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Tesi di Dottorato

"Dirofilaria immitis and *Angiostrongylus vasorum* in dogs: epidemiological and diagnostic challenges"*

Candidato

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PhD Thesis

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Chapter 1
Literature review on canine heartworm (*Dirofilaria immitis*)

Chapter 2
Literature review on *Angiostrongylus vasorum* in dogs

Research objectives

Chapter 3
Dirofilaria immitis and *Angiostrongylus vasorum*: the contemporaneous detection in kennels

Chapter 4
Angiostrongylus vasorum in stray and owned dogs in southern Italy: prevalence and clinical findings

%	Percentage
AHS	American Heartworm Society
APHS	Acid phosphatase histochemical staining
APP	Acute phase protein
BID	bis in die, twice a day
CK-MB	Creatine kinase-MB
CPA	Cardiopulmonary angiostrongylosis
CPD	Cardiopulmonary dirofilariosis
CRP	C-reactive protein
CT	Computed tomography
cTnI	Cardiac troponin I
CVBDs	Canine vector-borne diseases
DIC	Disseminated intravascular coagulation
DNA	Deoxyribonucleic acid
DPI	Days post infection
e.g.	For example
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ESCCAP	European Scientific Counsel Companion Animal Parasites
ESDA	European Society of Dirofilariosis and Angiostrongylosis
FDA	Food and Drug Administration
g	Gravity
GDD	Growing degree-days
HRCT	High resolution computerized tomography scanning
IFAT	Immunofluorescence antibody test
Kg	Kilogram
LPG	Larvae per gram
L1	First-stage larvae
L2	Second-stage larvae
L3	Third-stage larvae
L4	Forth-stage larvae
L5	Fifth-stage larvae
mg	Milligram
MKT	Modified Knott's technique
ml	Millilitre
MLs	Macrocyclic lactones
Mm	Millimetre
MPI	Months post infection

List of abbreviations

MRI	Magnetic resonance imaging
OD	Optical density
ºC	Celsius Degrees
PCR	Polymerase chain reaction
per os	Oral administration
SCD	Subcutaneous dirofilariosis
SCI	Science Citation Index
UK	United Kingdom
USA	United States of America
VCS	Vena cava syndrome
vs	Versus
WSP	*Wolbachia* surface protein
µm	Micrometer

1.1. Male and female adults of *Dirofilaria immitis* (Mappe Parassitologiche 8)

1.2. Ventral and lateral view of *Dirofilaria immitis* female (Mappe Parassitologiche 8)

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Cardiopulmonary nematodes, *Dirofilaria immitis* and *Angiostrongylus vasorum*, are severe and life-threatening parasites increasingly reported in dogs throughout Europe. However, the lack of country-wide epidemiological data regarding these two vector-borne helminths in Italy could make more difficult the awareness by practitioners and thus the implementation of effective prevention and control strategies. According to this, the present thesis presented new epidemiological and diagnostic data on dirofilariosis and angiostrongylosis underlying their clinical impact in dogs in Italy as in other European countries. In order to reach our purpose, four objectives were fulfilled: (i) the prevalence of *D. immitis* and *A. vasorum* in kennel dogs from southern Italy by serological analysis; (ii) the extent and clinical relevance of *A. vasorum* infection in owned and stray dogs from southern Italy by using the FLOTAC technique; (iii) the prevalence of *D. immitis* through ELISA antigen testing before and after heat treatment, in dogs from eastern Romania; (iv) the evaluation of single and multiple vector-borne infections (*Dirofilaria, Leishmania* and *Ehrichia*) in dogs from Spain and Italy by ematological and serological diagnosis by also testing the possibility of a *D. immitis* quantitative ELISA test to reverse false negatives due to antigen-antibody complexes.

The PhD thesis entitled "*Dirofilaria immitis* and *Angiostrongylus vasorum* in dogs: epidemiological and diagnostic challenges" consists of two parts, according to the European standard requirements. The first part - entitled "Literature Review" - is divided into two chapters and summarizes information from literature about aetiology, epidemiology, clinical implications, diagnostic concerns and treatment approaches of heartworm diseases and angiostrongylosis in dogs. The second part entitled - "Own Research" - presents the general and specific aims of the thesis followed by four original studies conducted in Italy, Romania and Spain, focused on epidemiology, diagnosis and clinical relevance of *Dirofilaria* and *Angiostrongylus* infection in dogs, with conclusions and recommendations. The literature review in **Chapter 1** provides an overview of the main cardiopulmonary nematode (*Dirofilaria immitis*) infection 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 diagnostic concerns of the infection. **Chapter 2** provides literature data on aetiology, life cycle and other aspects of the clinical, diagnostic and control challenges for canine angiostrongylosis. Subsequently, we also analyse the current

distribution of canine angiostrongylosis in Europe and Italy. This review provided herein, indicates a lack of detailed studies on the prevalence of both *D. immitis* and *A. vasorum* pathogens in southern Italy, as well as data regarding the clinical importance of these nematodes.

Chapter 3 assesses the seroprevalence of *D. immitis* and the faecal presence of first stage larvae (L1) of *A. vasorum* in dogs from 68 kennels of the Campania region (southern Italy). Antigens of *D. immitis* were detected in 24/537 (4.4%) dogs in 6 out of the 68 kennels (8.8%). *A. vasorum* L1 were detected in dogs from 9 out of the 68 kennels (13.2%). Pooled faecal samples from 25 boxes (epidemiological unit in the kennel) out of the 1360 analyzed (1.8%) resulted positive to *A. vasorum* L1. The results indicated that cardiopulmonary nematodes are present in southern Italy in symptomatic dogs as well as in asymptomatic ones. Therefore, regular parasitological surveillance, appropriate treatment strategies and high-quality standard of hygiene are required to guarantee the health and welfare of kennel dogs.

Chapter 4 reports the findings of a survey conducted in southern Italy in order to evaluate the extent and clinical relevance of *A. vasorum* infection in owned and stray dogs using the FLOTAC technique. *A. vasorum* was detected in 29 out of 1499 faecal samples examined (1.9%). Out of 656 owned dogs examined, seven (4 females, 3 males) were positive for *A. vasorum* (1.1%); age ranged between 18 and 48 months; the LPG values detected varied between 10-560 (mean value = 216 LPG). Out of 843 stray dogs investigated, 22 (14 females, 8 males) were positive for *A. vasorum* (2.6%). Age ranged between 12 and 84 months and the LPG count varied between 10-668 (mean value = 254.8 LPG). The prevalence in stray dogs (2.6%) was significantly higher than the prevalence detected in owned dogs (1.1%). Seven dogs resulted positive to *A. vasorum* by FLOTAC showed clinical evidences compatible with the lungworm infection. As expected, clinical signs of *A. vasorum* infected animals were: gagging, coughing, anorexia, weight loss, exercise intolerance. The most consistent findings at the radiological exam were: generalized interstitial and alveolar pattern and enlargement of tracheobronchial lymph nodes. The findings showed a spread of *A. vasorum* in southern Italy, demanding the necessity of stimulating concern on this infection among vet practitioners, which should always include angiostrongylosis on differential diagnosis when signs are consistent.

Chapter 5 provides results of a study aimed to determine infection prevalence for *D. immitis*, through ELISA antigen testing before and after heat treatment, in dogs from eastern Romania where both *D. immitis* and

Abstract

Dirofilaria repens are endemic. Of 194 dogs sampled from four cities in Romania, *D. immitis* circulating antigens were found in 16 (8.2%) non-heated samples and in 52 (26.8%) heated samples. Of the 108 dogs examined by Knott test, 24 dogs (22.2%) were positive for circulating microfilariae (mf). Fifty% of dogs with circulating *D. immitis* mf had positive antigen tests before and after heating, while the other 50% reverted to positive only after heat treatment. Sixty% of dogs with mixed *D. immitis/D. repens* infection were antigen positive before and after heating, while the other 40% converted to positive after heating. Antigen testing for *D. immitis* in the 12 dogs with only *D. repens* mf gave conflicting results. Only two dogs (16%) were antigen negative both before and after heat treatment. Six dogs (50%) became antigen positive after heating and four dogs (30%) were antigen positive both before and after heat treatment. Results would suggest that: false negative result for antigen testing can be reverted by heating of the serum sample; dogs infected with *D. repens* may have also an occult infection with *D. immitis*; heat treatment of serum from *D. repens*-infected dogs can reveal an occult infection with *D. immitis.*

Chapter 6 aimed to evaluate the prevalence of single and multiple vector-borne infections (*Ehrlichia, Dirofilaria* and *Leishmania*) in dogs from Spain and Italy by ematological and serological diagnosis. In particular, a quantitative ELISA test was evaluated before and after serum heat treatment in order to: detect *D. immitis* antigen; verify the cross-reactivity with other pathogens; verify the possibility to reverse false negatives due to antigen-antibody complexes. A total of 46 blood samples (23 from Madrid, Spain and 23 from Molise region, Italy) were randomly collected from stray, hunting and owned dogs. All the dogs were screened for *D. immitis*, *D. repens*, *Leishmania infantum* and *Ehrlichia canis*, using the following laboratory techniques: Knott test, quantitative ELISA test (Petcheck®, Idexx) before and after heat treatment, antibody test (SNAP 4Dx Plus Test). The results of this study showed: i) the presence of *L. infantum*, *E. canis* and *D. immitis* in central Spain as well as in central Italy; ii) the lack of occult infection with *D. immitis* probably due to the fact that vector-borne pathogens other than *D. immitis* (e.g. *Leishmania* and *Ehrlichia*) even if induce hyper-gammaglobulinemia, not always can affect antigen test results as expected; iii) the increase of OD in heartworm positive samples after heating, confirming the hypothesis that canine serum and plasma from some dogs may contain inhibitors of *D. immitis* antigen detection, so the heat treatment of these samples prior to testing could improve the sensitivity of these assay in some dogs.

In chapter 7, the present assessments and future perspectives on dirofilariosis and angiostrongylosis in dogs are discussed with particular focus on the epidemiological and diagnostic challenges. Promoting awareness among practitioners and dog owners is one of the priority areas for an integrated parasite control in pets as recommended by the European Scientific Counsel Companion Animal Parasites (ESCCAP) and the European Society of Dirofilariosis and Angiostrongylosis (ESDA).

Cardio-pulmonary nematode infections in dogs

The cardiopulmonary nematodes *Dirofilaria immitis* and *Angiostrongylus vasorum* are increasingly reported in dogs and are responsible for two diseases with overlapping endemic areas, especially in Europe: dirofilariosis and angiostrongylosis (Morgan et al., 2010; Otranto et al., 2013; Maya et al., 2015). The reasons for their apparent emergence have been discussed in several recent studies: increased disease awareness, the availability of better diagnostic tools and climatic changes are considered the main causes of the recent spread of these parasites (Genchi et al., 2005; Morgan et al., 2014; Helm et al., 2015; Tolnai et al., 2015). Noteworthy, global warming seems to represent a fundamental factor for the seasonality and the spread of *D. immitis* and could be involved also in the development of infectious stages in the intermediate hosts of *A. vasorum* (Genchi et al., 2009; Traversa et al., 2010).

Current epidemiological data confirm the expanding trend of *D. immitis* in Italy, as observed in the rest of Europe (Genchi et al., 2009; Otranto et al., 2013). Today, *D. immitis* is present not only in the hyperendemic area of the Po River Valley (Genchi et al., 2005; Otranto et al., 2013), but from northern to southern regions of the Italian peninsula, including islands where the infection is now endemic (Cringoli et al., 2001; Otranto et al., 2009; Pipia et al., 2014).

