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New insights into diagnosis and control of *Dirofilaria* infection in dogs

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List of abbreviations

AT	Acceleration time
CI	Confidence interval
CPG	Cysts per gram of faeces
ELISA	Enzyme-linked immunosorbent assay
EPG	Eggs per gram of faeces
ET	Ejection time
HWD	Canine heartworm disease
LPG	Larvae per gram of faeces
OPG	Oocyst per gram
PA	Pulmonary artery
PCR	Polymerase Chain Reaction PE Parasitic Elements
SD	Standard deviation

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Abstract

Dirofilariosis are an important vector-borne diseases affecting primarily domestic dogs and wild canids, as well as cats, other species of wild mammals and humans. The most important species responsible of dirofilariosis are *Dirofilaria immitis* and *Dirofilaria repens* (Spirurida, Onchocercidae) which cause canine heartworm disease and subcutaneous dirofilariosis, respectively. The majority of dogs diagnosed with heartworm infection are asymptomatic (or have only mild symptoms such as intermittent cough) and go through adulticide therapy without complication. Complications that occur with heartworm infection and during its treatment are usually directly directly attributable to damage to the pulmonary vascular and parenchymal injury inflicted by *D. immitis*. Early diagnosis and treatment are essential for dogs with heartworm disease, as the risk of these complications increase with disease progression. Although *D. repens* is the main zoonotic species and its presence is increasingly reported in Europe, it has received less attention by researchers than *D. immitis*, therefore it is under-reported and still neglected. Diagnosis of *D. repens* in dogs is a challenge: many infected dogs are asymptomatic and there is a limited number of reliable diagnostic tools available. Indeed, diagnosis is mostly based on the detection of circulating microfilariae during patent infection by Knott's test, followed by morphometric or molecular species identification.

Heartworm disease is a parasitosis that is increasing its geographical range over time, for reasons related to the parasites, the hosts and the environment. Southern Italy, once considered low-risk, is increasingly becoming the site of autochthonous outbreaks. Currently, *D. repens* occurs in southern Italy (Campania and Molise regions), especially in the coastal areas of the two regions. However, the lack of country-wide epidemiological data on these two vector-borne filariosis in Italy could hamper the awareness of practitioners and thus the implementation of effective prevention and control strategies.

In order to deepen the knowledge on *D. immitis* and *D.repens* infections in dogs, the present thesis entitled "New Insights into the Diagnosis and Control of *Dirofilaria* Infection in Dogs" had four specific objectives: i) to improve of the Knott's test method for identifying the microfilariae of *Dirofilaria immitis* and *D. repens*; ii) to advance the knowledge in the immune response of *D. repens* in experimentally infected dogs; iii) to evaluate the slow kill therapy with doxycycline and various commercially available formulations of

moxidectin in dogs naturally infected with *D. immitis*; iv) to update the distribution of *D. immitis* and *D. repens* in dogs from an endemic area of southern Italy.
The PhD thesis consists of two parts, according to the European standard requirements. The first part - entitled "Literature Review" - summarizes information from the literature about aetiology, epidemiology, clinical signs, diagnostic concerns and control approaches on dirofilaria infections in dogs. The second part entitled - "Own Research" - presents the general and specific objectives of the thesis followed by four original studies addressing the epidemiology, diagnosis, clinical relevance and treatment of *Dirofilaria spp.* infections in dogs.

The literature review in **Chapter 1** provides an overview of both pathogens *Dirofilaria immitis* and *D. repens* in dogs in Europe. Data regarding aetiology, life cycle and other biological aspects, pathogenesis, clinical diagnosis, treatment and prevention are discussed in detail with emphasis on the geographical distribution and diagnostical concerns of the infection. This review provided herein, indicates a lack of detailed studies on the prevalence of both *D. immitis* and *D. repens* pathogens in southern Italy, as well as data regarding the clinical importance of these diseases. Moreover, the need to expand our current knowledge on *D. repens* biology and the immune response of the infected host is mandatory for a better prevention and an accurate diagnose of this canine infection.

Chapter 2 reports the suitability of different reagents as safe alternatives to 2% formalin in the modified Knott's test for the diagnosis of subcutaneous (*D. repens*) and cardiopulmonary (*D. immitis*) dirofilariosis. A total of 61 blood samples from dogs naturally infected with *D. immitis* and *D. repens* were collected and analysed in two different laboratories (Lab 1, University of Parma and Lab 2, University of Napoli). For each blood sample the modified Knott's method was performed to identify and measure the mean length and width of the microfilariae (mfs) using 2% formalin (A), 2% acetic acid (B), 2% glacial acetic acid (C), 10% saponin (D) and distilled water (E). All alternative reagents caused more marked haemolysis compared to formalin, improving readability of slides. The values of the mean length and the mean width of *D. immitis* and the mean width of *D. repens* mfs obtained with formalin and distilled water were statistically different ($P < 0.005$) between the

two laboratories. Results suggest that distilled water could replace formalin in the modified Knott's test, as a safer reagent that allows morphology-based species differentiation of *Dirofilaria* spp.

Chapter 3 provides new insights from experimental infections of dogs with *D. repens*, focusing on the evaluation of: 1) the pre-patent period and 2) the antibody response against *D. repens* somatic antigens and against the Wolbachia endosymbiont. Briefly, on Day 0, twenty purpose-bred Beagle dogs were experimentally infected with 50 infective larvae (L3) of D. repens. Starting from Day 58 until the last day of the study (Day 281), blood samples were collected on a monthly basis for detection of antibodies against *D. repens* (Dr) and recombinant *Wolbachia* surface protein (rWSP) by non-commercial IgG- ELISAs. Additional samples were collected on Days 220, 245 and 281 for the detection of microfilariae (mff) using the modified Knott's test and biomolecular analysis, following two PCR protocols: Gioia et al. (2010; protocol A) and Rishniw et al. (2006- protocol B). Overall, the outcome of the study revealed that out of the 20 dogs experimentally infected with *D. repens*, 16 (80 %) were microfilaraemic, 17 (85 %) were positive at DNA detection in the blood, 18 (90 %) had *D. repens* antibodies and 16 (80 %) had *Wolbachia* antibodies on the last day of the study. The overall k agreement between Knott's and PCR protocol B was 0.442 (P = 0.0001) and increased throughout the study, reaching 0.828 (P = 0.0001) on Day 281. Results would suggest that the development of an immunological response to infection could lead to application in epidemiological studies, risk assessment and as an aid in the diagnostic approach in dogs, in particular for early infections without mff.

Chapter 4 aimed to evaluate the adulticide effect of oral, topical and extended-release injectable formulations of moxidectin when combined with doxycycline in dogs naturally infected with *D. immitis* from a shelter located in southern Italy. A total of 30 dogs with naturally acquired *D. immitis* infection were divided in three groups (G) and treated either with oral moxidectin (G1) once a month for 9 consecutive months, topical moxidectin (G2) once a month for 9 consecutive months or with an extended release moxidectin injectable (G3) at enrolment and again at 6 months (Day 180). All treatment groups received doxycycline for the first 30 days. Microfilarial concentration in 1 ml (mff/ml) of blood were determined monthly for 9 months, with the

modified Knott's test. A clinical scoring system was employed for each dog enrolled in the study based on thoracic radiography and cardiac ultrasound (CU) exams performed at Day -15 (before treatment) and Day 180. In general, mff loads decreased markedly in all dogs from all groups at Day 30, and all but one dog were negative at Day 60. Results from the present study suggest that efficacy (evaluated at Day 270) is related to the moxidectin formulation used and that injectable moxidectin showed superior efficacy compared to topical and oral injectable formulations. Overall, the treatment with moxidectin and doxycycline combination was effective and almost all the dogs from the treatment groups were cleared of pulmonary abnormalities by six months from the beginning of treatment (p-value=0.000). Although the therapy proved to be an effective adulticide, the echocardiographic parameters studied were not able to show a marked improvement of cardiac function after the treatment.

Chapter 5 aimed to investigate the occurrence of *Dirofilaria* spp. in southern Italy. For this purpose, a local dog shelter in Castel Volturno (Caserta province, southern Italy) was selected and dogs were screened for the presence of *Dirofilaria* spp. A total of 260 blood samples were examined for identification of microfilariae (mff) and for detection of *Dirofilaria immitis* antigens. Moreover, all the dogs that showed co-infections with both *D. immitis* and *D. repens* mff were confirmed with molecular analyses. In addition, data regarding the length of stay of the dogs in the shelter at the time of sampling were also recorded. The dogs were divided into four age classes (class 1: dogs ≤ 2 years; class 2: dogs $> 2 \leq 6$ years; class 3: dogs $> 6 \leq 10$ years; class 4: dogs > 10 years old) and three groups of dogs based on the length of stay in the shelter at the time of sampling (group 1-new arrivals: dogs that have been received in the shelter in the last four months; group 2- dogs that were housed in the shelter for more than four months up to 2 years; group 3-dogs that were housed for more than 2 years). The modified Knott's test revealed that 188 dogs (72.3%; 95%CI=66.4-77.6) were positive for circulating mff of *Dirofilaria* spp. Specifically, 113 (60.1%; 95%CI=52.7-67.1) dogs were positive for *D. immitis* mff and 75 (39.9%; 95%CI=32.9-47.3) were positive to *D. repens* mff. In addition, 58 (30.8%; 95% CI=24.4-38.1) dogs presented *both D. immitis* and *D. repens* mff. Antigen testing showed 98/260 (37.7%; 95%CI=31.8-43.9) dogs positive to *D. immitis*. However, 13% (95%CI=6.5-19.2) of the dogs with *D. immitis* mff were antigen-negative.

Abstract

PCR testing confirmed the co-infections with both pathogens in all 58 dogs. The prevalence was almost twice as high in males (65.4%; 95%CI=58.1-72.1) as in females (34.6%; 95%CI=27.9-41.8). As expected, prevalence was lowest in age class 1 (16.5%; 95%CI=11.6-22.7) and higher in age classes 2, 3 and 4 (25.5%; 95%CI=19.5-32.5; 27.6%; 95%CI=21.5-34.7 and 30.3%; 95%CI=23.9-37.5 respectively) but these differences were not significant. There was a significant difference in relation to the length of stay of the dogs in the shelter, reflecting mainly an increase in prevalence in the group 1 (45.2%; 95%CI=39.0-53.7; P=0.012) in which all dogs were new arrivals in the shelter since four months and their origin was from different localities of the Campania region, including the city center of Naples. We conclude that dog shelters from southern Italy constitute hot spots for *Dirofilaria spp.* transmission and we strongly recommend education and veterinary guidance regarding regular testing and systematic treatments.

The thesis ends with an overall discussion based on the four studies presented herein with particular emphasis on epidemiological status of *Dirofilaria* spp. infection in dogs in southern Italy, diagnostic challenges and slow-kill treatment of *Dirofilaria* spp. infection in dogs.

Introduction

Dirofilariosis is an important vector-borne disease affecting primarily domestic dogs and wild canids, as well as cats, other species of wild mammals and humans. The most important species responsible of dirofilariosis are *D. immitis* and *D. repens* (Spirurida, Onchocercidae) that cause canine heartworm disease and subcutaneous dirofilariosis, respectively (McCall et al., 2008). Different species of culicid mosquitoes of the genera *Culex, Aedes*, and *Anopheles* act as vectors for dirofilariosis. The epidemiological scenario of dirofilariosis is currently rapidly changing, due to several factors as climatic changes, increasing presence of competent vectors, as well as, the increasing number of dogs travelling with their owners, and the presence of reservoirs such as jackals and foxes (Ciuca et al., 2016). The prevalence of canine dirofilariosis is, indeed, rising in endemic areas, and the infections are spreading into new areas, as the central and southern Italy (Otranto et al., 2009; Genchi et al., 2011; Simón et al., 2012; Kartashev et al., 2014; Ionica et al., 2015; Kartashev et al., 2015; Tasic-Otasevic et al., 2015; Simón et al., 2017; Capelli et al., 2018; Gizzarelli et al., 2019; Genchi et al., 2019). In dogs, heartworm disease by *D. immitis* usually develops a chronic progression, first showing vascular and pulmonary effects and eventually affecting the right chambers of the heart, with death of the dog (Venco et al., 2017). *D. repens* infections, instead, often are asymptomatic, although cutaneous disorders such as pruritus, dermal swelling, subcutaneous nodules, as well as conjunctivitis can be observed (Genchi et al., 2017). Rare cases of unusual localizations of *D. repens* have been reported in dogs in the pelvic cavity and mesentery, bulbar conjunctival mass, and testicle (Hermosilla et al., 2006; Ravindran et al., 2016; Mircean et al., 2017; Agapito et al., 2018; Omeragić et al., 2018; Napoli et al., 2019; Barlozzari et al., 2021). In humans, infection leads to the formation of nodules around dead or dying worms. In most patients, *D. immitis* is in lungs, causing pulmonary dirofilariosis. *D. repens* can be present as nodules in the subcutaneous tissue in various human body areas (e.g., facial regions, perioral or periorbital tissues, forehead, skin of lower leg, hand, fingers, hypogastrium, neck, scrotum and testicles, breasts, etc.) or as free worms under the conjunctiva (ocular dirofilariosis) (Simón et al., 2012; Capelli et al., 2018).

Although *D. repens* is the main zoonotic species and its presence is increasingly reported in Europe, it has received less attention by researchers than

D. immitis, therefore it is under-reported and still neglected (Capelli et al., 2018). Diagnosis of *D. repens* in dogs is a challenge: many infected dogs are asymptomatic and there is a limited number of reliable diagnostic tools available. Indeed, diagnosis is most often based on detection of circulating microfilariae (mff) during patent infection by Knott's test, followed by morphometric or molecular species identification (ESDA-Guidelines, 2017). Molecular detection and identification of *D. repens* mf by multiplex PCR, based on amplification of cytocrome c oxidase subunit 1 (cox1), the intergenic spacer (ITS) regions, or 12S rRNA regions, resulted a highly sensitive and specific diagnostic technique (Rishniw et al., 2006, Gioia et al., 2010; Latrofa et al., 2012; Ciuca et al., 2016), but requires specialized laboratories and experienced personnel. However, all the above-mentioned techniques do not permit to detect the mf during the pre-patent period. Other diagnostic options include ultrasound examination of subcutaneous nodules and fine needle aspirate cytology (Giori et al., 2010; Albanese et al., 2013; Manzocchi et al., 2017; Capelli et al., 2018). Therefore, the lack of any commercially available test for serological diagnosis is likely one of the most important limitations and drawbacks for *D. repens* diagnosis (Simón et al., 2012; Capelli et al., 2018). Many epidemiological studies have been published in different endemic areas, both in humans, and in cats, using a non-commercial ELISA for the detection of IgG antibodies against adult somatic antigens of *D. repens* (Simón et al., 1991; Simón et al., 1999; Santamaria et al., 1995 a,b; Prieto et al., 2000; Simón et al., 2003; Kramer et al., 2007; Grandi et al., 2008; Morchón et al., 2010; Gonzalez-Miguel et al., 2010; Kartashev et al., 2011; Cabrera et al., 2018; Ciuca et al., 2018). Mounting evidence suggests that cross-reactivity may be present between the somatic antigens of *D. immitis*, *D. repens* and those of other helminths. Therefore, a complementary immunological assay to detect the antibodies against *Wolbachia*, a bacterial endosymbiont present only in filarial nematodes, may increase diagnostic specificity (Cancrini et al., 1999; Prieto et al., 2000; Simón et al., 2003; Grandi et al., 2008; Cabrera et al., 2018; Ciuca et al., 2018). Gonzales-Miguel et al. (2010) identified 23 immunoreactive proteins of *D. immitis* and 15 of *D. repens* in human patients with pulmonary and subcutaneous dirofilariosis. These results showed the existence of a differential antigenic recognition (in terms of number and types) between these two species. Instead, Olega et al. (2009) identified 19 immunoreactive proteins of *D. immitis* in dogs. Comparing the results obtained from Olega et al. (2009) and Gonzales-Miguel et al. (2010),

it seems that the human host presents a greater reactivity than the dog against this species, which is consistent with its limited capacity of development in humans, when compared to the animal reservoir (Gonzalez-Miguel et al., 2010). There are few studies reporting the antibody response to *D. repens* somatic antigen in experimentally and naturally infected dogs (Cancrini et al., 2000; Joekel et al., 2017; Ciuca et al., 2020). Response to crude somatic antigens from adults of *D. repens* showed dogs becoming antibody positive as early as 24 days post-infection. A further diagnostic challenge for dirofilariosis will be the development of a serological assay that can discriminate between *D. immitis* and *D. repens*. Commercial antigenic tests resulted in cross-reactions in dogs with *D. repens* mono-infections (Ciuca et al., 2016). In addition, it has been proved that concomitant infections with other vector-borne canine pathogens (e.g. *Leshmania infantum*, *Ehrlichia canis, Anaplasma* spp., *Babesia* spp.) can affect serological test results. This would have important implications for prevalence studies in areas where these infections co-exist. Indeed, as reported by Little et al. (2014), the serum of a hypergammaglobulinemic infected dog with other parasites can block the antigen detection of *D. immitis*. Therefore, to develop a sensitive and specific serological assay that could be an important tool for veterinary practitioners to detect *D. repens*, firstly cross-reactions with *D. immitis* and other canine vector-borne diseases (CVBDs) should be evaluated. Such a new serological test could support the current knowledge of *D. repens* epidemiology and allow the screening of dogs moving from non-endemic into endemic areas, helping to prevent the spread of subcutaneous dirofilariosis. However, there are not information regarding the immunoreactive proteins of dogs infected with *D. repens*.

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Chapter 1

Literature review on *Dirofilaria* infections in dogs: aetiology, biology epidemiology, clinical signs, diagnosis and control

Miron L, Acatrinei D, Iacob O, Ivanescu L, **Ciuca L**, Roman C, Mindru R, Lupu A, Andrei Cimpan, Martinescu G, Velescu E, Matei MC, Manciuc DC, Manole A, Azoicai D, Miron FM, Massari GA, Colibaba A, Colibaba C, Colibaba S, Gheorghiu E, Ionel A, Gheorghiu I, Antonita C, Colibaba A, Turk N, Milas Z, Franic ZK, Karalis T, Karalienė R, Jarulė V, Norviliene L, Katinaite D, Malinauskiene D, Pascucci I, Pediconi O, Giordano A. Dirofilariosi: guida delle principali malattie infettive trasmesse dagli animali non umani agli umani - la dirofilariosi negli umani e negli animali. Zoonoses Online Education, Biblioteca nazionale lituana / Soros International House - https://zoeproject.eu/documents/study/IO3_Diro_IT.pdf

1.1 Aetiology

The first description of *Dirofilaria repens* was made by Bonvicini in Italy in 1910. Later, in 1911, the same worm was studied in France by Railliet and Henry. However, *Dirofilaria immitis* was first described by the noble Francesco Birago in the 17th century when he identified in the heart of his hunting dog a filarial worm which he erroneously described as *Dyoctophyma renale* (Simone et al., 2012).

In 1937, Faust proposed the classification of *Dirofilaria* genus into *Dirofilaria* subgenus with affinity for the cardiovascular system (*Dirofilaria immitis*) and *Nochtiella* subgenus with predilection in the subcutaneous tissue (*Dirofilaria repens*). Currently, the most studied parasites of dogs are: *D. immitis* (Leidy, 1856) and *D. repens* (Railliet and Henry, 1911) (*Spirurida: Onchocercidae*), which cause cardiopulmonary and subcutaneous dirofilariosis, respectively. Both species are viviparous and microfilariae spread in the bloodstream of their definitive host (Otranto et al. 2013).

Recent studies show the presence of an endosymbiont, *Wolbachia pipientis*, a Gram negative bacterium belonging to the Rickettsiales Order, which resembles to other bacteria of the same order (*Ehrlichia spp., Anaplasma spp*.). This bacterium plays an important role in the parasite embryogenesis as well as triggering immunological reactions. The study of this endosymbiont provides better knowledge of the parasite's biology and the pathological mechanisms determined by these filaria, as well as important aspects in the treatment of filariosis (Dingman et al., 2010, Belanger et al., 2010, McHaffie et al., 2012).

The presence of vectors in the lifecycle of the *Dirofilaria* spp. is determined by global climate change (Genchi et al., 2001; Sassnau et al., 2014).

The generic name of "dirofilariosis" joins together all the helminthoses produced by de species of the *Dirofilaria* genus Railliet & Henry, 1910 (*Spirurida: Onchocercidae*) in humans and animals. The *Dirofilaria* genus comprises around 50 species but only 27 have been validated, divided in two subgenera: *Dirofilaria* (5 species with cardio-vascular affinity) and *Nochtiella* (22 species with subcutaneous and conjunctival tropism). Of the validated species (table 1.1), only six have proved zoonotic potential (*D. immitis, D. repens, D. tenuis, D. ursi, D. striata* and *D. spectans*). Due to the frequency with which they were diagnosed and to their zoonotic potential, *D. immitis* and *D. repens* are considered the most important and consequently the most studied.

Tab. 1.1 Valid species of the Dirofilaria genus (Dantas-Torres, F., Otranto, D., 2013)

	Genera, Sub-genera and species	Definitive host (Families)	Geographical area
Dirofilaria	*D. ailure* (Ryjikov and Románova,1961)	Procyonidae	China
	D. freitasi (Machado de Mendonca,1949)	Bradypodidae	Brazil
	D. immitis (Leidy, 1856)	Canidae, Felidae, Hominidae, and many others	Cosmopolitan
	D. lutrae (Orihel, 1965)	Mustelidae	USA
	D. spectans (Freitas and Lent, 1949)	Hominidae (single case), Mustelidae	Brazil
	D. acutiuscula (Molin, 1858)	Canidae, Caviidae, Felidae, Tayassuidae	South America, USA
Nochtiella	*D. bonnie* (Vogel and Vogelsang,1930)	Muridae	Java
	D. cancrivori (Eberhard, 1978)	Procyonidae	Guyana
	D. corynodes (Linstow, 1899)	Cercopithecidae	Africa, Thailand
	D. genettae (Baylis, 1928)	Felidae, Viverridae	Nigeria
	D. granulosa (Linstow, 1906)	Felidae	Africa, Asia
	D. incrassata (Molin, 1858)	Bradypodidae, Procyonidae	Brazil and Central America
	D. linstowi (Dissanaike, 1972)	Cercopithecidae	Sri Lanka
	D. macacae (Sandground, 1933)	Cercopithecidae	Indochina
	D. macrodemos (Eberhard, 1978)	Bradypodidae	Guyana, Panama
	D. magnilarvata (Price, 1959)	Cercopithecidae, Hominidae, Hylobatidae	Malaya
	D. minor (Sandground, 1933)	Felidae	Vietnam
	D. pagumae (Sandground, 1933)	Viverridae	Indochina

Continued

	Genera, Sub-genera and species	Definitive host (Families)	Geographical area
Nochtiella	*D. panamensis* (Eberhard, 1978)	Bradypodidae	Panama
	D. repens (Railliet and Henry, 1911)	Canidae, Felidae, Hominidae, Viverridae	Europe, Asia, Africa
	D. sachsi (Shoho, 1974)	Bovidae	East Africa
	D. striata (Molin, 1858)	Canidae, Felidae, Hominidae (single case), Tayassuidae	Brazil, Venezuela, USA
	D. subdermata (Mönnig, 1924)	Erethizontidae	North America, South Africa
	D. sudanensis (Linstow in Schipley, 1902)	Felidae, Hyaenidae	Sudan
	D. tawila (Khalil, 1932)	Cercopithecidae	Africa
	D. tenuis (Chandler, 1942)	Hominidae, Procyonidae	North America
	D. ursi (Yamaguti, 1941)	Felidae Ursidae Hominidae	Asia, North America

1.1.1 Biology

Dirofilaria immitis has a smooth, whitish cuticle, with only the male showing striae and ridges on the ventral face of the last caudal spindle. Males measure 12-18 cm in length and 0.6-0.9 cm in width, and the tail (Figure 1.1) resembles a corkscrew (ESCCAP, 2012). The spicules are unequal, with many spirurids, the left one is 300-355 µm long and the right one 175-226 µm (Fülleborn et al., 1912; Vogel et al., 1927). Adult females are 25-31 cm long and 1-1.3mm wide.

