 2019; Mendoza- Roldan et al., 2021; Miterpáková et al., 2022). Fairly recently, a study conducted in humans confirmed the diagnosis of dirofilariosis through PCR in. tumor-like lesions extracted from the right lung (Kuthi et al., 2024). It should be pointed that human patients can be asymptomatic, on occasions though, they present clinical manifestations such as pulmonary nodular lesions which are erroneously diagnosed as neoplasias, leading to unnecessary, invasive procedures, such as cirurgical extraction, which may have severe side implications (Miterpáková et al., 2022). In order to circumvent this situation, new diagnostic approaches, such as sensitive and specific serologic tests, should be explored.

In short, the research work carried out demonstrates that the practice of veterinary medicine is not limited to the identification and analysis of clinical aspects related with the diagnosis and treatment of diseases. Veterinary science is much more comprehensive, it is based on the scientific method and requires constant monitoring of the evolution and dynamics of ecosystems, evaluating it as a whole so that the paradigm that establishes the pathology associated with vectors can be explored in the light of the One Health approach, that recognises and never neglects the interaction between people, animals and the environment that surrounds them.

Concluding Remarks

All the main objectives of this work were achieved. It contributed to a better understanding of dirofilariosis in Portugal, provided information on the fundamental aspects of the epidemiological distribution of *Dirofilaria* spp. in both domestic and wild canids, and demonstrated for the first time microfilaremia in wolves. This work has also shown that the use of molecular methods is becoming increasingly important, not only for new diagnostic approaches, but also for expanding knowledge about the spread of genetic variants responsible for resistance to treatments. In view of the high prevalence observed, it is important that animal owners are made aware of this problem and that veterinarians step up their efforts to implement effective prevention and control measures to minimise the risk of infection in both animals and humans, always taking into account the One Health approach.

Future Prospects

It is important to fully characterise the dirofilariosis situation throughout the country, not only in dogs but also in wild canids, both to gain knowledge in areas that have never been studied, and to update the information already available through the studies that have been conducted so far.

Given the crucial role that mosquitoes play in the spread of dirofilariosis and its volatile dynamics, it would be essential to conduct an in-depth study on the current status of existing mosquitoes.

Climate change is another significant factor affecting vector and parasite populations, and further studies are needed to improve our understanding of this impact.

Due to the paucity of information on cats and why they can be affected by dirofilariosis, a large-scale study would be highly recommended.

Since wild canids develop both antigenemia and microfilaremia, the question arises as to how preventive control and treatment programmes could be implemented not only to prevent the spread of the disease via vectors, but also to prevent these animals from developing clinical signs.

Given the number of cases of *Dirofilaria* spp. detected in human medicine and the fact that diagnosis is challenging, epidemiological, parasitological and clinical information as well as clinical case reports are needed to improve and standardise the clinical management of dirofilariosis in humans.

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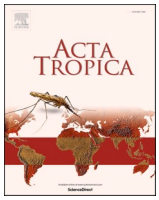

Assessment of the circulation of *Dirofilaria immitis* in dogs from northern Portugal through combined analysis of antigens, DNA and parasite forms in blood

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ABSTRACT

Dirofilariasis is a vector-borne disease frequent in many countries. Not only infected dogs, but also cats and wild canids (including wolves and foxes), represent important sources of infection for mosquitoes, which are the pathogen vectors. The disease is endemic in Mediterranean countries with increasing incidence in Italy, France, Greece and Spain, but limited epidemiological data is available from Portugal regarding its distribution and impact. Aiming to clarify this, canine whole blood samples ($n = 244$) from the north of Portugal were tested for *Dirofilaria* spp. antigens by use of a commercial rapid immunomigration test. Polymerase chain reaction (PCR) and the modified Knott test were also used to assess the presence of microfilariae. Results were also compared to assess the performance of each test used. Of the 244 animals tested, 118 (48.4%) were positive for *Dirofilaria immitis* (heartworm) in the serological adult worm rapid antigen detection test, and 36 (14.8%) had circulating microfilariae, identified as *D. immitis*. A combined positivity of 51.6% (126/244) was found. Results indicate that the risk of exposure to *D. immitis* in dogs is high in this region of Portugal, and that prophylaxis against the parasite is advisable to decrease the occurrence of canine infection and disease. The present study highlights the diagnostic value of serological and molecular tests in determining the prevalence of *D. immitis*.

1. Introduction

Cardiopulmonary dirofilariasis caused by heartworm *Dirofilaria immitis,* also known in dogs as canine heartworm disease, and subcutaneous dirofilariasis caused by *D. repens* are zoonoses whose agents have adapted to canine, feline and human hosts, with distinct biological and clinical implications (Dantas-Torres and Otranto, 2020; Capelli et al., 2018; Diakou et al., 2019; Bozidis et al., 2020). *Dirofilaria* spp. are transmitted by mosquitoes belonging to different genera of the family Culicidae (i.e., *Aedes, Anopheles, Culex* and *Ochlerotatus*) (Alho et al., 2014; Dantas-Torres and Otranto, 2020; Edgerton et al., 2020; Macchioni et al., 2020).

Dirofilaria immitis is a parasitic nematode that infects domestic and wild canids as well as other mammals, including humans. Cardiopulmonary dirofilariasis, the associated disease, typically occurs in temperate, tropical, and subtropical areas of the world, with the agent being transmitted by several mosquito species especially from the genera *Culex, Aedes*, and *Anopheles* (Barriga, 1982). These mosquitoes deposit infective larvae (stage L3) at the biting site on the host's skin. These larvae moult into the next stage (L4) 3–12 days post-infection (dpi), later moulting into preadult worms at 50–70 dpi, which migrate to the pulmonary artery and right ventricle (70–85 dpi), then reaching sexual maturity at 120 dpi. Female worms initiate the production of microfilariae (L1) 6-9 months post-infection, with adults potentially

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living more than 7 years and microfilariae up to 2 years (Simón et al., 2012).

Recently, the epidemiology and distribution dynamics of elements of the genus *Dirofilaria* has suffered modifications due to several factors, including the influence of climate change, human modification of environment, such as irrigating lands for farming, transboundary movements of animals and humans, and changes in the parasite-host relationship such as the outburst of new species of vector mosquitoes (Alho et al., 2014; Capelli et al., 2018; Fontes-Sousa et al., 2019; Esteban-Mendoza et al., 2020; Farkas et al., 2020). Several cases of pulmonary heartworm and ectopic infections have been reported in humans, despite the fact that these are considered as accidental hosts for *Dirofilaria* spp. (Capelli et al., 2018; Djaković et al., 2019; Bozidis et al., 2020; Esteban-Mendoza et al., 2020). The presence of *D. immitis* in dogs represents a risk for the human population (Morchón et al., 2012). Although cardiopulmonary dirofilariasis has a worldwide distribution, with considerable prevalence in the American continent, Australia and Japan (Dantas-Torres and Otranto, 2013; Esteban-Mendoza et al., 2020), the disease is also endemic in Mediterranean countries with increasing incidence in Italy, France, Greece and Spain, where the causative agent is considered one of the most widespread animal parasites (Diakou et al., 2019; Laidoudi et al., 2020; Macchioni et al., 2020; Mendoza-Roldan et al., 2021).

Worms live in the pulmonary arteries and right cardiac ventricle of domestic and wild canids. Infection may cause a wide variety of clinical signs, which go from cough to dyspnoea and exercise intolerance to severe vascular and pulmonary disease with hearth failure that may cause death (Alho et al., 2018). The zoonotic implication of these infections is important because, not long after the inoculation by the mosquitoes, there is the possibility of larval migration along human tissues (Gabrielli et al., 2021). This may provoke ocular, skin and also pulmonary nodular lesions which are frequently and wrongly diagnosed as pulmonary carcinomas (Alho et al., 2018; Fontes-Sousa et al., 2019; Gabrielli et al., 2021). Information about the prevalence and distribution of cardiopulmonary parasites is essential for the control of animal diseases and, in the case of *D. immitis*, for the control of potentially associated illnesses in humans. Some studies refer that the presence of higher seroprevalence of *D. immitis* might be due to the hiperendemic status of canine dirofilariasis (Fontes-Sousa et al., 2019; Esteban-Mendoza et al., 2020; Mendoza-Roldan et al., 2021). There are cases of lesions which were initially incorrectly identified has malignant neoplasm and therefore required invasive procedures prior to reaching the correct diagnosis (Gabrielli et al., 2021). Also, the absence of circulating microfilariae disables the use of parasite DNA detection in peripheral blood samples as a feasible tool for diagnosis, chiefly basing its identification in histopathological findings of extracted nodules (Gabrielli et al., 2021). For these reasons, understanding the distribution and occurrence of human cases of dirofilarioses is quite relevant in the differentiated diagnosis of subcutaneous and/or pulmonary nodules or other suspected clinical manifestations (Fontes-Sousa et al., 2019; Mendoza-Roldan et al., 2021).