Similarly, *A. vasorum* is undoubtedly spreading from northern to southern Italy, especially in the central regions that offer ideal environmental and epidemiological conditions for the expansion of this parasite and the establishment of further new endemic *foci* (Di Cesare et al., 2011; Schnyder et al., 2011; Guardone et al., 2013; Rinaldi et al., 2014). However, there are limited data and insufficient understanding of the spread of these two infections to predict further range expansions. No national or international surveillance mechanisms are in place to determine the prevalence and distribution of dirofilariosis and angiostrongylosis, although both of them are currently considered important emerging diseases of dogs (Morgan et al., 2012; Simòn et al., 2012; Elsheikha et al., 2014).

The overall aim of the thesis was to evaluate the epidemiological scenario of *Dirofilaria immitis* and *Angiostrongylus vasorum* in domestic dogs in southern Italy with a particular emphasis on the clinical and diagnostic challenges.

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Chapter 1

Literature review on *Dirofilaria immitis* infection in dogs

1.1 Aetiology

Dirofilaria immitis is a filiform nematode responsible of a vector-borne parasitic infection. The parasites are whitish, the female measuring 25-31 cm x 1.0-1.3 mm and the male 12-20 cm x 0.7-0.9 mm. (Figure 1.1.;1.2.) Even though the typical host is considered to be the domestic dog, *Canis familiaris*, it has also been reported in several wild canids: wolves (*Canis lupus*), red wolves (*Canis rufus*), dingoes (*Canis lupus dingo)*, coyotes (*Canis latrans*), jackals (*Canis aureus*), raccoon dogs (*Nyctereutes procyonoides*), dholes (*Cuon alpinus*), maned wolves (*Chrysocyon brachyurus*), crab-eating foxes (*Cerdocyon thous*), red foxes (*Vulpes vulpes*) and gray foxes (*Urocyon cinereoargenteus*) (Anderson, 2000; Bowman & Atkins, 2009). Occasionally, it has been reported also in felids (*Felis catus*, *Felis concolor*, *Leopardus pardalis*, *Neofelis neburosa*, *Uncia uncial*, *Panthera tigris*, *Panthera leo*), mustelids (*Mustela putorius*), wolverines (*Gulo gulo*), raccoons (*Procyon lotor*), bears (*Ursus arctos*), pandas (*Ailuropoda melanoleuca*), red pandas (*Ailurus fulgens*), beavers (*Castor* spp.), muskrats (*Ondatra zibethicus*), coatis (*Nasua nasua*), rabbits (*Leoporus* spp), horses (*Equs caballus*), seals (Phocidae), sea-lions (Otariidae), non-human primates and humans (Abraham, 1988; McCall et al., 2008). Usually, humans are dead-end hosts for *D. immitis,* which is responsible for human pulmonary dirofilariosis (Simon et al., 2012). Adult *D. immitis* worms occur in the pulmonary arteries and right heart chambers (Fig.1.3), causing severe condition known as canine and feline heartworm (HW) disease. The life cycle consists of five larval stages developing in an intermediate mosquito host, that also acts as a vector. The adult females release embryos (microfilariae) into the blood of the definitive host. The intermediate hosts are mosquitoes of the family Culicidae that become infected when taking a blood meal from a microfilaraemic host (Mc Call et al., 2008). *Dirofilaria* infective larvae are transmitted by several mosquito genera, including *Culex*, *Aedes*, *Anopheles* (Cancrini et al., 1995). However, *Aedes vexans*, *Culex pipiens* and *Aedes albopictus* are mainly implicated as natural vectors of filarial worms in Europe (Cancrini et al., 2006, 2007).

Fig.1.1. Male and female adults of Dirofilaria immitis *(Mappe Parassitologiche 8).*
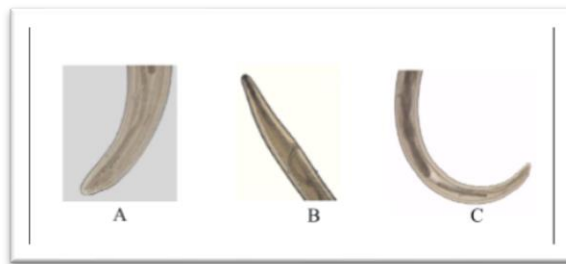
Fig. 1.2. Ventral and lateral view of Dirofilaria immitis *female: A) Anterior end of female, ventral view; B) Cephalic end of female, ventral view; C) Posterior end of female, lateral view (Mappe Parassitologiche 8).*
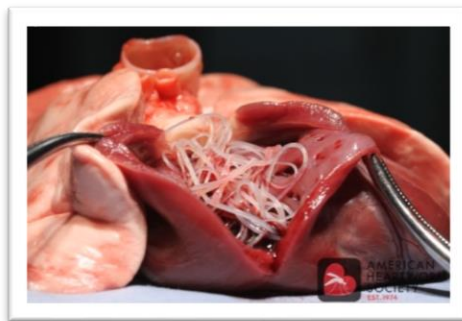
Fig.1.3. Dirofilaria immitis *adult in right chamber of the heart in dog (AHS, 2014).*

1.2 Life Cycle

1.2.1   Development in mosquitoes

The life cycle of *D. immitis* is relatively long (usually 7-9 months) compared with most parasitic nematodes. The susceptible mosquito becomes infected when taking a blood meal from a microfilaraemic host. The microfilariae (270-365 μm long and 6-8 μm wide) remain in the mosquito midgut for approximately 24 hours before migrating into the large cells of the Malpighian tubules. The larvae then become stouter as they develop into the 'sausage' stage. By the fifth day, a gut may be differentiated, consisting of an oesophagus, intestine and rectum. On days 6-7, the larvae leave the Malpighian tubule cells and enter the lumen of tubules. The larvae molt to the second stage 8-10 days after infection and again to the third stage 2-3 days later. After 1-2 days, L3 perforate the distal ends of the tubules and migrate via the haemocoel (body cavity) to the head and mouthparts where they become infective (1100-1300 μm long) (Taylor, 1960; Abraham, 1988; Cancrini and Kramer, 2001). The time required for the development of microfilariae to the infective third stage is temperature-dependent (Table 1.1). At 27°C and 80% relative humidity, development takes about 10-14 days (Orihel, 1961; Mc Call, 1981).

Table.1.1. Temperature-dependent development of Dirofilaria immitis *in some vector species.*

Mosquito vector	Temperature-dependent development of *D. immitis*		References
	T (°C)	Days	
Aedes aegypti	24-27	≥ 12	Webber and Hawking, 1955
Aedes vexans	30	8-9	Fortin and Slocombe, 1981
	26	10-14	
	22	17	
Aedes albopictus	26	14-18	Cancrini et al., 1995

1.2.2. Development in animals

The biological component of the episystem of dirofilariosis is extremely complex as *Dirofilaria* spp. parasitize a wide range of vertebrate species and vectors (Fig. 1.4) (Simon et al., 2017), all of which have their own level of adaptation. Infective L3 are transmitted to the definitive host while the mosquito is taking a blood meal and are deposited on the skin of the host via the puncture wound made by the blade-like stylets in the mouthparts (McGreevy et al., 1974). Three days after infection of the dog, most of the larvae are found in the subcutaneous (SC) tissues near their entry site. By day 21, most of them have migrated to the abdomen of the dog, and by day 41, they may be recovered from either the abdomen or the thorax. Worms reach the heart as early as day 70 and all have arrived at the heart by day 90-120. Apparently, L3 and L4 travel between muscle and eventually the jugular, or other veins, directing them towards the heart (Kume and Itagaki, 1955; Orihel, 1961; Kotani and Powers, 1982). The molt from L3 to L4 begins as early as day 3 (Kotani and Powers, 1982; Lichtenfels et al., 1985) and as late as day 9-12 (Orihel, 1961). L4 molt to the final stage at day 50-70; the first worms entering the heart on day 70-85 are 2-4 cm in length. After reaching the heart, the female worms will increase in length by almost tenfold. They become sexually mature about day 120 post-infection. Dogs develop patent infections (i.e., have microfilariae circulating in their blood) as early as 6 months (Call, 1981) but usually by 7-9 months post-infection (Orihel, 1961; Kotani and Powers, 1982).

When juvenile heartworms first reach the heart and lungs, the pressure of venous blood forces them into the small pulmonary arteries (Rawlings et al., 1978). As they grow and increase in size, they progressively migrate upstream into larger arteries until the worms become fully mature. The eventual location of the mature adult worms appears to depend mainly of the worm burden. A dog with a low worm burden (i.e., 10) usually has worms mainly in the lobar arteries and main pulmonary artery. As the worm burden increases, worms are also located in the right ventricle. Dogs with more than 40 worms are more likely to have caval syndrome, where most of the worms migrate into the right ventricle, right atrium and the caudal vena cava, thus interfering with valvular function and /or blood flow (Jackson, 1975; Ishihara et al., 1978; Atwell and Buoro, 1988).
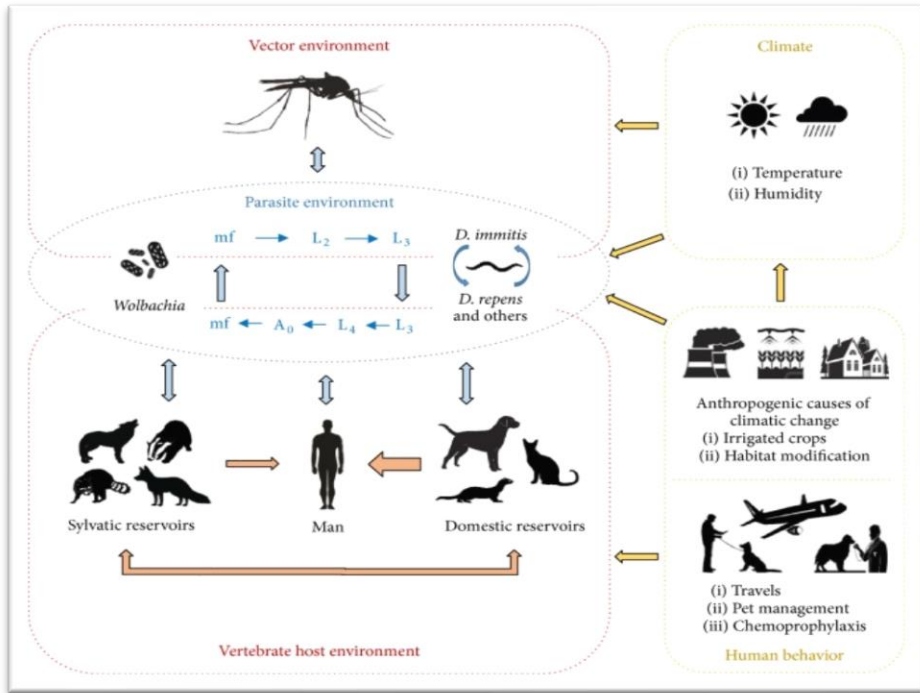
Fig. 1.4. The episystem of dirofilariosis. Main interactions among organisms involved, climate and human-derived behavior factors (Simon et al., 2017).