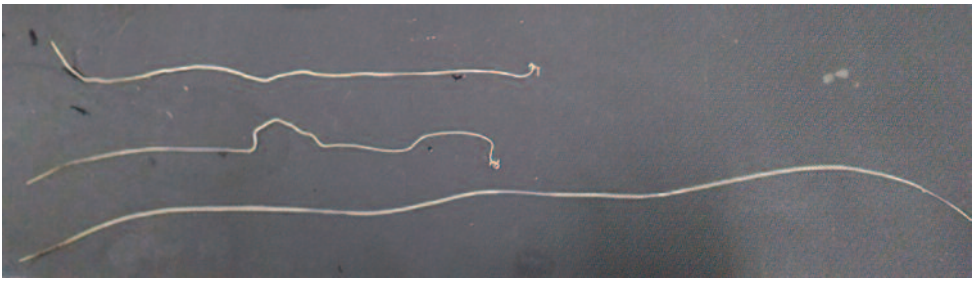

Fig. 1.1 Two males (up) and one female (down) of Dirofilaria immitis.

The microfilaria sizes are: 301.77 ± 6.29 average length and 6.30 ± 0.26 mean width. The maximum and minimum dimensions fall within the following ranges: 180-340 µm in length and 5-7 µm in width (Taylor et al., 1960a). The microfilaria does not present a sheath, the anterior extremity is tapered and the posterior one is straight with a sharp tail (Magnis et al., 2013).
Dirofilaria repens adults have a white cuticle with different longitudinal and transversal striations and ridges. Adult females measure 10-17 cm in length and 0.46-0.65 mm in width, while males are 5-7 cm long and 0.37-0.45 mm wide. The adult nematodes are located in subcutaneous connective tissues and intramuscular interstices, where they are difficult to detect, they migrate sometimes and produce a subcutaneous nodule of about 1 cm in size (Genchi et al., 2011). The microfilaria of *Dirofilaria repens* measure 325-375 µm in length and 6-8 µm in width. In the microscopic examination, the larvae of *Dirofilaria repens* exhibit an anterior obtuse end, the tail resembling an umbrella handle, and the absence of the cephalic hook (Magnis et al., 2013). The microfilaria aspects of both species are shown in Figure 1.2.

Fig. 1.2 First stage microfilaria of D. immitis (left) and D. repens (right) isolated from canine blood using Knott technique. Note the shape of the tail of D. repens first stage larva, resembling an umbrella handle. Light microscopy, 1000x.

1.2 Life Cycle

The lifecycle of *Dirofilaria spp*. is of two-host type and it is spent between a vertebrate (definitive host) and an arthropod vector (mosquitos from the *Culicidae* family). The species of some genera, such *as Aedes, Culex, Culiseta, Mansonia, Ochlerotatus, Coquillettidia and Anopheles (Aedes aegypti, Ae. albopictus, Ae.notoscriptus, Culex vexans, Cx. quinquefasciatus, Cx. tritaeniorhynchus, Cx erythrothorax, Culiseta incidens, Cu. inornata, Coquillettidia richiardii, Anopheles maculipennis* group) were found to be competent vectors for *D. immitis* (Cancrini et al., 2003, 2006, Fuehrer et al., 2016, Loftin et al., 2015, Smith et al., 2013, Vezzani et al., 2005, Lai et al., 2001, Konichi E., 1989, Yildirima et al., 2011, et | al.,1992). The period of adult development of both *D. immitis* and *D. repens* in the definitive host is relatively long (7-9 months) compared to other nematodes (McCall et al., 2008).

The first stage microfilaria (L1) are ingested by the mosquito vector when feeding on a definitive host. Within 8-10 days (Venco et al., 2011) microfilaria migrate in the Malpighian tubes and molt to L2. The second molting process occurs three days later and L3 have to leave the Malpighian tubules in another 2 days to became infective in the mouthparts of the mosquito. The infective L3 is 1mm long and grows to 1.5mm after being inoculated in the definitive host`s subcutaneous connective tissue (Cancrini and Kramer, 2001; Taylor et al., 1960; Manfredi et al., 2007).

The development of L1 to infective L3 inside the mosquito depends on the environmental temperature and is favored by the presence of the *Wolbachia pipientis* symbiont. The development process occurs in 10-14 days at a temperature of 27º C and 80% humidity (Orihel, 1961). The number of infested larvae is limited by antigenic recognition and vector- based humoral and cellular defense mechanisms (Castillo et al., 2011).

The infection with L3 of the definitive host is performed during mosquito feeding, when about 10 larvae can be inoculated in a single feeding session. In subcutaneous connective tissue, adipose tissue and muscle tissue of the definitive host, *D. immitis* larvae (L3) develop actively for 70 days. During this period two moltings take place (L4 and L5 are 1-2 cm long) until the pre-adult stage. These stages are able to migrate into the vascular system and from here to the heart and lungs where they localize and undergo final maturation, and become capable of reproduction within 120 days post-infection (McCall et al., 2008; Manfredi et al., 2007).

Dirofilaria immitis is located in the pulmonary arteries, with a predilection for the caudal lobes, but also in the right ventricle, the right atrium and occasionally in the cava vein. Adult females begin to produce the first larvae (L1 microfilaria) after 6-9 months post-infection. Adult longevity in the host may be longer than 7 years, and the microfilaria's lifespan more than 2 years (Venco et al., 2011). Adults of *D. repens* remain in the connective tissue, the abdominal cavity and the muscular fascia of the definitive host (Genchi et al., 2011). The prepatent period in the dog is 6-9 months, when new microfilariae are released by the adult female (Venco et al., 2011). After infesting a host, the microfilaria continues to live in the blood for several months, up to 3 years. Adults can live for 4 years or more at the site of inoculation. *Dirofilaria repens* can be located in the subcutaneous tissue in the nodules and may also invade the ocular region (Paes-de- Almeida et al., 2003; Mircean et al., 2017). Incidentally, both filarial species can also be found in other anatomical regions other than those described above (Pampiglione et al., 2000; Theis et al., 2005).

After the blood meal, mosquito females lay eggs in rafts- shaped groups or solitary eggs on the surface of water, humid soils or in tree hollows. As a rule, the larvae develop at temperatures below 18 degrees Celsius, but they can also adapt to higher temperatures (Cancrini et al., 1988).

Once ingested by the mosquito, the microfilaria are temperature-dependent throughout the development process up to the infective larval stage (L3). Thus, for larvae (L1) it is necessary to reach the optimal temperature within 30 days to get to the infestation stage, a process called extrinsic incubation period (Slocombe et al., 1989; Medlock etal., 2007). The time required for the development of larval stages in the mosquito is influenced by temperature: 8-10 days at 28-30 °C, 11-12 days at 24 °C and 16-20 days at 22 °C.

The minimum temperature at which the larvae's growth process can be carried out is 14°C (Lok and Knight, 1998; Slocombe et al., 1989; Vezzani and Carbajo, 2006; Medlock et al., 2007; Genchi et al., 2011). Taking into account the period and temperaturerequired for the development of the infesting larva (L3), Slocombe et al. (1989) developed a model that estimates the initial and final period for the transmission of dirofilariosis as well as the number of generations of *Dirofilaria*.

Thus, the complete development of the larva (L3) requires 130 "degrees-days". The extrinsic incubation period is also called "Dirofilariosis Development Units" (HDUs). Another important rule of the extrinsic incubation

period is the accumulation of HDUs within 30 consecutive days, the maximum survival time of the mosquito.

1.2.1 Epidemiology

The epidemiological situation of dirofilariosis is currently undergoing accelerated changes. Canine dirofilariosis is rising in endemic areas as well as spreading into new areas previously reported as dirofilariosis-free (Genchi et al., 2011; Ionica et al., 2015; Kartashev et al., 2014; Kartashev et al., 2015; Otranto et al., 2009; Simon et al., 2012; Simon et al., 2017; Tasic-Otasevic et al., 2015). The highest prevalence of *D. immitis* was found in the Canary Islands and Madeira and Mediterranean countries (22-40%). Prevalence of *D. repens* ranged from 23 to 49% in Southwestern Russia and from 25% to 38% in some central and northern European countries (Simon et al., 2012; Demiaszkiewicz et al., 2014). *D. immitis* has been found in feline populations in Portugal, Spain, and Italy, with prevalence rates between 3 and 27%, and there have been frequent reports in France (Traversa et al., 2010; Vieira et al., 2015). Its presence has also been increasingly reported in European populations of foxes (3.7%–35%), jackals (7.7%–23.3%), and raccoon dogs (31.1%) and occasionally in wolves, while *D. repens* has been found in foxes, wolves, jackals, and badgers with prevalence rates that come close to 10% in some of the hosts (Kravchenko et al., 2016; Cirović et. al., 2014). Currently new endemic areas have been identified and confirmed in Austria, Czech Republic, Germany, Hungary, Poland, Russia, Ukraine, Slovakia, Turkey, and the Balkan Peninsula (Albania, Bosnia, Bulgaria, Croatia, Greece, Macedonia, Romania, Serbia) (Genchi et al., 2011; Tasic et al., 2015; Ciuca et al., 2016). It is noteworthy that in several eastern European countries *D. repens* prevalence is higher than *D. immitis*: for example, in Romania, where the heartworm prevalence ranges 02% to 2% and the *D. repens* prevalence range is from 7% to 20% (Ilie et al., 2012; Ionica et al., 2015). In the Balkan Peninsula, the more recent data showed a mean prevalence of 7% for *D. immitis* and 11% for *D. repens* in Kosovo and in a survey carried out in 1995–1996, Rapti and Rehbein (2010) reported a heartworm prevalence of 83% in dogs presenting cardiopulmonary signs and 10% in "inconspicuous" dogs. In Serbia, two extensive, recent surveys showed a prevalence of 17–49% for *D. repens* microfilaremic dogs and of 1.6–7% for *D. immitis* microfilaremic dogs, depending on the surveyed area (Tasic et

al., 2008, 2012); including occult infections, the actual prevalence of heartworm was 12% (Tasisc et al., 2012).
Many studies showed the influence of the changing climatic factors by using prediction model tools such as Geographic Information Systems (GIS), Global Positioning (GPS), Remote Sensing (RS) on the dynamic and trends of dirofilariosis in different ways such as: increase in vector density, in the duration of their annual activity period and agressiveness, the introduction of invasive species of competent vectors in endemic areas (e.g. *Aedes albopictus, A. koreicus*), and the shortening of the extrinsic incubation period of the parasite (Genchi et al., 2005, 2011; Montarsi et al., 2015; Rinaldi et al., 2006).
Although infestation with *D. immitis* has been diagnosed in more than 30 mammalian species: wild and domestic carnivores, domestic and wild felines, mustelids, monkeys, marine mammals, rodents and ungulates (Otto, 1975), dogs are most frequently infested with a large number of parasites (Genchi et al., 1988), being the most competent reservoir of infection. Humans and cat are less susceptible hosts to infection due to changes in the process of development of filaria in their bodies (McCall et al., 2008). In the natural infection (Figure 1.3), the number of adults parasiting increases with the dog's age (about 150 parasites/dogs in the endemic areas) (Genchi et al., 1988, Miller et al., 2011, Bolio Gonzales et al., 2007).
The literature provides many epidemiological studies that estimate the distribution of dirofilariasis over time as well as the number of generations of dirofilaria in different regions by using the predictive model described above (Slocombe et al., 1989) and the temperatures recorded at meteorological sta-

Fig. 1.3 Adults of D. immitis removed from the heart and pulmonary artery of a 12-year-old male mongrel dog at necropsy.

tions (Lok and Knight, 1998, Genchi et al., 2005, 2009, 2011, Vezzani and Carbajo, 2006, Medlocket et al., 2007, Mortarino et al., 2008, Rinaldi et al., 2013b; Kartashev et al., 2014; Sassnau et al., 2014; Simón et al., 2014).The capacity of geographic information systems to predict the distribution and epidemiology of dirofilariosis in different geographic areas has already been demonstrated by empirical epidemiological data obtained at continental level (Genchi et al., 2009; and Kartashev et al., 2014), national (Medlock et al., 2007; Simón et al., 2014) and regional (Mortarino et al., 2008; Montoya-Alonso et al., 2015). Geographic information systems could become an important tool for managing dirofilariosis in endemic and non-endemic countries. In dirofilariosis, the host-parasite relationship is complex mainly due to the capacity of the two, *D. immitis* and *D. repens*, to infect various vertebrate hosts in which the filaria develop and give rise to different pathologies, as well as to the presence of the symbiotic bacterium Wolbachia in the larval stages and in the adult stages of both species above. Receptive hosts are exposed to both antigenic, nematode and *Wolbachia* bacteria; the response induced by these antigens correlates directly with the survival or death of the nematode and the inflammatory process developed in dirofilariosis. From an epidemiological point of view, dirofilariosis is considered an emerging parasitic disease of humans and animals. Significant and continuous change in the distribution and prevalence of canine reservoirs hosts is reported worldwide, and these changes in turn alter the epidemiological parameters in the dirofilariosis with humans and cats. Global warming influences the stages of the parasite's lifecycle, and pet management and human intervention in the environment affect vertebrate hosts and vectors, which might explain the substantial increase in the *Dirofilaria* infection.

Despite efforts to prevent infestation, especially in dogs, the disease appears to spread to previously non-endemic areas (Genchi et al., 2007), so many countries are now considered endemic to dirofilariosis (Genchi et al., 2011; Genchi et al., 2019; Genchi and Kramer, 2020). The spread of cardiopulmonary dirofilariosis in Europe may be due to several factors such as global warming (Genchi et al., 2001; Sassnau et al., 2014), the presence of vectors and favorable climatic conditions for its development, new species of mosquitoes which are competent vectors of filariasis (Madon et al., 2002; Cancrini et al., 2003; Roiz et al., 2007), the growing number of dogs traveling with their owners, for example on holidays, as well as the increasing role of infection reservoirs, such as jackals and foxes (Tolnai et al., 2014).

Subcutaneous dirofilariosis is considered a widespread zoonosis. The prevalence of this disease seems to be growing, and new cases are reported in South-East, Central and Western Europe, Asia and Africa (Tarello, 2010). The *D. repens* infection is considered an emergent zoonosis in several European countries: France, Italy, Hungary, Russia (Kramer et al., 2007; Genchi et al., 2009), where the main host and reservoir is considered to be the dog. The highest prevalence was reported in dogs in Sri Lanka (60%) and Italy in the Po River Valley (30%), Spain 9%, Greece 22%, Serbia 49.22%, Belgrade 19.26%, Hungary 14%, France 22%. Although there are various specific and sensitive diagnostic methods, effective prophylaxis, dirofilariosis in dogs is still prevalent in large areas (McCall, et al., 2008). This disease affecting animals and humans is more and more frequently detected in Mediterranean countries (Genchi et al., 2005). Spain, Portugal, Italy and France were endemic before 2001 and remain in this situation. However, in these regions, the distribution of cardiopulmonary dirofilariosis is generally reported only sporadically or not reported at all (Morchon et al., 2012). Dirofilaria species have spread to eastern and northeastern Europe, but limited epidemiological information from these countries is available (Genchi et al., 2009, 2011).
The prevalence of *Dirofilaria* spp. infections in dogs and humans in the Balkan Peninsula suggests that ecological factors, the climate and an abundance of vectors favor the full development and transmission of the infection (Tasic-Otasevic et al., 2015).

1.2.2 Pathogenesis – Dirofilaria immitis

The cardiovascular dirofilariosis in dogs is characterized by acute and chronic inflammatory lesions in the lungs and other organs due to the presence of adults and microfilaria. *Dirofilaria immitis*, like most filarial worms, has its metabolism conditioned by the presence of an intracellular rickettsian symbiont which has been found in abundance in the Malpighi tubes of mosquitoes (Sironi et al., 1995). *Wolbachia* would appear to have a major role in filarial physiology because the literature reports a massive decrease in the number of larvae in peripheral blood when the definitive host is treated with tetracyclines, especially doxycycline, which is most active against these bacteria McCall et al., 2008). Kramer et al. (2014) show that by sequencing the Wolbachia symbiont genome and by comparing it with the *Dirofilaria* species, it has been proved that the two entities are closely linked, each of them being

able to encode proteins, enzymes, vitamins, nucleotides that the other cannot encode. Darby (2012) suggested that for *Onchocerca ochengi, Wolbachia* plays the role of mitochondria, providing the energy required for organic processes and muscle contraction. The pathophysiological response in cardiovascular dirofilariosis is mainly due to the presence of *D. immitis* parasites in the pulmonary arteries. The first lesion occurs in the pulmonary artery (Figure 1.4) and in the pulmonary parenchyma due to intravascular adult localization; pulmonary hypertension occurs, which then leads to congestive heart failure. Another syndrome is the blood circulation disorder due to the location of the *Dirofilaria* in the right side of the heart (Figure 1.5), at the level of the tricuspid valve. These disorders lead to massive haemolysis and hemoglobinuria, being responsible for the cave vein syndrome (Ishihara et al., 1978; Kitagawa et al., 1987).

The microfilariae appear to play a minor pathogenic role, but they can cause pneumonitis and glomerulonephritis. Some individuals may develop a hypersensitivity to microfilaria, so they disappear from the peripheral blood. Occasionally, parasites may have ectopic locations, such as the anterior chamber of the eye (Weiner et al., 1980). Massive infestations can result in obstruction of the right ventricle and pulmonary artery (Figure 1.6), and fragments of dead parasites as well as microfilaria can cause emboli in the pulmonary capillaries and coronary arteries. Microfilaria can reach the encephalus, the spinal cord, the eye vessels, and even the anterior or posterior chamber of the eye. Toxic and antigenic action is caused by the substances produced by adult parasites in the arteries, the right side of the heart, and especially thromboxanes released by the blood platelets in contact with parasites (Uchida and Saida, 2005).

The heartworms act mechanically through their large body and tend to block, in particular, the right ventricle and the pulmonary artery, while the hematophagous regimen produces anemia and irritation. It forms emboli (the appearance and circulation in the bloodstream of foreign particles of the normal blood morphochemical composition) by pushing parasite fragments into the bloodstream and causing the sudden death of the animal due to breakage of cerebral vessels (Kitagawa et al., 2003).

The caval syndrome is a severe clinical form of dirofilariosis in a dog. The main mechanisms of this syndrome are: severe and acute tricuspid regurgitation, reduced cardiac output, and intravascular haemolysis. In this situation, a large number of *D. immitis* adults (over 60) migrate from the right side of

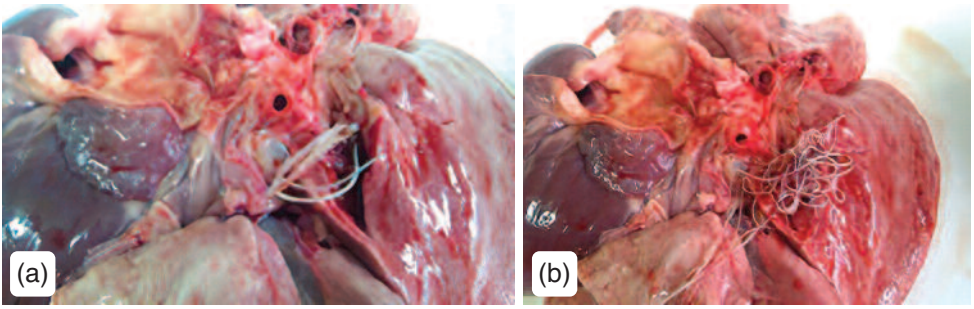

Fig. 1.4 Adults of Dirofilaria immitis before (a) and after mechanical extraction (b) from a nodule from the traject of the right diaphragmatic lobar branch of the pulmonary artery in a male mongrel dog aged 12 years.

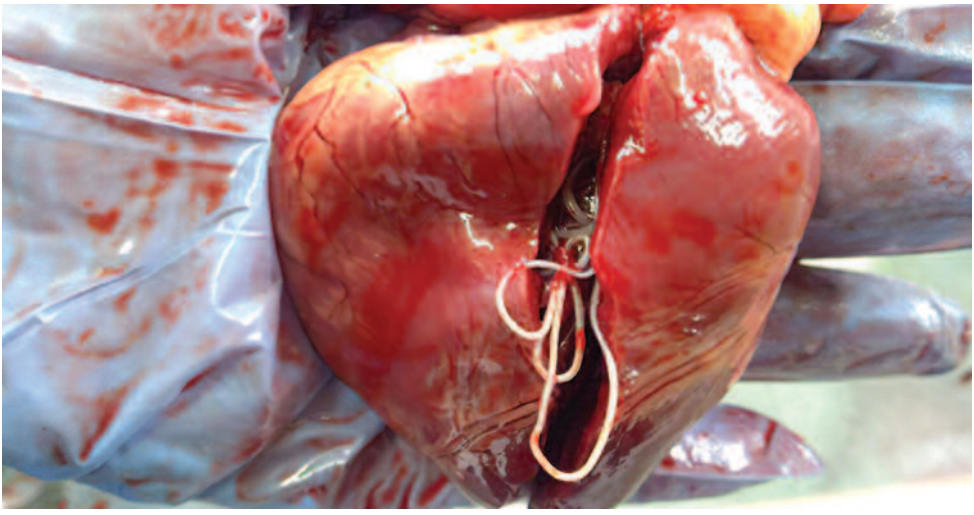

Fig. 1.5 Adults of D. immitis in the right chambers of the canine heart.

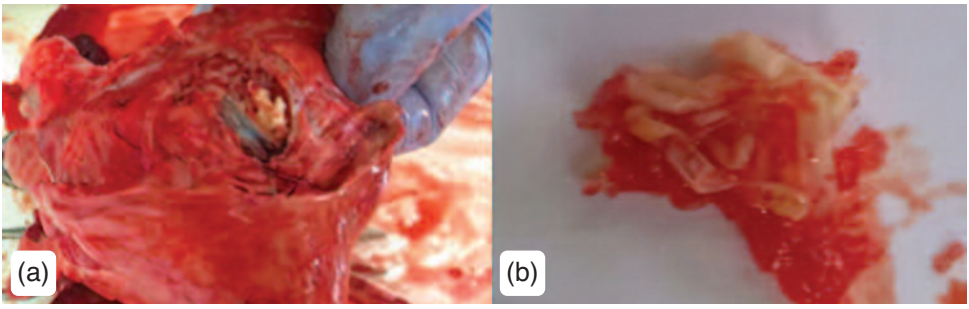

Fig. 1.6 Nodule on the traject of the lobar branch of the pulmonary artery filled with organic debris of dead D. immitis worms (a). The aspect of the content removed from the nodule (b).

the heart to the large vessels. Sudden shock, collapse and destruction of red blood cells, usually without early symptoms, occur. Death usually occurs within 1-2 days and the only effective treatment is to open the jugular vein and extract the worms with a special forceps. Survival of the dog depends on the surgical extraction of a sufficient number of adults so that blood circulation can be restored (Marck et al., 1998). Adults reaching the right ventricle are located in the tricuspid system and migrate to the right atrium. Their simple presence in the tricuspidian system produces severe damage of valves (Figure 1.7), followed by tricuspid regurgitation and aggravated by preexisting pulmonary hypertension. Very soon, there is right heart failure with right systolic murmur, hepatomegaly, splenomegaly, abdominal ascites (Wendy et al., 2007). Pulmonary hypertension as well as tricuspid regurgitation lead to reduced peripheral arterial circulation and reduced pulmonary venous circulation, and implicitly to decreased left heart volume with decreased cardiac output, decreased diastolic volume etc. (June et al., 1998).
Intravascular haemolysis caused by canine heartworm remains a matter of speculation. Endothelial cell disruption and denudation of the intima are the first lesions that occur a few days after the parasites occupy the blood vessels. Evidence suggests that endothelial damage occurs as soon as the parasite is in place, too early for the host to develop an immune response.