Epidemiological data available in Portugal show that *D. immitis* is present in the country, but little information is known regarding its distribution impact and potential acquisition of new ecosystems (Alho et al., 2014, 2018; Maia et al., 2015; Ferreira et al 2017). Tests for the detection of antigens provide a convenient, sensitive and specific way to identify *D. immitis* infection in routine veterinary patients (Genchi et al., 2018; Henry et al., 2018; Laidoudi et al., 2020; Panarese et al., 2020), while the polymerase chain reaction (PCR) is a valuable tool for the screening and diagnosis of filarial infections in dogs, due to the rapid and accurate detection and differentiation of *Dirofilaria* spp. from other concomitant blood microfilariae, allowing species confirmation by specific primers or sequencing (Hou et al., 2011; Ferreira et al., 2017; OH et al., 2017; Little et al., 2018; Gomes-de-Sá et al., 2022a). Considering that the climate in Portugal is compatible with the development, reproduction and survival of the vectors, it is appropriate to define Portugal as a country in which canine dirofilariasis by *D. immitis* is endemic (Alho et al 2018). Caminha, located in the North of Portugal, with a population of about 16,000 human inhabitants, shares the same propitious climate as the rest of the country. Up to this moment there has been no study of dirofilariasis conducted in this municipality.

Wild animals can be sylvatic reservoirs for *D. immitis* by supporting the transmission of these parasites to domestic animals (Gomes-de-Sá et al., 2022b). At the country level, a study revealed a prevalence of 3.2% of *D. immitis* detected by necropsy in red foxes (*Vulpes vulpes*) (Eira et al., 2006); moreover, in a national serological survey conducted in red foxes in Portugal, 8.5% were positive for *D. immitis* (Alho et al., 2016). *Dirofilaria immitis* was also found in three Eurasian otters (*Lutra lutra*) in Portuguese natural habitats (Torres et al., 2004; Saraiva et al., 2013), afterwards in a collection of pinnipeds: common seals (*Phoca vitulina*), California sea lions (*Zalophus californianus*) and South African fur seals (*Arctocephalus pusillus pusillus*) in a Portuguese oceanographic park (Alho et al., 2017) and more recently antigenemia was detected with an occurrence of 15.8% in red foxes and also, for the first time, microfilaremia in Iberian wolves (*Canis lupus signatus*), which was revealed by PCR, in the National Park of Peneda Gerês (Gomes-de-Sá et al., 2022b). Epidemiological studies are important to follow the spread of canine heartworm disease and the availability of diagnostic tools options contributes greatly to the reduction of the number of severe clinical cases through timely drug treatment.

To add knowledge in Portugal, we assessed the presence of *Dirofilaria* spp. in dogs from the municipality of Caminha, northwest part of the country, through combined analysis of antigens, DNA and parasite forms in blood.

2. Materials and methods

Blood samples were collected from a total of 244 dogs from the municipality Caminha, located in the northern border of Portugal with Spain. Blood was taken at the request of a public kennel as part of the surveillance and control scheme for vector-borne diseases. Canine profiles, including sampling date, age, gender, breed and use of antiparasitic drugs, were recorded. In terms of age, the dogs were divided into three groups: [1–2], [3–6] and [7–11] years (Table 1). The sample consisted of 140 neutered females and 104 neutered males, all tested dogs were of mixed breed and none of the dogs had received antiparasitic drugs. Two milliliter of blood was taken from the cephalic vein and immediately transferred to a tube containing EDTA.

For the initial screening of *D. immitis* infection, RIM kits (Uranotest

Table 1
Prevalence of *Dirofilaria immitis* infection in dogs from the municipality of Caminha (northwestern Portugal) as determined by adult worm RIM, PCR and combined results.

Variable/ category	Dogs sampled (n)	Relative distribution (%)	% (n) antigen positivity	% (n) PCR positivity	% (n) combined positivity
Sex			$p = 0.642$	$p = 0.431$	$p = 0.784$
Female	140	57.4	50.0 (70)	12.9 (18)	53.6 (75)
Male	104	42.6	46.2 (48)	17.3 (18)	51.0 (53)
Age group (years)			$p = 0.026$	$p = 0.528$	$p = 0.014$
[1–2]	34	13.9	32.4 (11)	8.8 (3)	35.3 (12)[a]
[3–6]	83	34.0	42.7 (35)	14.5 (12)	47.0 (39)
[7–11]	127	52.0	55.9 (71)	16.5 (21)	60.6 (77)[a]
Total	244	100	48.4 (118)	14.8 (36)	51.6 (126)

PCR: polymerase chain reaction

[a] $p = 0.042$. Bonferroni's correction has been incorporated by multiplying a previously significant pairwise p value (0.014) by 3. Only statistically significant differences are shown for pairwise comparisons of age group categories (i.e. [1–2] and [7–11]).

Dirofilaria ®, Barcelona, Spain) were used, according to the manufacturer's instructions. To determine if the antigen-positive dogs were a source of *D. immitis* for mosquitoes, the dogs were tested by PCR and the modified Knott test. For DNA detection, nucleic acids were extracted from blood using the QIAamp® DNA Mini Kit (Qiagen, Valencia, CA, USA), using an automated QIAcube nucleic acid extractor (Qiagen GmbH, Germany). DNA was stored at -20 °C until further analysis. A negative extraction control was processed along with each batch of 12 samples. DNA specimens were initially screened for the presence of microfilariae by using a conventional PCR targeting the ITS2 region. Briefly, pan-filarial primer pair – DIDR-F1 and DIDR-R1 – amplifying products with distinct molecular weights were used to differentiate nine filarial species (Rishniw et al., 2006). For all reactions, a total of 5 µl of genomic DNA was added to 12.5 µl Xpert Fast Hotstart Mastermix (2X) with dye (KAPA Biosystems, Woburn, MA, USA; Grisp, Porto, Portugal), 5.5 µl of deionized sterile water and 1 µl (10 µM) of each of the primers in a 25 µl final volume of the reaction mixture. The reactions were carried out in an automatic DNA thermal cycler 100 (Bio-Rad Laboratories, Hercules, CA, USA), including negative and positive controls (extracted from an adult *D. immitis* female). The PCR amplification products were visualized by Xpert green (Grisp) fluorescence after electrophoresis in a 1.5% agarose gel at 100 V for 40 min. To confirm species identification, all amplicons of expected size were sequenced bidirectionally for genetic characterization. Briefly, amplicons were purified with GRS PCR & Gel Band Purification Kit (Grisp), and bidirectional sequencing was performed by Sanger method, using the respective primers. Sequences were manually corrected using the BioEdit Sequence Alignment Editor v 7.1.9 software package, version 2.1 (Ibis Biosciences, Carlsbad, CA, USA) and further analysis were performed by comparison with the sequences available in the NCBI (GenBank) nucleotide database (http://blast.ncbi.nlm.nih.gov/Blast). Phylogenetic analysis was performed using MEGA version 6.0 software (Tamura et al., 2013). The obtained consensus sequences in this study and representative sequences for *O. volvulus, D. immitis* and *D. repens* obtained from GenBank were used for the phylogenetic analysis. Maximum likelihood (ML) method was applied. The ML bootstrap values were estimated using 1000 replicates with Tamura 3-parameter as the correction model, estimated as the best substitution model by MEGA version 6.0 software. We deposited the 5.8S-ITS2-28S of *D. immitis* sequences recovered in this study in GenBank.

The modified Knott test was also performed on blood samples from the 118 dogs that were positive for *D. immitis* antigen, according to described methods (Magnis et al., 2013; Soares et al., 2022).

The chi-square test was used to compare seropositivity values between different categories of the same independent variables (i.e. sex and age group). Cohen's kappa coefficient (κ) measured agreement beyond chance between diagnostic test results from paired samples (i.e. from the same animal). A probability (p) value < 0.05 was regarded as statistically significant. Statistical analyzes were done with IBM® SPSS® Statistics 26.0® software.