1.3 Epidemiology

1.3.1   Distribution and prevalence of *Dirofilaria immitis* in dogs

Dirofilaria immitis is present in tropical and temperate regions throughout the world, including canine populations from Africa, America, Asia, Australia and Europe (Genchi, et al., 2005; AHS, 2014; Simón et al., 2017). In the Americas *D. immitis* predominates, having been detected in the majority of the countries. The highest prevalence has been reported in the Eastern states of USA, the Caribbean coast of Mexico, Caribbean Islands and areas of Brazil and Argentina (20.4% to 70%). *D. immitis* also predominates in the canine populations of Africa and Australia (1% to15%) (Alvasen et al., 2016). In the European continent the highest prevalence of *D. immitis* was found in the Canary Islands and Madeira and in Mediterranean countries (22-40%) (Pereira da Fonseca et al., 1991; Simon et al., 2017). Nevertheless, comparisons of the current and past

epidemiological data show significant changes in the distribution pattern and prevalence of dirofilariosis, highlighting the establishment of new foci and increasing prevalence throughout the world (Fig. 1.5) (Simón et al., 2012). Epidemiological surveys and recent clinical reports describe a significant expansion of canine autochthonous infections by *D. immitis* particularly in central and northern European countries, areas where dirofilariosis was not reported or only few sporadic cases were documented (Genchi et al., 2005; Svobodova et al., 2005; Duscher et al., 2009; Overgaauw et al., 2009; Pantchev et al., 2009). In Italy, *D. immitis* has recently spread into northern, central and southern areas of the peninsula, where temperatures are more favourable for larval development in mosquitoes. For instance, *D. immitis* prevalence in dogs is 0.01% in Sicily (Giannetto et al., 1997; Giannetto et al., 2007) and 4.4% in the Campania region (Del Prete et al., 2015). However, the spread of *Aedes albopictus* in Italy and the evidence that this mosquito species can act as a natural vector for *D. immitis* could enhance the risk of transmission from animals to humans, considering the aggressive anthropophilic behaviour of the species (30-48 bites/h) (Cancrini et al., 2003).

Fig.1.5. Geographic distribution of the different species of Dirofilaria *in the animal hosts in the world.* D. immitis *in pets (blue);* D. repens *in pets (green);* D. immitis *and* D. repens *in pets (striped); without information (white); (∗) sporadic subcutaneous infections (Simon et al., 2017).*

1.3.2 Distribution and prevalence of *D. immitis* in Human

In Europe there are reported almost 4250 cases of human infections caused by *D. immitis* and *D. repens*. Of these, 35 are pulmonary cases attribuited to *D. immitis* (Simon et al., 2012). In Asia, Sri Lanka and India only 3 pulmonary cases were detected (Kini et al., 2015) while in Japan 280 cases of dirofilarial infection are registered (Suzuki et al., 2015). In the Americas 175 cases of pulmonary infection in humans have been approximately reported, located in USA (119 cases) and Brazil (close to 50 cases) (Biswas et al, 2013; Bublitz et al., 2012). (Fig. 1.6).
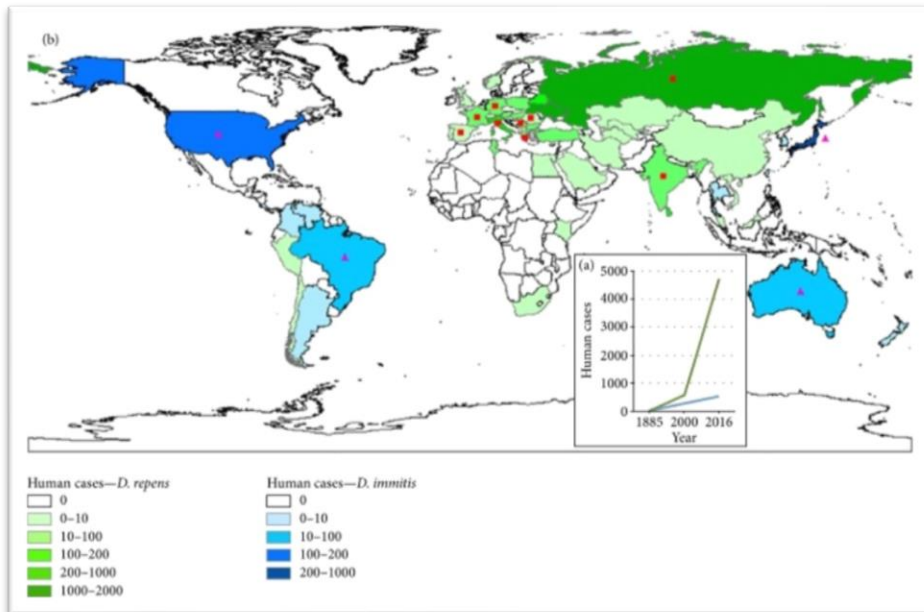
Fig. 1.6. Changes in the incidence of human dirofilariasis reported cases (a). Geographic distribution of human dirofilariasis (reported cases) (b). Pulmonary dirofilariasis (blue); subcutaneous/ocular dirofilariasis (green); sporadic cases of subcutaneous/ocular dirofilariasis in areas where pulmonary dirofilariasis predominates (fuchsia triangles); sporadic cases of pulmonary dirofilariasis in areas where subcutaneous/ocular dirofilariasis predominates (red squares) (Simon et al., 2017).

1.3.3 Climate-based model and seasonality

The transmission of *Dirofilaria immitis* depends on several factors, such as: sufficient numbers of infected and microfilaremic dogs, competent mosquito species and suitable climatic conditions that allow the extrinsic incubation of the parasites in the vector mosquitoes (Medlock et al., 2007). Forecast models based on growing degree days (GDD) have been used to predict the occurrence and seasonality of *D. immitis* in different parts of the world (Slocombe, 1989; Genchi et al., 2005; Genchi et al., 2009) (Fig.1.7). The model is based on evidence that there is a threshold temperature of 14°C below which *Dirofilaria* development will not occur in mosquitoes. Furthermore, there is a requirement of 130 GDD for larvae to reach infectivity and a maximum life expectancy of 30 days for mosquitoes (Lok and Knight, 1998; Slocombe, 1989). Models have allowed to estimate the number of annual *Dirofilaria* generations, as well as the length of infection risk periods in several regions of Europe (Genchi et al., 2005; 2009; Genchi et al., 2011). Overall, the length of the transmission season is critically dependent on the accumulation of sufficient heat to incubate larvae to the infective stage in the mosquito (Lok & Knight, 1998). In the northern hemisphere, the peak months for transmission are typically July and August. Although the transmission decreases in the winter months, the risk never reaches zero, due to the presence of microenvironments in urban areas. According to some authors, early predictions estimated that dirofilariosis would have the conditions to be introduced into central and northern Europe (Svobodová et al., 2002; Jacsó et al., 2009; Kartashev et al., 2011; Tasić-Otašević et al., 2015; Fuehrer et al., 2016). Indeed, the ongoing climate changes are lengthening the annual periods of mosquito activity and shortening larval developmental stages, with a consequent increase in the transmission in several areas. Moreover, it's important to consider the introduction of new species of competent mosquitoes, like *Aedes albopictus* (the Asian tiger mosquito), a highly adaptable species. This vector is native from south-eastern Asia and western Pacific, but has already spread to Africa, America and Europe, becoming adapted to colder climates (Roiz et al., 2007). Other examples of invasive species introduced in Europe are *Aedes koreicus* and *Aedes japonicus*, which are enhancing the risk of spreading *D. immitis* in endemic and non-endemic areas (Montarsi et al., 2015; Silaghi et al., 2017).
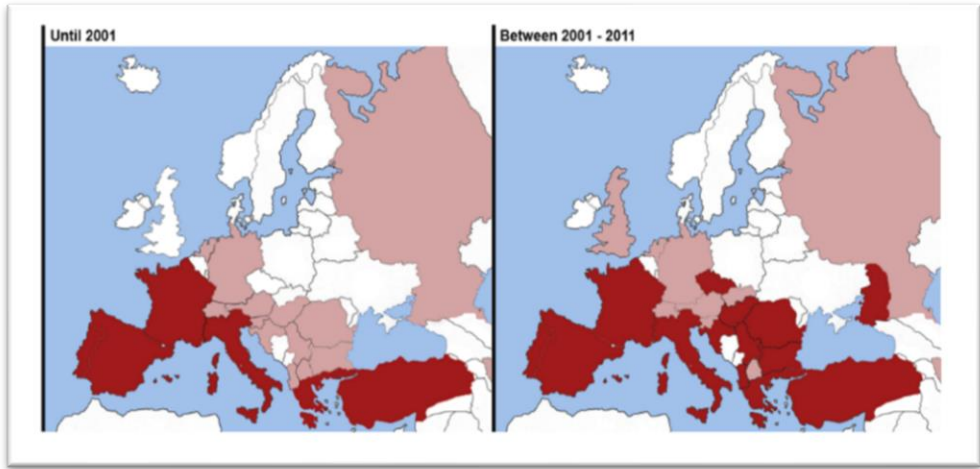
Fig. 1.7. Comparison on geographical distribution in Europe of heartworm disease observed in dogs between 2001 and 2011. Illustrated map taking into account Genchi et al. (2005, 2009), (ESCCAP).

1.4   Risk factors and populations at risk

The distribution of dirofilariosis is prevalent in regions that showed in the last years a climatic change and high humidity. Generally, the prevalence in rural and peri-urban areas is higher than in urban ecosystems. This is usually explained by a set of factors: the largest number of irrigated fields in rural areas that attract vectors and allow their proliferation; the abundant presence of wildlife animals that may carry the infection and spread the disease; the highest number of stray dogs in rural areas along with sporadic pet chemoprophylaxis, contributing to the perpetuation of the disease (Genchi et al., 2011). Regarding the risk populations, both outdoor and indoor pets represent a threat of dirofilariosis transmission. Nevertheless, outdoor animals or those who spend more time outside (e.g., kennels, shepherd, military, hunting or guard dogs) particularly during peak mosquito hours, in areas of high endemicity, are naturally an increased risk of infection (Simón et al., 2012; AHS, 2014).

1.5 Pathogenesis

Canine heartworm disease is characterized by both acute and chronic inflammatory lesions in the lungs and other organs caused by the presence of adult worms and circulating microfilariae. The pathophysiological response to heartworm infection is mainly due to the presence of adult *D. immitis* in the pulmonary arteries. The primary lesions in this disease occur in the pulmonary arteries and lung parenchyma. They are mostly attributable to the intravascular adult parasites; they cause pulmonary hypertension, that if not treated progresses inevitably to congestive heart failure (CHF). Other syndromes are related to the disturbance of blood flow due to the location of heartworms in the right atrium at the level of the tricuspidal valve. This event causes massive haemolysis and related haemoglobinuria, responsible for the vena caval syndrome (Ishihara et al., 1978; Kitgawa et al., 1987). Microfilariae appear to play a relatively minor pathogenic role but may cause clinically significant pneumonitis and glomerulonephritis. Some individuals develop a hypersensitivity to microfilariae which then disappear from the blood. Occasionally, aberrant migration results in the parasites becoming trapped in ectopic locations, such as the anterior chamber of the eye (Weiner et al., 1980) or systemic arteries (Liu et al., 1966; Slonka et al., 1977). Data regarding immunopathogenesis and the role of cytokines, pro-inflammatory mediators as well as cellular components of the immune system in the development of heartworm-related lesions has been recently reviewed (Grandi et al., 2005). Although the spectrum of pathologies related to chronic heartworm disease is broad, the most important clinical manifestation in dogs is CHF (cor pulmonale; a change in structure and function of the right ventricle of the heart as a result of respiratory disorder). Heartworm are, despite what their physical presence could suggest, primary agents of vascular disease, rather than simply the cause of obstruction and/or blood flow disturbances. Intimal proliferation occurs in arteries both of which may completely obstruct segments of the pulmonary arteries. These effects, both leading to pulmonary hypertension, are strongly correlated to worm burden, which in turn is related to the degree of their distribution through lung parenchyma (Knight, 1987).