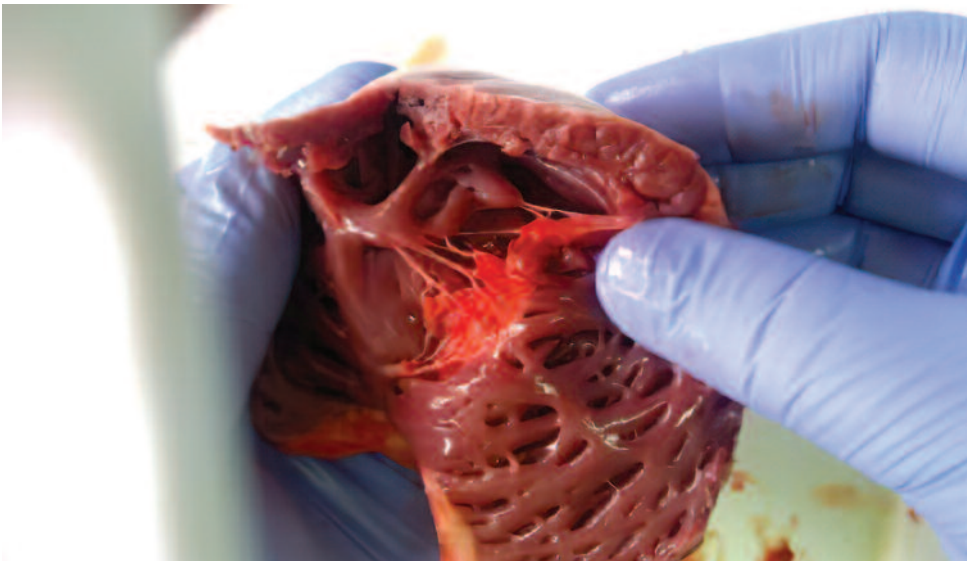

Fig. 1.7 Dog endocharditis due to chronic heartworm disease.

1.2.3 Pathogenesis – Dirofilaria repens

The pathogenicity of this nematode to the dog is very poorly known, as this infection is considered asymptomatic. Adults located in the subcutaneous tissue of the dog may cause dermatological clinical signs such as pruritus, erythema, papules, alopecia, hyperkeratosis, acanthosis, eczema or may well develop asymptomatically.

Serious infections with allergic reactions, possibly due to microfilaria, have also been reported. Generally, 85% of dogs with subcutaneous dirofilariosis exhibited at least one lesion of the subcutaneous tissue in the dorsal part of the body, in the lumbosacral region, the posterior limbs, or in the perianal region (Mandelli, Mantovani, 1966). Recent reports

indicate the association of subcutaneous dirofilariosis with other diseases, such as babesiosis (100%), granulocytic erlichiasis (60%) leishmaniosis, most commonly in the Italian region (Tarello, 2010).

1.2.4 Clinical signs-heartworm dirofilariosis

Normally, the expression of the cardiovascular dirofilariosis symptoms appears in the chronic form. The disease may develop asymptomatically over a period of several months or even years, the appearance of clinical signs being dependent on the number of adults in the heart or pulmonary artery, individual reactivity and physical activity of the dog (lesioning of the artery walls is directly proportional to the physical activity of the animal) (Dillon et al., 1995a). Ideally, the infection with *D. immitis* should be identified by serological testing prior to the appearance of clinical signs. However, at the earliest, antigenemia and microfilaemia do not occur until up to 5 and 6.5 months, respectively, after the infection. When dogs do not receive prophylactic treatment and are not properly tested, the infection is not detected and it progresses as the number of adults of *D. immitis* increases. Clinical signs such as cough, exercise intolerance, apathy, dyspnea, cyanosis, hemoptysis, syncope, epistaxis and ascites (right congestive heart failure) may occur. The frequency and severity of clinical signs correlate with pulmonary pathology and the physical activity level of animals. In sedentary dogs, signs are often not observed even though the number of adults of *D. immitis* in the heart may be relatively large. Infected dogs experiencing a dramatic increase in physical activity, such as during the hunting season, may show obvious clinical signs. Also, parasite death and thromboembolies precipitate expression and wors-

ening of clinical signs (McCall, et al., 2008).
In congestive heart failure, the following are usually noted: abdominal distension, edema of the limbs, anorexia, weight loss and dehydration. At this stage of the disease, there are sounds of heart murmur on the right side of the chest due to tricuspid valve insufficiency, and abnormal heart rhythm due to atrial fibrillation. Sudden death happens very rarely and dogs usually die due to respiratory emergency or cachexia. Occasionally, acute episodes can also be observed during the chronic period of disease progression, so after severe adult death severe thromboembolism may occur, and dogs may display acute dyspnea and hemoptysis with a fatal outcome. Based on the assessment of the number of adults in the right side of the heart, animal health, and age and lifestyle, a dog may be classified as having a low or high risk for the development of clinical signs of infection with *D. immitis* (Furlanelloet al., 1998; Calvert et al., 1985; Venco et al., 2001).
There is also a more complex classification system in which dogs are classified from I to IV based on the severity of clinical signs: Class I dogs with mild infection; dogs in Class II have coughing; Class III dogs are severely affected and show cough, haemoptysis, weight loss, lethargy, exercise intolerance, dyspnoea, heart failure (ascites), and radiographic findings suggestive of cardiovascular infection (large primary pulmonary arteries and lobar pulmonary arteries are truncated, arteries pulmonary sinuous lung and infiltrated lymphadenopathy). Class IV dogs are those with caval syndrome characterized in principle by haemodynamic changes (AHA, 2014). The main signs are: dyspnoea, tricuspid heart murmur, acute intravascular haemolysis, and the sign considered pathognomonic for caval syndrome is hemoglobinuria. In this situation, in the absence of surgery to eliminate the heart parasites, the animal will not survive (Atwell and Buoro, 1988; Kitagawa et al., 1986, 1987; Venco, 1993).
Dogs aged 5-7 are at a higher risk of infection with a high number of *D. immitis* adults, probably due to increased exposure time and the development of the disease. There are also other factors that affect the evaluation of the risk of *D. immitis* infection, such as cardiopulmonary disease or other systemic diseases and pathologies of other organs. Another important aspect is the extent to which the physical activity of the animal can be restricted during the treatment period (Venco et al., 2001).
In the very rare cases in which *D. immitis* adults are in the right side of the heart, an abnormal lsound may be heard due to tricuspid valve insufficiency

and galloping heart rate (Atkins et al., 1998a). Neurological signs such as ataxia, syncope, blindness can be observed when the ectopic localization of the filaria occurs (Atkins et al., 1998a, Dillon et al., 1996, 1997a, b, 1998, McCall et al., 1994). Although rarely observed clinically, pulmonary edema, pneumothorax, or chylothorax were reported in cat dirofilariosis (Atkins et al., 1998b, Dillon et al., 1997b, Glaus et al., 1995, Treadwell et al., 1998). In principle, there are two phases of clinical expression in the evolution of dirofilariosis in the cat: the first stage in which *D. immitis* larvae L5 reach the pulmonary arteries and die, and the second stage is marked by the death of *D. immitis* adults (Atkins et al., 1998a Dillon et al., 1995b).

1.2.5 Clinical signs-subcutaneous dirofilariosis

Subcutaneous dirofilariosis in dogs is usually asymptomatic. Clinical manifestations are classified in two clinical syndromes: multifocal nodular dermatitis, which is generally located on the face and prurigo papularis dermatitis. Numerous dermatological manifestations such as: pruritus in 100% of animals, erythema (79%), papules (62%), focal or multifocal alopecia (55%), hyperkeratosis (14%), nodules (12%), acanthosis (5%), eczema (3%), pyoderma (3%) and edema (1%) have been noted. Extradermal localisations of *D. repens* adults include: conjunctivitis (46%), anorexia (35%), vomiting (26%), fever (25%), lethargy (20%), enlarged lymph nodes (10%) (Simone et al., 2012; Simone et al., 2017). A recent study conducted by Mircean (2017) reveals the implications of *D. repens* microfilaria in kidney and liver imbalances and the presence of adults in abdominal and ocular cavities. These changes and injuries have been attributed to both mechanical and immunopathological processes. Consequently, experimental investigations on pathogenic mechanisms of subcutaneous dirofilariosis are required.

1.2.6 Diagnosis

Paraclinical diagnosis

In optimal conditions, the lifecycle lasts 184-270 days, so that the dog can become microfilaremic within ca. 7-9 months after the infection. Not all infected dogs become microfilaremic (in unisex infestations, when administering drugs that induce sterility of *Dirofilaria immitis* females, in individual situations of occurrence of immune-mediated reactions leading to the death

of microfilaria) (McCall JW, et al., 2008).
The diagnosis of dirofilariosis is based on the presence of circulating microfilaria and / or circulating antigens from adult females. Not all microfilaria found in the blood of dogs are *Dirofilaria immitis* (Acantocheilonema reconditum, *Dirofilaria repens*, *Dipetalonema* dracunculoides and, very rarely, *Dipetalonema grassi*). Adults of *Dirofilaria immitis* live between 5-7 years. Transplacentary trasmitted microfilaria or those transmitted by haematransfusion are incapable of developing in adults (Castillo JC, et al., 2011).
The antigen detection test was first described in the early 1980s. Weil et al. (1984) showed the detection of adults of *D. immitis* by counterimmune electrophoresis (CIE). Subsequently, the authors described the ELISA based on monoclonal antibodies (Weil et al., 1985). Both techniques are characterized by high specificity and sensitivity to the detection of circulating microfilaria. Also, the antigen screening test was able to assess the degree of infestation. Indeed, Brunner et al. (1988) showed that the sensitivity of the tests was not affected by the presence of circulating microfilaria of *D. immitis*, but was largely influenced by the large number of *D. immitis* adults.
Tests with false negative results may be due to the presence of male or female worms (unisex infections are extremely rare in dogs, Rishniw et al., 2012), elimination by means of immune system mechanisms or the use of macrocyclic lactones (LM, Rawlings et al., 1982). Antigen screening tests, which can be performed on whole blood, plasma or serum, can also produce false negative results because of antigen-antibody complexes that inhibit immunoassay tests to identify antigens and develop subsequent colorimetric reaction (Tonelli et al., 1989). Recently, it has been found that long-term use of monthly macrocyclic lactone in infected dogs (so-called “slow killing”) can also cause false negative test results for antigen detection, probably due to an intense antibody response to antigens released from adults of *Dirofilaria immitis* that die (Drake et al., 2015).
Interestingly, most of the heartworm screening tests used an antigen recovery method to minimize the effects of immune complex formation on the performance of the test (Little et al., 2014). Also, the use of chemicals (e.g., pepsin and acid treatment) has been reported to remove antigen-detectable inhibitors (Rodríguez-Iglesias et al., 1992).
It has been argued that pretreatment of heat serum samples before testing for antigens is able to reverse false negative results due to antigen-antibody complexes in hosts infected with *D. immitis*, both experimentally and naturally

(Little et al., 2014a; Little et al., 2014b; Velasquez et al., 2014; Ciuca et al., 2016). Thus, heat treatment disrupts antigen-antibody complexes and releases antigen which is subsequently made available for detection. This may have important consequences for the diagnosis of clinical disease but also for epidemiological studies, especially in areas where the prevalence of infection is not well known.

Diagnosis of *D. repens* infection is based on the presence of circulating microfilaria or on parasite observation in the subcutaneous nodules, as there are currently no screening tests available for antigens for serological diagnosis. *D. immitis* and *D. repens* can also be identified by histochemical staining of the anatomical regions of microfilaria with phosphatase activity and amplification of microfilaria DNA by the PCR method. *D. immitis* microfilaria shows two areas of phosphatase activity near anal and excretory pores, while mff. *D. repens* have only one area of phosphatase activity near the anal pores. Recently, a duplex real-time PCR method capable of detecting and differentiating the two filaria as well as the multiplex PCR method for simultaneous detection of filaria in the dog have been described.

There have been several studies published on the prevalence of *D. immitis* and *D. repens* infection in dogs living in endemic areas for both parasites (Pantchev et al., 2009, 2011; Demiaszkiewicz et al., 2014; Ionică et al., 2015). The Knott method, along with the antigen detection test and the PCR technique were used to determine the state of monoinfection or co-infection of the final hosts. However, many dogs in which the antigen detection test had a negative result, whether or not microfilaremic, were considered either uninfected or infected only with *D. repens* on confirmation by the PCR method. None of these studies subjected the serum samples to heat treatment, probably underestimating the actual prevalence.

The skin lesions associated with this parasitosis are frequently erroneously detected with dermatological problems of different aetiology and, moreover, the identification methods are often invalidated by the experience of the operator carrying out the test.

The communion between the absence of valid diagnostic methods and the possibility of asymptomatic progression of this pathology, allowed a distribution of this parasite, particularly in the southern regions of Europe, where it is now endemic.

As mentioned above, the diagnosis is a real challenge for both clinicians and researchers due to the lack of efficient diagnostic tools.

Those are mainly based on the identification of circulating microfilariae, followed by morphometric or molecular species identification.
Although there are publications about diagnosis and biology, there are not many reports about the pre-patent period of the disease in dogs.
The values obtained from the latter are numerous and different, for example early data such as 164 days post infection (Petry et al., 2015) or late data such as 239 days p.i.(Cancrini et al., 1989) while there are other intermediate data such as Webber and Hawking which in 1955 reported a pre-patent period of 182 days.
This enormous variability due to the fluctuations of the pre-parent period and the differences in the clinical manifestation of the pathology makes unreliable the diagnostic importance of the identification of circulating microfilaria in the bloodstream.
One of the main identification methods is the Modified Knott's Test, which, however, needs an experienced operator to allow a differential diagnosis between *D.repens, D.immitis and A.reconditum*.
Diagnosis by PCR is the only method with good sensitivity and specificity, using nucleotide sequences of subunit 1 of cytochrome c-oxidase, of the intergenic spaces regions and 12S RNA (Rihniw et al., 2006; Gioia et al., 2010; Latrofa et al., 2012; Ciuca et al., 2016). However, these techniques require specialised laboratories with experienced staff.
A further lack in the diagnosis can be seen in the absence of a specific serological test for this species, as commercially available ELISA tests exist only for *D. immitis* although non-commercial ELISA tests have recently been developed for the identification of the immune response against adult *D. repens* antigen in human medicine, for people living in endemic areas.
However, there are tests to assess the humoral response to *D.immitis* and *Wolbachia*, an endosymbiont bacterium, present in the filarias (Ciuca et al., 2018). The author suggests that a possible cross-reactivity between *D.immitis* and *D.repens* antigens and an increase in *Wolbachi*a positivity may increase the diagnostic specificity of these tests.
Serological evaluations were carried out only through commercial kit, which showed positive *D.immitis* and also in case of co-infestation by *immitis* and *repens*.

Clinical diagnosis

The evaluation by thoracic radiography, echocardiography and electrocardiography provides a perspective on the clinical condition of each patient with cardiopulmonary dirofilariosis. Chest radiographs identify pulmonary artery enlargement, lung parenchymal changes, and right cardiomegaly in the advanced stages of the disease. This technique cannot be used to evaluate parasitic burden. Echocardiography is an examination by means of which the adults can be visualized in the right heart chambers, the caudal vena cava, the main pulmonary artery, and the proximal tract of both caudal lung arteries. Adults can be identified as short, double linear, floating in the right heart chamber or in the lumen of the vessels (Moise, 1988; 276). Cardiac ultrasound also provides information about heart parasite load and disease status, important factors in establishing appropriate therapy. An important aspect is that cardiac ultrasound should be considered in cases where clinical and imaging characteristics suggest a severe infection. The Doppler echocardiography can accurately determine the presence and severity of pulmonary hypertension. The electrocardiogram is a useful exam through which abnormalities of the electrical action of the heart (right electrical axis deviation, atrial fibrillation) can be identified, but these changes are usually found in the last severe stage of the disease (McCall et al., 2008).

1.3 Control

The treatment in cardiopulmonary dirofilariosis is complex and difficult to establish in conditions where adulticides can cause thromboembolism and death of the patient. In conclusion, the therapy schedule should be used depending on the animal's health status and burden with adults of *D. immitis*, and the association with other competing diseases. In principle, the treatment is aimed at eliminating microfilaria from the blood and disrupting the development of larval stages in adults and the elimination of preexisting adults. Assessment for the treatment of adulticides and the risk of thromboembolism should be performed individually for each infected animal. If so far the disease was considered to have a 4-stage evolution (Di Sacco and Vezzoni, 1992), researchers have now reduced the disease to two categories of evolution: mild (low risk of thromboembolism) and severe (high risk of thromboembolism).

Dogs with low risk of thromboembolism include: a small number of adults without clinical signs, normal radiological profile, cardiac ultrasound does not reveal the presence of adults in the right side of the heart, low titre of circulating antigens or negative antigen test, but the presence of microfilaria in blood, the absence of concurrent disease association, andthe availability of physical activity restriction (owner involvement) (Venco et al., 2004; Ciuca et al, *in press*).
Dogs with high risk of thromboembolism include: high adult loads, clinical signs specific to the disease (cough, abdominal distension), adult observation in the right side of the heart to cardiac ultrasound, severe pulmonary changes, high circulating antigens, absence of restriction of physical activity (absence of owner involvement) (Venco et al., 2011, Maccal et al., 2008).
The supportive therapy has the role of reducing and controlling pulmonary inflammation, pulmonary edema and reducing complications resulting from adulticide therapy (Dillon et al., 1995). Corticosteroid use (prednisolone 1-2 mg / kg for 4-5 days), diuretics (furosemide 1mg / kg) and digoxin can only be used when atrial fibrillation is present.

1.4 Adulticide therapy

The only substance approved and recommended by American Heartworm Society (AHA) is melarsomina, used in a 2.5 mg / kg dose, two doses at 24-hour intervals. Recently, the AHA recommendation (2005) proposes two-phase melarsomine therapy to reduce the risk of pulmonary thromboembolism consisting of intramuscular injection of two doses at 24 hours followed by a third dose at 30 days. This treatment scheme involves the initial elimination of 90% of adult males and 10% of adult females, reaching a 50% reduction in the total number of adults. The third dose eliminates the remaining adults, so reducing the risk of thromboembolism and shock when adult death is achieved gradually (AHA, 2012). Generally, adulticidal therapy causes pulmonary thromboembolism, especially if parasitic load is high. Pulmonary thromboembolism can be controlled by movement restriction at least one month after adulticide therapy, administration of heparin and corticosteroids to reduce pulmonary inflammation and to avoid severe respiratory shock due to adult elimination (Venco et al., 1998).
Numerous studies suggest that macrocyclic lactone therapy (ivermectin),

which has been shown to be partially adulticidal when used in doses of 6-12 mcg / kg every month for 16 months or even 30 months, has an efficacy of 100% (McCall et al., 2001). In contrast, other studies demonstrate a worsening of the animal's health when adult elimination is achieved slowly and over a long period of time (Venco et al., 2004).

Surgical extraction is recommended in dogs with a large parasitic charge as the only safe method of eliminating adults without the risk of pulmonary thromboembolism (Morini et al., 1998). The surgical extraction of *D. immitis* adults is performed with a Flexible Forceps Alligator (Fuji Photo Optical LTD, Japan) which is inserted along the jugular vein with guidance provided by fluoroscopy (Ishihara et al., 1990).

Doxycycline therapy (10mg/ kg) for a period of 4-6 weeks, followed by macrocyclic lactone administration in the usual doses for microfilaricide therapy, leads to female sterilization and amicrofilaremia, prevention of reinfestation and slow killing of adults. Adult death, not susceptible to efflux of endosimbionts, is characterized by low risk of thromboembolism and inflammation (Kramer et al., 2014).

Prevention of *D. repens* with macrocyclic lactones is questionable and, to date, drugs containing continuous release moxidectin appear to be effective, according to experimental studies (Genchi et al., 2019). Monthly prophylaxis with macrocyclic lactones is the only effective option for cats living in endemic areas of dirofilariosis in dogs. The monthly doses of prophylactic substances are as follows: 24 µg / kg body weight of ivermectin, 2 mg / kg milbemycin oxima, 1 mg / kg moxidectin and 6 to 12 mg / kg selamectin, starting at 8 weeks of age (Genchi et al. 2007).

Canine heartworm disease (HWD) is caused by the filarial nematode *Dirofilaria immitis*, a vector-borne parasite transmitted by several mosquito species and is endemic in many parts of the world (Genchi et al., 2014). The presence of adult worms in the pulmonary arteries of infected dogs causes changes in arterial structure and function that can lead to pulmonary hypertension and, eventually, to right-sided congestive heart failure (Balbo et al., 1968; Bowman and Atkins, 2009). Melarsomine dihydrochloride is the only approved adulticidal drug for treatment of HWD. Several studies in both experimentally and naturally infected dogs have reported the adulticide effect of a combination of macrocyclic lactones (ML) and doxycycline against *D. immitis*, showing that these protocols are safe and effective (Bazzocchi et al., 2008; Grandi et al., 2010; Mavropoulou et al., 2014; Bendas et al., 2017; Savadelis

et al., 2017; Genchi et al., 2019a; Paterson et al., 2020; Vörös et al., 2022). Doxycycline targets the bacterial endosymbiont *Wolbachia*, whose reduction contributes to worm infertility and death (Kramer et al., 2007; Louzada-Flores et al., 2022). This activity, combined with the known detrimental effects of ML, may eliminate adult worms and lessens the inflammatory reaction against dead and dying worms (Kramer et al., 2011). The American Heartworm Society (AHS, 2018) and the European Society of Dirofilariosis and Angiostrongylosis (ESDA, 2017) currently suggest that in cases where treatment with melarsomine is not possible or is contraindicated, a monthly treatment based on ML along with doxycycline for a 4-week period might be considered (ESDA, 2017; AHS guidelines, 2018; Vörös et al., 2022). It has been shown that the combination of moxidectin/doxycycline has superior adulticide efficacy compared to ivermectin/doxycycline (Jacobson and DiGangi et al., 2021). Grandi et al. (2010) reported 73% efficacy 10 months after the beginning of doxycycline (daily for 1 month) combined with oral ivermectin every 15 days for 6 months. More recently, Savadelis et al. (2017) reported a 95.9% adulticide efficacy in experimentally infected dogs after 1 month of doxycycline combined with 10 monthly treatments with a topical formulation of moxidectin. Genchi et al. (2019a) reported that 15/16 naturally infected dogs became antigen negative at 9 months with the same protocol. Paterson et al. (2020) reported 93.0% of dogs treated with the same protocol were antigen-free at month 15.

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Objective of the thesis

To achieve the overall objective of the PhD thesis entitled “New Insights into the Diagnosis and Control of *Dirofilaria* Infection in Dogs”, the following specific objectives were pursued:

- To improve of the Knott's test method for identifying the microfilariae of Dirofilaria immitis and *D. repens*.

- To advance the knowledge in the immune response of *D. repens* in experimentally infected dogs.

- To evaluate the slow kill therapy with doxycycline and various commercially available formulations of moxidectin in dogs naturally infected with *D. immitis*.

- To update the distribution of *D. immitis* and *D. repens* in dogs from an endemic area of southern Italy.

Chapter 2

Evaluation of alternative reagents on the performance of the modified Knott's test

Marco Genchi, **Lavinia Ciuca**, Alice Vismarra, Elena Ciccone, Giuseppe Cringoli, Laura Kramer, Laura Rinaldi, 2021. Evaluation of alternative reagents on the performance of the modified Knott's test. Veterinary Parasitology 298 (2021) 109555

2.1 Abstract

The aim of the present study was to evaluate the suitability of different reagents as safe alternatives to 2% formalin in the modified Knott's test for the diagnosis of subcutaneous (*Dirofilaria repens*) and cardiopulmonary (*D. immitis*) dirofilariosis. A total of 61 blood samples from dogs naturally infected with *D. immitis* and *D. repens* were collected and analysed in two different laboratories (Lab 1, University of Parma and Lab 2, University of Napoli). For each blood sample the modified Knott's method was performed to identify and measure the mean length and width of the microfilariae (mfs) using 2% formalin (A), 2% acetic acid (B), 2% glacial acetic acid (C), 10% saponin (D) and distilled water (E). When compared to 2% formalin, there was no significant difference ($P > 0.05$) among the mean length and width of either *D. immitis* or *D. repens* mfs with distilled water (E). The lengths and widths of mfs, however, were significantly reduced ($P < 0.05$) when using B, C, D likely due to more pro- nounced parasite dehydration. Despite differences in measurements, the morphological features of the head and tail of the two species were maintained, suggesting that all the solutions tested could be a suitable alternative to formalin. All alternative reagents caused more marked haemolysis compared to formalin, improving readability of slides. The values of the mean length and the mean width of *D. immitis* and the mean width of *D. repens* mfs obtained with formalin and distilled water were statistically different ($P < 0.005$) between the two laboratories. The difference in mf measurements between the two labs could be due to the use of reagents purchased from different manufacturing companies.
Results suggest that distilled water could replace formalin in the modified Knott's test, as a safer reagent that allows morphology-based species differentiation of *Dirofilaria* spp.