3. Results

Of the 244 shelter dogs tested, 118 (48.4%) were positive for *D. immitis* based on the RIM for adult worms, and 36 (14.8%) were found to be positive by PCR to detect microfilaremia (Fig. 1). Moreover, the use of this latter test optimized the differentiation of *D. immitis* from eight other filarioids.

When comparing results positive by RIM and/or PCR, combined positivity showed to be significantly higher in dogs of the [7–11] year group (60.6%) when compared with those in the group of [1–2] years (35.3%) ($p = 0.042$; Table 1). The difference between the number of female (53.6%) and male (51.0%) dogs positive for *D. immitis* was not considerable.

Eighty-two of the 118 antigen-positive animals were not detected for *D. immitis* DNA, a circumstance which reveals 69.5% occult infections.

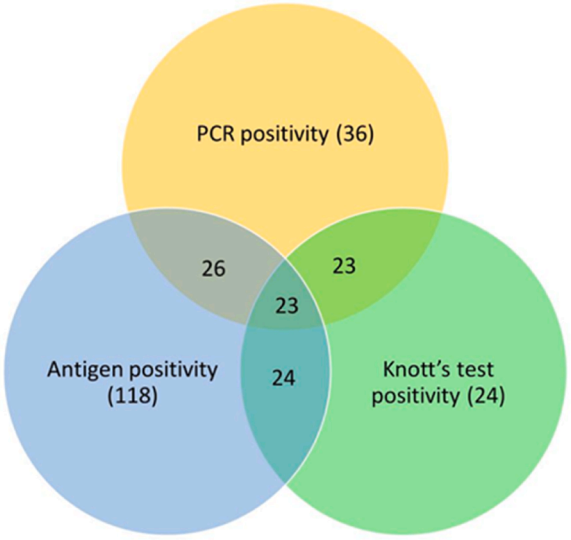

Fig. 1. Venn diagram on dirofilariasis diagnosis by RIM, PCR and Knott tests. Numbers in parentheses indicate the intersection positivity of tests.

On the other hand, 10 (7.9%) of the 126 antigen-negative samples yielded positive results for the presence of *D. immitis* microfilariae DNA.

Amplicon size assessment suggested that all detected microfilariae were from *D. immitis*. Bidirectional sequencing revealed 98.3% -100% identity with the *D. immitis* reference sequences (GenBank MN596213, KX932106, MW019916, KX932113, MN332198, KY863453, MF962487).

The following accession numbers were assigned to the sequences obtained in this work: OK632232-OK632250, OK632252-OK632268. Phylogenetic analysis based on the 460 nt partial region of the 5.8S-ITS2-28S regions showed that sequences OK632235, OK632240, OK632243, OK632250, OK632263 shared 99.44–100% identity between them and clustered with a *D. immitis* sequence retrieved from a mosquito in Turkey and a *D. immitis* sequence from a dog in Iran (Fig. 2). On the other hand, sequences OK632232- OK632234, OK632236-OK632239, OK632241-OK632242, OK632243-OK632249, OK632252-OK632262 and OK632264-OK632268 showed to be 90.91%-100% identical between them and clustering with a *D. immitis* sequence from a mosquito in Portugal and a *D. immitis* sequence from a Red Panda in China Fig. 2.

The decision of not using the Knott test in all the tested animals one of the limitations of this study. It was assumed that PCR would be more reliable for the detection of microfilaremia, because it allows differentiation from other filarial species and Knott test does not. As the RIM is aimed at detecting adult forms, Knott test would be used to detect microfilaremia in samples which presented antigenemia.

Microfilariae were detected in blood samples of 20.3% (24/118) dogs by the modified Knott's technique (Fig. 1). These 24 dogs were found to host microfilariae with characteristic morphology of *D. immitis*. The agreement between modified Knott and PCR results for the same 118 blood samples had a κ value of 0.879 ($p < 0.001$), which represents an almost perfect agreement beyond chance.

4. Discussion

From the total amount of 244 dogs, 118 tested positive for adult forms antigens, 36 showed microfilaremia through PCR, and all detected microfilariae were of *D. immitis*. Eighty-two (i.e. 118 minus 36) occult infections were identified and the largest percentage of infections were from the group of [7–11] years.

In the diagnosis of human dirofilariasis, indirect methods are not useful because L1, which is the stage mostly responsible for activating an

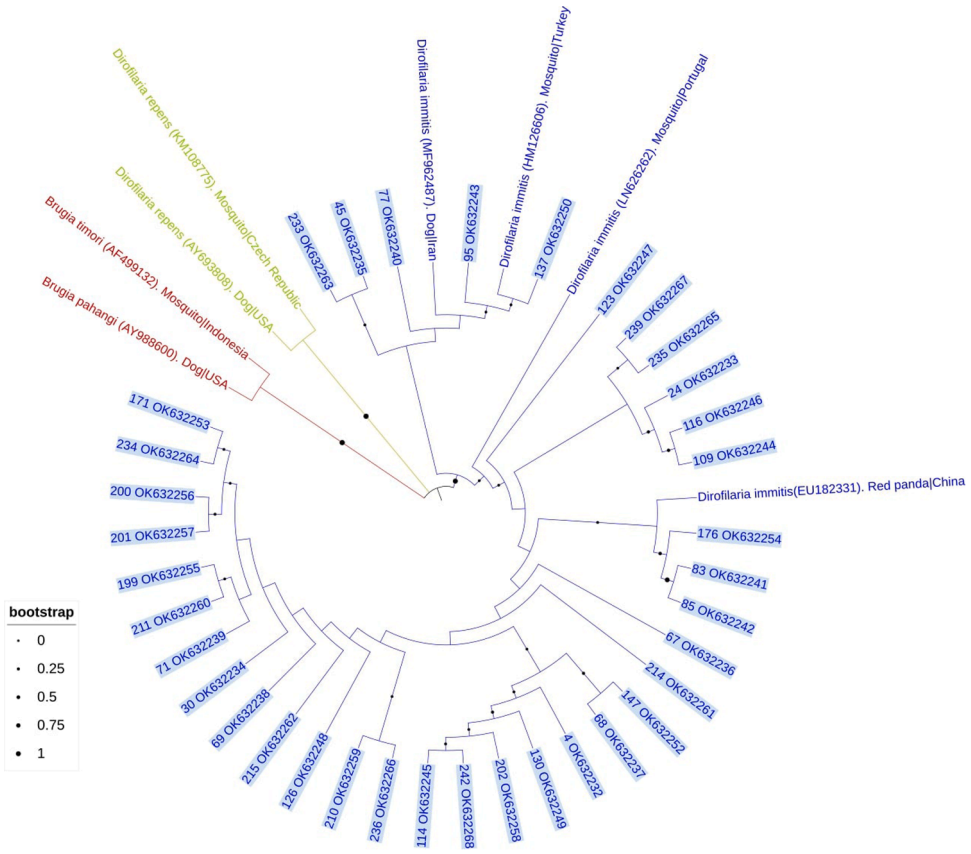

Fig. 2. Phylogenetic analysis of *Dirofilaria immitis* found in dogs in Portugal. The evolutionary history was inferred by using the ML method based on the Tamura et al. (2013) 3-parameter model.

immunological reaction in infections with filarial nematodes in humans, seldom appears in human dirofilariasis, and therefore the detection of antibodies against filariae as well as the utilization of DNA in peripheral blood samples of the majority of patients is not possible (Gabrielli et al., 2021). For that reason, the majority of reports of human dirofilariasis have been based on histopathological findings of extracted nodules, in which biopsy is considered the conclusive diagnostic method, even though its invasive nature could be a limitation. For a biopsy, and only when the parasite extraction is possible, the methods of molecular diagnosis, based on direct sequencing of the parasites' DNA, play a fundamental role in the identification of the etiological agent involved (Fontes-Sousa et al., 2019; Gabrielli et al., 2021).

The examination of microscopic morphological features of human dirofilariasis should surely be taken into consideration for solitary nodules of uncertain nature in subcutaneous tissues or mucous membranes, especially when patients live in areas where high infection prevalence in dogs is reported (Gabrielli et al., 2021). In order to avoid the use of invasive diagnostic means, in-house tests – i.e. non-invasive test currently available – would be an adequate option in defining the nature of the pulmonary cysts, so that the presence of antibodies to *D. immitis* and its symbiotic bacteria *Wolbachia* spp. could be detected (Fontes-Sousa et al., 2019).