The juvenile heartworms are only about 2.5 cm long when they reach the systemic venous circulation. They passively embolize the pulmonary arteries and are disbursed in proportion to the lobar blood flow. Generally, the larger and more readily accessible right caudal lobar artery accumulates

more worms than the left (Atwell and Rezakhani, 1986). Contact between the parasite and the intima of the pulmonary arteries is an important, if not essential, initial step in the development of the endovascular lesions. The earliest lesions are limited to the small peripheral branches where the worms first come to rest. As the parasite grows, lesions occur in more proximal segments. Intimal thickening and narrowing of the vessel lumen is small peripheral branches of the pulmonary arteries are the major cause of obstructed blood flow and pulmonary hypertension. The intimal proliferation is caused by migration of medial smooth muscle cells through the internal elastic lamina (Munnel et al., 1980; Schaub et al., 1981).

The pathogenesis of the arteritis caused by heartworms remains a matter of speculation. Disruption of the endothelial cell junction and denuding of the intimal surface are characteristics of the first lesions that occur only a few days after the worms occupy the vessels. The evidence suggests that injury to the endothelium occurs immediately upon arrival of the parasite, too soon for the components of an acquired immune response to fall into place without prior sensitization.

Macrophages, granulocytes and platelets are attracted to the site of endothelial damage and adhere to the exposed subendothelium. Shortly after their arrival, vascular smooth muscle cells migrate into the intima and a very active process of myointimal proliferation produces rapid growth of the lesions. The prominence of platelets in the acute lesions and their documented ability to stimulate growth of the vascular smooth muscle, through the release of platelet-derived growth factors (Ross, 1986), are hypothesized to be a likely mechanism for triggering and sustaining the growth of these lesions (Schaub and Rawlings, 1980; Schaub et al., 1981). Although the lesions thicken the wall of these large elastic vessels and produce a rough texture on the intimal surface, they do not obstruct blood flow by narrowing the lumen. On the contrary, the large distributing arteries actually dilate as pulmonary hypertension becomes increasingly severe. Pulmonary blood flow is impeded primarily by the reduction in cross-sectional area of the arterial vascular bed, caused by obliterative endarteritis of small peripheral branches. Recently, in heartworm infected dogs, it has been demonstrated that there is a markedly increased plasma level of endothelin-1, a mediator that induces acute vasoconstriction and chronic vascular remodelling. It is probable that both of these events contribute in turn to the development of pulmonary hypertension (Uchide and Saida,

2005). Thrombosis and thromboembolism may compromise the pulmonary circulation further. As worms accumulate, lesions also develop in the large distributing arteries, which dilate and become stiffer, and the pulmonary blood pressure rises. The decreased distension of the large vessels significantly increases cardiac work by coupling the right ventricle directly to the high vascular resistance in the obstructed peripheral vasculature. Right ventricular hypertrophy is a compensatory response to the increased pressure load. As heartworm disease impedes flow in an increasing number of branches, the pulmonary vascular reserve diminishes. For a time, normal pulmonary blood pressure is preserved at rest and rises only modestly during exercise as patent arteries reach full distension. Eventually, the pulmonary arterial tree is restricted to the point that it assumes that characteristics of a system of rigid tubes and pulmonary vascular resistance become fixed. At this stage, pressure rises in direct proportion to further increase in flow. Consequently, the more severe the disease and active the patient, the more cardiac work must be performed. In advanced cases of heartworm disease, low-output CHF develops as a result of the right ventricle's inability to generate and sustain the high perfusion pressures required to move blood through the lung. Recently, a decrease on extracellular collagen matrix has been observed in the myocardium of heartworm- infected dogs that may contribute to ventricle dilatation, thereby markedly affecting the systolic and diastolic functions of the heart (Wang et al., 2005). Frequently, dogs at this stage experience syncope (a temporary loss of consciousness and posture) when attempting to suddenly increase cardiac output. Right-sided congestive heart failure (R-CHF) with ascites, hepatomegaly and cachexia is a late sequela and may be precipitated by an acute episode of pulmonary thromboembolism.

1.6   *Wolbachia* Endosymbiosis

Dirofilaria immitis, the causative agent of canine and feline heartworm disease, harbours intracellular bacteria named *Wolbachia pipientis.* Indeed, most filarial species studied, with very few exceptions, contain these micro-organisms that are thought to play an essential role in the biology and reproductive functions of their filarial hosts (Sironi et al., 1995). Indeed, as Gram-negative bacteria, *Wolbachia* have a potential to play an important role in the pathogenesis and immune-response to filarial infection. The immunopathology of filarial disease is extremely complex and the clinical manifestations of infection are strongly dependent on the type of immune

response elicited by the parasite. Furthermore, the fact that adult parasites can survive for years in the otherwise immunocompetent hosts is likely due to the parasite's ability to avoid/modulate the immune response. It is therefore extremely important to identify which components of the parasite interact with the host's immune system, including *Wolbachia*.

W. pipientis, the only species thus far identified in the genus, are gram negative bacteria belonging to the order Rickettsiales. They are closely related to other bacteria belonging to the same group, such as *Ehrlichia* spp. and *Anaplasma* spp. (Bandi et al., 2001). Electron microscopy, histology and immunohistochemistry have offered a clear description of the distribution of *Wolbachia* in *D. immitis* (Bandi et al., 1999; Sacchi et al., 2002; Kramer et al., 2003; Kozek, 2005). They are found throughout all the stages of the life cycle of the nematode although they occur in varying proportions between individual worms and different developmental stages. In adult *D. immitis*, *Wolbachia* is predominantly found throughout the hypodermal cells of the lateral cords. In females, *Wolbachia* is also present in the ovaries, oocytes and developing embryonic stages within the uteri. They have not been demonstrated in the male reproductive system (Sacchi et al., 2002), suggesting that the bacterium is maternally transmitted through the cytoplasm of the egg and not through the sperm. All current evidence suggests that *Wolbachia* is a symbiont in filarial worms (Taylor et al., 2005), that is, the presence of bacteria is essential for the filarial worm's survival. The phenomenon of bacterial endosymbiosis is well known in arthropods, but less so in nematodes. There are, however, several features of the relationship between *Wolbachia* and filarial worms (including *D. immitis*) that suggest its symbiotic nature: (1) in those species of filarial worms that have been identified as harbouring *Wolbachia*, all of the individuals are infected (100% prevalence); (2) the evolution of the bacteria match that of the filarial worms, as shown by phylogenic studies; (3) the bacteria are transmitted from female to offspring, thus the symbiont guarantees its own future by increasing the fitness of the host that is involved in its transmission and (4) removal of *Wolbachia* (antibiotics/radiation) leads to sterility of females and eventual death of adults heartworms.

Adverse reactions to filaricidal therapy (ivermectin, diethylcarbamazine) are associated with *Wolbachia* and/or its DNA in the bloodstream and peak levels of *Wolbachia* correlate with levels of pro-inflammatory cytokines. Furthermore, a major surface protein in *Wolbachia* (WSP) from *D. immitis*

has been shown to provoke chemiokinesis and IL-8 production in canine neutrophils in vitro. It should be noted that *Wolbachia* can be eliminated from filarial worms through antibiotic therapy of the infected host. Such depletion of *Wolbachia* is often followed by clear anti-inflammatory effects, thus antibiotic treatment may be used concomitantly with the use of adulticidal therapeutic agents. *D. immitis* infected dogs also come into contact with *Wolbachia* either through microfilarial turnover or natural death of adult worms. Intense staining for the WPS was observed in various tissues from dogs who had died from natural HW disease (Kramer et al., 2005). It is still unclear, however, exactly what *Wolbachia* does to make it so important for its filarial host. Several hypotheses have been suggested. Studies on population dynamics in *Brugia malayi* (Fenn and Blaxter, 2004) have shown that the numbers of bacteria remain stable in microfilariae and in the larval stages L2 and L3 within the mosquito. After approximately a week following infection of the mammalian host, bacteria numbers increase rapidly throughout L4 development. This means that the major period of bacterial population growth occurs within the first month of infection of the definitive host, suggesting a role in evasion of mammalian immunity, the long-term survival of adult worms and possibly in molting (McGarry et al., 2004).

1.7   Clinical diagnosis

The clinical presentation of canine heartworm disease is usually chronic. Most infected dogs do not show any symptoms for months or years, depending on the worm burden, individual reactivity and exercise, as arterial damage is more severe in dogs with intensive exercise than in dogs at rest (Dillon et al., 1995). Signs of the disease develop gradually and may begin with a chronic cough and coughing may be followed by moderate to severe dyspnoea, weakness and sometimes lipothymia after exercise or excitement. Infected dogs may also exhibit intolerance to exercise or syncope related with increased physical activity or excitement. Physical examination may reveal evidence of weight loss, right sided heart murmur of tricuspid insufficiency, split-second heart sound and cardiac gallop (Atkins, 2010). Whenever right heart failure is present, jugular venous ingurgitation and pulsation, along with hepatosplenomegaly and ascites may occur. Pulmonary manifestations include cough, dyspnoea, pulmonary crackles, muffled lung sounds and eventually cyanosis. If pulmonary thromboembolism occurs, dyspnoea may worsen and fever and haemoptysis

may be noted. Although sudden death is rare, it can occur due to cardiorespiratory insufficiency or severe thromboembolism (Venco et al., 2011). The presence of adult worms in the pulmonary artery cause pulmonary hypertension and massive haemolysis and consequent haemoglobinuria, responsible for the "caval syndrome", which is fatal unless surgical intervention (Kitagawa et al., 1987; Venco, 1993). Infection by *D. immitis* may cause also renal dysfunction and glomerulonephritis induced by the deposition of immune complexes triggered by the antigens of adult worms and larval stages (Abramowsky et al., 2003). Infected dogs may develop eczematous dermatitis (secondary to kidney disorders) and eosinophilic pneumonia, due to an eosinophilic reaction against microfilarial antigens. Other organs may also be affected by ectopic localizations, such as the eyes, brain, liver and peritoneal cavity, with related pathologies.

1.8   Laboratory diagnosis

Canine dirofilarial infections can be diagnosed by several methods. However, it must be kept in mind that: I) it is always necessary to identify the microfilaria species by morphological features; II) a certain number of dogs are amicrofilaraemic and other test should be used in case of clinical suspicion; III) both thoracic radiography (x-R) and echocardiography (ECHO) are necessary to assess the severity of cardiopulmonary disease and to visualize adult worms (Genchi et al., 2007).

1.8.1   Fresh Blood Smear

A drop of fresh venous blood is placed on a microscopic slide, covered with a coverslip and examined under low microscopic power. Microfilariae are seen through the movement they cause to the red blood cell layer. This method is quite rapid and inexpensive but the sensitivity is very low, false negatives are frequent and is not possible to differentiate microfilariae (no species diagnosis) (Genchi et al., 2007).