2.2 Introduction

Dirofilariosis, caused by *Dirofilaria immitis* and *Dirofilaria repens,* are two mosquito-borne diseases that are (re-) emerging and spreading in several countries (Genchi and Kramer, 2020; Mendoza-Roldan et al., 2020; Széll et al., 2020; Brianti et al., 2021; Deksne et al., 2021). Various factors have facilitated this expansion, including climate change and globalization (Genchi et al., 2009), that allowed the introduction of new competent mosquito species such as Aedes albopictus, together with the movement of pets to or from endemic areas. Furthermore, misdiagnosis and lack of prevention (in particular for *D. repens,* which is usually asymptomatic (Genchi et al., 2019), likely contributes to underestimation of risk for infection among the veterinary practi- tioners). Finally, both *D. immitis* and *D. repens* are important and emerging agents of vector-borne zoonosis, in particular *D. repens* (Simo´n et al., 2012).

Correct diagnosis of *Dirofilaria* spp. infection is an essential part of disease management and in controlling the spread to other animals and humans. The current American Heartworm Society guidelines (Amer- ican Heartworm Society, 2020) recommend testing for both circulating microfilariae (mfs) and antigens to confirm heartworm infection. The European Society of Dirofilariosis and Angiostrongylosis (ESDA, 2017) and the European Scientific Counsel Companion Animal Parasites guidelines (ESCCAP, 2019) indicate detection of circulating mfs as the best and most sensitive and specific option for diagnosis of *D. repens* infection, even when compared to molecular methods such as PCR (Ciuca et al., 2020). Moreover, the ESCCAP guidelines (ESCCAP, 2019) recommend that dogs are checked for circulating mfs before beginning annual preventive treatment in order to reduce the risk of selecting resistance against *D. immitis*. Identification of mfs is also important due to potential cross-reactivity of enzyme-linked immunosorbent assay (ELISA) and immunochromatographic tests for *D. immitis* with other filarial nematodes such as *D. repens, Angiostrongylus vasorum* and *Spi- rocerca lupi* (Schnyder and Deplazes, 2012; Aroch et al., 2015; Panarese et al., 2020a).

The modified Knott's test (Knott, 1939) is an easy and inexpensive technique based on concentration, staining, detection and morphome-

tric identification of circulating mfs of different species. The technique foresees the dilution of 1 mL of EDTA venous blood with 9 mL of 2% formalin (ESDA, 2017). However, formaldehyde, the substance contained in formalin, has been shown to be mutagenic and genotoxic in several experimental models, both *in vivo* and *in vitro* (National Toxi- cology Program (NTP), 2010; Bernardini et al., 2020). The toxicity of formaldehyde is thought to be due to its high water solubility and reactivity in interactions with nucleophilic groups of proteins, DNA and RNA molecules (Katsnelson et al., 2013). In 2012, the International Agency for Research on Cancer (IARC) classified formaldehyde as a human carcinogen (International Agencyfor Research on Cancer, 2012). In addition, the ECHA (European Chemical Agency) identifies this sub- stance as fatal if inhaled, toxicif swallowed, toxic following contact with skin, capable of causing severe skin burns and eye damage. Studies on dosimetry modelling of inhaled formaldehyde in humans have shown that more than 95 % of the inhaled formaldehyde is predicted to be retained by the respiratory tract (Overton et al., 2001). Moreover, formalin requires special precautions in handling, storage and disposal and should be employed only under a chemical hood. It would therefore be of interest to find alternative fixation methods in order to increase the use of the Knott's test, especially among veterinary practitioners.

Therefore, the aim of this study was to evaluate the suitability of different reagents, i.e. acetic acid-vinegar, glacial acetic acid, saponin and distilled water, as safer alternatives to formalin to use in the modified Knott's test for the diagnosis of subcutaneous (*D. repens*) and cardiopulmonary (*D. immitis*) dirofilariosis.

Tab. 2.1 Comparison of mean length of Dirofilaria immitis *using different solutions for the modified Knott's test: Formalin (A), Acetic acid-vinegar (B), Glacial acetic acid (C), Saponin (D), Distilled water (E).*

Solution	No. Samples	Mean	Min.	Max.	SD	*P* value (post hoc analysis)
A	28	312.34	300.20	335.22	8.48	A vs. B = 0.0000
B	27	285.12	271.65	312.83	11.09	A vs. C = 0.0000
C	24	290.09	274.89	326.22	9. 78	A vs. D = 0.0000
D	25	277.91	258.35	297.89	9.38	A vs. E = 0.0789
E	25	307.75	287.99	325.87	7.95	B vs. C = 0.0620 B vs. D = 0.0660 B vs. E = 0.0000 C vs. D = 0.0000 C vs. E = 0.0000 D vs. E = 0.0000

Tab. 2.2 Comparison of mean width of Dirofilaria immitis *using different solutions for the modified Knott's test: Formalin (A), Acetic acid-vinegar (B), Glacial acetic acid (C), Saponin (D), Distilled water (E).*

Solution	No. Samples	Mean	Min.	Max.	SD	*P* value (post hoc analysis)
A	28	6.30	5.70	6.60	0.22	A vs. B = 0.0000
B	27	5.84	5.40	6.90	0.35	A vs. C = 0.0000
C	24	5.84	5.27	6.34	0.23	A vs. D = 0.0000
D	25	5.70	5.40	6.63	0.25	A vs. E = 0.0983
E	25	6.25	5.70	7.13	0.39	B vs. C = 1.0000 B vs. D = 0.5060 B vs. E = 0.0400 C vs. D = 0.0231 C vs. E = 0.0010 D vs. E = 0.0000

Tab. 2.3 The comparison of mean length of Dirofilaria repens *using different solutions for the modified Knott's test: Formalin (A), Acetic acid-vinegar (B), Glacial acetic acid (C), Saponin (D), Distilled water (E).*

Solution	No. Samples	Mean	Min.	Max.	SD	*P* value (post hoc analysis)
A	31	368.24	347.45	386.97	8.64	A vs. B = 0.0000
B	24	344.17	326.09	353.65	6.63	A vs. C = 0.0000
C	32	339.21	321.80	354.55	8.56	A vs. D = 0.0000
D	21	331.84	307.82	351.35	13.63	A vs. E = 1.0000
E	23	368.77	352.48	387.54	10.11	B vs. C = 0.0121 B vs. D = 0.0060 B vs. E = 0.0000 C vs. D = 0.0204 C vs. E = 0.0000 D vs. E = 0.0000

Tab. 2.4 The comparison of mean width of Dirofilaria repens *using different solutions for the modified Knott's test: Formalin (A), Acetic acid-vinegar (B), Glacial acetic acid (C), Saponin (D), Distilled water (E).*

Solution	No. Samples	Mean	Min.	Max.	SD	*P* value (post hoc analysis)
A	20	7.72	6.40	9.18	1.12	A vs. B = 0.0000
B	20	5.96	5.05	6.55	0.33	A vs. C = 0.0010
C	21	6.48	5.70	7.85	0.48	A vs. D = 0.0000
D	20	5.94	5.29	6.62	0.29	A vs. E = 0.0869
E	20	8.04	6.48	9.13	0.98	B vs. C = 0.0200 B vs. D = 1.0000 B vs. E = 0.0000 C vs. D = 0.0010 C vs. E = 0.0000 D vs. E = 0.0000

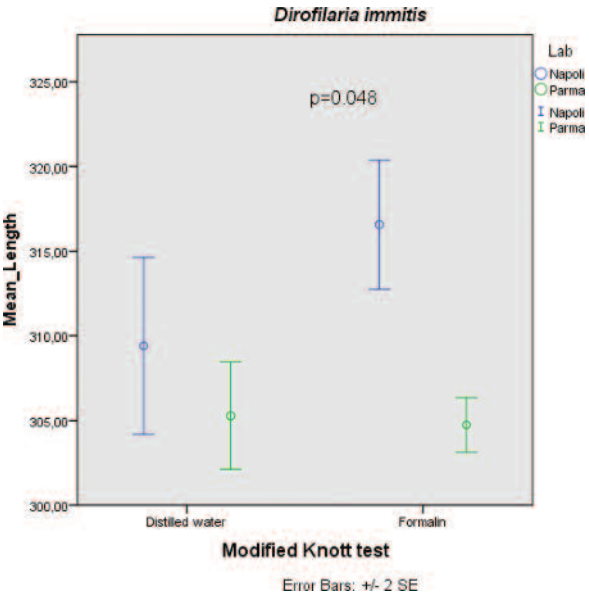

Fig. 2.1 Comparison of modified Knott's tests using formalin and distilled water between two laboratories: Napoli (n = 18) and Parma (n = 10). The circles represent the mean of length of D. immitis mfs.

Notes: Modified Knott's test with distilled water-Napoli: mean = 309.411; Standard Deviation (SD) = 9.408; Standard error of mean (SE) = 2.429; Parma: mean = 305.282; SD = 4.429; SE = 1.400; Modified Knott's test with formalin-Napoli: mean = 316.568; SD = 7.663; SE = 1.586; Parma-mean = 304.742; SD = 2.259; SE = 0.714.

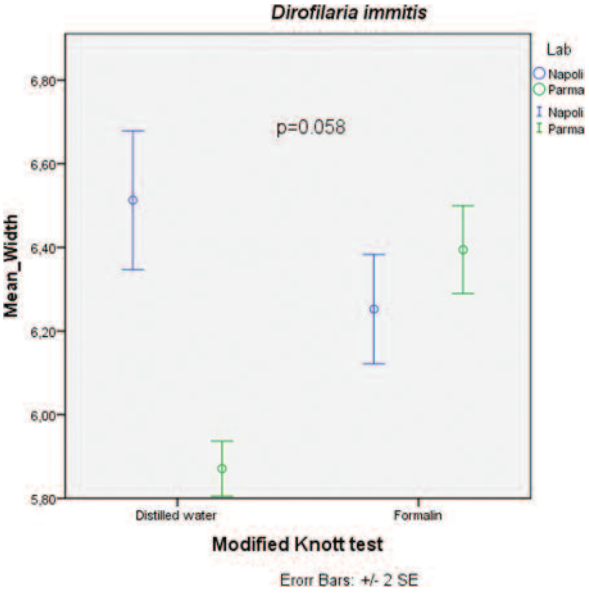

Fig. 2.2 Comparison of modified Knott's tests using formalin and distilled water between two laboratories: Napoli (n = 18) and Parma (n = 10). The circles represent the mean of width of D. immitis *mfs.*

Notes: Modified Knott's test with distilled water-Napoli: mean = 6.153; SD = 0.299; SE = 0.077; Parma: mean = 5.871; SD = 0.091; SE = 0.029; Modified Knott's test with formalin-Napoli: mean = 6.252; SD = 0.254; SE = 0.061; Parma-mean = 6.394; SD = 0.146; SE = 0.046.

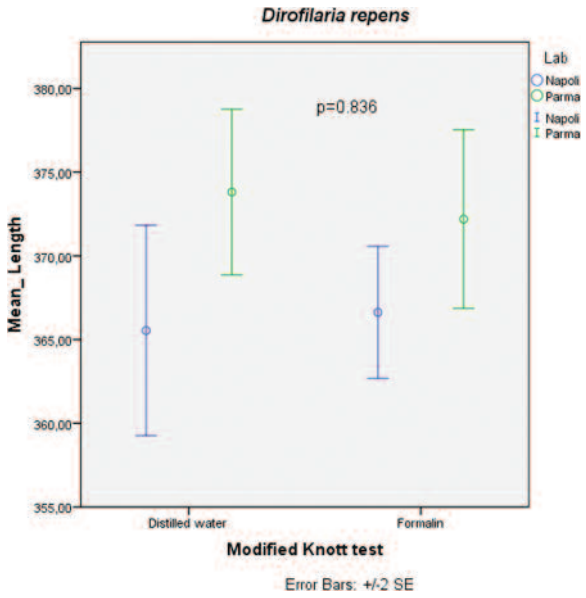

Fig. 2.3 Comparison of modified Knott's tests using formalin and distilled water between two laboratories: Napoli (n = 24) and Parma (n = 9). The circles represent the mean of length of D. repens *mfs.*

Notes: Modified Knott's test with distilled water-Napoli: mean = 365.539; SD = 6.437; SE = 2.145; Parma: mean = 373.808; SD = 6.437; SE = 2.145; Modified Knott test with formalin-Napoli:mean = 366.626; SD = 8.890; SE = 1.895; Parmamean = 372.195; SD = 6.941; SE = 2.313.

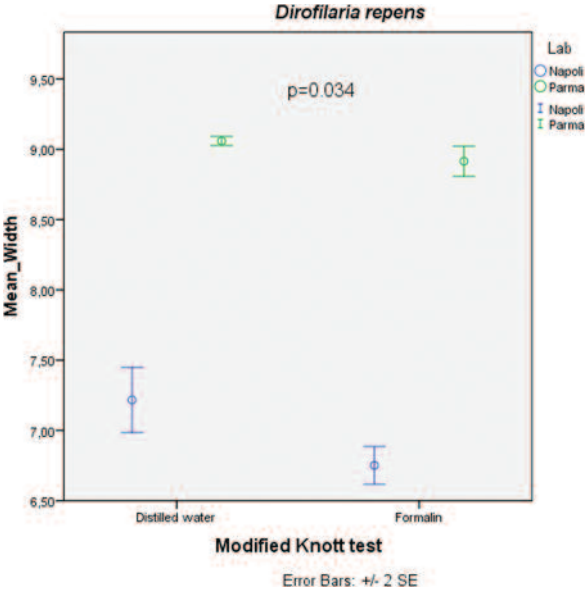

Fig. 2.4 Comparison of modified Knott's tests using formalin and distilled water between two laboratorires: Napoli (n = 24) and Parma (n = 9). The circles represent the width of D. repens *mfs.*

Notes: Modified Knott's test with distilled water-Napoli: mean = 7.217; SD = 0.384; SE = 0.116; Parma: mean = 9.059; SD = 0.048; SE = 0.016; Modified Knott's test with formalin-Napoli: mean = 6.751; SD = 0.224; SE = 0.061; Parma-mean = 8.915; SD = 0.160; SE = 0.053.

2.3 Materials and Methods

2.3.1 Sampling and modified Knott's tests

Between January and September 2020, blood samples from 61 dogs of different breeds, sex and age, previously found to be microfilaremic to *Dirofilaria* spp. using the modified Knott's test, by different practitioners. All the blood samples were sent to two parasitology laboratories for the species identification: Lab 1, University of Parma (19 samples) and Lab 2, University of Napoli (42 samples).

Approximately 7 mL of venous blood were collected from each dog and placed in EDTA vacutainer tubes. The blood samples were delivered by the practitioners to the laboratories within 24 h. Moreover, all the blood samples were constantly kept refrigerated at 3 5 °C and analyzed on the day of arrival in the laboratory.

All samples were analyzed with the modified Knott's test. One ml of EDTA blood was mixed with 9 mL of 2% formalin solution (A) in a 15 mL tube. The tube was gently inverted 4 times to mix the solution and centrifuged for 3 min at 1500 xg. The supernatant was poured off and 1–2 drops of 1% methylene blue were added (ESDA, 2017). A drop of the sediment was placed on a glass slide and covered with a coverslip. The slide was examined under the microscope at 10x to assess the presence of mfs, and at 40x to observe the morphology features.

The same procedure was applied to all the samples by replacing formalin with the following reagents: 2% acetic acid-vinegar (B), 2% glacial acetic acid (C), 10 % saponin (D), distilled water (E).

Ten mfs were randomly selected from the slides prepared with each of the 5 reagents body length and width were measured and the morphology of the head and tail were determined (Magnis et al., 2013). All evaluations were done by two of the authors (MG, LC), in blind. Morphometric analyses were conducted with standard diagnostic microscopes (Lab 1 used a Nikon Eclipse E200 with Zeiss Axiocam Erc 5c; Lab 2 used a Leica DM 1000 LED with ICC50 W camera system). Moreover, an antigen test for detection of *D. immitis* (PetCheck Heartworm PF Antigen, IDEXX) was carried out on all blood samples to confirm diagnosis of *D. immitis* infection.

In order to determine the correspondence of mfs measurements taken by the two laboratories, the same histological section obtained from a

naturally *D. immitis* infected dog showing mfs within the pulmonary ar- teries was examined by each laboratory. The microscopes to be used for the study were calibrated with a stage micrometer, measure- ments ob- tained by the two laboratories were recorded and compared.

2.3.2 Statistical analysis

The arithmetic mean, standard deviation (SD), standard error of the mean (SE) and ranges (min and max) of lengths and widths of *D. immitis* and *D. repens* mfs were calculated for each of the following reagents: Formalin (A), Acetic acid-vinegar (B), Glacial acetic acid (C), Saponin (D), Distilled water (E). Multiple comparisons of mean ranks depending on the fulfillment of the test assumptions between the values obtained in the A, B, C, D, E solutions were performed using a parametric test, i.e. the one-way analysis of variance (ANOVA) along with post hoc testing. Before applying post-hoc test (in between the groups), the homogeneity of the variances among the groups (Levene's test) was tested. If vari- ances were homogeneous (P 0.05) or not homogeneous ($P < 0.05$) different tests (Bonferroni and Games-Howell, respectively), were used. For all comparisons, a level of α 0.05 was assumed, and the obtained P values were rounded to two decimal places. Moreover, Error Bars graphics were constructed in order to compare the results of measure- ments (length and width) of mfs of both *D. immitis* and *D. repens* using formalin and purified water in two laboratories (Napoli, Parma). Sta- tistical analysis and graphs were performed using SPSS Statistics v.23 (IBM, Armonk, NY, USA).

2.4 Results

Mini-FLOTAC was shown to be a simple and rapid technique for detecting the number of eggs/cysts/oocysts per gram of feces. Among the 70 pools an- alyzed, 80% (24/30; 95% confidence interval [CI] = 30-54%) were positive for at least one parasite (Table 2.1).

Parasite intensity express in eggs, larvae, cysts and oocyst per gram (EPG, LPG, CPG and OPG) of feces detected in zoo mammals are showed in Table 2.2.

Higher prevalence of parasites was detected in the zoos in central Italy (Lanciano 47% and Aprilia 37.5%) than in the zoos in southern Italy (Naples 37.5% and Pesco Sannita 20.1%).

Several genera of helminths and protozoa were detected in mammals at the four zoos. Among the nematodes, GI strongyles were the most frequent (40%), followed by *Trichuris* spp. (23.3%), *Parascaris* spp. (13.3%) and *Capillaria* spp. (3.3%). With regard to protozoa, most of the samples were positive for *Blastocystis* spp. (6.6%), followed by *Giardia* spp. (3.3%) and *Eimeria* spp. (3.3%).

Widespread presence of GI parasites was confirmed in the four zoos. Helminthic infections were more common than protozoan infections in all the mammal orders examined and this was concordant with the findings from two other Italian zoos located in Apulia and Tuscany, where the overall prevalence of parasitic infection was 61.5% (96/156) by flotation test using a low-specific-gravity solution, sedimentation and a modified McMaster technique (Fagiolini et al., 2010).

All of the gastrointestinal parasites identified in our study were described previously in zoo animals by other authors, and these parasites are known to be pathogenic to both animals and humans (animal handlers and zoo visitors) (Fontenot et al., 2008; Fagiolini et al., 2010; Tahas & Diakou et al., 2013; Maesano et al., 2014; Nosal et al., 2016; Cringoli et al., 2017). Gastrointestinal parasites of zoo mammals include zoonotic species and therefore their presence raises public health concerns, particularly *Blastocystis* spp. and *Giardia* spp. (Goossens et al., 2005; Levecke et al., 2007).

Some authors have reported the possibility that these protozoa may be transmitted to humans or other animals (Rajah Salim et al., 1999). Transmission between animals and humans in association with clinical outbreaks among animal keepers has been reported in various studies (Miller et al., 2004; Levecke et al., 2007; Berrilli et al., 2011). The presence of zoonotic parasites emphasizes the need to use rapid copro-microscopic techniques and specific devices that protect the operator.

This was the first study in which Mini-FLOTAC in combination with Fill-FLOTAC was used for rapidly diagnosing parasitic infections in zoo mammals. Currently, to make diagnoses of GI parasites in zoo animals, routine coprological procedures such as direct and indirect wet-mount preparations or the McMaster method are used (Malan et al., 1997; Perez Cordon et al., 2008; Levecke et al., 2010). Achieving accurate rapid diagnoses is a critical

point in enabling control over parasitic infections.
In conclusion, Mini-FLOTAC in combination with Fill-FLOTAC was shown to be user-friendly and safe, with a wide diagnostic range (protozoa and helminths). These features are particularly useful for monitoring and control programs in which large numbers of fecal samples need to be processed rapidly and safely.
In this study, we showed the ability of the Mini-FLOTAC technique to detect GI parasites in different species of zoo mammals. The different flotation solutions used in this study appeared to increase the detected recovery rates for eggs, cysts and oocysts in all samples. This is a big advantage in comparison with other copro-microscopic methods, in which only one solution is used to perform the tests.
Mini-FLOTAC in combination with Fill-FLOTAC ensured a high level of safety for the operator. In fact, Fill-FLOTAC equipped with a collector/homogenizer allows sampling and processing of fecal samples without coming into contact with them. It operates as a closed system and therefore not only provides protection for the operator but also enables the possibility of sample preservation in formalin for subsequent processing.
Furthermore, the present study highlighted the presence of parasitic elements in samples from zoo mammals despite regular diagnosis.

2.5 Discussion and conclusions

Reliable diagnosis of filarial infections in companion animals is fundamental for the prevention and control of disease and for moni- toring the spread of these parasites with zoonotic potential to non- endemic areas (Genchi and Kramer, 2017; Capelli et al., 2018).
The modified Knott's test is the only available diagnostic test for *D. repens* due to the lack of a commercially available serological test. However, the discrimination between different species can be challenging in cases of mixed infections with *Dirofilaria* spp. or in cases of low parasitaemia. In these cases, as well as for discrimination between *Dirofilaria* and Acanthocheilonema spp., either molecular methods (e.g. multiplex PCR) or histochemical staining are required. The use of formalin, however, is of concern and likely limits the application of the test in private practice and in diagnostic laboratories.

Indeed, the final report of National Toxicology Program (NTP), lists formaldehyde in the Eleventh Report on Carcinogens (RoC), and indicates that there is suf-ficient evidence for the carcinogenicity of formaldehyde in humans (NTP, 2010). Moreover, a recent study has shown that exposure to formaldehyde can damage fundamental biomolecules such as DNA and proteins (Reingruber and Pontel, 2018).