In the present work, a RIM test was used to detect adult worms, and 118 positive animals were found positive from a sample of 244 shelter dogs. According to the manufacturer's information, this test consists of an immunochromatographic technique aiming at the qualitative detection of *D. immitis* antigen in blood, based on a 14 kDa antigen detection not exclusively related to the feminine genital apparatus, infections with a load of only one adult parasite of any type (males, adult females or immature females), with sensitivity and specificity of 94% and 100%, respectively, when compared to necropsy data. This method is known to be sensitive for screening a population of apparently healthy dogs or for confirming a clinically suspected *D. immitis* infection presenting a sensitivity greater than 90% in dogs infected with one adult female worm and 100% in dogs infected with more than one adult female (Genchi et al., 2018; Henry et al., 2018; Laidoudi et al., 2020; Panarese et al., 2020). Although these are highly specific and sensitive for *D. immitis*, recent work has demonstrated limitations in specificity due to cross-reactions with *D. repens* or *Angiostrongylus vasorum* (Schnyder and Deplazes, 2012; Venco et al., 2017; Alho et al., 2018). In these cases, diagnosis depends on examining the microfilariae, usually applying a modified Knott test and PCR. In the present work, the RIM test detected 89 amicrofilaremic infections (75.4%), because only 29 of the 118 antigen-positive animals had microfilaremia as detected by PCR. These results demonstrate that RIM also make it possible to identify amicrofilaremic infections. Contrarily to our study, heartworm antigens were detected by Borthakur et al. (2016) in 141 dogs, but with only 32 (22.7%) being considered as occult infections, because 109 animals with *D. immitis* microfilariae were detected by PCR.

The absence of circulating microfilariae depends on the prepatent period of the parasite (about 7 months) and on the low concentration of microfilariae in the samples (Panarese et al., 2020). Twelve months after infection, due to the development of an immune response or the aging of adult females in the absence of reinfections, amicrofilaremia can also occur (Panarese et al., 2020). This circumstance can lead to false negative results and undiagnosed occult *D. immitis* infections (Genchi et al., 2018; Miterpáková et al., 2018). In the present study, 10 animals despite being negative for the rapid antigen detection test, were PCR positive. This can be found when the parasite load of adult females of *D. immitis* is low, if there is formation of antigen-antibody complexes or even if there is persistence of microfilariae after death of the adult forms (Velasquez et al., 2014; Little et al., 2018; Panarese et al., 2020).

Antigenemia can be suppressed up to about 9 months post-infection in infected dogs receiving chemoprophylaxis based on macrocyclic lactones. If the test is performed before the end of the pre-patent period (7 months), the possibility of false negatives also increases (Little et al., 2018; AHS, 2020).

Several studies have demonstrated that molecular methods can be a highly sensitive and specific analytical tool for the simultaneous diagnosis and characterization of infections, providing more reliable data when compared to serological and parasitological methods (Hou et al., 2011; Ferreira et al., 2017; Mircean et al., 2017; Little et al., 2018). The modified Knott test allows the detection and differentiation of *D. immitis* from other filarial species (Magnis et al., 2013; Genchi et al., 2018; Moreira et al., 2019). There are also cases in which incorrect identification of microfilariae occurs, namely when there is a high concentration of *Dirofilaria* spp. or *Acanthocheilonema* spp. in a given area, failure in fixation techniques, variation of reference values and relying on specialist training to accurately differentiate the filariae (Rishniw et al., 2006; Magnis et al., 2013; Little et al., 2018). The PCR test was chosen for the detection of circulating microfilariae and, of the 244 tested, 36 dogs were considered positive for *D. immitis*. Twenty-six samples (22.0%) from 118 *D. immitis* antigen positive dogs were considered positive for *D. immitis* microfilariae DNA. Subsequently, sensitivity of PCR was compared with that of the modified Knott test, revealing 24 animals with microfilaremia. In the present study, there were no significant differences between parasitological and molecular tests. Similar results were obtained in studies in which the modified Knott test was considered effective, sensitive and compatible with PCR (Ferreira et al., 2017; Soares et al., 2022). In order to obtain accurate results, a combination of several diagnostic methods should be used, because although microfilaremia does not necessarily compromise the health of an infected patient, this can still act as a reservoir for further infection of other dogs if the intermediate mosquito host is present.

Although filarial species (e.g. *D. immitis, D. repens, A. reconditum, A. dracunculoides,* can be distinguished using molecular tools (Rishniw et al., 2006), in the present study species other than *D. immitis* were not detected both in modified Knott or PCR tests. The same happened in studies in which no other filarial species were detected, probably due to the low pressure of suitable vectors in the environment (Ferreira et al., 2017, Panarese et al., 2020).

In the present study, the difference between the number of female and male dogs positive for *D. immitis* was not considerable, which is in agreement with other studies that showed that *D. immitis* infection related to the sex of the canids was not statistically significant (Hou et al., 2011; Anvari et al., 2019; Miterpáková et al., 2018; Esteban-Mendoza et al., 2020). The present study showed a significantly higher prevalence (combined positivity) in older dogs than in younger ones. Similar results were obtained by other authors (Hou et al., 2011; Ferreira et al., 2017; Anvari et al., 2019; Miterpáková et al 2018). The gradual increase of infection associated with increasing age of dogs can be explained by the cumulative probability of exposure to mosquito bites in older animals, prolonged proliferation of microfilariae in the blood, and insufficient immunity against adult parasites (Hou et al., 2011; Anvari et al., 2019).

These results suggest that the risk of exposure to *D. immitis* in dogs is high in Caminha and this can be explained by the fact that all animals tested had access to outdoors and, therefore, had a greater degree of exposure to vectors. It should also be noted that they lived in areas close to water sources and none of the animals tested were subjected to any prophylactic measure. Prophylaxis against the parasite is advisable to reduce the occurrence of infection and canine disease, so the application of ectoparasiticides and repellents is essential.

The role of wolves and foxes as *D. immitis* reservoir hosts and their contribution to disease transmission are acknowledged throughout Europe and therefore, understanding infection and disease prevalence in wild canids is especially important because these may act as reservoirs, thus increasing the risk of infection for domestic pets, including urban canids (Morchón et al., 2012; Otranto and Deplazes, 2019; Pluemer et al., 2019; Gomes-de-Sá et al., 2022b). While in endemic areas regular chemoprophylactic treatments of domestic dogs reduce the general prevalence of the infection, wild canids are likely to play a decisive role in the maintenance of infection (Otranto and Deplazes, 2019), recently a study assessed *D. immitis* occurrence in a population of Portuguese wolves and foxes by searching for *D. immitis* antigens and microfilaremia and confirmed the existence of antigenemia in these wild animals, with microfilaremia being detected for the first time in wolves (Gomes-de-Sá et al., 2022b).

5. Conclusion

The present study confirms a substantial circulation of filarioid parasites in stray and shelter dogs of Caminha, highlighting the diagnostic value of serological tests and identification of microfilariae of *D. immitis* by PCR. Considering that *D. immitis* is a zoonotic parasite, the application of ectoparasiticides and repellents is highly recommended for dogs in this region. Systematic monitoring studies are required to better understand the environmental risk factors and to identify the competent mosquito vectors in the local epidemiology of dirofilariasis

Ethics approval and consent to participate

All procedures complied with the Portuguese legislation for the protection of animals used for scientific purposes (i.e. Decree-Law no. 113/2013 of 7 August 2013), which transposes European legislation (i.e. Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010).

CRediT authorship contribution statement

Sónia Gomes-de-Sá: Writing – review & editing, Data curation, Formal analysis, Methodology, Resources, Writing – original draft. **Sérgio Santos-Silva:** Methodology, Data curation, Formal analysis. **Alícia de Sousa Moreira:** Methodology, Data curation, Formal analysis. **Patrícia Ferreira Barradas:** . **Irina Amorim:** Methodology, Data curation, Formal analysis. **Luís Cardoso:** Methodology, Data curation, Formal analysis. **João R. Mesquita:** Data curation, Formal analysis, Methodology, Resources, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The funders had no role in the design of the study; in the collection, analyzes, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

Data availability

Data will be made available on request.

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Parasites & Vectors

SHORT REPORT Open Access

Dirofilaria immitis antigenemia and microfilaremia in Iberian wolves and red foxes from Portugal

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Abstract

Background: *Dirofilaria immitis* is a parasitic nematode endemic in the Mediterranean countries, which causes cardiopulmonary dirofilariosis in wild and domestic animals. Despite being recognized hosts of *D. immitis*, wild carnivores such as wolves and foxes are frequently disregarded when considering a potential role in the transmission of these zoonotic nematodes. In Portugal, studies available regarding *D. immitis* circulation are scarce, likely underestimating its relevance. To add knowledge on this, we sought to assess Iberian wolves (*Canis lupus signatus*) and red foxes (*Vulpes vulpes*) from northern Portugal for *D. immitis* antigenemia and microfilaremia.