1.8.2 Modified Knott Test

The most commonly used method for microfilariae identification is the Modified Knott's technique (MKT). It is an easy, quick and inexpensive diagnostic concentration method. Briefly, 1 ml of EDTA blood is mixed with 9 ml of 2% formalin and centrifuged for 5 minutes at 500×g. After that, the supernatant is poured off, one drop of blue methylene is added and the sediment is observed under the light microscope (Venco et al., 2011; Magnis et al., 2013). Given the variety of canine filarial species presenting blood microfilariae [(with *D. immitis*, *D. repens*, *Acanthocheilonema dracunculoides* and *A. reconditum* as the most important in Europe (Genchi et al., 2011)] it is essential to perform a morphometric analysis (Fig.1.8) to obtain a correct diagnosis and select the appropriate treatment. Magnis et al. (2013) validated morphometric criteria for the identification of microfilariae in the dog's blood using the MKT, allowing a clear distinction between *D. immitis* (302 µm average length, 6 µm average width, with a conical front end and a straight rear end), *D. repens* (369 µm average length, 9 µm average width, with a conical front end and curved caudal end), *A. dracunculoides* (259 µm average length, 5 µm average width, with a round front and straight caudal end) and *A. reconditum* (265 µm average length, 5 µm average width, with a blunt front end and a small hook in the rear end). Due to the overlapping size ranges of *A. dracunculoides* and *A. reconditum*, biochemical or molecular methods are required to distinguish these two species (Magnis et al., 2013).
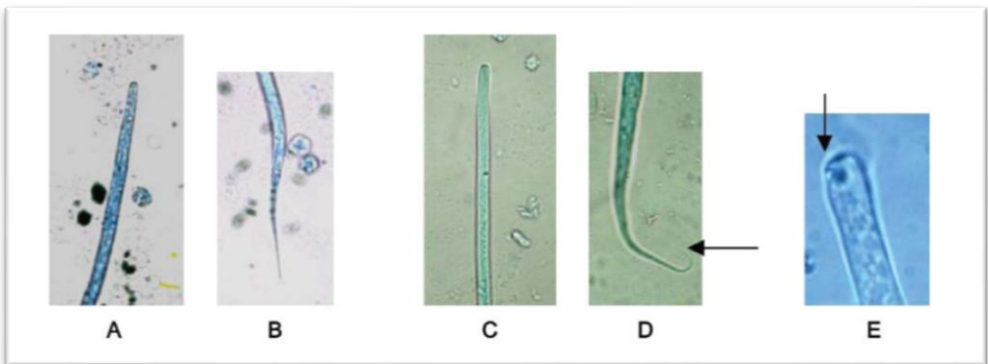
Fig.1.8. Cranial and caudal extremity of microfilariae species- A) cephalic end (x400) D. immitis, *B) caudal end (x1000)* D. immitis, *C) cephalic end (x400)* D. repens, *D) caudal end (x1000)* D. repens, *E) cephalic end (x1000)* Acantocheilonema reconditum.

1.8.3   Filter Test

One ml of venous blood anticoagulated with either EDTA or heparin, is added to approximately 10 ml of lysate solution. The mixture is injected into a Millipore filter chamber. The filter is removed from the chamber, placed on a glass slide, stained, and examined under a microscope. This method is rapid and sensitive and does not need a centrifuge apparatus. However, it is quite expensive. The lysate solution causes the microfilariae and new measurement standards are needed to help differentiate species (Genchi et al., 2007).

1.8.4   Histochemical stain

One ml of venous blood collected in EDTA is injected into 10 ml of deionized water and centrifuged at 1500 rpm for 15 minutes. The supernatant is discarded and the sediment placed on a slide and air-dried. The smear is then fixed in absolute acetone, air-dried, and covered with acide phosphatase substrate. The substrate needs to be either fresh, as described by Chalifoux and Hunt (1971), or frozen at -80°C in aliquot portions. After two hours at room temperature, the slide is airdried and covered with a coverslip. The two *Dirofilaria* species show different distribution patterns of acid phosphatase activity (highlighted with red spots).

1.8.5   ELISA and Immunochromatographic tests for adult female HW circulating antigens

Several ELISA and immunochromatographic kits are commercially available to detect presence of adult female circulating antigens in serum, plasma and blood of dogs and cats. They are very specific, quite sensitive, rapid and easy to perform (Fig. 1.9). Most of them are in-clinic test kits for single diagnosis but ELISA plates for multiple runs are also available. Manufacturers consider the result positive if one adult female worm has infected the animal. Male worms are not detectable using antigen tests.

 In dogs, detectable antigenemia develops about 5 to 6.5 months post infection (Genchi et al., 2007).

Because of the rather rapid clearance of antigens at the death of worms, these techniques can be used to assess the efficacy of adulticide therapy. In order

to confirm the success of the adulticide therapy, dogs have to be retested at five and nine months post- treatment. If the test at five months is negative, testing at nine months is not necessary.

Because unisex infections consisting of only male worms or symptomatic immature are not uncommon in cats, none of the presently available antigen tests can be relied upon to rule out HW disease in cats. In cats with heavy infections, detectable antigenemia develops at about 5.5 to 8 months post infection (Mc Call et al., 2008).

It is worth mentioning that amount of antigen in circulation, determined by semiquantitative and laboratory ELISA tests (not immunochromatographic tests), have a direct, but imprecise, relationship to the adult female worm burden. The utility of the ELISA tests for assessing the degree of parasitism may be limited by the transient increase in antigenemia associated with recent worm death. When animals are given monthly preventive medication during the HW transmission season, it is advisable to retest each year before initiating the preventive treatment for the following season (Drake et al., 2015).

If the chemioprophylactic treatment is performed with a sustained-release injectable drug (only for dogs), periodic testing (every 2 or 3 years) will ensure that there have been no gaps in efficacy. Such tests are very specific and sensitive (gold standard: when positive, the test is the definitive proof of HW infection in dogs and cats). However, they are costly and not available for other filarial infections (e.g. *D. repens*). (AHS, 2014; Genchi et al., 2007).

Fig.1.9. Canine heartworm Antigenic test

1.8.6   Molecular diagnosis

Other diagnostic options are the amplification of microfilaria DNA by polymerase chain reaction (PCR) (Favia et al., 1997). PCR is a sensitive and accurate tool to discriminate microfilariae from the different filarial worms.

A duplex real-time PCR was developed to detect and differentiate infection by *D. immitis* and *D. repens* in dogs and mosquitoes and a multiplex PCR was described for the simultaneous detection of canine filarioids (Latrofa et al., 2012). The specific primers (12SF/12SRdeg/12SF2B/12SR2) are used to amplify a portion of the small subunit ribosomal RNA gene of the mitochondrion (12S rDNA) to perform a multiplex PCR. The amplification of the conserved region is 500bp (12SF/12SRdeg) for canine filarial samples, and for the simultaneous amplification of *D. immitis* and/or *D. repens* the specific fragment is 204 bp (12SF2B/12SRdeg) and 327bp (12SF/12SR2) respectively (Gioia et al., 2010).

1.8.7   Other diagnostic aids

Thoracic radiograph, echocardiography and electrocardiography provide insights regarding the clinical status, severity and prognosis of cardiopulmonary disease secondary to heartworm infection. Characteristic

(nearly pathognomonic) radiographic features are enlarged, tortuous, truncated peripheral intralobar and interlobar branches of the pulmonary arteries (particularly on the diaphragmatic lobes), accompanied by pulmonary parenchymal disease, right heart cardiomegaly in advanced stages, and pleural effusion following right heart congestive failure (Rawlings, 1986; Bowman and Atkins, 2009). Echocardiography allows the visualization of the worms, which are seen as two parallel hyperechoic lines in the main pulmonary artery, interlobe branches, right heart atrium and ventricle (Badertscher et al., 1988). Besides, allows the assessment of cardiac anatomy and functional capacity, providing conclusive confirmation of the vena cava syndrome (VCS) when heartworms are located in the tricuspid valve. However, echocardiography is not an efficient method of making this diagnosis, particularly in lightly infected dogs, as the worms are frequently limited to the peripheral branches of the pulmonary arteries, thus beyond the echocardiographic field of view. Electrocardiography can reveal alterations in the electrical axis and rhythm (deviations to the right side of the axis and atrial fibrillation) in dogs in terminal stages that exhibit severe enlargement of the right atrium (Venco et al., 2011).

1.9 Diagnostical concerns

Although filarial species can be identified by microfilarial size and morphology, these features are challenging, particularly when low parasitaemia or mixed infections are present. Besides, both Modified Knott (MKT) and Acide Phosphatase Stain (APHS) only work when heartworm-infected dogs have a detectable microfilaraemia. This happens only in 2/3 of the cases, as 30-40% of infected dogs remain or become amicrofilaremic despite a persisting infection with adults (Deplazes et al., 2016). Moreover, APHS reagents have a limited shelf life and require fresh samples to yield interpretable results (Peribáñez et al., 2001).

Diagnoses exclusively based on circulating antigen, may give a false negative result in infections with low parasite burden. Furthermore, in some dogs, antigen–antibody complexes may entrap antigens, hampering the immunological detection. As circulating antigens are only detectable when *D. immitis* reached the adult stage, antigen testing should not be carried out earlier than 7 months after exposure to infection. The combination of the serological techniques with MKT or APHS allows an accurate detection of dirofilariosis. A positive microfilaria test with a positive antigen test

confirms an infection with *D. immitis*. A positive antigen test without circulating microfilariae indicates an amicrofilaremic or occult infection by *D. immitis* that may be due to pre-patency, unisex infection by female worms, drug-induced sterility of adult filariae, or even immune-mediated clearance of microfilariae (Genchi et al., 2007). A positive microfilaria test with a negative antigen test may indicate an infection caused by a species apart from *D. immitis* that may be confirmed by MKT or molecular techniques. If it is *D. immitis* microfilariae, it might be due to antigen-antibody complex formation that can interfere with the antigen detection (Little et al., 2014), low female worm burden, or the persistence of microfilariae following the natural or the pharmacological death of adults (Atkins, 2003). Overall, diagnostic techniques must be taken together (testing for both microfilaria and antigens) and interpreted along with the results from clinical examination (thoracic radiography and echocardiography) to achieve a reliable diagnosis. Annual testing of dogs is important not only to ensure that prophylaxis is correctly being performed but also to provide early treatment with lower pathological effects when an infection is present (AHS, 2014).

1.10   Prognosis

The prognosis of subclinical infected dogs with cardiopulmonary dirofilariosis is generally good. Although the prognosis for severely infected dogs should be guarded, a large number can be successfully managed. Nevertheless, dogs with severe disseminated intravascular coagulation, vena cava syndrome massive embolization, pulmonary eosinophilic granulomatosis, severe pulmonary artery disease or heart failure, have a poor prognosis (Rawlings, 1986).

1.11   Treatment

The main goal of *D. immitis* treatment is to eliminate all forms of the parasite (e.g., adults, juveniles, larval stages and microfilariae) and improve the animal's clinical conditions and welfare, with minimal complications (AHS, 2014). This can be achieved pharmacologically using a multimodal approach, combining melarsomine dihydrochloride (an adulticide drug) with macrocyclic lactones (MLs) (microfilaricide) and doxycycline (antibiotic against *Wolbachia* spp. organisms). Mechanical heartworm removal is also indicated as a method of eliminating as many adult worms

as possible before pharmacological treatment is initiated (AHS, 2014). Despite the range of therapeutic options, it's important to underline the complexity and inherent risk associated with the treatment of cardiopulmonary dirofilariosis, given the massive worm destruction in the bloodstream and its multiple side effects. Before starting therapy, the staging and risk of thromboembolic complications of each animal should be assessed, considering age, dog size, parasite load, severity of pulmonary disease and possibility to restrict dog's physical activity (Venco et al., 2004).