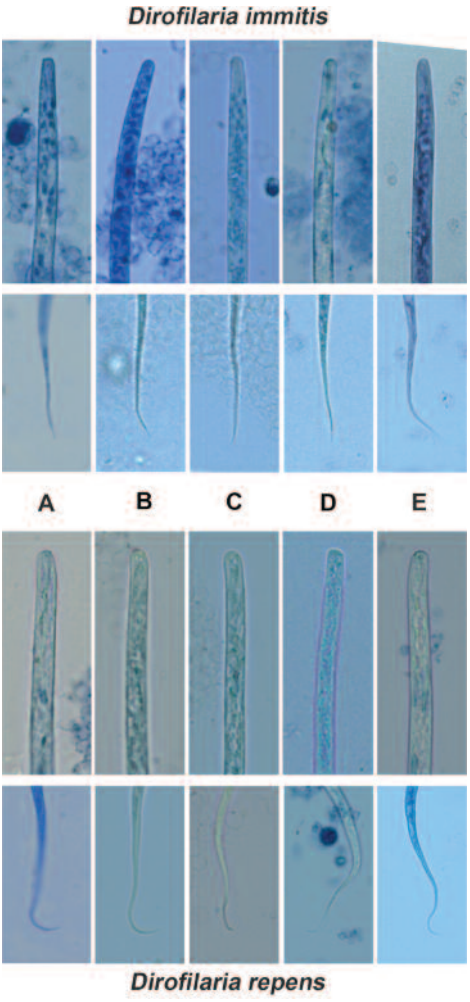

Fig. 2.5 Morphology of Dirofilaria immitis *and D. repens mfs with the five different reagents: Formalin (A), Acetic acid-vinegar (B), Glacial acetic acid (C), Saponin (D), Distilled water (E), used for modified Knott's test.*

The results of the present study suggest that distilled water maintains the same morphological characteristics and dimensions of mfs as those observed with formalin, while the lengths and widths of mfs were significantly reduced when using the other evaluated reagents. This may be due to more pronounced dehydration. Similar results were recently reported by Evans et al. (2019) with acetic acid (vinegar) and Long et al. (2020) with glacial acetic acid. However, despite the statistically sig- nificant differences ($P < 0.05$) of mfs length and width observed with reagents B, C and D, the morphological features of the head and tail of the two species were maintained (Fig. 2.5) We can therefore affirm that all solutions could be suitable and safe alternatives to formalin for the identification of circulating mfs in clinical settings with Knott's test, but that distilled water performs best in maintain-

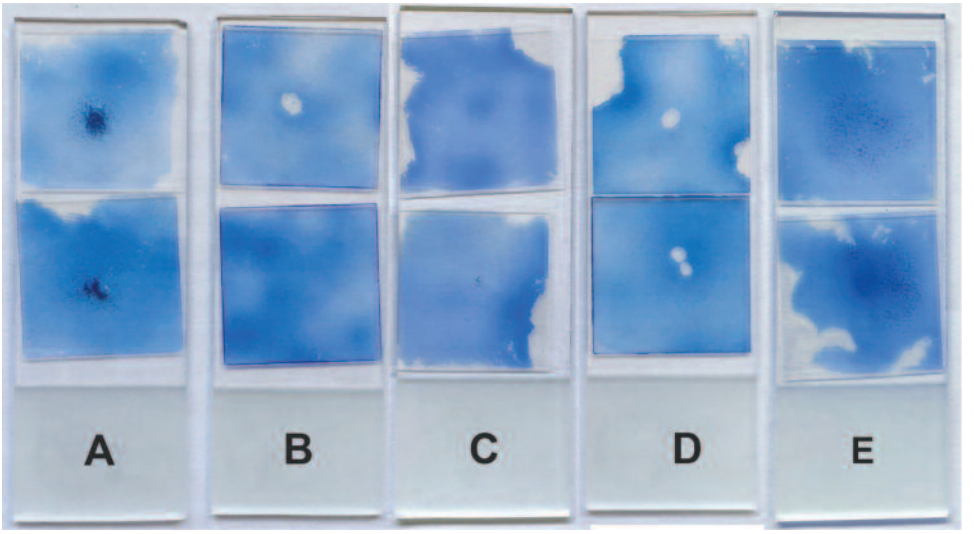

Fig. 2.6 Readability of the slides, using formalin (A), acetic acid-vinegar (B), saponin (C) and distilled water (E) in modified Knott's test.

ing mfs' dimensions, which are useful for the identification of the mfs species.

Hemolysis is an important factor in the modified Knott's test, as it improves the readability of the slides by removing a large fraction of red blood cells from the sample, thus making the identification and enumeration of mfs easier. Even though the present study did not evaluate the effect of the different reagents on heamolysis, the read- ability of slides was considered improved by each operator (Fig. 6). Indeed, stronger hemolytic activity with all the alternative reagents was observed when compared to 2% formalin.

The difference in mfs measurements between the two labs cannot be fully explained. The authors performed additional Knott's tests to evaluate the influence of storage time of the blood samples (4 °C for 24 h, 48 h, 72 h and 96 h) as this was the only difference identified for sample handling between the 2 laboratories. However, differences in mfs measurements between the laboratories remained (data not shown). Moreover, the measurements recorded by Lab 2 in the present study did not agree with a previous study conducted in the same area in 2001 (Cringoli et al., 2001) (Table 5). Furthermore, the measures

obtained by both laboratories were not in agreement with several previous studies (Magnis et al., 2013; Longo et al., 2020), but were in agreement with others that reported broader size ranges of both *D. immitis* and *D. repens* mfs (Sloss et al., 1994; Taylor et al., 2007; Traversa et al., 2010; Liotta et al., 2013; ESCCAP, 2019; Panarese et al., 2020b). The authors can only hypothesize that the variability among studies could be due to the influence of examination methods or to the use of reagents purchased from different manufacturing companies. We can conclude that distilled water could successfully replace formalin in the modified Knott's test for species differentiation of *Dirofilaria* spp.

Tab. 2.5 Comparison of mean length and width of Dirofilaria immitis *and* D. repens *between the results obtained by the authors from the present study (Lab 1, Parma and Lab 2, Napoli) and other authors from previous studies, using modified Knott test with 2% formalin.*

Dirofilaria immitis mfs		*Dirofilaria repens* mfs		Authors
Mean length µm	Mean width µm	Mean length µm	Mean width µm	
304.74	6.39	372.20	8.92	Laboratory 1(Parma)
315.31	6.27	365.05	6.75	Laboratory 2 (Napoli)
311.30	5.69	366.20	6.40	Cringoli et al., 2001
311.96		364.53		Panarese et al., 2020a,b Site 1
294.97		354.93		Panarese et al., 2020a,b Site 2
316				Evans et al., 2019
295 to 325				American Heartworm Society, 2020
301.77	6.30	369.44	8.87	Magnis et al., 2013
259.2±28.3		322.7±21.1		Liotta et al., 2013
306.2	5.5			Long et al., 2020
260-340	5-7.5			Taylor et al., 2007

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Chapter 3

New insights into the biology, diagnosis and immune response to *Dirofilaria repens* in the canine host

L. Ciuca, A. Vismarra, W. Lebon, F. Beugnet, R. Morchon, L. Rinaldi, G. Cringoli, L. Kramer, M. Genchi. New insights into the biology, diagnosis and immune response to *Dirofilaria repens* in the canine host.Veterinary Parasitology: X 4 (2020) 100029

3.1 Abstract

Dogs are the primary host for *Dirofilaria repens*, therefore it is mandatory to accurately diagnose the canine infection and to expand our current knowledge on parasite biology and the immune response of the infected host for a better prevention.Thus, the aim of the present study was to provide new insights from experimental infections of dogs with *D. repens*, focusing on the evaluation of: 1) the pre-patent period and 2) the antibody response against *D. repens* somatic antigens and against the *Wolbachia* endosymbiont. Briefly, on Day 0, twenty purpose-bred Beagle dogs were experimentally infected with 50 infective larvae (L3) of *D. repens*. Starting from Day 58 until the last day of the study (Day 281), blood samples were collected on a monthly basis for detection of antibodies against *D. repens* (Dr) and recombinant *Wolbachia* surface protein (rWSP) by non-commercial IgG- ELISAs. Additional samples were collected on Days 220, 245 and 281 for the detection of microfilariae (mff) using the modified Knott's test and biomolecular analysis, following two PCR protocols: Gioia et al. (2010; protocol A) and Rishniw et al. (2006- protocol B). The results were analysed by univariate statistical analyses using 2 × 2 contingency tables and K Cohen was calculated to assess the agreement among all the diagnostic techniques. Overall, the outcome of the study revealed that out of the 20 dogs experimentally infected with *D. repens*, 16 (80 %) were microfilaraemic, 17 (85 %) were positive at DNA detection in the blood, 18 (90 %) had *D. repens* antibodies and 16 (80 %) had *Wolbachia* antibodies on the last day of the study. The overall k agreement between Knott's and PCR protocol B was 0.442 (P = 0.0001) and increased throughout the study, reaching 0.828 (P = 0.0001) on Day 281. To the authors knowledge, this is only the second study reporting antibody response to *D. repens* somatic antigen in experimentally infected dogs. ELISA results showed that an antibody response de- velops before the onset of patency, and steadily increases with time. Results would suggest that the development of an immunological response to infection could lead to application in epidemiological studies, risk assessment and as an aid in the diagnostic approach in dogs, in particular for early infections without mff.

3.2 Introduction

Dirofilaria repens (Spirurida, Onchocercidae) is among the most widespread vector-borne helminths in dogs and is an emerging zoonosis in Europe (Otranto et al., 2013; Genchi and Kramer, 2020). However, despite its emergence and zoonotic impact, *D. repens* continues to be a neglected parasite, when compared to others like *D. immitis,* the cause of a serious and potentially fatal canine heartworm disease (Genchi and Kramer, 2017), due to the development of pulmonary and cardiac pa- thologies (Venco, 2007). Subcutaneous dirofilariosis caused by *D. repens* is commonly associated with the presence of the adults in subcutaneous tissues and/or subcutaneous nodules. The infection usually progresses asymptomatically (Grandi et al., 2007). Therefore, the clinical relevance of *D. repens* infections in dogs is relatively minor compared with the one induced by *D. immitis*. Even though there is evidence of geographical spread of *D. repens*, there is still a need to further evaluate pathogenicity, effective treatment options and better diagnostic options (Capelli et al., 2018).
The geographic distribution of *D. repens* is changing rapidly, as testified by the increasing prevalence in endemic areas (e.g. Italy, France, Spain) and the spreading into previously unaffected areas (e.g. Genchi et al., 2011; Iglódyová et al., 2012; Ionică et al., 2015; Joke- lainen et al., 2016; Kartashev et al., 2015; Miterpáková et al., 2010; Simón et al., 2012; Şuleşco et al., 2016; Tasić-Otašević et al., 2015; Simón et al., 2017). A recent review on *D. repens* (Capelli et al., 2018), including analysis of current geographical distribution, epidemiology, and zoonotic impact, highlights the increased prevalence and the spread of *D. repens* from endemic areas of Southern Europe towards countries in Central Europe. Several factors are likely responsible for the spread of infection into new areas, including the movement of infected dogs from endemic areas, climate change, and the lack of diagnostic tools that do not rely on mff identification. Indeed, the asymptomatic nature of canine subcutaneous dirofilariasis may lead to under-diagnosis and consequent risk of infected dogs, the main reservoir for both canine and human infections, to go unobserved and untreated. (Genchi and Kramer, 2017; Simón et al., 2017; Capelli et al., 2018; Genchi et al., 2019).

Diagnosis of *D. repens* in dogs is a challenge: many infected dogs are asymptomatic and there is a limited number of reliable diagnostic tools available. Indeed, diagnosis is most often based on detection of circulating mff during patent infection, followed by morphometric or molecular species identification (ESDA-Guidelines, 2017). There are, however, only a few published studies on the prepatent period of *D. repens* in dogs. Reported values vary and mff have been observed in experimentally infected dogs as early as 164 days post-infection (p.i.) (Petry et al., 2015) to as late as 239 days p.i. (Cancrini et al., 1989), while Webber and Hawking (1995) reported a pre-patency of 182 days. This wide variability in pre-patency often makes detection of mff an unreliable diagnostic tool.

Molecular detection and identification of *D. repens* mff by multiplex PCRs, with cytocrome c oxidase subunit 1 (cox1), the intergenic spacer (ITS) regions, and 12S rRNA as the most common gene targets, has been reported as being both sensitive and specific (Rishniw et al., 2006; Gioia et al., 2010; Latrofa et al., 2012; Ciuca et al., 2016), but requires specialized laboratories and experienced personnel. Other diagnostic options include ultrasound examination of subcutaneous nodules and fine needle aspirate cytology (Giori et al., 2010; Albanese et al., 2013; Manzocchi et al., 2017; Capelli et al., 2018).

The lack of a commercially available test for serological diagnosis is likely one of the most important limitations for *D. repens* diagnosis (Simón et al., 2012; Capelli et al., 2018). A non-commercial ELISA has recently been used to evaluate the antibody response against *D. repens* adult somatic antigens in humans living in endemic areas (Ciuca et al., 2018). The same study also evaluated humoral responses against *D. immitis* and against the bacterial endosymbiont *Wolbachia*, found in filarial nematodes (Ciuca et al., 2018). The authors suggest that cross-reactivity may be present between the somatic antigens of *D. immitis, D. repens* and those of other helminth infections and that an associated positive serology against *Wolbachia* may increase diagnostic specificity (Ciuca et al., 2018).

Therefore, the aim of the present study was to provide new insights from experimental infections of dogs with *D. repens*, focusing on the evaluation of: 1) the pre-patent period and 2) the antibody response against *D. repens* somatic antigens and against the *Wolbachia* endosymbiont.

3.3 Materials and methods

The study was performed in accordance with VICH Guideline 9 'Good Clinical Practice' (July 2001). Since there is no guideline that includes specific recommendations for *D. repens*, the infection protocol was based on scientific knowledge and experience from previous experimental studies (Genchi et al., 2010, 2013; Petry et al., 2015). The study started in December 2018 and ended November 2019 and was performed in a Boehringer Ingelheim Animal (BI-AH) Health Research Centre, in France.

3.3.1 Animals

Twenty purpose-bred Beagle dogs (12 males and 8 females), aged between 2.9 and 4.4 months and weighing 3.1–9.6 kg at time of experimental infection (Day 0) were included in the study. The accli- mation ofthe animals to the study conditionsstarted on Day -7. The dogs were managed similarly and with due regard for their well-being, as approved by the BI–AH Ethics Committee, and other local applicable regulations and requirements. No animal had been treated with macrocyclic lactones within three months before infection. Animals were group-housed in cages by sex throughout the animal phase. Dogs were examined by a veterinarian during acclimation and all animals were confirmed healthy and suitable for inclusion in the study. In order to confirm that the animals were negative for *D. repens* infection (and/or *D. immitis*) before the experimental infection, blood and sera samples were collected on Day -5 and tested by biomolecular and serological analysis as described bellow.

General health observations were conducted once daily from the first day of acclimation to the end of the experimental phase. Each animal was also evaluated for presence or absence of skin nodules.

3.3.2 Dirofilaria repens experimental infection

The infective *D. repens* third stage larvae (L3) were obtained as follows: a D. repens microfilaraemic blood sample was collected from a naturally infected dog from the province of Naples (Italy, 2018). The

microfilaria density was assessed at 60 mff /20 µL of blood and the heparinised blood was used for the artificial meal of mosquitoes (Aedes aegypti, Liverpool strain). *D. repens* L3 were obtained from experimentally infected *Aedes aegypti* as described by McCall (1981). The blood was maintained at 37 °C in a feeding apparatus and the mosquitoes were allowed to feed for 60 min in a room with controlled temperature at 27 °C and 80 % relative humidity. After 14 days the infected mosquitoes were killed following exposure to cotton containing ether and 50 *D. repens* larvae (L3) for each vial, were manually collected using a glass pipette and transferred to 20 vials containing 1.5 mL RPMI-medium. On Day 0, each dog was injected subcutaneously in the neck region between the shoulder blades, with 50 infective *D. repens* larvae (L3) using a sy- ringe with a 20-gauge needle. All the dogs were infected with *D. repens* L3 sampled from the same dog from Naples (Italy, 2018).

3.3.3 Blood sampling

Starting from Day 58, blood samples were collected on a monthly basis for *D. repens* antibody testing. Blood was placed in plain tubes and serum was obtained and stocked at -20 °C until testing. Additional samples were collected on Days 220, 245 and 281 for the detection of mff using the modified Knott's test and biomolecular analysis. Samples were collected and placed in EDTA tubes. Sampling was always per- formed in the morning before 10:00 am. The experimental design (study days and laboratory techniques used) is reported in Table 3.1.

Tab. 3.1 Study days and laboratory techniques used on the dogs experimentally infected by Dirofilaria *repens on Day 0.*

Laboratory techniques	Days
Non-commercial IgG-ELISA for detection of antibodies against Dirofilaria repens (Dr) and recombinant Wolbachia surface (rWSP) (Cabrera et al., 2018; Ciuca et al., 2018)	- 5*, 58, 91, 121, 148, 178, 220, 245, 281
Modified Knott's test (Knott, 1939; Magnis et al., 2013)	220, 245, 281
Multiplex PCR (Gioia et al., 2010; Rishniw et al., 2006)	- 5, 220, 245, 281

*Also detection of IgG antibodies of *Dirofilaria immitis*.

3.3.4 Serology

All the sera samples were analyzed with a non-commercial ELISA to detect the IgG antibody response using adult *D. repens* somatic antigens and rWSP as described by Kramer et al. (2005, 2007) with some modi- fications. For ELISA IgG anti *D. repens* analysis, 96-well microplates (Corning® 3369, 96 Well EIA/RIA Assay Microplate) were incubated overnight at 4 °C with 0.8 µg of an extract of *D. repens* adult worms (200 µL/well), prepared as previously described (Kramer et al., 2007). In brief, *D. repens* adult worms obtained from the nodules of naturally infected dogs were macerated and sonicated in PBS pH 7.2. The homogenate was centrifuged at 10,000 rpm/30 min and the sediment discarded. The concentration of the antigen obtained was 1 µg/µl by BCA (Bicinchoninic Acid Kit) for protein determination methods. Finally, this extract was stored at -80 °C until processing. Serum samples were analyzed at 1:100 dilution using a dilution buffer (145 mM NaCl; 15 mM Na_2HPO_4; 2,5 mM NaH_2PO_4; 4% BSA; 0,025 % Tween 20) and incubated (100 µl/well) at 37 °C during 1 h. Goat anti-dog IgG (H + L) conjugated to horseradish peroxidase (Sigma-Aldrich, Spain) was used at 1:5,000 dilution (100 µl/well) with dilution buffer and incubated at 37 °C during 2 h. The reaction was revealed with substrate solution (25 mM $C_6H_8O_7$; 45 mM Na_2HPO_4; 1,5 mM OPD; 004 % H_2O_2; pH 5) (100 µl/well) during 7 min. The cut-off for *D. repens* (OD = 0.8) was established using the mean value ±3 Standard Deviation (3 SD) (of 30 serum samples from clinically healthy blood donors (negative con- trols) living in a *D. repens*-free area, from Leòn, Northwest of Spain. After each incubation step, the wells were washed with washing buffer (PBS pH 7; $Tween_{20}$ 0.05 %) for 3 times (200 µl/well).

For ELISA IgG anti-WSPr analysis, 96-well microplates (Corning® 3369, 96 Well EIA/RIA Assay Microplate) were incubated overnight at 4 °C with 0.3 µg of rWSP, (100 µl/well) which was produced in recom- binant form and purified as described by Diosdado et al. (2017). All serum samples were analyzed at a 1:20 dilution with dilution buffer and incubated at 37 °C for 1 h. Anti-human peroxidase-conjugated IgG was diluted 1:5,000 with dilution buffer (100 µl/well) and incubated at 37 °C for 2 h. The reaction was revealed with sub-

strate solution (100 µl/well) for 10 min. After each incubation step, the wells were washed with washing buffer (PBS pH 7; $Tween_{20}$ 0.05 %) for 3 times (200 µl/well). The cut-off (OD = 0.5) was established, using the mean value ±3 SD of 45 serum samples from clinically healthy blood donors (negative controls) living in a *D. repens*-free area. Optical densities were measured at 492 nm with iMark™ Microplate Absorbance Reader for both ELISAs.

The serum samples of naturally infected dogs (diagnosed by the presence of adults and mff of *D. repens*) were used as positive controls and serum samples from healthy dogs living in a *D. repens*-free area as negative controls, for both ELISAs (*D. repens* and *Wolbachia* antibodies), *Modified Knott's test and Multiplex PCR* A modified Knott's test was used for the detection of circulating mff of *D. repens* (Knott, 1939; Magnis et al., 2013) as follows. One mL of EDTA blood was mixed with 9 mL of formalin 2% and centrifuged for 3–5 minutes at approximately 1500 rpm. The supernatant was removed from the tube and the content was stained with 1–2 drops of 1% methylene blue. A drop was placed on a microscope slide covered with a cover slip and observed under an optical microscope at 100x. For each positive sample, the level of microfilariaemia was quantified using the full content of the tube (mff/mL).

For molecular determination of microfilaria species, genomic DNA was extracted from 200 microliters of each blood sample using the DNeasy® Blood and Tissue kit (Qiagen, Germany), following the manufacturer's instructions. Molecular analyses were performed following two different protocols of multiplex PCR for simultaneous detection of the different Dirofilaria species, i.e. the protocol described by Gioia et al. (2010; protocol A) and the protocol described by Rishniw et al. (2006; protocol B). For the latter, the PCR reactions were increased to 25 µL of total volume, containing 5 µL of genomic DNA for each sample amplification.

The sensitivity range for the multiplex PCR protocol according to Gioia et al. (2010) is reported as allowing successful amplification for *D. repens* with the highest naturally infected blood samples of 100,000 mff/mL and the lowest with 4 mff/mL. The multiplex PCR protocol according to Rishniw et al. (2006) does not report data regarding the highest or the lowest microfilarial loads in the positive samples.

3.3.5 Statistical analysis

The results were analyzed by univariate statistical analyses using 2 x 2 contingency tables and K Cohen was calculated to assess the agreement among all the diagnostic techniques. Kappa (k) statistic was employed to determine the strength of agreement using the following criteria (Altman, 1991): ≤0.2 = poor; 0.21-0.40 = fair; 0.41-0.60 = moderate, 0.61-0.80 = good and ≥0.80 = very good. All the dogs were divided into four groups based on the number of the microfilariae on Days 245 and 288 (0 = negative; 1-50; 51-400; 401-850) and analyzed by 2 x 2 contingency tables, in order to assess the significant associations with the biomolecular and serological analyses (i.e. multiplex PCR protocol B, IgG-ELISA *D. repens* and IgG-ELISA rWSP) performed on the same study days.

The level of significance was set at a p-value < 0.05. The statistical analysis was performed using SPSS Statistics v.23 (IBM, Armonk, NY, USA).

3.4 Results

3.4.1 Modified Knott's test, PCR and serological analyses

The results of Knott's test, multiplex PCRs and serology are reported in Tables 3.2 and 3.3.

Knott's tests were negative for all the dogs on day 220, positive on day 245 in thirteen dogs (65.0 %) and positive on day 288 in sixteen dogs (80.0 %). Four dogs remained negative for the entire study period. Molecular analyses performed on day -5 were negative for all 20 dogs included in the study. On day 220, 3 samples (15.0 %) were positive with protocol A and 12 samples (60.0 %) gave positive results with protocol B. On day 245, 10 samples (50.0 %) were positive with protocol A and 15 samples (75.0 %) were positive with protocol B. Finally,12 samples (60.0 %) were positive with protocol A and 17 (85.0 %) were positive with protocol B by day 281.

Table 3.3 reports the results of anti-*D. repens* antibody response in experimentally infected dogs. Beginning at day 58, an increasing number of dogs had antibodies against somatic antigens of *D. repens* and, from day 148, a total of eighteen out of twenty dogs were positive and

Tab. 3.2 Results of Knott' test and multiplex PCR on different study days in the 20 dogs experimentally infected with Dirofilaria repens.

Dog ID	Modified Knott's (mff/mL)				Multiplex PCR (protocol A)[a] (+/-)				Multiplex PCR (protocol B)b (+/-)			
	Study Days				Study Days				Study Days			
	-5	220	245	281	-5	220	245	281	-5	220	245	281
1	na	0	0	3550	–	–	+	+	–	+	+	+
2	na	0	0	0	–	–	–	–	–	–	–	+
3	na	0	600	100	–	–	+	+	–	+	+	+
4	na	0	0	350	–	–	+	+	–	+	+	+
5	na	0	0	0	–	–	–	–	–	–	–	–
6	na	0	0	0	–	–	–	–	–	–	–	–
7	na	0	400	3650	–	+	+	+	–	+	+	+
8	na	0	300	3250	–	+	+	+	–	+	+	+
9	na	0	0	25	–	–	–	–	–	–	–	+
10	na	0	250	275	–	–	+	+	–	+	+	+
11	na	0	100	4900	–	–	–	+	–	+	+	+
12	na	0	0	100	–	–	–	–	–	–	+	+
13	na	0	300	150	–	–	–	–	–	–	+	+
14	na	0	250	25	–	–	–	–	–	+	+	+
15	na	0	150	3750	–	–	+	+	–	+	+	+
16	na	0	400	4900	–	–	+	+	–	+	+	+
17	na	0	850	1900	–	+	+	+	–	+	+	+
18	na	0	100	250	–	–	–	+	–	+	+	+
19	na	0	600	2300	–	–	+	+	–	–	+	+
20	na	0	0	0	–	–	–	–	–	–	–	–
Pos (%)/total tested = 20	na	0 (0%)	12 (60.0%)	16 (80.0%)	0 (0%)	3 (15.0%)	10 (50.0%)	12 (60.0%)	0 (0%)	12 (60.0%)	15 (75.0%)	17 (85.0%)

na = not assessed; (+/-)= (positive/negative); [a]Multiplex PCR using the protocol by Gioia et al. (2010) (protocol A); [b]Multiplex PCR using the protocol by Rishniw et al. (2006) (protocol B).

Tab. 3.3 Results of serological diagnosis on Dirofilaria repens *and its* Wolbachia *on different study days (-5 to 281) in the 20 dogs experimentally infected with* Dirofilaria repens.