Methods: Blood samples from 42 Iberian wolves and 19 red foxes were collected, during 2010–2012, in Peneda-Gerês National Park. Antigenemia was searched for by rapid antigen detection test kits (Uranotest Dirofilaria ®). Microfilaremia was assessed by polymerase chain reaction (PCR). Nucleic acids were extracted from blood using QIAamp® DNA Mini Kit (Qiagen), and DNA was screened for the presence of microfilaria using a conventional PCR targeting the 5.8S-internal transcribed spacer 2–28S regions, followed by bidirectional sequencing, Basic Local Alignment Search Tool analysis and phylogenetic analysis.

Results: Three red foxes had antigenemia, with an occurrence of 15.8% (95% confidence interval [CI] 3.4–39.6), while showing no evidence for the presence of microfilaremia. No wolf samples presented evidence for *D. immitis* antigenemia. Nevertheless, two wolves were positive for *D. immitis* microfilaremia (4.8%; 95% CI 0.6–16.2%) as revealed by PCR and confirmed by bidirectional sequencing.

Conclusions: Although *Dirofilaria* microfilaremia in wolves does not necessarily correlate to an endangerment of the infected animal's health, positive individuals can act as a reservoir for further infection if the intermediate mosquito hosts are present. To the best of our knowledge, one single study had reported that wolves were suitable *Dirofilaria* hosts, but microfilaremia have never been reported.

Keywords: *Dirofilaria immitis*, Foxes, Wolves, Portugal, Wildlife

Background

Dirofilaria immitis (Rhabditida; Onchocercidae) is a parasitic nematode, which causes cardiopulmonary dirofilariasis in wild and domestic canines and felines, and also pulmonary dirofilariasis in humans [1]. Mosquitoes (Diptera; Culicidae) are vectors to *Dirofilaria* spp., making the parasite distribution susceptible to changes, as

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well as to rapid and significant variations in defined geographic regions, such as movement of infected animals, the introduction of new mosquito species and anthropogenic activities in ecosystems [2, 3].

Dirofilariosis is endemic in the Mediterranean countries, including Portugal, particularly because of the appropriate geographic and climatic conditions [4]. The climate in Portugal is typically temperate with warm and dry summers. It is considered to be divided in subtypes, namely the first with hot summers and average temperatures > 22 °C in the warmest months and the second with warm summers with average temperatures ≤ 22 °C in the warmest months and with ≥ 4 months with the average temperatures > 10 °C [5].

Wildlife carnivores have long been overlooked when considering their possible role in transmitting zoonotic nematodes [6]. However, increasing human activities have continuously promoted wild environment invasion, ultimately redefining domestic and wild interface boundaries and consequently increasing the contact between humans and wild animals [6]. Moreover, wild animals are frequently exposed to vector-borne pathogens to such an extent that wild carnivores like the gray wolf (*Canis lupus*), red fox (*Vulpes vulpes*) and golden jackal (*Canis aureus*) are now recognized hosts of *D. immitis* [7].

Wolves and foxes' role as *D. immitis* reservoir hosts and their contribution to disease transmission are acknowledged throughout Europe [8]. However, in Portugal, little information is known regarding *D. immitis* circulation, and the studies available are limited to a few surveys and case reports, possibly underestimating the relevance of these nematodes [3]. Moreover, the extent to which wild carnivores remain reservoirs for *D. immitis* is still unknown. As such, the aim of the present investigation was to assess the presence of *D. immitis* in wolves and foxes in Portugal by searching for both *D. immitis* antigens, using a rapid immunomigration test (RIM), and for *D. immitis* microfilaremia, by using a species-specific polymerase chain reaction (PCR) assay optimized for the simultaneous detection and differentiation of *D. immitis* and eight other concurrent filarioids.

Methods

Blood samples were collected from a total of 61 wild carnivores, 42 wolves (*C. lupus signatus*) and 19 foxes (*V. vulpes*), during 2010–2012, from a National Park located at the northern border of Portugal (Peneda-Gerês National Park). Samples from these animals were collected as part of a carnivore protection program, an initiative that manages the collection of recently deceased animals suspected to be poisoned. Tissues and other matrices are available for studies considered to be of value for the assessment of animal population morbidities and mortalities.

For the initial detection of *D. immitis* infection, rapid antigen detection test kits (Uranotest Dirofilaria ®, Barcelona, Spain) were used, according to the manufacturer's instructions. This test consists of an immunochromatographic technique aiming at the qualitative detection of the *D. immitis* antigen in blood, which resources to 14 kDa antigen detection not related to the parasite's feminine genital apparatus, hence detecting both male and female parasites. The platform detects infections with a load of only one adult parasite of any type (males, adult females, immature females), with sensitivity and specificity of 94% and 100%, respectively, compared to necropsy, according to the manufacturer's information.

To detect microfilaremia, DNA was extracted from blood using the QIAamp® DNA Mini Kit (Qiagen, Valencia, CA, USA), using an automated QIAcube nucleic acid extractor (Qiagen GmbH, Hilden, Germany). DNA was stored at − 20 °C until further analysis. A negative extraction control was processed along with each batch of 12 samples. DNA specimens were initially screened for the presence of microfilaria by using a conventional PCR targeting the 5.8S-internal transcribed spacer (ITS) 2–28S regions of the genome. Briefly, pan-filarial primer pair − DIDR-F1 and DIDR-R1 − amplifying products with distinct molecular weights were used to differentiate nine filarial species, namely *Acanthocheilonema dracunculoides* (584 base pairs [bp]), *Acanthocheilonema reconditum* (578 bp), *Brugia malayi* (615 bp), *Brugia pahangi* (664 bp), *Brugia timori* (625 bp), *Dirofilaria immitis* (542 bp), *Dirofilaria repens* (484 bp), *Mansonella ozzardi* (430 bp) and *Onchocerca volvulus* (470 bp). For all reactions, a total of 5 µl of genomic DNA was added to 12.5 µl Xpert Fast Hotstart Mastermix (2×) with dye (GRiSP, Porto, Portugal), 5.5 µl of deionized sterile water and 1 µl (10 µM) of each of the DIDR-F1 and DIDR-R1 primers in a 25-µl final volume of the reaction mixture. The reactions were carried out in an automatic DNA thermal cycler 100 (Bio-Rad Laboratories, Hercules, CA, USA), including negative and positive controls (extracted from an adult female *D. immitis*). The PCR amplification products were visualized by Xpert Green DNA Stain direct (GRiSP, Porto, Portugal) fluorescence after electrophoresis in a 1.5% agarose gel at 100 V for 40 min. To confirm species identification, all amplicons of expected size were sequenced bidirectionally for genetic characterization. Briefly, amplicons were purified with GRS PCR & Gel Band Purification Kit (GRiSP, Porto, Portugal), and bidirectional sequencing was performed by Sanger method, using the respective primers. Sequences were manually corrected using the BioEdit

Sequence Alignment Editor v 7.1.9 software package, version 2.1 (Ibis Biosciences, Carlsbad, CA, USA), and further analyses were performed by comparison with the sequences available in the NCBI (GenBank) nucleotide database (http://blast.ncbi.nlm.nih.gov/Blast). Phylogenetic analysis was performed using MEGA version 6.0 software [9]. The obtained consensus sequences in this study and representative sequences for *O. volvulus*, *D. immitis* and *D. repens* obtained from GenBank were used for the phylogenetic analysis. Maximum likelihood (ML) method was applied. The ML bootstrap values were estimated using 1000 replicates with Tamura 3-parameter as the correction model, estimated as the best substitution model by MEGA version 6.0 software. We deposited the 5.8S-ITS2-28S of *D. immitis* sequences recovered in this study in GenBank.