1.11.1 Anthelmintic therapy

The only adulticidal drug available and approved by the Food and Drug Administration (FDA) for *D. immitis* is melarsomine dihydrochloride, an arsenic compound. According to the American Heartworm Society (AHS), the three-dose protocol of melarsomine (one injection of 2.5 mg/kg followed one month later by two injections of 2.5 mg/kg, 24 hours apart) is the recommended regimen regardless the stage or severity of the disease (with the exception of VCS) (Tab. 1.2). The three-dose protocol has proven to have an overall increased safety and efficacy (98% *vs* 90%) in comparison to the two-injection protocol (two injections of 2.5 mg/kg, 24 hours apart) (AHS, 2014). Strict exercise restriction for 30 to 40 days after adulticide treatment is crucial for minimizing cardiopulmonary complications, such as pulmonary thromboembolisms, which constitute an inevitable consequence of successful adulticide therapy. Whereas mild embolism in healthy lung areas may be clinically unapparent, severe embolism may cause life-threatening respiratory distress. Signs of embolism are usually evident within 7 to 10 days after completion of adulticide administration and may include low fever, cough, haemoptysis and exacerbation of right heart failure (Hirano et al., 1992). As melarsomine has incomplete efficacy against young adult worms (less than 4 months old) (Dzimianski et al., 1989; Dzimianski et al., 1990), MLs should be administered monthly to eliminate existing larvae. As MLs may cause a rapid decrease in microfilariae numbers, it should be used with caution in dogs with high microfilarial counts, coupled with antihistamines and corticosteroids to minimize potential reactions. Treatment approaches using only MLs as a slow-kill adulticide are not recommended (AHS, 2014). The administration of a tetracycline antibiotic is useful to reduce the number of *Wolbachia* organisms and their metabolites such as the *Wolbachia* surface protein (WSP), a major responsible for the pathogenesis of filarial diseases.

Doxycycline (at 10 mg/kg, BID, for 4 weeks) should be given before the administration of melarsomine so that *Wolbachia* organisms and metabolites are reduced or absent when the worms die and fragment. Indeed, pre-treatment with ivermectin and doxycycline prior to melarsomine in experimentally infected *D. immitis* dogs, had shown to reduce pulmonary pathology associated with the death of the heartworms (reviewed by McCall et al., 2008; Kramer et al., 2011).

Table 1.2- Recommended treatment and management protocol for Dirofilaria immitis *infections in dogs (AHS, 2014)*

Day	Treatment
Day 0	Dog diagnosed and verified as heartworm positive: ➢ Positive antigen (Ag) test verified with microfilaria (MF) test ➢ If no microfilariae are detected, confirm with 2th Ag test from a different manufacturer Begin exercise restriction. ➢ The more pronounced the signs, the stricter the exercise restriction If the dog is symptomatic ➢ Stabilize with appropriate therapy and nursing care ➢ Prednisone prescribed 0.5 mg/kg BID 1st week, 0.5mg/kg SID 2nd week, 0.5mg/kg EOD 3rd and 4th weeks
Day 1	Administer heartworm preventive ➢ If microfilariae are detected, pretreat with antihistamine and glucocorticosteroid, if not already on prednisone, to reduce risk of anaphylaxis ➢ Observe for at least 8 hours for signs of reaction
Days 1-28	Administer doxycycline 10mg/kg BID for 4 weeks. ➢ Reduces pathology associated with dead heartworms ➢ Disrupts heartworm transmission
Day 30	Administer heartworm preventive.
Day 60	Administer heartworm preventive. First melarsomine injection 2.5mg/kg intramuscularly (IM) Prescribe prednisone 0.5 mg/kg BID 1st week, 0.5 mg/kg SID 2nd week, 0.5mg/kg EOD 3rd and 4th weeks. Decrease activity level even further. ➢ Cage restriction/on leash when using yard
Day 90	Administer heartworm preventive. Second melarsomine injection 2.5mg/kg IM
Day 91	Third melarsomine injection 2.5mg/kg intramuscularly (IM) Prescrive prednisone 0.5mg/kg BID 1st, 0.5 mg/kg SID 2nd week, 0.5mg/kg 3rd and 4th weeks. Continue exercise restriction for 6 to 8 weeks following last melarsomine injections.
Day 120	Test for presence of microfilariae ➢ If positive treat with a microfilaricide and retest in 4 weeks Establish year-round heartworm prevention.
Day 271	Antigen test 6 months after completion; screen for microfilariae.

1.11.2 Supportive therapy

Supportive therapy is indicated in dogs before receiving adulticide or surgical therapy for thrombosis prophylaxis, as well as on those in which causal therapy is not recommended. Corticosteroids, diuretics, vasodilators, positive inotropic agents and fluid therapy might be used (AHS, 2014).

1.11.3 Surgical intervention for *Dirofilaria immitis* worm's extraction

Surgical therapy is advised in dogs severely infected with *D. immitis*. These dogs are poor candidates for immediate melarsomine treatment because of the immune reaction triggered by the rapid killing of the worms that can culminate in pulmonary thromboembolism. Surgical therapy is also indicated in the case of VCS, a life-threatening condition that ends fatally within 2 days if worm's extraction is not pursued promptly (AHS, 2014).

Surgical removal can be accomplished using distinct devices, including rigid or flexible alligator forceps (Ishihara et al., 1988) or intravascular retrieval snares (Yoon et al., 2013). These devices are introduced via the right external jugular vein, aided by fluoroscopic guidance, to access the right cardiac chambers and the major pulmonary arteries. Unlike adulticide treatments, surgical removal of filariae can potentially avoid the risk of pulmonary thromboembolism. The principal advantages are the shorter duration of general anaesthesia and the reduced invasiveness of the procedure with lower damage of the vascular endothelium. Generally, the intraoperative mortality risk is low, and survival and recovery rates are positively correlated with the number of parasites removed (Morini et al., 1998). After few weeks following surgery, chemotherapy is recommended to eliminate any remaining worms (AHS, 2014).

1.12 Prevention

The most safety and effective prophylactic option for the control of the infection is the administration of macrocyclic lactones (LM) such as ivermectin, milbemycin oxime, moxidectin or selamectin. Although, LM do not prevent the inoculation of larvae, they impede larval development in the vertebrate host. Prevention through injectable long- lasting formulation or monthly oral or spot-on administration should start one month prior the mosquito season (early spring), and should be continued until one month

after this period ends (late autumn). However, in endemic areas and regions where the climate conditions allow transmission throughout the year, continuous annual protection against heartworm is recommended (Simón et al., 2012). Regular application of mosquito repellents, emptying standing water collections, installation of window screens and avoidance of areas and day periods in which mosquitoes are most active, are important control measures to prevent mosquitoes bites and to reduce *D. immitis* infection (ESCCAP, 2012; AHS, 2014).

1.13   Public Health concerns

Human dirofilariosis is currently considered an emerging disease in some areas of the globe (Ermakova et al., 2017), due to the high increase of subcutaneous and ocular cases reported in the recent years, contradicting the idea that human dirofilariosis is infrequent (Kramer et al., 2007; Avellis et al., 2011; Simón et al., 2012; Agrawal et al., 2017; Walters et al., 2017). Human pulmonary dirofilariosis, usually associated with *D. immitis*, is characterized by pulmonary nodules triggered by an inflammatory response around the immature worms that reach the pulmonary artery. The most frequent presentation is a single spherical or ovoid nodule located in peripheral areas like the subpleural region. Infection is usually asymptomatic, although cough, thoracic pain, haemoptysis, dyspnoea, fever and malaise have been reported (Muro and Cordero, 2001). These nodules are frequently misdiagnosed with malignant lesions (Simón et al., 2005).

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Chapter 2

Literature review on *Angiostrongylus vasorum* in dogs

2.1 Aetiology

Angiostrongylus vasorum (Fig. 2.1) is a metastrongyloid nematode that primarily infects Canidae, especially domestic dogs and the common fox (*Vulpes vulpes*). Natural infection has also been reported in the wolf (*Canis lupus*), coyote (*Canis latrans*), jackal (*Canis aureus*), European otter (*Lutra lutra*), ferret (*Mustela putoris*), Eurasian badger (*Meles meles*), red panda (*Ailurus fulgens fulgens*), pampas fox (*Pseudalopex gymnocerus*), hoary zorro (*Pseudalopex vetulus*), and crab-eating fox (*Dusicyon thous*). Experimentally, the Nile rat (*Arvicansthis niloticus*) and domestic cat have also been infected after oral inoculation with L3 larvae (Guilhon et al., 1965; Guilhon 1973; Poli et al., 1984; Bolt et al.,1994; Lima et al., 1994; Segovia et al., 2001; Torres et al., 2001; Bourque et al., 2005; Fiorello et al., 2006; Dias et al., 2008; Patterson- Kane et al., 2009). Strains of the parasite found in South America are genetically distinct from those found in Europe and North America, and might represent a different species (Jefferies et al., 2009). Other metastrongyloid parasites of the *Angiostrongylus* genus include *Angiostrongylus cantonensis* and *Angiostrongylus costaricensis*, which are known to infect humans. *A. cantonensis* was described in rats in China in 1933 and the first identification as a human pathogen was in Taiwan and in the Pacific Islands during World War II (Wang et al., 2008). Its definitive host is the rat, with the adult worm residing in the pulmonary arteries. Humans, dogs, and macropods that ingest larvae in snails (or other vectors), or in contaminated water and vegetable matter can develop signs of eosinophilic meningitis (Alicata et al.,1962; Collins et al., 1992). *A. costaricensis* was first described in 1967(Cspedes et al., 1967) and determined intestinal eosinophilic granuloma in children. It is especially prevalent in Latin America (Loria-Cortes et al., 1980).

Fig.2.1. Adult stage of Angiostrongylus vasorum

2.2 Life cycle

The dioecious adult *A. vasorum* lives in the pulmonary arteries and heart of the definitive hosts, i.e. dogs and other canids, and produces eggs that hatch to first-stage larvae (L1). These penetrate the alveoli, migrate up to oropharynx, after which they are swallowed and then eliminated in the faeces. A definitive host can shed as many as 280 000 larvae per gram of faeces (Martin et al., 1993). These L1 must infect a gastropod mollusc intermediate host (slug or snail), for the development of the infective third-stage larvae (L3), which occurs within 10-16 days under optimum conditions (Guilhon et al., 1963; Morgan et al., 2005). Until now virtually all investigated snails and slugs have proven to be able to function as intermediate host to this parasite. Frogs act as paratenic hosts, following the ingestion of infected snails or slugs, and can also act as intermediate hosts (Bolt et al., 1993). L3 of *A. vasorum* are able to infect the dog (*Canis familiaris*) either directly, through ingestion of L3 present in the environment, or indirectly, through ingestion of an intermediate gastropod or paratenic (transport) host. Then, L3 penetrate the gut wall and migrate to the abdominal lymph nodes, where they moult to fourth-stage larvae (L4); then enter the portal circulation, migrate through the liver parenchyma and eventually reach the right ventricle and pulmonary arteries, where they become adults. The pre-patent period, is reported to be 38-57 days; however, it can range widely from 28 to 108 days (Bolt et al.,1994).

2.3 Wildlife hosts

The most significant wildlife reservoir in Europe for *A. vasorum* is the red fox (*V. vulpes*) (Bolt et al., 1992; Morgan et al., 2008; Gerrikagoitia et al., 2010). In wolves, *A. vasorum* is rare, with only few cases described (Segovia et al., 2001; Eleni et al., 2014). Indeed, the lack of genetic diversity between the *A. vasorum* samples obtained from dogs, foxes and coyotes support the hypothesis that transmission occurs between wild and domestic canids (Jefferies et al., 2009). It is suggested that an increase density of infected foxes in areas populated with dogs may increase the contamination of the environment with L1. This may consequently lead to a higher number of infected gastropods and potential paratenic hosts, or even to an increased number of free L3 in the environment and easily accessible to dogs. In fact, in several countries like Canada, England or Scotland the first identification of the parasite was reported in foxes before it emerged in dogs from the same

area, suggesting the potential that infected wild canids may have in domestic dog's infection when geographic overlap occurs (Smith et al., 1973; Bourque et al., 2002; Morgan et al., 2008; Helm et al., 2009; Schnyder et al., 2013). Furthermore, recent serological investigations showed that foxes may develop a non-protective immunity of the parasite, allowing its long-term survival, therefore contributing to the establishment and dissemination of the disease (Gillis-Germitsch et al., 2017).