Dog ID	Non-commercial IgG-ELISA results (+/-) *Dirofilaria repens* (Dr) and recombinant *Wolbachia* surface (rWSP)								
	Study Days								
	-5	58	91	121	148	178	220	245	281
1	-/-	-/-	-/-	+/-	+/-	+/-	+/-	+/+	+/+
2	-/-	-/-	-/-	-/+	-/+	-/+	-/+	-/+	-/+
3	-/-	-/+	-/-	+/-	+/-	+/-	+/-	+/-	+/-
4	-/-	-/-	-/-	+/-	+/-	+/-	+/-	+/-	+/-
5	-/-	-/-	+/-	+/-	+/-	+/-	+/+	+/+	+/+
6	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
7	-/-	-/-	+/-	+/-	+/+	+/-	+/+	+/+	+/+
8	-/-	-/-	-/-	+/-	+/-	+/-	+/-	+/-	+/-
9	-/-	+/-	+/-	+/+	+/-	+/+	+/+	+/+	+/+
10	-/-	-/-	-/-	+/+	+/+	+/-	+/-	+/-	+/-
11	-/-	-/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-
12	-/-	-/-	+/-	+/-	+/+	+/+	+/+	+/+	+/+
13	-/-	+/-	-/+	-/+	+/+	+/+	+/+	+/+	+/+
14	-/-	-/-	+/-	+/-	+/-	+/+	+/-	+/-	+/-
15	-/-	-/-	-/-	+/+	+/+	+/+	+/+	+/+	+/+
16	-/-	-/-	-/-	+/-	+/-	+/-	+/-	+/-	+/-
17	-/-	-/-	+/-	+/-	+/-	+/+	+/+	+/-	+/-
18	-/-	-/-	-/-	+/-	+/-	+/-	+/-	+/-	+/-
19	-/-	-/-	+/+	+/+	+/+	+/+	+/+	+/+	+/+
20	-/-	-/-	+/-	+/-	+/-	+/+	+/+	+/+	+/+
Total positive Dr (%)/rWSP (%)	0 (0%)/ 0(0%)	2 (10.0%)/ 1(5.0%)	9 (45.0%)/ 2(10.0%)	17(85.0%)/ 6(30.0%)	18(90.0%)/ 7(35.0%)	18(90.0%)/ 9(45.0 %)	18(90.0%)/ 10(50.0%)	18(90.0%)/ 10(50.0%)	18(90.0%)/ 10(50.0%)

(+/-)= (positive/negative).

remained so until the end of the study (day 281). Two dogs maintained OD values under the cut-off (0.8) during the entire study.
The anti-WSP antibody responses started on Day 58, with one positive dog, and increased until the last day of the study with sixteen positive dogs.
The overall findings revealed that all the positive dogs had an increase of OD values for the antibodies against *D. repens* somatic antigens and against *Wolbachia* endosymbiont with peaks on Days 220, 245 and 281 (Fig. 3.1).
Overall, the outcome of the study revealed that out of the 20 dogs experimentally infected with *D. repens*, 16 (80.0 %) were micro- filaraemic, 17 (85.0 %) were positive at DNA detection in the blood, 18 (90.0 %) had *D. repens* antibodies and 16 (80.0 %) had *Wolbachia* anti- bodies on the last day of the study.
The overall k agreement between Knott's and PCR protocol B was 0.442 (P = 0.0001) and increased throughout the study, reaching 0.828 (P = 0.0001) on Day 281. Analyses were not carried out comparing Knott's with protocol A, given that this protocol resulted in a lower number of positive samples.
The results of the univariate statistical analysis are reported in Ta- bles 4 and 5. Briefly, a statistical significant association ($p < 0.05$) was observed between the number of positive dogs with the Knott's test on Day 245 and the outcome of protocol B multiplex PCR as well as with the number of the positive dogs at *D. repens* IgG-ELISA ($p < 0.005$).

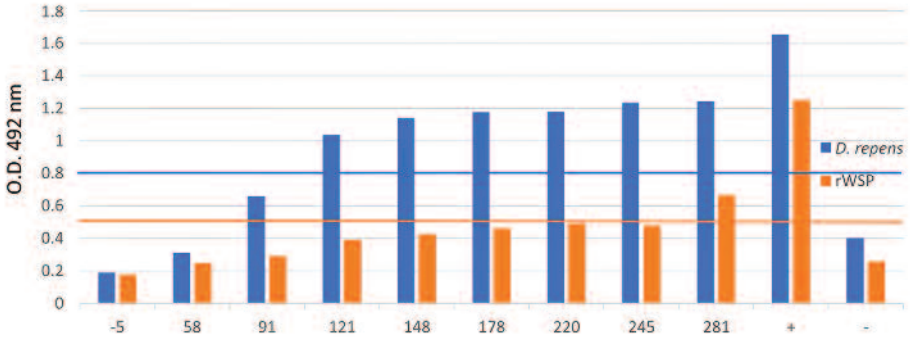

*Fig. 3.1 Immunological responses of anti-*Dirofilaria repens *and anti-*Wolbachia *in 20 dogs experimentally infected with* Dirofilaria repens. *Cut-off for ELISA IgG anti-*D. repens *(OD = 0.8); cut-off for ELISA IgG anti-WSPr (OD = 0.5); "+ "=positive control; "-"=negative control.*

Similarly, on Day 281, the Knott's test outcome was statistically associated ($p < 0.05$) with both molecular and *D. repens* IgG-ELISA tests. Instead, there was no statistically significant association ($p < 0.05$) between the results of the Knott's test and the results of the *D. repens* IgG and IgG anti- WSPr ELISAs on either study day (245, 281).

3.4.2 Clinical features

The vast majority of dogs remained clinically healthy throughout the study period. Skin nodules were observed in two dogs on Day 220: one dog had a nodule on each ear pinna and the other dog had a nodule on the right anterior paw. On Day 245, one of the ear pinna nodules had resolved, while the other nodules remained until the end of the study.

Tab. 3.4 Results of molecular and serological analyses compared to Knott's test outcomes (mff/mL) on study day 245 (onset of patency).

Knott's test		Multiplex PCR (protocol B)	IgG-ELISA_Dr	IgGELISA_rWSP
mff/mL	No. positive dogs (% and 95%CI)	No. positive dogs (% and 95%CI)	No. positive dogs (% and 95%CI)	No. positive dogs (% and 95%CI)
0	7 (35.0%) (16.3-59.1)	3 (42.9%) (11.8-79.8)	5 (71.4%) (30.3-94.9)	5 (71.4%) (30.3-94.9)
1-50	1 (5.0%) (0.3-26.9)	0 (0%)	1 (100%) (5.5-89.2)	1 (100%) (5.5-89.2)
51-400	9 (45.0%) 23.8-67.9)	9 (100%) (62.9-98.9)	9 (100%) (62.9-98.9)	3 (33.3%) (9.0-69.1)
401-850	3 (15.0%) (3.9-38.9)	3 (100%) (31.0-96.8)	3 (100%) (31.0-96.8)	1 (33.3%) (1.8-87.5)
Total no. positive dogs/ no. examined (% and 95%CI)	13/20 (65.0%) (40.9-83.7)	15/20 (75.0%) (50.6-90.4)[a]	18/20 (90.0%) (66.9-98.3)[b]	10/20 (50.0%) (27.9-72.2)

[a]Indicates statistically significant difference: chi-square test = 5.934, $p < 0.0015$, 95 % CI = 50.6-90.4%; [b]chi-square test = 4.127, $p < 0.0042$, 95 % CI = 66.9–90.0 %; Multiplex PCR_245: (Rishniw et al., 2006); Total number of examined dogs = 20.

Tab. 3.5 Results of molecular and serological analyses compared to Knott's test outcomes (mff/mL) on study day 281 (final day of study).

Knott's test		Multiplex PCR (protocol B)	IgG-ELISA_Dr	IgGELISA_rWSP
mff/mL	No. positive dogs (% and 95%CI)	No. positive dogs (% and 95%CI)	No. positive dogs (% and 95%CI)	No. positive dogs (% and 95%CI)
0	4 (20.0%) (6.7-44.3)	1 (25.0%) (1.3-25.0)	2 (25.0%) (9.2-90.8)	3 (75.0%) (21.9-98.7)
1-50	2 (10.0%) (1.8-33.1)	2 (100%) (19.8-95.1)	2 (100%) (19.8-95.1)	2 (100%) (19.8-95.1)
51-400	6 (30.0%) (12.8-54.3)	6 (100%) (51.7-98.5)	6 (100%) (51.7-98.5)	5 (83.3%) (36.5-99.1)
401-4900	8 (40.0%) (19.9-63.6)	8 (100%) (59.8-98.8)	8 (100%) (59.898.8)	6 (75.0%) (35.6-95.6)
Total no. positive/ no. examined	16/20 (80.0%) (55.8-93.4)	17/20 (85.0%) (61.1-96.0)[a]	18/20 (90.0%) (66.9-98.3)[b]	16/20 (80.0%) 55.7-93.9)

[a]Indicates statistically significant difference: chi-square test = 5.934, $p < 0.0015$, 95 % CI = 50.6-90.4%; [b]chi-square test = 4.127, $p < 0.0042$, 95 % CI = 66.9–90.0 %; Multiplex PCR_245: (Rishniw et al., 2006); Total number of examined dogs = 20.

3.5 Discussion

In the present study, 16/20 dogs were successfully infected with *D. repens*, resulting in patent infections (based on the presence of mff) beginning at 245 days p.i., with all 16 positive for circulating mff by day 281.

Different factors may influence the time necessary for parasites to develop to the adult stage and become fertile. *Dirofilaria repens* dwells in the subcutaneous tissue and migration away from the site of inoculation is likely random. The presence of adult worms of both sexes at the same anatomical location may indeed be a question of "chance", thus explaining the wide variation in reported pre-patent periods (as early as 4.5 and as late as 8 months p.i.).

This wide variation in pre-patency renders the Knott's test, currently

the most commonly used diagnostic method for *D. repens* infection, highly prone to false-negative results. In order to find a more sensitive alternative, and to assist in the differentiation between *D. repens* and *D. immitis*, several molecular methods have been developed in recent years.

In the present study, two PCR protocols were used and their per- formance compared to the Knott's test. Results would suggest that both PCR protocols are able to detect infection earlier than the Knott's test and that the protocol described by Rishniw et al. (2006) is more sensitive compared to the protocol of Gioia et al. (2010). All of the 12 dogs that were Knott's negative/DNA positive on day 220 became Knott's positive by the end of the study, as did the remaining 4 dogs that were Knott's negative/DNA positive on day 245. When microfilarial counts are too low to allow identification with the Knott's test, biomolecular analysis can be considered a sensitive alternative. Ciuca et al. (2018) reported the use of Knott's testing combined with multiplex PCR for the diagnosis of *D. repens* in naturally infected dogs and showed that using biomole- cular analysis the infection status can be confirmed. Infections not only with *D. repens* and *D. immitis* have to be differentiated, but also with other apathogen filaroids (for e.g. *Acanthocheilonema reconditum).* In this context, there are alternative multiplex PCRs (Latrofa et al., 2012) or, in absence of technical equipment, the measurement of mff (Magnis et al., 2013) should be considered. In order to develop a sensitive and specific serological assay that would offer veterinary practitioners a powerful tool for screening asymptomatic dogs, the serological test performed in the present study should first be evaluated for cross-reactions with *D. repens* and other non-pathogenic filarial infections.

Such a test would support the current knowledge of *D. repens* epidemiology, and also allow the screening of those dogs moving from non-endemic into endemic areas, therefore helping to prevent the diffusion of *D. repens*. Simón et al. (1997) described a group of poly- peptides of *D. repens* (range of 26–40 kDa) that, when used in ELISA, are specifically recognized by sera from human patients with subcutaneous dirofilariosis. A further study of patients with dirofilariosis reported better performance of these peptides in Western Blot compared to serology (Cancrini et al., 1999).

To the authors knowledge, this is only the second study reporting antibody response to *D. repens* somatic antigen in experimentally infected dogs. Joekel et al. (2017) evaluated the antibody response in dogs naturally infected with several different filarial species. Three *D. repens*-experimentally infected dogs were also analyzed. Response to crude somatic antigen from adult *D. repens* showed dogs becoming antibody positive as early as 24 days post-infection, much earlier than in the present study. The increasing antibody titres were observed until approximately 5–6 months post-infection, following the same trend as seen in the present study. Cancrini et al. (2000) also looked at antibody responses in naturally infected dogs, using an adult somatic antigen. The authors reported positive serology for all PCR/Knott positive dogs and also for a portion of PCR/Knottnegative dogs. The authorsconclude that a combination of parasitological, biomolecular and serological tech- niques might increase the diagnostic reliability for naturally infected dogs.

In the present study, ELISA results showed that an antibody response develops before the onset of patency, and steadily increases with time. On the last study day, 90% of infected dogs were seropositive on anti-*D. repens*. In the present study, antibody response was not always asso- ciated to patent infection, with 4 dogs being seropositive, but without circulating mff by the end of the study. It is very interesting that both studies mentioned above, used somatic/crude antigens, the same in the present study, for detecting the antibody response. They also reported cross reactions on serology in naturally infected dogs with different filarial infections (*A. reconditum* and *Dipetalonema dracunculoides*).

In the present study, this was not a concern given that the dogs were experimentally infected with *D. repens* and were free from any other filarial infections. However, it would have been interesting to explore the cross-reactions and the specificity of the serological test to better evaluate its suitability for diagnostic purposes. The lack of such analyses represents a limitation of the study that warrants further investigation. The most problematic issue regarding the use of serology is the po- tential cross-reactivity between the somatic antigens of *D. immitis/D. repens* and those of other parasites that may be present in a dog popu- lation (e.g. ascarids, hookworms). It has been reported that

most in- dividuals exposed to *D. immitis* or *D. repens* infection produce anti-WSP antibodies (Grandi et al., 2008; Cabrera et al., 2018). Since *Wolbachia* is present only in filarial nematodes and not in other helminths (e.g. *Toxocara, Ascaris* or hookworms, etc), the presence of anti-WSP anti- bodies would be highly suggestive of exposure of *Dirofilaria* spp. in humans living in endemic areas. A further challenge will be the devel- opment of a serological assay that can discriminate between *D. immitis* and *D. repens.* This problem is already noted with the *D. immitis* antigen test which has been reported as cross reacting when used in dogs with mono-infections with *D. repens* (Ciuca et al., 2018).

It is possible that the immune system of the dogs having *D. repens* mono-infection eliminated the larvae, leaving however an antibody response behind. This "trace" of past infection has also been suggested by Cancrini et al. (2000).

Interaction between *Wolbachia* and host's humoral immune system has been reported by several authors in different hosts infected or immunized with different species/extracts of filariae (Bazzocchi et al., 2000; Punkosdy et al., 2001; Simón et al., 2003; MMarcos-Atxutegi et al., 2003; Kramer et al., 2005). Exposure of the immune system to *Wolbachia* is thought to occur when the parasite dies. To the author's knowledge, there are no studies that could state clearly if *Wolbachia* of *D. immitis* is different or similar with *Wolbachia* of *D. repens*. It has been shown in dogs with *D. immitis* that antibodies to WSPr are associated with circulating mff and their natural attrition (Morcho´n et al., 2012). In cats, the antibody response to *Wolbachia* is thought to follow immune-mediated elimination of infective larvae (Morcho´n et al., 2004). In the present study, 12 of the 16 dogs with circulating mff had antibodies against *Wolbachia*. Interestingly, all 4 amicrofilaraemic dogs were also positive for *Wolbachia*, suggesting that the parasites died before reaching maturity. This is the first report of the IgG response against *Wolbachia* in *D. repens*-experimentally infected dogs.

3.6 Conclusions

Results from both ELISAs, anti-*D. repens* and anti-WSPr, confirm that the development of serological tests for *D. repens* infection could be a starting point for application in epidemiological studies and as an aid in the diagnosis of infection in dogs, in particular for early stage infections and in absence of mff. Indeed, only 1 dog in the present study was negative for all tests carried out.
The identification of specific immune-active proteins for *D. repens* infection could be useful in diagnosing, for example, the presence of adult parasites in the absence of both clinical signs and circulating mff. They could also be useful in monitoring the efficacy of adulticide treatment. It would also be necessary to evaluate the present tests in terms of specificity in the field, given the risk of cross reactivity with other filarial nematodes.

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Chapter 4

Comparison of oral, topical and extended-release injectable formulations of moxidectin combined with doxycycline for adulticide treatment in *Dirofilaria immitis*-naturally infected dogs

Lavinia Ciuca, Alice Vismarra, Dario Constanza, Antonio Di Loria, Leonardo Meomartino, Paolo Ciaramella, Giuseppe Cringoli, Marco Genchi, Laura Rinaldi, Laura Kramer

4.1 Abstract

Several studies in both experimentally and naturally infected dogs have reported the adulticide effect of a combination of macrocyclic lactones and doxycycline against *Dirofilaria immitis*, showing that these protocols are safe and effective. The present study evaluated the adulticide effect of oral, topical and extended-release injectable formulations of moxidectin when combined with doxycycline in dogs naturally infected with *D. immitis* from a shelter located in southern Italy. A total of 30 dogs with naturally acquired *D. immitis* infection were divided in three groups (G) and treated either with oral moxidectin (G1) once a month for 9 consecutive months, topical moxidectin (G2) once a month for 9 consecutive months or with an extended release moxidectin injectable (G3) at enrolment and again at 6 months (Day 180). All treatment groups received doxycycline for the first 30 days. Microfilarial concentration in 1 ml (mff/ml) of blood were determined monthly for 9 months, with the modified Knott's test. A clinical scoring system was employed for each dog enrolled in the study based on thoracic radiography and cardiac ultrasound (CU) exams performed at Day -15 (before treatment) and Day 180. In general, mff loads decreased markedly in all dogs from all groups at Day 30, and all but one dog were negative at Day 60. Results from the present study suggest that efficacy (evaluated at Day 270) is related to the moxidectin formulation used and that injectable moxidectin showed superior efficacy compared to topical and oral injectable formulations. Overall, the treatment with moxidectin and doxycycline combination was effective and almost all the dogs from the treatment groups were cleared of pulmonary abnormalities by six months from the beginning of treatment (p-value=0.000). Although the therapy proved to be an effective adulticide, the echocardiographic parameters studied were not able to show a marked improvement of cardiac function after the treatment. This aspect could be related with the absence of symptomatic dogs enrolled in the study. However, in a limited number of animals (4/30), a progressive improvement of cardiac function was observed after therapy.

4.2 Introduction

Canine heartworm disease (HWD) is caused by the filarial nematode *Dirofilaria immitis*, a vector-borne parasite transmitted by several mosquito species and is endemic in many parts of the world (Genchi et al., 2014). The presence of adult worms in the pulmonary arteries of infected dogs causes changes in arterial structure and function that can lead to pulmonary hypertension and, eventually, to right-sided congestive heart failure (Balbo et al., 1968; Bowman and Atkins, 2009). Melarsomine dihydrochloride is the only approved adulticidal drug for treatment of HWD. Several studies in both experimentally and naturally infected dogs have reported the adulticide effect of a combination of macrocyclic lactones (ML) and doxycycline against *D. immitis*, showing that these protocols are safe and effective (Bazzocchi et al., 2008; Grandi et al., 2010; Mavropoulou et al., 2014; Bendas et al., 2017; Savadelis et al., 2017; Genchi et al., 2019a; Paterson et al., 2020; Vörös et al., 2022). Doxycycline targets the bacterial endosymbiont *Wolbachia*, whose reduction leads to worm infertility and death (Kramer et al., 2007; Louzada-Flores et al., 2022). This activity, combined with the known detrimental effects of ML, may eliminate adult worms and lessens the inflammatory reaction against dead and dying worms (Kramer et al., 2011). The American Heartworm Society (AHS, 2018) and the European Society of Dirofilariosis and Angiostrongylosis (ESDA, 2017) currently recommend that in cases where treatment with melarsomine is not possible or is contraindicated, a monthly treatment based on ML along with doxycycline for a 4-week period might be considered (AHS guidelines, 2018; ESDA guidelines, 2017; Vörös et al., 2022). It has been shown that the combination of moxidectin/doxycycline has superior adulticide efficacy compared to ivermectin/doxycycline (Jacobson and DiGangi et al., 2021). Grandi et al. (2010) reported 73% efficacy 10 months after the beginning of doxycycline (daily for 1 month) combined with oral ivermectin every 15 days for 6 months. More recently, Savadelis et al. (2017) reported a 95.9% adulticide efficacy in experimentally infected dogs after 1 month of doxycycline combined with 10 monthly treatments with a topical formulation of moxidectin. Genchi et al. (2019a) reported that 15/16 naturally infected dogs became antigen negative at 9 months with the same protocol. Paterson et al. (2020) reported 93.0% of dogs treated with the same protocol were antigen-free at month 15. There is only one report on the efficacy of extended-release injectable moxidectin combined with doxycycline

in naturally infected dogs, in which 90.0% of the dogs were free of adult worms within one year of treatment with 1 month of doxycycline and administration of 2 doses of injectable moxidectin at 6 and 12 months (Alberigi et al., 2020). To the authors knowledge, there is no data regarding the evaluation for adulticidal efficacy of oral moxidectin combined with doxycycline. The aim of the present study is to evaluate the adulticide effect of oral, topical and extended-release injectable formulations of moxidectin when combined with doxycycline in dogs naturally infected with *D. immitis* from a shelter located in southern Italy.

4.3 Material and methods

4.3.1 Animals

The study started in July 2020 and ended September 2021.
The protocol of this study was approved by the ethical committee of animal experiments of the Department of Veterinary Medicine and Animal Production, University of Federico II Naples, Italy (Approval Number 0093204/2022). Shelter owner's consent was obtained prior to any study activities.
The study design is reported in Fig. 4.1.

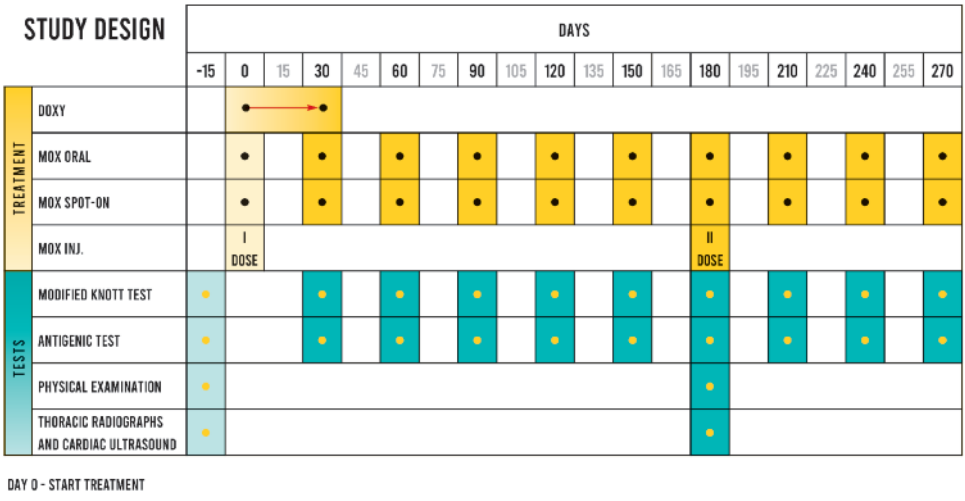

Fig. 4.1 Study design.

A total of 30 dogs with naturally acquired *D. immitis* infection were enrolled from a municipal shelter located in southern Italy (Castel Volturno, Campania region; geographical coordinates 41°0'57"24 N and 13°56'49"56 E). Inclusion criteria included dogs of any breed/sex, weighing at least 1 kg of bodyweight, being >6 months of age and not having been treated within 2-3 months prior with any macrocyclic lactone or doxycycline. Dogs were considered positive for infection when a circulating antigen test (PetChek® HTWM PF, IDEXX) and/or microfilariae (modified Knott's test) was positive for *D. immitis.*

4.3.2 Treatment protocols

The 30 dogs were allocated at random into three treatment groups (10 dogs for each group) based on the moxidectin formulations used for the treatment protocols as described below.

Group 1 (G1) - Oral moxidectin (3µg/kg SID; Afilaria®, Fatro-Italy) once a month for 9 consecutive months, together with doxycycline (10 mg/kg SID; Ronaxan®, Boehringer Ingelheim Animal Health, Italy) for the first 30 days.

Group 2 (G2). Topical 10% imidacloprid + 2.5% moxidectin (Advocate®, Elanco, Italy) once a month for 9 consecutive months, together with doxycycline (10 mg/kg SID) for the first 30 days.