Results and discussion

For the initial detection of *D. immitis* infection, out of the 61 wild carnivores screened by rapid antigen detection, three had *D. immitis* antigen circulation in blood (4.9%; 95% confidence interval [CI] 1.0 − 13.7). All three wild carnivores showing antigenemia were foxes, yielding an occurrence of 15.8% (95% CI 3.4−39.6). The same 61 animals were again tested for microfilaremia through conventional PCR, and only two wolves (1.6%; 95% CI 0.0−8.8) tested positive, with a prevalence in the whole sampled lupine population of 4.8% (95% CI 0.6 − 16.2). Both positive samples yielded amplicons with 542 bp, being presumptively positive for *D. immitis.* After bidirectional sequencing, the consensus sequences showed to be identical. Basic Local Alignment Search Tool confirmed the identity (100%) of *D. immitis* in both lupine samples. The following accession numbers were assigned to the sequences obtained in this work: OK632269 and OK632270. One of the sequences obtained (OK632270) spanned only 77 nucleotides (nt); hence, phylogenetic analysis was performed with the other sequence. Phylogenetic analysis based on the 460 nt partial region of the 5.8S-ITS2-28S regions showed clustering with *D. immitis* (Fig. 1).

The present study evaluated *D. immitis* occurrence in a population of Portuguese wolves and foxes by searching for both *D. immitis* antigens and *D. immitis* microfilaremia, using a RIM test and a species-specific PCR assay followed by sequence confirmation, respectively. *Dirofilaria immitis* is usually detected by specific antigen testing and/or identification of microfilariae [1]. Despite the negative heartworm antigen test result, the infected animals may still show microfilaremia in the blood [1]. In Europe, eight species of filarioids, including zoonotic species, have been reported mainly in domestic dogs, and occasionally in wild carnivores [10]. Species discrimination is of high clinical and epidemiological importance because of zoonotic concerns and therapeutic implications [1]. The application of molecular analysis targeting filarial DNA is a highly sensitive and specific analytical tool for the diagnosis and simultaneous characterization of filarial infections, thus being an extremely valuable approach [1].

In this study, none of the 42 wolf samples presented evidence for *D. immitis* antigenemia. Nevertheless, two wolves were positive for *D. immitis* microfilaremia by PCR (4.8%; 95% CI 0.6–16.2%). Although *Dirofilaria* microfilaremia does not necessarily correlate to an endangerment of the infected animal's health, the individual can act as a reservoir for further infection if the intermediate mosquito host is present [1]. To the best of our knowledge, only one study has reported that wolves were suitable *Dirofilaria* hosts and appeared exposed to infection similarly to sympatric unprotected dogs [7]. Until today, microfilaremia had never been found, thus hampering the assessment of the impact of wolves on infection maintenance.

Interestingly, the present study shows that none of the foxes presented microfilaremia, but three were positive for *D. immitis* antigenemia (15.8%; 95% CI 3.4–39.6). Variable occurrences have been reported, namely 6.4% in Australia [11], 32.3% in Spain [12], 1.6% in Serbia [13], 3.7% in Hungary [14], 25.2% in Bulgaria [15], 0.3% in Romania [10] and 2% in France [16]. In Portugal, the prevalence of *D. immitis* detected by necropsy of red foxes ranged from 3.2% in northern-central locations, such as Coimbra district [17], to 11.8% in southern and central districts, such as Santarém and Setúbal [18]. In a serological survey conducted in red foxes in Portugal, 8.5% (10/118) were positive for *D. immitis* circulating antigens, with positive animals found in northern and southern areas [3]. It should be noted that the lack of microfilaremia can be related to several factors, including unisexual infections, pre-patency or the host's immune response leading to the elimination of microfilariae. Comparisons between studies should be made with caution as different assays with distinct sensitivities/specificities were used.

Conclusions

The present study provides molecular and serological evidence for *D. immitis* infection in wild carnivore species present in Portugal, supporting their potential epidemiological role. While in endemic areas frequent chemoprophylactic treatments of domestic dogs reduce the overall prevalence of the infection, wild canids

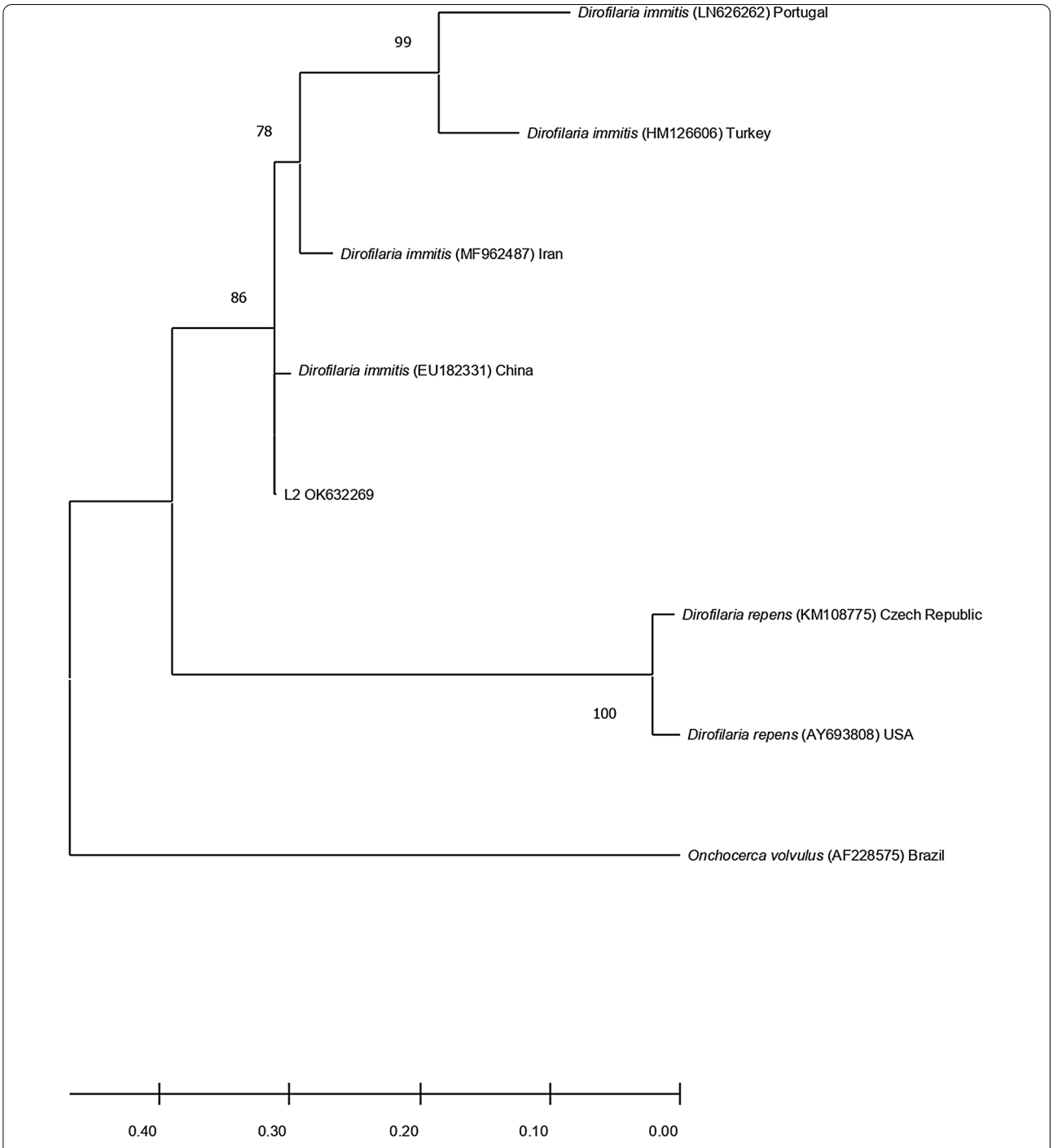

Fig. 1 Phylogenetic analysis of *D. immitis* found in wolves in Portugal. The evolutionary history was inferred by using the ML method based on the Tamura 3-parameter model. The analysis involved eight sequences. The *D. immitis* sequence characterized in this study is represented by sample code (L2) followed by the GenBank accession number (OK632269). Other sequences are represented with the species name followed by the corresponding accession number and country. Branch lengths are measured as the number of substitutions per site. Reliability of internal branches was assessed using the bootstrapping method (1000 replicates)

might play a crucial role in the maintenance of infection. Understanding infection and disease prevalence in wild canids is especially important because these may act as reservoirs, increasing the risk of infection for domestic pets, including urban canids [19]. Infected microfilaremic carnivores may, in the presence of competent

vector species, also act as reservoir hosts. To the best of our knowledge, this is the first detection of *D. immitis* microfilaremia in wolves, supporting that these animals can have a role as *D. immitis* reservoirs.