2.4 Epidemiology

2.4.1 Distribution of *A. vasorum* in dogs

Angiostrongylus vasorum is recognised as having a worldwide distribution. But, when focussing on the enzootic prevalence of *A. vasorum* globally there are limited data from which to draw conclusions. No national or international surveillance mechanisms are used for assessing the prevalence and global distribution of *A. vasorum* infection. The geographical distribution of *A. vasorum* in the Americas and Africa is less defined than in Europe, thus preventing the assumption of global "trends" in infection. Nonetheless, canine angiostrongylosis is considered endemic in certain areas of Europe, including regions of Denmark, Germany, Hungary, Finland, France, Ireland, Italy, the Netherlands, Poland, Slovakia, Spain, Sweden, Switzerland, Turkey and the United Kingdom, in Canada, in South America (Brazil and Colombia), and in Uganda in Africa. The country list is growing as the *A. vasorum* geographic distribution continues to evolve in several countries of Europe (Traversa et al., 2010; Rinaldi et al., 2014), and there is a growing risk of establishment in the Americas (Conboy et al., 2011). Studies carried out within dog populations in the United Kingdom, Denmark, Germany and Greece estimate the prevalence of *A. vasorum* to range from 0.3% to 9.8% (Morgan et al., 2010). Estimated prevalence varies widely between different canine populations (e.g. pet, hunting or stray dogs), health status (e.g. clinically affected or healthy), and methods used (e.g. variations on coprological methods) this is in contrast to studies within fox populations in Canada, Denmark, Hungary, Italy and Spain, which estimate prevalence of *A. vasorum* to be often higher at 5-56% (Koch et al., 2009). In Italy *A. vasorum* is undoubtedly spreading from northern (0.6% from Liguria region) (Guardone et al., 2013) to southern (3.4% from Sardinia region) (Pipia et al., 2014) of the country, especially in the central regions (0.96% and 2.48% from Marche and Abruzzo regions, respectively)

(Di Cesare et al., 2010) that offer ideal environmental and epidemiological conditions for the expansion of this parasite and the establishment of further new endemic foci. Knowledge of the epidemiology and clinical importance of *A. vasorum* has grown in the last 20 years and is correlated especially with increased urbanization of the red fox (*Vulpes vulpes*) which acts as a reservoir host representing a potential infection for dogs (Santoro et al., 2015; Taylor et al., 2015).

2.5 Pathogenesis

Infection with *A. vasorum* may be asymptomatic in some cases or may result in a wide spectrum of clinical signs, ranging from mild respiratory manifestations to severe forms, which are characterized by coagulative, respiratory or neurological disorders.

2.5.1   Respiratory signs

Respiratory signs are the most common clinical manifestations, with coagulation disorders being less common but more likely to be fatal. The most significant of these are coughs (either productive or unproductive), and dyspnoea, with or without tachypnoea. Related clinical signs that are less commonly reported are intolerance to exercise and lethargy. It is expected that a dog positive for infection with *A. vasorum* shows pulmonary changes upon lateral or ventrodorsal radiography. General findings include increased interstitial, peribronchial and alveolar patterns, pneumothorax, subcutaneous emphysema and an abnormally wide cranial mediastinum. The most common signs noted upon radiographic analysis are alveolar infiltrate and bronchial thickening (Gallagher et al., 2012). The classic respiratory signs are due to a verminous pneumonia resulting from L1 migration and the presence of ova throughout the host's pulmonary bed (Traversa et al., 2008). This typically causes coughing, dyspnea, tachypnea, or gagging. Auscultation of the lung fields is usually un- remarkable; however, in severe cases crackles and other adventitious sounds may be heard (Chapman et al., 2004). Pulmonary hypertension and cor pulmonale may occur, with the presence of adult worms in the pulmonary vasculature causing pulmonary vessel thrombosis and vascular smooth muscle hypertrophy (Bourque et al., 2002; Nicolle et al., 2006). These outcomes are perhaps more likely with heavy or very chronic infections, but are less a feature of angiostrongylosis than classic heartworm disease caused by

Dirofilaria immitis. The relationships between the development of disease, host factors, and the level of infection have not, however, been determined.

2.5.2   Cardiovascular signs

Coagulopathies represent the second most common reported manifestation of *A. vasorum* infection, approximately 35% of cases in a UK study of 23 dogs had hemorrhagic diatheses. It is thought that *A. vasorum* triggers a form of disseminated intravascular coagulation (DIC) with numerous studies demonstrating a prolonged activated partial thromboplastin time, or prothrombin time (Ramsey et al., 1996; Cury et al., 2002; Garsosi et al., 2005; Whitley et al., 2005; Wessmann et al., 2006; Helm et al., 2009), with thrombocytopenia and increased circulating D-dimer and fibrin degradation products concentrations. A secondary immune-mediated thrombocytopenia (Gould et al., 1999), acquired von Willebrand factor deficiency (Whitley et al., 2005), and a decrease in FV and FVIII (Cury et al., 2002) are also described. Currently, the exact pathophysiologic mechanism for the derangements of coagulation is not understood; it may be that a protein is secreted or re- leased by the parasite (either the adult or the larvae) that then triggers a coagulopathy, but at present there are no molecular studies confirming this (Schelling et al., 1986). It is also possible that endothelial disruption by the migrating larvae and adult parasites could result in DIC. The clinical signs demonstrated in cases affected with coagulopathy are diverse, and include petechiae, ecchymoses, scleral hemorrhage, sublingual hemorrhage, and hematomas (Gould et al.,1999; Ribiere et al., 2001; Chapman et al., 2004; Whitley et al., 2005). Hemoabdomen (Humm et al., 2008; Willesen et al., 2008), hemothorax (Sasanelli et al., 2007), and exsanguination due to a ruptured femoral artery in a dog experimentally infected with L3 *A. vasorum* (Cury et al., 1996), have also been described. Continued bleeding following elective surgery in infected dogs has been reported in practice and these cases might be presented as emergencies. It is notable that the severity of bleeding correlates poorly with the extent of detectable coagulation abnormalities, such that some dogs exhibit severe signs with apparently normal coagulation profiles. Thus, screening for infection and performing a coagulation profile before surgery in known hyperendemic areas is indicated.

2.5.3   Neurological signs and other signs

Neurologic manifestations of the disease occur in a proportion of affected dogs. These signs can be diverse, ranging from seizures, circling, ataxia, alterations in behavior, and cranial nerve deficits, to paresis or paralysis. Multiple reports exist of neurologic signs secondary to intracranial or intraspinal hemorrhage. It is also possible that neurologic signs may result from cerebral hypoxia secondary to chronic heart failure or respiratory disease (Bourque et al., 2008).

Ocular signs are also described; these include hemorrhage of the sclera, retina or iris (King et al., 1994; Manning et al., 2007), uveitis and the presence of larvae or adults in the anterior chamber (Perry et al., 1991).

Nonspecific signs including weight loss, lethargy, depression, and inappetance may be a feature, either as a sole finding or in conjunction with the above signs. Signs of gastrointestinal dysfunction may also accompany infection (Schelling et al., 1986). In dogs experimentally infected with 100 L3 larvae per kilogram, vomiting and excessive abdominal movement were recorded within an hour of oral administration of larvae (Oliveira et al., 2006). Some authors suggest that it may be the ingestion of the intermediate host (snails) that causes this gastric irritation (Rosen et al.,1970; Pennisi et al., 1994), but in the above study dogs fed non infected snails showed no adverse gastrointestinal signs (Oliveira et al., 2006). A case of gastric dilatation secondary to *A. vasorum* infection, likely due to aerophagia and the frequent swallowing of expectorated blood, is also reported (Koch et al., 1992). Sudden death following infection can occur, most commonly in young dogs with a high worm burden (Bolt et al., 1994; Ramsey et al., 1996). There is also evidence that dogs can remain asymptomatic following *A. vasorum* infection (Koch et al., 2009). There are few published data on the prevalence of asymptomatic infection in pet dogs in endemic areas, but anecdotally this appears to be low.

Aberrantly, migrating larvae have been found in a multitude of organs and tissues (including the liver, skeletal muscle, myocardium, gastrointestinal tract, spleen, thyroid, kidneys, skin, eye, and diaphragm) (Helm et al., 2010), and ectopic adult worms have been detected in the eye, pericardium, and urinary bladder. This implies a potential for clinical signs related to parasitic infection of any of these organs and it is likely that the full spectrum of

clinical signs related to *A. vasorum* infection in dogs has not yet been described. The wide variety of clinical signs potentially associated with infection makes it impossible to confirm or exclude *Angiostrongylus* infection based on clinical signs alone.

2.6 Diagnosis

2.6.1 Parasitological diagnosis

Qualitative Baermann migration-sedimentation test is the technique of choice for the diagnosis of *A. vasorum*, allowing the recovery and the consequent identification of L1 based on its morphological features (Fig. 2.2 B) (Koch et al., 2009). *A. vasorum* L1 are 310–399 µm in length and 14–16 µm in diameter (Zajac et al., 2012), have an anterior cephalic button, and the tail ends in a sinus wave S-shaped curve (severe kink) with a dorsal spine (McGarry et al., 2009). Accurate identification of the larvae must be performed to exclude other potential lungworms (e.g. *Crenosoma vulpis* and *Oslerus osleri*, intestinal parasites and free-living soil and plant nematodes that can also be present in dog faeces (Conboy et al., 2009; Zajac et al., 2012). Important limitations of Baermann test are its limited sensitivity on a single faecal examination, the high variability in shedding of larvae, the time involved (up to 24 hours) and the fact that is too laborious to be used in large scale epidemiological studies (Verzberger-Epshtein et al., 2008). Sensitivity can be improved by pooling faeces from three consecutive days (Koch et al., 2009). Other coprological methods such as direct faecal smear and faecal flotation to detected L1 may also be performed but with lower sensitivity (up to 54-61%) (Humm et al., 2010; Morgan et al., 2010). FLOTAC, a recent improved flotation-based coprological method that allows the visualization of parasitic elements in faecal samples, showed higher sensitivity than the Baermann method for the detection of *A. vasorum* L1 (Schnyder et al., 2011). Larvae may also be detected in bronchoalveolar lavage (Barçante et al., 2008), although with lower sensitivity and higher risk, especially in dyspnoeic patients.

Fig.2.2. First stage larvae of A) Crenosoma vulpis*, B)* Angiostrongylus vasorum*, C)* Oslerus osleri

2.6.2   Immunological diagnosis

Serological detection of circulating *A. vasorum* antigen (Verzberger-Epshtein et al., 2008; Schnyder et al., 2011b) and parasite specific antibodies (Schucan et al., 2012) has shown to be more sensitive than faecal examination tests in dogs. Serological tools are currently considered useful techniques, either relevant to clinical diagnosis and to epidemiological surveillance studies (Schnyder et al., 2013b). The antigen detection using monoclonal and polyclonal antibodies in a sandwich ELISA has a high specificity (94%) and sensitivity (95.7%) (Schnyder et al., 2011b). Additionally, a rapid in-clinic assay (IDEXX AngioDetect[TM] Test) (Fig.2.3) is now available for routine detection of circulating antigens (specificity of 100% and sensitivity of 84.6%) (Schnyder et al., 2014). An antibody detection ELISA, using *A. vasorum* adult somatic antigen purified by monoclonal antibodies has shown high sensitivity (81.0%) and specificity (98.8%) (Schucan et al., 2012) and has been applied along with antigen detection in several serological surveys. However, it is worth mentioning that when using antibody detection as a clinical diagnostic tool, persistence of antibodies in dogs from areas with high background levels of exposure to *A. vasorum*, could lead to false positives. Thereby, the exclusive use of antibody test alone is of limited value and should only be used to screen for exposure. The option of combining antigen and antibody detection by ELISA in clinical and epidemiological settings or combining ELISAs with copromicroscopic techniques is the best approach to increase sensitivity and the capacity to detect an early infection (Schnyder et al., 2013a).