Group 3 (G3). Extended release moxidectin injectable (Afiliara SR®, Fatro, Italy) every six months for two administrations, together with doxycycline (10 mg/kg BW, SID; Ronaxan®, Boehringer Ingelheim Animal Health, Italy) for the first 30 days.

4.3.3 Parasitological evaluation

Blood samples were collected once a month for nine months from all dogs enrolled in the study. Microfilarial concentration in 1 ml (mff/ml) of blood were determined with the modified Knott's test (Knott, 1939) following the protocol by Genchi et al. (2021). Serum samples were tested for circulating antigens using PetChek® HTWM PF (IDEXX), following the manufacturer's instructions. A subjective scoring system, based on the intensity of the color reaction, was used to express the results of each test as negative (-), weak positive (+) and strong positive (++). "First negative" was defined as a minimum of two consecutive negative tests, according to Paterson et al (2020).

4.3.4 Radiological evaluation

The radiological evaluation was performed at Day-15 (before the treatment) and at Day 180 (after 6 months of treatment) by the same operator. The thoracic radiographs included right-lateral, left-lateral, and dorso-ventral views. All the radiographs were obtained on manually restrained and unsedated patients during peak inspiration. The parameters that were emphasized in the radiographic evaluation were: size and shape of the pulmonary arteries and its branches (vascular pattern), dilatation of the main pulmonary artery, enlargement of the right atrium, enlargement of the left atrium, generalized cardiac enlargement evaluated both subjectively and objectively using the vertebral heart score (VHS) (Buchanan, 2000). Furthermore, the presence and distribution of alterations affecting the pulmonary interstitium, the alveolar space and the bronchial walls were classified using common radiographic patterns: interstitial, alveolar, and bronchial pattern, respectively, or mixed if two or more patterns were present (Thrall, 2018). Based on the presence and degree of extension of one or more of the radiographic alterations, a clinical scoring system was considered as follows: score 0 (normal, without radiographic alterations consistent for heartworm disease), score 1 (mild pulmonary and/or cardiac alterations), score 2 (moderate pulmonary and/or cardiac alterations) and score 3 (severe pulmonary and/or cardiac alterations, signs compatible with thrombo-embolism/pneumonia) (Thrall et al., 1980; Mavropoulou et al., 2014).

4.3.5 Cardiac ultrasound evaluation

All the dogs underwent complete echocardiographic examination at Days -15 and 180 by the same operator. The exams included transthoracic 2-D, M-mode, and spectral and color-flow Doppler echocardiographic evaluations using transducer arrays of 1-4 MHz, 3-8 MHz (ESAOTE, Italy). Examinations were performed in conscious unsedated dogs during a period of quiet breathing, with the animals in left and right lateral recumbency. At least five consecutive cardiac cycles were acquired and stored for off-line measurements. As for radiological procedures, a clinical scoring system was employed based on ultrasound findings, according to Schober and Baade (2006), Kellum and Stepien (2007), and Mavropoulou et al. (2014). Briefly, pulsed wave (PW)-spectra signals for calculation of STIs were acquired from the

right parasternal short axis view of the pulmonary artery with the sample volume at the valve level; the Acceleration time (AT) of pulmonary artery (PA) flow was measured from the onset of the pulsed Doppler PA flow signal to peak flow velocity. The right ventricular Ejection time (ET) was measured from the onset to the end of the Doppler PA flow signal, moreover the AT/ET ratio was calculated. When present tricuspid regurgitation and/or mitral regurgitation, CW-spectra were acquired from the left apical cranial four-chamber views. Considering AT, ET, AT/ET and TRV, each dog was assigned a score from 0-3 for pulmonary hypertension (Mavropoulou et al., 2014).
The radiological and echocardiographic examinations were performed by two independent investigators that were blinded to dog identification and clinical and parasitological data.

4.4 Statistical analysis

The non-parametric Wilcoxon signed- rank test was used to determine the level of significant differences between pre- (Day -15) and post-treatment (Day 180) based on the radiological and cardiac scores in the three treatment groups (G1, G2 and G3). Statistical analysis was performed using Windows SPSS® (version 17.0). The level of significance was set at a p-value < 0.05.

4.5 Results

4.5.1 Parasitological findings

Table 4.1 summarizes the results of Knott's test of all dogs at enrolment, while table 4.2 reports results of antigen testing scores. All dogs except three were positive for circulating mff, with values ranging from 50-83,600 mf/ml of blood. Circulating antigens were present in all but two dogs, who were however positive for circulating microfilariae.
Twenty-two/30 dogs (73.3%) were also positive for *D. repens* mf (range 100-78,200 mff/ml).
Figure 4.1 reports the results for reduction of circulating microfilariae induced by the three treatment protocols evaluated in the study. In general, mff loads decreased markedly in all dogs from all groups at Day 30, and all but one dog were negative at Day 60. The one positive dog remaining was from the in-

Tab. 4.1 Dirofilaria immitis *microfilarial count at enrolment (mff/ml) evaluated 374 through Knott's test.*

Dog	Group 1. Moxidectin oral	Dog	Group 2. Moxidectin spot-on	Dog	Group 3. Moxidectin injectable
1	0	1	62500	1	39400
2	2500	2	0	2	13800
3	2900	3	1100	3	100
4	10150	4	22800	4	15900
5	200	5	3100	5	8600
6	200	6	40800	6	3900
7	0	7	5600	7	200
8	50	8	70350	8	11200
9	0	9	83600	9	31500
10	200	10	33800	10	12200

Tab. 4.2 Circulating antigen scores at enrolment (Days –15).

Dog	Group 1. Moxidectin oral	Dog	Group 2. Moxidectin spot-on	Dog	Group 3. Moxidectin injectable
1	+ +	1	+ +	1	+ +
2	+ +	2	+ +	2	+ +
3	+ +	3	+	3	-
4	+ +	4	+ +	4	+ +
5	+ +	5	+ +	5	-
6	+ +	6	+ +	6	+ +
7	+ +	7	+ +	7	+ +
8	+ +	8	+ +	8	+ +
9	+ +	9	+	9	+ +
10	+ +	10	+ +	10	+ +

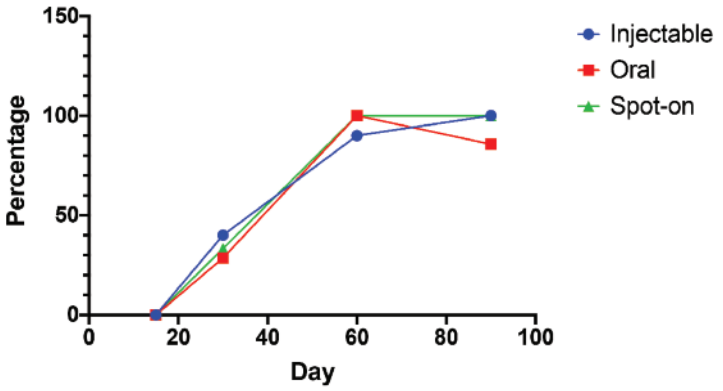

Fig. 4.2 Reduction of circulating microfilariae.

jectable formulation group (G3) and became negative at Day 90. Interestingly, dog no. 3 from the oral formulation group (G1) became negative at Day 60 (from 2900 mff/ml at enrolment) and then became once again positive for mff at Day 90 (100 mff/ml). The dog was negative at all subsequent time points. All dogs with *D. repens* mf were negative at Day 90 (data not shown).
Table 4.3 reports the results for antigen testing for each group at each time point until Day 270. There was a clear reduction of antigen concentration for all treatment groups, beginning as early as Day 30. At Day 270, 9/10 dogs (90.0%) from G1, 6/10 dogs (60.0%) from G2 and 8/10 dogs (80.0%) from G3 had at least 2 consecutive negative tests. Several dogs from each group reverted to positive antigen status at different time points following negativization.

4.5.2 Radiological and cardiac ultrasound findings

Table 4.4 reports the radiological and cardiopulmonary scores obtained by all the dogs in the three treatment groups before treatment (Day-15) and after treatment (Day 180).
The results of radiological examination showed that at Day-15, 5/30 (16.6%) were assigned to score 0 (2 dogs from G1; 1 dog from G2; 2 dogs from G3), 12/30 (40%) to score 1 (4 dogs from G1; 3 dogs from G2; 5 dogs from G3) and 13/30 (43.4%) to score 2 (4 dogs from G1; 6 dogs from G2; 3 dogs from G3). No dog was assigned to score 3. At Day 180, all the dogs with score 0

Tab. 4.3 Results of the antigen testing for each treatment groups (G 1, G2 and G3) from Day 30 to Day 270.

Dog	Moxidectin formulation	Days								
		30	60	90	120	150	180	210	240	270
1	Oral	++	+	++	-	++	-	-	-	-
2	Oral	-	-	++	-	+	-	-	-	-
3	Oral	++	++	++	++	++	++	+	++	++
4	Oral	++	+	+	+	-	-	-	-	-
5	Oral	++	++	++	++	++	++	++	++	++
6	Oral	++	++	++	++	++	+	+	+	++
7	Oral	++	++	++	-	+	++	+	++	-
8	Oral	++	++	++	+	-	-	-	-	-
9	Oral	-	-	-	++	-	+	-	-	++
10	Oral	++	++	++	-	+	-	-	-	++
1	Spot-on	++	++	++	++	++	++	++	++	-
2	Spot-on	++	++	+	+	+	-	-	+	-
3	Spot-on	-	++	++	-	-	-	-	+	-
4	Spot-on	++	++	++	++	+	-	-	-	-
5	Spot-on	++	+	+	-	+	++	-	-	-
6	Spot-on	++	+	++	+	+	-	-	-	-
7	Spot-on	+	++	+	+	+	-	-	n.d.	++
8	Spot-on	+	+	++	++	+	++	-	-	-
9	Spot-on	-	-	-	++	+	-	-	-	-
10	Spot-on	++	++	++	++	++	++	++	++	++
1	Injectable	++	++	++	++	+	-	++	-	-
2	Injectable	++	++	++	++	++	++	++	++	++
3	Injectable	-	++	+	n.d.	n.d.	-	-	n.d.	-
4	Injectable	++	++	++	++	++	-	-	-	-
5	Injectable	+	+	-	+	-	-	-	-	-
6	Injectable	++	++	++	++	+	+	-	-	++
7	Injectable	-	++	+	+	+	-	-	-	-
8	Injectable	++	++	++	+	+	-	-	-	-
9	Injectable	-	-	-	-	-	-	-	-	-
10	Injectable	+	+	++	+	-	-	++	-	++

Tab. 4.4 Results of radiological and cardiac ultrasound evaluation (scores) for all the dogs in each treatment groups (G1, G2, G3) at Day-15 (pre-treatment) and Day 180 (post-treatment).

Treatment-Groups (dogs)	Radiological evaluation (scores)								Cardiac ultrasound evaluation							
Group 1 (oral moxidectin)	Day-15				Day 180				Score 0-3 (Days-15)				Score 0-3 (Days 180)			
	0	1	2	3	0	1	2	3	0	1	2	3	0	1	2	3
1		x			x				x				x			
2		x				x				x				x		
3	x				x				x				x			
4			x		x				x				x			
5		x			x					x				x		
6	x				x				x				x			
7			x			x					x			x		
8			x		x				x				x			
9			x		x				x				x			
10		x			x					x				x		
Total dogs	2	4	4	0	8	2	0	0	6	3	1	0	7	3	0	0

Treatment-Groups (dogs)	Radiological evaluation (scores)								Cardiac ultrasound evaluation							
Group 2 (spot-on moxidectin)	Day-15				Day 180				Score 0-3 (Days-15)				Score 0-3 (Days 180)			
	0	1	2	3	0	1	2	3	0	1	2	3	0	1	2	3
1	x				x				x				x			
2			x		x					x				x		
3		x			x				x				x			
4		x			x				x				x			
5			x		x				x				x			
6			x			x			x				x			
7		x			x				x				x			
8			x		x					x				x		
9			x		x					x				x		
10			x		x				x				x			
Total dogs	1	3	6	0	9	1	0	0	7	3	0	0	8	2	0	0

Treatment-Groups (dogs)	Radiological evaluation (scores)								Cardiac ultrasound evaluation							
Group 3 (injectable moxidectin)	Day-15				Day 180				Score 0-3 (Days-15)				Score 0-3 (Days 180)			
	0	1	2	3	0	1	2	3	0	1	2	3	0	1	2	3
1		x			x				x				x			
2	x				x				x				x			
3			x			x				x				x		
4		x				x			x				x			
5			x		x					x				x		
6		x			x					x				x		
7			x			x				x			x			
8	x				x				x				x			
9		x			x				x				x			
10		x				x			x				x			
Total dogs	2	5	3	0	6	4			6	4	0	0	7	3	0	0

Legend of colours:
Blue: Improvement (score 2 to score 1; score 1 to score 0; score 2 to score 0)
Red: Without changes (score 1 to score 1; score 2 to score 2)
Grey: Normal score 0 to score 0

from the previous examination were assigned to the same score. Furthermore, 9/12 (75%) dogs assigned to score 1 at Day -15, had a substantial improvement and consequently changed into score 0 (3 dogs from each group) at Day 180 (Fig. 4.3). Additionally, the other 3/12 (12%) dogs had almost static radiographic appearance and remained in the same score 1 (1 dog from G1 and 2 dogs from G3), as before treatment (Fig. 4.4). Moreover, all the 13 dogs initially with score 2 had a radiographic improvement of the pulmonary and/or of the cardiac condition. Indeed, 4/13 (30.7%) dogs had a partial resolution of the radiological alterations and were moved into score 1 (1 dog from G1; 1 dog from G2 and 2 dogs from G3) and 9/13 (69.3%) had a substantial improvement and therefore were moved from score 2 into score 0 (3 dogs from G1; 5 dogs from G2 and 1 dog from G3).

The results of cardiac ultrasound examination showed that at the beginning of the study (Day-15), 11/30 (36.7%) dogs showed altered pulmonary blood

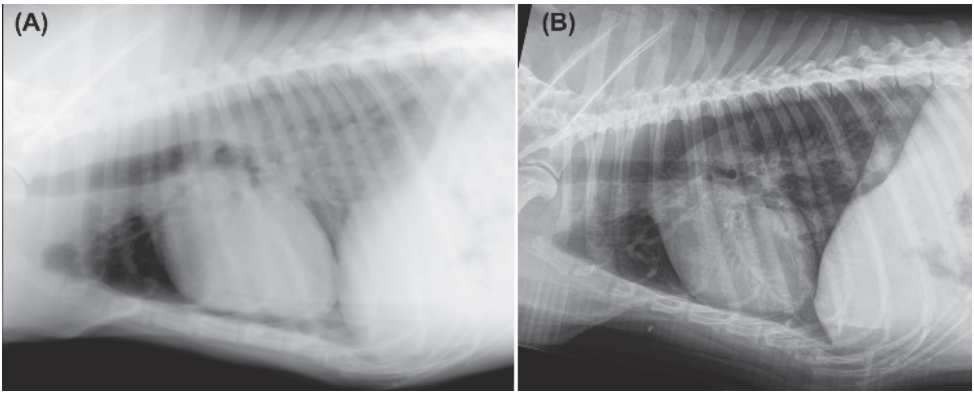

Fig. 4.3 Right-lateral radiographs pre- (A) and post-treatment (B) of the same dog. Figure (A) shows a diffuse mixed alveolar/unstructured interstitial pattern. This increase in pulmonary opacity involves especially the caudal lung lobes where multiple air-bronchograms are visible. In figure B, there is a partial resolution of the radiographic alterations involving the lung lobes, although there is a residual unstructured interstitial pattern associated with mild to moderate thickening of the bronchial walls. This dog was initially assigned in score 2, but after the six months of therapy was moved to score 1.

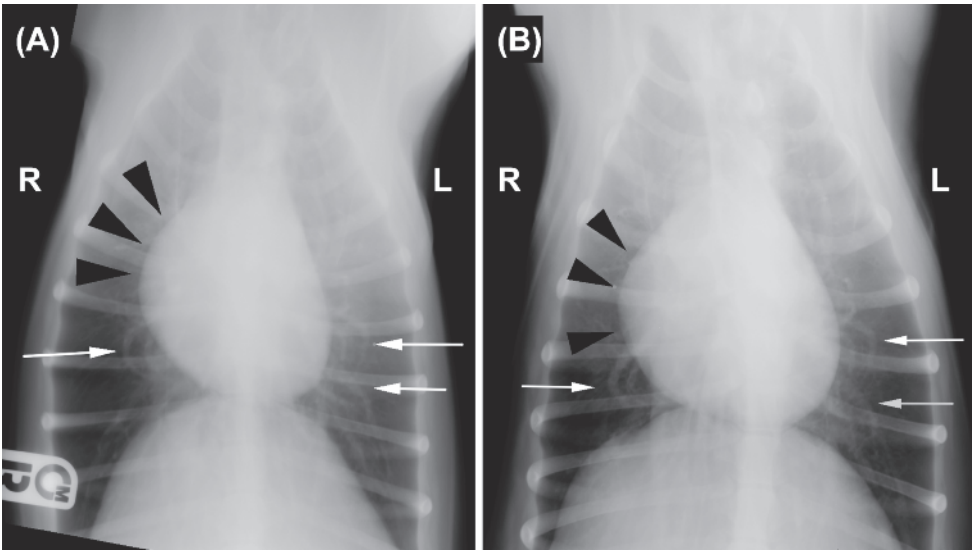

Fig. 4.4 Dorsoventral (DV) radiographs pre- (A) and post-treatment (B) of the same dog. In both views, there is a mild, diffuse, unstructured interstitial pattern associated with mild dilatation of the right atrium (black arrowheads). Furthermore, some caudal lobar arteries (white arrows) have a convoluted shape. After the first thoracic exam, this dog was assigned in score 1, and since the radiographic findings were almost unaltered, after six months of therapy was re-assigned in the same score1. Legend: R= right, L= left.

flow, ranging from score 1 (3 dogs from G1; 3 dogs from G2; 4 dogs from G3) to score 2 (1 dog from G3). Moreover, 19/30 (63.3%) dogs were assigned in score 0 (6 dogs from G1; 7 dogs from G2 and 6 dogs from G3). In particular, 12/30 (40%) dogs were classified with chronic degenerative mitral disease (CDMD), representing a comorbidity for the enrolled animals (Keene et al., 2019). Specifically, two of the dogs classified in score 1, one dog in score 2 and three dogs in score 0 showed enlarged left atrium and concomitant hemodynamic significative mitral regurgitation and were classified in stage B2 of CDMD. Moreover, stage B1 of CDMD was recorded in 5 dogs classified in score 0 and in 3 dogs classified in score 1. Six dogs with CDMD in stage B2 (6/30) received oral administration of benazepril 0.25 mg/Kg SID.

Subsequent echocardiographs tests carried out after 180 Days from the first examination except for the 4/11 dogs that showed a progressive improvement, did not show any significant variation of scoring previously assigned. In particular, 3 dogs with slight alteration assigned in score 1 become normal (one dog from each group) while one dog (from G1) with moderate alteration assigned in score 2 showed slight modifications and was assigned in score 1.

Results of statistical analysis on the radiological scores obtained before treatment (Day -15) and after treatment (Day 180) showed that the therapy with moxidectin/doxycycline was effective for all the animals in the three treatment groups (p-value=0.000). There was no significant difference among the three groups (p-value=0.564) post-treatment at Day 180. Regarding the cardiac evaluation, the Wilcoxon test showed statistically significant higher rank of post-treatment scores than the pre-treatment rank scores (p-value=0.045).

4.6 Discussion

In the present study we evaluated the adulticide efficacy of different formulations of moxidectin combined with doxycycline in dogs naturally infected with *D. immitis*. According to Genchi et al. (2019b), even though melarsomine is still widely used by veterinary practitioners in Italy, a monthly macrocyclic lactone together with doxycycline is currently being used by nearly 30% of surveyed veterinary facilities. The use of moxidectin is a valid alternative to ivermectin, as shown by several studies (Savadelis et al., 2017; Genchi et al., 2019a; Paterson et al., 2020; Kryda et al., 2020; Jacobson and DiGangi et al., 2022).

The adulticidal efficacy of the injectable formulation of moxidectin was 90% at 9 months, showing slightly superior efficacy to the spot-on formulation (80%) and clear superiority to the oral formulation (60%). This may be due to its pharmacokinetics. Lok et al. (2001), in an early pharmacokinetic study of the formulation used here (0.17 mg moxidectin/kg), reported that effective serum moxidectin levels peak eight days after injection and remain at this level for six months. McCall et al. (2001) studied the retroactive activity of moxidectin extended release on immature worms and reported 85.9% efficacy against 4-month-old *D. immitis* infections and efficacy was even higher (97.2%) when a second treatment was given 6 months later. There is only one previous study that assessed the adulticide efficacy of an extended-release injectable formulation of moxidectin combined with doxycycline, in which 2 doses six months apart resulted in 90% of dogs becoming antigen negative (Alberigi et al., 2020).

The spot-on formulation was efficacious in 80% of dogs at 9 months. Bowman et al. (2016) reported that topical moxidectin reaches steady-state serum concentrations at levels that are much higher than those needed for heartworm prophylaxis. The exposure of adult parasites to high concentrations of moxidectin likely contributes to the efficacy of this protocol.

Oral moxidectin combined with doxycycline gave the lowest percentage of dogs (60%) that became negative for circulating antigens by 9 months. It has been reported that, when compared to oral ivermectin, moxidectin has lower total body clearance and higher volume of distribution, which results in a prolonged elimination half-life (Al-Azzam et al., 2007). However, it may be that oral administration is not always followed by optimal gastro-intestinal absorption, leading to under-dosing and lack of efficacy. It is well known that bioavailability of orally administered drugs depends on a multitude of factors, including gastric pH and emptying time, small intestinal fluid properties, changes in gastrointestinal integrity, etc (Vinarov et al., 2021).

In all treatment groups, there was an observed variability from month to month in the concentration of heartworm antigen detected, as reported previously (Savadelis et al., 2017; Paterson et al., 2020). For this reason, the authors of the present study defined “first negative” as a minimum of two consecutive negative tests, according to Paterson et al. (2020). The return to antigen-positive status following negativization may be due to the gradual death and consequent antigen release from dying worms, as suggested by Genchi et al. (2019a). The authors of the present study chose not to preheat

the serum samples, due to the risk of cross-reactivity and false positive reactions due to co-infection with *D. repens*, as previously reported by others (Venco et al., 2017; Sobotyk et al., 2021).

Treatment regimens were also effective against *D. repens* mff, with no differences observed among the three formulations used (data not shown). However, there is no current test to verify adulticide efficacy against *D. repens* and the clearance of circulating mf observed in the present study is not necessarily indicative of adult worm death. However, Petry et al. (2015) reported the adulticide effect of the same topical formulation used in the present study in *D. repens*-experimentally infected dogs.

In the present study, all treatment regimens were well tolerated. Only six dogs were treated with furosemide and benazepril for cough and mitral regurgitation during the entire study.

It has been reported that dogs treated with doxycycline can have gastrointestinal upset, while coughing has also been reported in dogs treated with the ML/doxycyline protocol (Paterson et al., 2020).

Radiographic alterations occurring during natural heartworms infection are related to the parasite load and time elapsed since the infection, with radiographic findings ranging from subclinical disease without apparent alterations in the lung fields and pulmonary vasculature to severe pulmonary and cardio-circulatory impairment (Losonsky et al., 2005; Venco et al., 2004; Savadelis et al., 2020). In our study, the most frequent radiographic findings in dogs with score 1 were a diffuse interstitial pattern and pulmonary vascular changes, similar to those reported by Mavropoulou et al. (2014) and Genchi et al. (2019a). These findings are considered common in cases of heartworm disease and the alterations in the lung parenchyma are attributed to eosinophilic bronchopneumonia, fibrotic changes and focal pulmonary consolidation (Venco et al., 2004; McCall et al., 2014).

The dogs classified as moderate diseased (score 2) had dilatation of the right atrium, among the most frequent alterations, probably related to infection with a higher or long-lasting parasite load and a more severe pulmonary interstitial pattern than dogs with score 1. Moreover, there were many dogs with enlargement of the left atrium or an overall increase in cardiac size, most likely related to concurrent mitral valve disease.