Abbreviations
bp: Base pairs; CI: Confidence interval; ITS: Internal transcribed spacer; ML: Maximum likelihood; nt: Nucleotide(s); PCR: Polymerase chain reaction; RIM: Rapid immunomigration.

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Authors' contributions
SGdS: conducted sample collection and testing and drafted the manuscript; SSS: conducted testing and data analysis; AdSM: conducted testing and data analysis; PFB, IA: supervised the study, conducted data analysis and drafted the manuscript; LC: supervised the study and interpreted data; JRM: supervised the study, designed the work and interpreted data. All authors have read and approved the final manuscript.

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Availability of data and materials
Data supporting the conclusions of this article are included within the article.

Declarations

Ethics approval and consent to participate
All procedures complied with the Portuguese legislation for the protection of animals used for scientific purposes (i.e. Decree-Law no. 113/2013 of 7 August 2013), which transposes European legislation (i.e. Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010).

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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Publisher's Note

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Communication

De Novo Assembly of the *Dirofilaria immitis* Genome by Long-Read Nanopore-Based Sequencing Technology on an Adult Worm from a Canine Cardiopulmonary Dirofilariosis Case

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Simple Summary: *Dirofilaria immitis* is a zoonotic parasite that infects canids and other vertebrates. We expanded the use of long-read nanopore-based sequencing technology by performing genomic de novo assembly of a *D. immitis* specimen retrieved from a canine cardiopulmonary dirofilariasis case by using the ONT MinION platform. We also identified loci previously characterized as being associated to macrocyclic lactone resistance selection pressure. The identification of a resistant zoonotic parasite alerts for the overuse of macrocyclic lactone in the region.

Abstract: *Dirofilaria immitis* is a zoonotic parasitic nematode that infects domestic and wild canids, among its vertebrate hosts. The genetic analysis of *D. immitis* nowadays transcends the need for genetic taxonomy of nematodes, such as the study of resistance to macrocyclic lactone. We expanded the use of long-read nanopore-based sequencing technology on nematodes by performing genomic de novo assembly of a *D. immitis* specimen retrieved from a canine cardiopulmonary dirofilariasis case using the ONT MinION platform, followed by the study of macrocyclic lactone resistance. The assembled genome of *D. immitis* consists of 110 contigs with an N50 of 3687191. The genome size is 87899012 and contains a total of 9741 proteins; 6 ribosomal RNAs, with three belonging to the small subunit (18S) and three to the large subunit (28S); and 73 tRNAs. Subsequent analysis of six loci previously characterized as being associated to macrocyclic lactone resistance selection pressure showed that four have a genotype associated with either some loss of efficacy or the resistance phenotype. Considering the zoonotic potential of *D. immitis*, the identification of a resistant parasite alerts for the overuse of macrocyclic lactone in the region, which poses a potential risk to both veterinary and human public health.

Keywords: *Dirofilaria immitis*; genome; macrocyclic lactone resistance; long-read

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1. Introduction

Dirofilaria immitis is a parasitic nematode that infects domestic and wild canids as well as other animals, including humans. The associated disease typically occurs in temperate, tropical, and subtropical areas of the world, with the agent being transmitted by several mosquito species such as those belonging to the *Culex*, *Aedes*, and *Anopheles* genera [1]. These mosquitoes deposit infective stage larvae (L3) at the biting site, which penetrate the host's skin. The L3 molt into L4 3–12 days post-infection (dpi), later molting into preadult worms 50–70 dpi, which migrate to the pulmonary artery and right ventricle 70–85 dpi, reaching sexual maturity 120 dpi [2]. Females initiate the production of microfilariae (first larval stage) 6–9 months post-infection, with adults living more than 7 years and microfilariae living up to 2 years [2].

Dirofilaria immitis worms can cause canine cardiopulmonary dirofilariasis (also known as heartworm disease in dogs), a widespread disease that can have a fatal outcome if animals are not treated [3]. Moreover, *D. immitis* in dogs represents a risk for the human population, who may suffer from pulmonary dirofilariasis and, in many cases, pulmonary nodules that can be misdiagnosed as malignant tumors [4].

Morphological analysis is commonly used for the differentiation of nematode species, but not without its drawbacks such as scarce distinguishable characters, a circumstance that may hamper their classification to the species or even genus level [5]. This is not necessarily the case for *D. immitis*, which can easily be distinguished from other filarioids, taking into account the cardiac location of adults. Nonetheless, genetic analysis nowadays transcends the need for genetic taxonomy of nematodes. An example is the requirement for ascertaining resistance to macrocyclic lactones, known to have a genetic origin [6]. Macrocyclic lactones such as milbemycin oxime, ivermectin, moxidectin, and selamectin are widely available drugs that are used to prevent the establishment of the L3–L4 *D. immitis* stages in dogs and cats. However, loss of efficacy has been described since 2005, and whole-genome analysis has been performed on *D. immitis* isolates to characterize their genetic profile and differences that could potentially be associated with evident loss of efficacy and resistance [6].

Nematode characterization based on markers such as the internal transcribed spacer (ITS) regions of the ribosomal RNA locus, the 28S large subunit ribosomal RNA gene (28S LSU rRNA), the 18S small subunit ribosomal RNA gene (18S SSU rRNA), and the cytochrome oxidase I gene (coi) followed by Sanger sequencing is a widespread, low-cost approach to ascertain genetic profiles [5]. However, the data generated are limited, and novel sequencing strategies such as single-molecule real-time sequencing or third-generation sequencing have reduced the expenses and the necessary hardware for obtaining thorough data on highly contiguous genome assemblies, permitting comprehensive, near-real-time biomonitoring of samples [7,8].

The aim of the present work was to expand the use of long-read nanopore-based sequencing technology on nematodes by performing genomic de novo assembly of a *D. immitis* specimen retrieved from a canine cardiopulmonary dirofilariasis case using the ONT MinION platform.

2. Materials and Methods

2.1. Extraction of Parasite DNA

Adult *D. immitis* worms (n = 32) were observed at routine parasitological investigation in the right heart and pulmonary artery of a dog that died from an unrelated cause in the municipality of Caminha, northern Portugal, November 2020. The city of Caminha is located by the mouth of the Minho River (circa 340 km in length), under a wet Atlantic climate, being the area of the country with the highest precipitation, reaching around 1800 mm on average and peaking at more than 3500 mm [9]. The dog hosting the *D. immitis* worms was kept in a municipal kennel since birth and had not received any macrocyclic lactone treatment. No ethics permission was obtained since the parasites were taken during regular post-mortem evaluation. One nematode was selected, washed three times

in phosphate-buffered saline at pH 7.2, frozen at −80 °C, and subjected to mechanical disruption with a disposable pestle. Nucleic acid extraction followed the previously described procedures [7]. Briefly, the homogenate was incubated with 20 µL proteinase K (Qiagen, Hilden, Germany) and 180 µL of Buffer ATL (Qiagen) for 48 h at 56 °C, with vortexing (200 rpm) in a thermoblock (Eppendorf Epp Thermomixer; Hamburg, Germany). High-molecular-weight (100–200 kb) DNA was then extracted using a magnetic-bead-based protocol (MagAttract HMW DNA kit; Qiagen) as described by the manufacturer. Eluted DNA (on 100 µL of 10 mM tris-HCL) was evaluated for size distribution on an agarose (0.8%) gel. DNA was assessed on a Nanodrop spectrophotometer (ThermoFisher, Waltham, MA, USA). Genomic DNA size selection was then performed using a 0.4× volume of AmpureXP beads (Beckman Coulter, Brea, CA, USA) in order to remove smaller fragments.

2.2. Library Preparation and Sequencing

The 1D genomic ligation (SQK-LSK109) library preparation kit (ONT, Oxford, UK) was used imputing 1.2 µg of extracted genomic DNA, and libraries were then developed as instructed by the manufacturer with a calculated final library quantity assessment at 467 ng. Then, 79.3 ng was loaded onto the MinION sequencer using an R9.4.1 flow cell managed by the MinKNOW software (version 18.12.9, ONT). The ONT MinION Mk1B platform was used with active channel selection performed at every 1.5 h, resourcing to no script modifications. Refueling of the flow cell was performed 24 h after initiation by first extracting excess liquid from the waste chamber, followed by the addition of 37.5 µL of SQB and 37.5 µL of H2O on the SpotON sample port. An additional 24-hour run was then performed.

2.3. Base-Calling, Genome Assembly, and Read Alignments

After completion of the sequencing run, Guppy (version 2.3.5, ONT) was used for base-calling signal data (.fast5 files), with the generated fastq files used to generate statistics with NanoPlot (version 1.19.0). Raw reads were processed using Porechop software (version 0.2.4) with the default parameters to trim sequences from all known Oxford Nanopore adapters. After trimming, all reads were used to perform a complete genome assembly using Flye software (version 2.8.3) with the parameters "-nano-raw" to specify the input type data and an expected genome size of 100 Mb. Contigs obtained at the assembly step were processed using Kraken2 to locate potential contaminants in the sample. Some contigs were identified as belonging to *Canis lupus*, *Homo sapiens*, or *Wolbachia* and therefore removed from the dataset for further analyses. Contigs potentially belonging to *D. immitis* were corrected by mapping the raw reads against the assembled contigs through the Pilon software (version 1.24). Confirmation of the species was double-checked by using the small subunit of the ribosomal RNA (18S) against the NR database using NCBI blast (E-value of 0.0 and 98.91% identity with accession AB973231.1) and blast against the SILVA SSU database, where the best match was again AB973231.1 belonging to *D. immitis*. The corrected contigs were compared against the three available *D. immitis* genomes at NCBI (GCA_009829315.1, GCA_001077395.1, and GCA_013365355.1) to verify the integrity of our assembly. The completeness of the assembly was verified through BUSCO software (version 4) using the nematode database. Final contigs were annotated in order to locate coding regions using Genemark-ES software (version 4.65) with self-training and default parameters. Non-coding regions belonging to rRNAs and tRNAs were also located with RNAmmer software (version 1.2) and tRNAscan-SE (version 2.0), respectively. Functional annotation for the coding regions was performed using Sma3s (version 2), retrieving GO terms and EC numbers for enzymes. Deposition of the Whole Genome Shotgun project was performed at DDBJ/ENA/GenBank under the accession code JAKNDB000000000, with the current version (described in the present paper) being JAKNDB010000000.

Single-nucleotide polymorphism (SNP) loci genotypes that differentiate loss of efficacy/resistant populations from susceptible *D. immitis* populations, previously referred to as markers for macrocyclic lactone resistance in *D. immitis* in the United States [6] and in

Australia [10], were identified in the *D. immitis* genome with the Basic Local Alignment Search Tool (BLAST) and Interactive Genome Viewer (IGV) for full characterization of the sample.

3. Results and Discussion

The assembled genome of *D. immitis* consists of 110 contigs with an N50 of 3687191. The majority of the contigs had a very good match with one or more of the compared genomes without any sign of fragmentation. The genome size is 87899012 and contains a total of 9741 proteins; 6 ribosomal RNAs, with three belonging to the small subunit (18S) and three to the large subunit (28S); and 73 tRNAs. The results obtained for BUSCO show that the genome is complete to a high level, showing the following values (C:93.9% [S:93.5%, D:0.4%], F:1.7%, M:4.4%, n:3131). We therefore present evidence that a single ONT MinION flow cell can produce sufficient data to assemble a contiguous, high-level, near-full-length genome of *D. immitis*. Noteworthily, parasite genome assemblies used as references are still today considered to be vastly fragmented, frequently hampering more in-depth analysis [11].

A total of 42 loci in the *D. immitis* genome, with the corresponding SNP associated with resistance selection pressure or at least loss of efficacy for various macrocyclic lactones [6,10], were studied. Subsequent analysis of the loci associated with macrocyclic lactone resistance selection pressure showed that 27 of the 42 loci have a genotype associated with either some loss of efficacy or the resistance phenotype (Table S1).

Interestingly, the dog hosting the *D. immitis* worm collected for this study was kept in a municipal kennel since birth and had not received any macrocyclic lactone treatment, a fact which suggests that resistance-associated SNPs were likely acquired from ancestral *D. immitis* previously circulating in the region. It is assumed that macrocyclic lactone resistance first appeared circa 1998. At that time, macrocyclic lactone *D. immitis* preventives had been recommended for use as the first line of treatment to prevent heartworm for over 10 years, having been registered as 100% effective [12,13]. Hence, it is generally assumed that the basis for genetic changes causing macrocyclic lactone resistance only arose and was selected in that decade. As such, resistant lines of *D. immitis* are suggested to have been circulating prior the use of macrocyclic lactone preventives [12,13].

True drug resistance has a genetic basis; hence, continuing efforts to analyze *D. immitis* whole genomes from resistant and susceptible lines could help detect genetic markers for resistance [6]. Long-read sequencing technologies allow for democratizing access to powerful sequencing options, reducing the cost of de novo genome assemblies of understudied organisms. The MinION platform was first made available in 2014, presenting a compact, novel, and lightweight sequencing platform that could produce long reads used for real-time base-calling [14]. The sequencer uses a nanopore that holds a biological membrane where DNA is driven while producing differences in electrical current that are measured and translated as different DNA bases [14–16]. The ONT MinION platform can not only provide a low-cost option for projects that were previously considered cost-prohibitive but also grants access to this technology in underdeveloped areas that do not have the infrastructural capacity for sequencing. Moreover, the sequence quality seems to allow for the profiling of drug resistance patterns, which allows for more informative treatment options [14].

4. Conclusions

In conclusion, this work provides, for the first time, the de novo assembly of the *D. immitis* genome using long-read nanopore-based sequencing technology. We show that a single ONT MinION flow cell can produce sufficient data to assemble a contiguous, high-quality genome from a complex nematode. The data from this study also provide information suggesting the circulation of macrocyclic-lactone-resistant *D. immitis* in northern Portugal. Considering the zoonotic potential of *D. immitis*, the identification of a resistant

parasite alerts for the overuse of macrocyclic lactone in the region, which poses a potential risk to both veterinary and human public health.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/ani12111342/s1, Table S1. Macrocyclic lactone resistance profile in a Dirofilaria immitis collected from a dog in northern Portugal, November 2020.

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LETTER TO THE EDITOR

Correspondence: "The One Health concept applied to dirofilariasis—a zoonotic disease"

To the Editor,

We have read with interest the paper by Silva et al.[1] "human pulmonary dirofilariasis: a pitfall in solitary pulmonary nodule".

After careful analyses, we would like to comment on certain statements from the article.

In this paper authors report the case of a 38-year-old man who presented to the emergency department with face edema, eosinophilia (2000/uL) and a chest X-ray showing a small peripheral solitary lung nodule on the right lung. Postoperative histopathological diagnosis was consistent with a central zone of necrosis surrounded by granulomatous inflammation and a fibrous wall. Besides that, a filarial worm was found in the lumen of an artery within the area of necrosis containing remnants of *Dirofilaria immitis*.

The zoonotic implication of *Dirofilaria* spp. infections is important since shortly after the inoculation of stage 1 larvae (L1) by vector mosquitoes, there is the possibility of larval migration along human tissues.[2] This can provoke ocular, skin and pulmonary nodular lesions, which are frequently and erroneously diagnosed as pulmonary carcinomas.[2] Moreover, the diagnosis of malignant neoplasm requires invasive procedures before reaching the correct diagnosis.[2]

The paper by Silva et al.[1] caught our attention as, upon biopsy, the morphological identification of dirofilariae parasites can be difficult to obtain, due to a loss of parasite integrity after tissue excision with consequent underdiagnosis, and a diagnosis based solely on the histological features only allows the determination of the genus *Dirofilaria*.[3] In addition, there are confirmed *Dirofilaria* spp. circulating in Portugal, namely *Dirofilaria repens*,[4] which has been reported to cause human pulmonary dirofilariasis with nodules that can be mistakenly diagnosed as malignant.[5] Noteworthy, Ferrari et al.[5] have reached definite diagnosis by multiplex-PCR targeting mitochondrial cytochrome oxidase subunit I gene (mtDNA cox1).

Determining *Dirofilaria* spp. based solely on histological diagnosis is yet to be confirmed and, when the parasite's DNA extraction is possible, the methods of molecular diagnosis based on sequencing can play a fundamental role in the identification of the etiological agent involved.[2] Hence, we would like to highlight the diagnostic accuracy of PCR followed by dideoxy chain termination sequencing as a valuable and affordable method to confirm worm species.

Dirofilariasis/dirofilariosis is not difficult to treat when diagnosed with accuracy; however, it remains an underdiagnosed infection and disease because of the complexity in identifying the parasites involved. The use of molecular biology techniques to detect and identify them is likely to overcome the complexity associated to the diagnosis.[3]

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