In our study, there was no evidence of worsening of the radiographic findings at the follow up after 6 months. Moreover, four of the dogs initially classified in score 2 showed a partial improvement and nine an almost complete reso-

lution. Similarly, all the dogs with score 1 improved. Overall, the treatment with moxidectin and doxycycline combination was effective and almost all the dogs from the treatment groups were cleared of pulmonary abnormalities by six months from the beginning of treatment. The combination of moxi/doxy was previously reported in experimentally treated animals to induce the reduction of pro-inflammatory antigen mass (Kramer et al., 2011). Similarly, Genchi et al. (2019a) reported that no dogs showed worsening of pulmonary patterns 12 to 24 months after the treatment with the same topical formulation of moxidectin combined with doxycycline for the first 30 days in dogs naturally infected by *D. immitis*.
In our study the echocardiographic examination showed important information regarding the diagnostic profile and the therapeutic follow-up. Although the therapy proved to be an effective adulticide, the echocardiographic parameters studied were not able to show a marked improvement of cardiac function after the treatment. This aspect could be related with the absence of symptomatic dogs enrolled in the study. However, in a limited number of animals (4/30), a progressive improvement of cardiac function was observed. Noteworthy we observed CDMD in comorbidity with heartworm disease in 40% of dogs enrolled and this could have influenced assignment of echocardiographic scores (Tai and Huang, 2013).

4.7 Conclusion

In conclusion, doxycycline/moxidectin combination treatment for HWD has been reported as being safe and effective. Results from the present study suggest that efficacy is related to the moxidectin formulation used and that injectable moxidectin showed superior efficacy compared to topical and oral formulations.

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Chapter 5

Emerging risk of *Dirofilaria* spp. infection in shelter dogs in southern Italy

Lavinia Ciuca, Valeria Caruso, Sergio Illiano, Antonio Bosco, Giuseppe Cringoli, Maria Paola Maurelli, Laura Rinaldi. Emerging risk of *dirofilaria* spp. infection in shelter dogs in southern Italy. Frontiers in Veterinary Science, 'Dog Filariosis: The Threat Walks Not Only in The Blood Stream'. Submitted, 2022.

5.1 Abstract

The aim of the present study was to better investigate the occurence of *Dirofilaria* spp. in southern Italy. For this, a local dog shelter from Castel Volturno in southern Italy was selected and screened for the presence of *Dirofilaria* spp. A total of 260 blood samples were examined for identification of microfilariae (mff) and for detection of *Dirofilaria immitis* antigen. The dogs were divided into four age classes (class 1: dogs ≤ 2 years; class 2: dogs > 2 ≤ 6 years; class 3: dogs > 6 ≤ 10 years; class 4: dogs > 10 years old) and three groups of dogs based on the length of stay in the shelter at the moment of sampling (group 1-new arrivals: dogs that have been received in the shelter in the last four months; group 2- dogs that were housed in the shelter for more than four months up to 2 years; group 3-dogs that were housed for more than 2 years). The modified Knott's test revealed that 188 dogs (72.3%) were positive for circulating mff of *Dirofilaria* spp. Specifically, 113 (60.1%) dogs were positive for *D. immitis* mff and 75 (39.9%) were positive to *D. repens* mff. In addition, 58 (30.8%) dogs presented *both D. immitis* and *D. repens* mff. Antigen testing showed 98/260 (37.7%) dogs positive to *D. immitis*. However, 13% of the dogs with *D. immitis* mff were antigen-negative. The prevalence was almost twice as high in males (65.4%) as in females (34.6%). As expected, prevalence was lowest in age class 1 (16.5%) and higher in age classes 2, 3 and 4 (25.5%; 27.6%; and 30.3%; respectively) but these differences were not significant. There was a significant difference in relation to the length of stay of the dogs in the shelter, reflecting mainly an increase in prevalence in the group 1 (45.2%; 95%CI=39.0-53.7; P=0.012) in which all dogs were new arrivals in the shelter since four months and their origin was from various locations of the Campania region, including the city center of Naples.

5.2 Introduction

Heartworm disease and subcutaneous dirofilariosis caused by *Dirofilaria immitis* and *D. repens* (Spirurida, Onchocercidae) respectively, are important vector-borne diseases especially for dogs and cats (Genchi and Kramer, 2020; Ciuca et al., 2021; Capelli et al., 2018). In addition, zoonotic infections, mostly due to *D. repens*, represents a major public health issue (Genchi and

Kramer, 2020). The epidemiological situation of canine dirofilariosis based on data reported in the recent review studies demonstrated that the prevalence of both *D. immitis* and *D. repens* are increasing in Europe and southern eastern regions of Asia and Africa (McCall et al., 2008; Capelli et al., 2018; Genchi and Kramer, 2020). However, there are studies that continuously reports changes in the prevalence of both *D. immitis* and *D. repens* pathogens by underling the non-endemic areas with an increased prevalence and the previously endemic/hyper-endemic areas with decreasing prevalence (Genchi et al., 2009; Genchi et al., 2019; Montoya-Alonso et al., 2010). In fact, in the northern part of Italy, the prevalence for *D. immitis* has been reduced in the last three decades from >40% to 8% in owned dogs (Genchi et al., 2009) due to the increased awareness of veterinary practitioners and better control and prevention strategies. In southern part of Italy, specifically in the Campania region, in the last twenty years, there have been mainly four reports in the last twenty years, demonstrating the occurrence of low prevalence of infections by *D. immitis* and *D. repens*. In fact, the prevalence of *D. repens* increased from 2% (Cringoli et al., 2001) to 10% (Ferrara et al., 2022). *D. immitis* and *D. repens* occurs in Campania, especially in the coastal areas of the region. Hunting dogs, stray dogs, and dogs housed in kennels are more exposed, with seroprevalences for *D. immitis* ranging from 0.2% to 4.4% (Del Prete et al., 2015; Piantedosi et al., 2017; Petruccelli et al., 2020). The results of the questionnaire survey on heartworm and subcutaneous dirofilariosis addressed to veterinary practitioners throughout Italy (Genchi et al., 2019) showed that at least one clinical case per year of cardiopulmonary dirofilariosis was diagnosed in dogs with frequent co-infestation by *D. immitis* and *D. repens* in Campania. Recent studies confirm the presence of autochthonous cases of *D. immitis* with a prevalence of approximately 0.1% (Santoro et al., 2019; Ferrara et al., 2022). Based on the last two reports of two cases of heartworm disease detected during post-mortem examination of two roaming dogs (Santoro et al., 2019) and a screening study that revealed ten positive dogs (out of 100 examined) for *D. repens* (Ferrara et al., 2022), both studies from the urban area of Castel Volturno in the Campania region of southern Italy the aim of the present study was to describe an outbreak of infections in a dog shelter from the same area.

5.3 Materials and methods

The study was conducted in a shelter located in Castel Volturno (Fig. 5.1) a deltaic coastal plain of the Volturno River that has been suspected, by veterinary practitioners, to have a high accidental incidence of *D. repens* (Ferrara et al., 2022).

5.3.1 Ethics aspects

The study started in July 2020 and ended July 2021. The protocol of this study was approved by the ethical committee of animal experiments of the Department of Veterinary Medicine and Animal Production, University of Federico II Naples, Italy (Approval Number 0093204/2022). Shelter owner's consent was obtained prior to the start of the study.

Fig. 5.1. Shelter located in Castel Volturno, southern Italy.

5.3.2 Animals and collection of blood samples

The screening for *Dirofilaria* spp. was performed on all dogs from the shelter with few exceptions, as follows. Dogs younger than nine months and dogs receiving doxycycline for the last month, at the time of sampling, were excluded from the study. The shelter housed a total of 285 dogs, and based on the above exclusion criteria, a total of 260 dogs (111 females, 149 males, age range: 3-14 years) were selected and sampled. The dogs were housed in outdoor boxes, each box hosting 3-4 dogs. The management of the shelter in terms of parasitic disease diagnosis and treatment strategy was as follows: i) all dogs (new arrivals and others with symptoms) were tested for *Ehrlichi*a/*Anaplasma* and *Leishmania infantum*; ii) all the were treated with a topical formulation (based on fipronil) against ectoparasites. Moreover, no screening for *Dirofilaria* spp, was performed until June 2020 (the start of the study) and no prophylactic treatment against *Dirofilaria* spp. was perfomed at the shelter. Anamnestic data were collected for the sampled dogs, including age, sex, health status (i.e. activity level, appetite, any health problems, any skin abnormalities). Data on the duration of the dogs' stay in the shelter at the time of sampling were also collected.

5.3.3 Laboratory analyses

All samples (N=260 EDTA blood and serum samples) were examined for identification of microfilariae (mff) using the modified Knott's test (Genchi et al., 2022) and for the detection of *Dirofilaria immitis* antigen using the Petcheck Canine Heartworm test (IDEXX). Moreover, molecular analyses (PCR protocol as described by Rishniw et al., (2006)) were used to confirm the diagnosis of dirofilariosis in the case of co-infections with both *D. immitis* and *D. repens*.

5.3.4 Modified Knott test and molecular analysis

A modified Knott's test was used for the detection of circulating mff of *Dirofilaria* spp. as follows. One mL of EDTA blood was mixed with 9 mL of distilled water and centrifuged for 3–5 minutes at approximately 1500 rpm. The supernatant was removed from the tube and the content was stained with 1–2 drops of 1% methylene blue. A drop was placed on a microscope slide covered with a cover slip and observed under an optical microscope at 100X (Genchi et al., 2022). For each positive sample, the level of microfilariaemia was quantified using the full content of the tube (mff/mL). For molecular de-

termination of microfilaria species, genomic DNA was extracted from 200 microliters of each blood sample using the DNeasy® Blood and Tissue kit (Qiagen, Germany), following the manufacturer's instructions. Molecular analyses were performed following the protocol of multiplex PCR for simultaneous detection of the different *Dirofilaria* species, described by Rishniw et al. (2006). The PCR reactions were increased to 25 μL of total volume, containing 5 μL of genomic DNA for each sample amplification (Ciuca et al., 2020).

5.3.5 Statistical analysis

All data of the dogs included in the study, gender, age, length of period (time) in the shelter at the moment of sampling, were analysed by univariate statistical analysis using the Pearson's Chi-square test for independence, to check its correlation with positivity for *D. immitis* and *D. repens*. For these, all dogs were divided into four age classes as following: class 1: dogs ≤ 2 years; class 2: dogs > 2 ≤ 6 years; class 3: dogs > 6 ≤ 10 years; class 4: dogs > 10 years old. In addition, three groups of dogs were formed based on the length of stay in the shelter at the time of sampling as follows: group 1-new arrivals (dogs entered the shelter in the last four months); group 2- dogs housed in the shelter for more than four months up to 2 years; group 3-dogs housed for more than 2 years. The Kappa (k) statistic was used to measure the concordance between the antigenic test and the Knott's test using the following criteria (Altman, 1991): ≤0.2 = poor; 0.21-0.40 = fair; 0.41-0.60 = moderate, 0.61-0.80 = good and ≥0.80 = very good. Hence, all the dogs were divided into four groups based on the number of the microfilariae (0 mff=negative (group 1); 1-100 mff (group 2); 101-500 mff (group 3); 501>mff (group 4). Moreover, multivariable (logistic regression) statistical analyses using the *Dirofilaria* exposure (positive/negative) as a dependent variable were performed. Only the independent variables that showed significance ($P < 0.01$) in the univariate test were used for the logistic regression model. If interaction between variables was suspected, logistic regression model was run with and without these variables in order to evaluate possible effect modification on their behalf (Hosmer and Lemeshow, 2000). The significance level was set at a p value < 0.05. The analysis of the data was performed using SPSS version 17 software, Chicago, IL, USA.

5.4 Results

The modified Knott's test revealed that 188 dogs (72.3%; 95%CI=66.4-77.6) were positive for circulating mff of *Dirofilaria* spp. Specifically, 113 (60.1%; 95%CI=52.7-67.1) dogs were positive for *D. immitis* mff and 75 (39.9%; 95%CI=32.9-47.3) were positive to *D. repens* mff. In addition, 58 (30.8%; 95% CI=24.4-38.1) dogs presented both *D. immitis* and *D. repens* mff. Antigen testing showed 98/260 (37.7%; 95%CI=31.8-43.9) dogs positive to *D. immitis*. However, 13% (95%CI=6.5-19.2) of the dogs with *D. immitis* mff were antigen-negative. PCR testing confirmed the co-infections with both pathogens in all 58 dogs. The prevalence was almost twice as high in males (65.4%; 95%CI=58.1-72.1) as in females (34.6%; 95%CI=27.9-41.8). As expected, prevalence was lowest in age class 1 (16.5%; 95%CI=11.6-22.7) and higher in age classes 2, 3 and 4 (25.5%; 95%CI=19.5-32.5; 27.6%; 95%CI=21.5-34.7 and 30.3%; 95%CI=23.9-37.5 respectively) but these differences were not significant. There was a significant difference in relation to the length of stay of the dogs in the shelter, reflecting mainly an increase in prevalence in the group 1 (45.2%; 95%CI=39.0-53.7; P=0.012) in which all dogs were new arrivals in the shelter since four months and their origin was from various locations of the Campania region, including the city center of Naples. The results regarding the groups with the number of microfilariae were as followed: 0 mff-negative=72 (group 1); 1-100 mff=34 (group 2); 101-500 mff=61 (group 3); 501>mff=93 (group 4). The overall k concordance between the Knott's test and the antigenic test was 0.260 (=fair) (P = 0.000).

The majority of the dogs had no health problems during the examination at the time of sampling. However, 13 dogs showed some symptoms: skin problems (skin lesions, poor quality of fur, itching) in which five dogs presented small soft nodules in subcutaneous tissues. All the dogs that presented nodules tested positive for *D. repens* in the subsequent PCR testing. Results from the logistical regression model showed a significant association between the *Dirofilaria* exposure and the time spent in the shelter at the moment of sampling indicating a higher value for the positive dogs that were housed in the shelter since four months (group 1), than other dogs from the following groups 2- dogs housed in the shelter for more than four months up to 2 years; group 3-dogs housed for more than 2 years, (45.2% OR=2.5; 95%CI=39.0-53.7; P=0.012 vs. 23.4% (P=0.089) and 30.3% (P=0.065).

5.5 Discussion and conclusions

Canine heartworm disease is a long-established cosmopolitan parasitosis. Several studies in the literature (Genchi et al., 2014; Montoya-Alonso et al., 2017; Vrhovec et al., 2017; Alho et al., 2018; Genchi et al., 2020) demonstrate its presence in Europe with different prevalence values. Indeed, we distinguish some so-called endemic/hyperendemic areas in countries such as Portugal, Spain and Greece with high prevalence values (4%-40%) and areas in countries such as Germany, France, the Netherlands and Poland where the prevalence values are generally low (< 1%) and confirmed cases of cardiopulmonary dirofilariosis are sporadic. Heartworm disease is historically present in the northern part of the Italian peninsula, especially in the area of the Po river where, in the last 30 years, thanks to the increasing attention of the veterinarians and the correct information provided to dog owners, an excellent decrease in prevalence has been achieved from 40% (Guerrero et al., 1989) to 8% (Genchi et al, 2009) to a maximum of 5-20 diagnosed cases per year of dirofilariosis in hyperendemic areas (Genchi et al., 2019). In the present study, the overall surveyed prevalence of *D. immitis* and *D. repens* in the population investigated was 72.3%. These results are significant and show evidence of an increased risk of dirofilariosis in southern Italy. Actually, there are few studies in the literature on the prevalence of *D. immitis*, especially in the Campania region, which showed an increase in seroprevalence in dogs over time: 0.2% out of 1335 dogs examined (Cringoli et al., 2001); 0.6% (Rinaldi et al., 2015); 8.8% (Del Prete et al., 2015). *D. repens* is a filarial nematode which, due to the subcutaneous localisation of the adult parasites, does not have a high pathogenic power in the definitive host; however, this filaria species has a high zoonotic potential and is often the cause of disease in humans. Indeed, *D. repens* is considered one of the most widespread zoonotic pathogens in Europe and the incidence of human cases goes hand in hand with infestation in dogs. The epidemiological situation in Italy regarding the prevalence of *D. repens* is following described. In the north, it is reduced and stable due to ten-year chemoprophylaxis, in the central and southern parts is still endemic and all the areas require adequate chemoprophylaxis also in view of the zoonotic potential. Our study confirmed the high prevalence of *D. repens* (39.9%) in dogs from southern Italy, in Campania regions. However, the most unexpected results of this study, was the predominance of *D. immitis* in the shelter dogs from Castel Volturno (60.1% prevalence). Fur-

thermore, almost half of the positive dogs had been infected prior to their arrival at the shelter, which means that the circulation of *Dirofilaria* spp. infections was widespread in other regions of Campania, including in the city center of Naples in southern Italy.

Currently, *D. repens* occurs in southern Italy (Campania and Molise regions), especially in the coastal areas of the two regions. The results of the questionnaire survey on dirofilariasis addressed to freelance veterinarians throughout Italy (Genchi et al., 2019) showed that in several veterinary facilities in Campania at least one clinical case per year of subcutaneous dirofilariasis in dogs was diagnosed with *D. immitis* and *D. repens*. Recent surveys confirmed the endemic status for D. repens in Campania with outbreak areas and regional prevalence ranging from 1.5% to 10%. (Santoro et al., 2019; Ferrara et al., 2022). The multicentre study by Traversa et al. (2019) did not reported microfilariemic dogs in either Campania or Molise; however, a recent survey performed on outdoor dogs (hunting, herding and stray) showed a prevalence of 0.8% for *D. repens* the Molise region (Gizzarelli et al., 2019).

The data obtained from this study are consistent with what has been described in the literature. Statistical analysis showed that male dogs were more infested with *D. immitis* than female individuals. In the literature to date there is no evidence exists to date to demonstrate an actual predisposition of sex for this parasitosis. The findings indicate that heartworm disease, known to be more endemic in Northern Italy, is an parasitosis also increasingly emerging in the southern regions of the peninsula. The epidemiology is influenced by several factors such as: 1) micro-macroclimatic changes that have favoured a greater spread of the vector, the selection of new vectors such as Aedes albopictus and Aedes koreicus (Montarsi et al., 2015) and have also modified and prolonged its seasonality of action; 2) the movement of animals, especially in European context (specifically in endemic areas for dirofilariasis), encouraged by the introduction of the pet travel scheme in 2000; 3) more number of dogs in kennels (Del Prete et al., 2015; Genchi et al., 2020), which represent the main reservoirs in the epidemiology of this parasitosis.

Furthermore, the municipality of Castel Volturno (Caserta), the site of our study, has several favourable environmental characteristics for the survival and development of the vector and thus the spread of *D. immitis*. It has a typical Mediterranean climate with long, hot summers and mild winters long, hot summers and mild winters. There are also several rural and marshy areas

populated by animals susceptible to infestation such as stray dogs and foxes that represent an ideal reservoir of infection (Alho et al., 2018).
Also, the diagnostic failures of this parasitosis by veterinarians should also be considered. In fact, in a study by Genchi et al. (2019), it became clear that diagnosis is often carried out using only a single method or associated methods that are not very sensitive, so that this parasitosis is easily underdiagnosed, especially in areas where notoriously less attention is paid to it. The results of this study have shown that serological testing alone (which is usually the most commonly used for the diagnosis of *D. immitis*) is not sufficient, as the Knott test was able to detect a greater number of positives in the sample tested. According to the ESDA and AHS guidelines, the correct diagnostic approach for dirofilariosis involves the combined use of both the serological test and the Knott's test and the use of instrumental investigations to establish the severity of the disease in progress. In conclusion, heartworm disease is a parasitosis that is increasing its range over time for the reasons mentioned above, and southern Italy, once considered low-risk, is increasingly becoming the site of autochthonous outbreaks. We conclude that dog shelters in southern Italy are hotspots for *Dirofilaria* spp. transmission and strongly recommend education and veterinary advice regarding regular testing and systematic treatment. Proper management of this parasitosis should be based on an effective and correct approach to diagnosis and updated therapy, as well as on new and practical prophylactic measures that protect both animal and public health.

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Overall discussion

The present thesis provides important insights into the diagnosis, epidemiology, immune response and treatment of *Dirofilaria immitis and D. repens* in the canine host.

Significant findings were obtained with respect to: i) the improvement of the Knott's test method for the identification of microfilariae of *Dirofilaria immitis* and *D. repens*; ii) the acquisition of knowledge on the immune response of *D. repens* in experimentally infected dogs; iii) the evaluation of slow kill therapy with doxycycline and various commercially available formulations of moxidectin in dogs naturally infected with *D. immitis;* iv) the distribution of *D. immitis* and *D. repens* in dogs from an endemic area of southern Italy.

The results on the improvement of the Knott's test method for the identification of microfilariae of *D. immitis* and *D. repens* (Chapter 2) indicate that distilled water maintains the same morphological characteristics and dimensions of the microfilariae (mfs) as those observed with formalin, while the lengths and widths of the mfs were significantly reduced when using the other reagents studied (i.e. 2% formalin, 2% acetic acid, 2% glacial acetic acid, and 10% saponin (D). This could be due to more pronounced dehydration. Similar results were recently reported by Evans et al. (2019) with acetic acid (vinegar) and Long et al. (2020) with glacial acetic acid. However, despite the statistically significant differences ($P < 0.05$) in the length and width of mfs observed with acetic acid, glacial acetic acid, and saponin, the morphological features of the head and tail of the two species were preserved. We can therefore affirm that all solutions could be a suitable and safe alternative to formalin for the identification of circulating mfs in clinical settings using the Knott's test, but that distilled water is the most suitable in maintaining mfs' dimensions, which are useful for the identification of mfs species. We can conclude that distilled water could successfully replace formalin in the modified Knott's test for species differentiation of *Dirofilaria* spp.

On the other hand, the findings presented in Chapter 3, revealed that the development of serological tests (namely two ELISAs, i.e. anti-*D. repens* and anti-WSPr) could be a starting point for application in epidemiological studies and as an aid in the diagnosis of infections in dogs, especially in early stage infections and in absence of mff. Indeed, only 1 dog in the present study was negative for all tests carried out. The identification of specific immune-

active proteins for *D. repens* infection could be useful in diagnosing, for example, the presence of adult parasites when neither clinical signs nor circulating mff are present in infected dogs. They could also be useful in monitoring the efficacy of adulticide treatment. Given the risk of cross-reactivity with other filarial nematodes, it would also be necessary to evaluate these serological tests in terms of their sensitivity and specificity in the field.
The results from Chapter 4, regarding the slow-kill therapy in dogs infected with *D. immitis*, showed that the adulticidal efficacy of the injectable formulation of moxidectin was 90% at 9 months post treatment, slightly superior to the spot-on formulation (80%) and highly superior to the oral formulation (60%). At 6-month follow-up, there was no evidence of worsening of the radiographic findings. Overall, the treatment with the combination of moxidectin and doxycycline was effective, and almost all dogs from the treatment groups were cleared of pulmonary abnormalities by six months from the beginning of treatment.
Finally, the last study (Chapter 5) revealed that heartworm disease is a parasitosis that is becoming more widespread over time for different reasons related to the parasites, the hosts and the environment. Southern Italy, once considered at low-risk of canine heartworm infection, is increasingly becoming the site of autochthonous outbreaks. We conclude that some coastal areas of southern Italy (e.g. Castel Volturno in the Campania region) are hotspots for *Dirofilaria* spp. transmission and strongly recommend education and veterinary advice regarding regular testing and systematic treatment. Proper management of this parasitosis should be based on an effective and correct approach to diagnosis and updated therapy, as well as on new and practical prophylactic measures that protect both animal and public health.
Some clinical issues on canine heartworm disease by *D. immitis* may affect their appropriate diagnosis. Some clinicians tend to perform therapeutic treatments without attempting a definitive diagnosis, ignoring important evidence about local epidemiological risk and the resulting data on treatment and prevention measures (Elsheikha et al., 2014). Standardization of diagnostic techniques such as the Knott's test and serology, as well as the use of these techniques should be more routinely employed in endemic or non-endemic areas, to improve the active surveillance of both infections and facilitate diagnosis and control.
Keeping in mind the impact that *D. immitis* may have on animal health, the zoonotic potential of *D. immitis* and *D. repens* and the geographical spread

of both infections, it is important to implement effective prophylactic and adequate vector control measures, as recommended by the American Heartworm Society (https://www.heartwormsociety.org) and the European Society of Dirofilariosis and Angiostrongylosis (http://www.esda.vet) .Therefore, promoting awareness among practitioners and dog owners is also one of the priority purpose for an integrated parasite control in companion animals as recommended by the European Scientific Counsel Companion Animal Parasites (www.esccap.org).

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