Fig.2.3. Antigen test of Angiostrongylus vasorum *in dogs (IDEXX Angio Detect ^{TM})*

2.6.3 Molecular diagnosis

As morphological studies are laborious and time consuming, molecular approaches such as the conventional PCR and the real-time PCR, have been used to identify nucleic acid sequences of *A. vasorum* and circulating DNA in definitive and intermediate hosts (Helm et al., 2009; Jefferies et al., 2011; Patel et al., 2014; Aziz et al., 2016). Although highly specific and requiring a blood sample rather than faeces, detection of *A. vasorum* DNA offered no great advantage over the Baermann test in terms of sensitivity, and is farless sensitive than detection of circulating antigens (Jefferies et al., 2011; Schnyder et al., 2015b). PCR has also been used to detect *A. vasorum* in coprological samples (Jefferies et al., 2009b; Al-Sabi et al., 2010), although PCR inhibitors present in faeces may limit the sensitivity with no obvious advantage over the Baermann or serological tests. PCR might be useful in the following situations: when faeces are stored for long periods with the consequent death of L1 which are not able to migrate in the Baermann apparatus; when Baermann is not suggestive of *A. vasorum* L1 given morphological alterations; or when clinical features are highly suspicious of *A. vasorum* infections but faecal analysis is not supportive. Recently, detection by quantitative PCR on bronchoalveolar lavage fluid has also been reported (Canonne et al., 2016).

2.7 Treatment

2.7.1   Anthelmintic therapy

Treatment should be started as soon as possible, as the earlier an infected dog is treated, the less severe are the pathological changes in the lungs (Schnyder et al., 2009). A variety of anthelmintics are efficacious against *A. vasorum* (Dodd, 1973; Martin et al., 1993; Conboy et al., 2004; Willesen, et al., 2007). Levamisole and ivermectin were routinely used in the past, but has now been replaced by safer licensed products. Fenbendazole remains a popular treatment of choice and is efficacious in many dose regimens, ranging from 25 to 50 mg/kg, per os, daily for three weeks (Martin et al., 1993; Chapman et al., 2004; Estèves et al., 2004; Garosi et al., 2005; Whitley et al., 2005). Fenbendazole produces a "slow kill" of the nematodes, therefore reducing the risk of anaphylaxis. However, in many territories is still not licensed as a treatment for *A. vasorum*. The risk of ivermectin toxicity has been reduced with the introduction of novel and safer formulations of macrocyclic lactones, namely selamectin, milbemycin oxime and moxidectin (Bishop et al., 2000; Novotny et al., 2000; Paul et al., 2000). Presently there are two licensed, highly efficacious parasite treatments effective against adult *A. vasorum* and immature stages: one is the combination of imidacloprid 10% moxidectin 2.5% (0.1 ml/kg) spot-on solution, that requires a single monthly spot-on application, showing a 85.2% efficacy against *A. vasorum* infection (Willesen et al., 2007); and the other is milbemycin oxime tablet, in combination with praziquantel, that requires a weekly oral administration for four weeks to treat *A. vasorum* infection (Conboy et al., 2004; Willesen et al., 2007; Schnyder et al., 2009).

As larval excretion may occur for up to three weeks after anthelmintic treatment, a three-day Baermann re-test is recommended earliest one month later the treatment to ensure that excretion of L1 has ceased. In addition, a new Baermann should be performed twice a year, particularly in endemic areas where re-infections are known to occur (Chapman et al., 2004).

2.7.2   Supportive therapy

Little is known about supportive treatment in *A. vasorum* infection as most of the information is retrieved from case reports or small case series. Depending on the clinical presentation, supportive treatment might be

indicated. Antibiotics, bronchodilators, oxygen, fluids, blood transfusions, corticosteroids, heparin and diuretics have been reported (Chapman et al., 2004; Estèves et al., 2004). Although dogs with life-threatening haemorrhages and coagulopathies may recover after 24-48 hours after the onset of anthelmintic treatment, transfusions of fresh frozen plasma or whole blood are advised concurrently with the anthelmintic treatment. Furthermore, hyperfibrinolysis and hypfibrinogenaemia can be treated with tranexamic acid combined with fresh frozen plasma transfusions (Sigrist et al., 2017). As complications, such as pneumothorax have been reported in the initial phases of treatment (Willesen et al., 2007), strict cage rest in the initial 2–3 days of treatment is often recommended for the most severely affected dogs (Koch and Willesen, 2009). Oxygen administration is also indicated in dogs with respiratory compromise. Anti-inflammatory doses of corticosteroids have been described to moderate potential anaphylactic reactions, to reduce pulmonary inflammation and to diminish secondary lung fibrosis (Manning, 2007). Immunosuppressive doses of corticosteroids are recommended in cases of immune-mediated thrombocytopenia (Soland and Bolt, 1996; Gould and McInnes, 1999). The use of adrenaline or antihistamines may also be considered to treat anaphylactic reactions. If the dogs are suffering from congestive heart failure, treatment with diuretics, angiotensinconverting-enzyme inhibitor and phosphodiesterase inhibitors are indicated to decrease pulmonary hypertension.

2.8   Control and prevention

Considering the wide presence of intermediate hosts and reservoirs of infection such as wild foxes, eradication of *A. vasorum* is unfeasible. Either way, measures to avoid dogs consuming L3, either from molluscs or from the environment, might be considered. Feeding dogs exclusively indoors, cleaning regularly outdoor bowls and toys or making them less accessible to slugs/snails, are possible strategies to reduce the risk of contamination with L3 (Elsheikha et al., 2014). In heavily contaminated areas, dog owners might be warned to avoid off-leash walking of dogs. The use of molluscicides is not advisable as many pesticides are not pet safe and may increase the availability of molluscs to dogs. Environmental control measures such as the use of nematophagous fungi have also been successfully used to destruct *A. vasorum* L1 (Braga et al., 2009; Koch and Willesen, 2009). To prevent environmental contamination with L1 and to break the life-cycle of *A. vasorum*, proper disposal of dog faeces is recommended, particularly in

public areas highly frequented by dogs. Nevertheless, data suggest that foxes are a much higher reservoir of infection when compared to domestic dogs (Elsheikha et al., 2014). The increasing reports of this parasite drive the need for effective anthelmintic treatment of infected dogs and even more importantly, regular prophylaxis to prevent the establishment of further infection (ESCCAP, 2010). Indeed, movement of infected and untreated dogs from endemic to non-endemic countries could present a serious threat to canine health and welfare and should be avoided. Monthly use of moxidectin and milbemycin oxime may be effectively used as *A. vasorum* prophylaxis (Schnyder et al., 2009; Böhm et al., 2014). The combination tablet of spinosad with milbemycin oxime was also shown to have 98.8% preventative efficacy against development of adult *A. vasorum* infection with a single treatment, and the potential to prevent the establishment of *A. vasorum* infections in dogs with monthly administrations (Böhm et al., 2014). Also, the combination of the insecticide and acaricide afoxolaner and the anthelmintic milbemycin oxime in a chewable tablet formulation, can prevent canine *A. vasorum* infection, with an efficacy of 94.9%, when administered at monthly intervals (Lebon et al., 2016). So far, no report has been published on resistance of *A. vasorum* to chemotherapeutic compounds. Alternatively, dogs may be regularly checked for the presence of *A. vasorum*, by the new serological diagnostic test or Baermann technique. As canine angiostrongylosis is increasingly being reported in regions where it was not traditionally found, veterinarians should remain abreast of latest development research and raise public awareness (Elsheikha et al., 2014).

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Research objectives

Cardiopulmonary nematodes (*Dirofilaria immitis* and *Angiostrongylus vasorum*) are severe and life-threatening parasites increasingly reported throughout Europe, constituting a major problem for domestic dogs. Information about their prevalence and distribution is essential for the control of animal diseases and, as concerns *Dirofilaria,* for the control of potentially associated zoonotic risk.

The overall aim of the thesis was to evaluate the epidemiological scenario of *D. immitis* and *A. vasorum* in domestic dogs in southern Italy with a particular emphasis on the clinical and diagnostic issues. The specific objectives were:

1. To study the contemporaneous distribution of *D. immitis* and *A. vasorum* in kennel dogs from southern Italy by serological analysis.

2. To study the prevalence and clinical relevance of *A. vasorum* in owned and stray dogs from southern Italy using new copromicroscopic techniques.

3. To evaluate the serum samples pre-heating process usefulness before quantitative ELISA test in naturally infected dogs with *D. immitis* in order to asses the real prevalence of heartworm disease in endemic and non-endemic areas from Europe.

4. To perform a study on dogs in southern Italy and Spain in order to evaluate the prevalence of single and multiple vector borne infections (*Dirofilaria, Leishmania* and *Ehrlichia*) and verify the possibility to avoid false negatives to *D. immitis* due to antigen-antibody complexes.

Chapter 3

Dirofilaria immitis and *Angiostrongylus vasorum*: the contemporaneous detection in kennels

3.1 Aim

The cardiopulmonary nematodes *Dirofilaria immitis* and *Angiostrongylus vasorum* are increasingly reported in dogs and are responsible for two diseases with overlapping endemic areas, especially in Europe: angiostrongylosis and dirofilariosis (Morgan et al., 2010; Otranto et al., 2013; Maia et al., 2015). The reasons for their apparent emergence have been discussed in several recent studies; increased disease awareness, the availability of better diagnostic tools and climatic changes are considered the main causes of the recent spread of these parasites (Helm et al., 2015; Tolnai et al., 2015). In particular, global warming may represent a fundamental factor for the seasonality and the spread of *D. immitis* (Genchi et al., 2009) and could be involved also in the development of infectious stages in the intermediate hosts of *A. vasorum* (Traversa et al., 2010).

In recent years, epidemiological surveys on *D. immitis* and *A. vasorum* prevalence have been conducted in many countries; however, no studies investigated the simultaneous detection of these cardiopulmonary nematodes in kenneled dogs. Therefore, the aim of this study was to investigate the distribution of *D. immitis* and *A. vasorum* in kenneled dogs of the Campania region of southern Italy. This is the first cross-sectional survey conducted at regional-scale in Italy and in Europe on the contemporaneous detection of *D. immitis* antigens and *A. vasorum* first stage larvae (L1) in kennels.

3.2 Methods

3.2.1   Study area and study kennels

The survey was conducted between June 2012 and August 2013 in 68 public kennels located in the Campania region of southern Italy (www.anagrafecaninacampania.it). The region (Latitude = 39°59′15″–41°30′25″; Longitude = 13°45′25″–15°48′23″) which extends over an area of 13,590 km² is mainly hilly and extends from 0 to 1,890 m above sea level. The climate is Mediterranean with dry summers and rainy winters.

As in other Italian regions, the kennels of the Campania region are facilities for roaming and abandoned animals; dogs are sheltered and then given in adoption, if possible. Indeed, the Italian regulation for dog

registration/identification (Law 281/1991) provides for setting up specific kennels to collect and re-home roaming and abandoned animals; for many disowned dogs, the kennel becomes a permanent shelter (Baldelli et al., 2011). These kennels are subjected to veterinary public service control. Usually, few anthelmintic treatments per season are used in these kennels, with the number of treatments given per year ranging from 1 (15%), 2 (75%) to 3 (10%) and using broad-spectrum antiparasitic drugs (Rinaldi et al., 2015). Routine prophylaxis for *D. immitis* is not performed in the region.

3.2.2   Blood sampling and analysis for *D. immitis* and *A. vasorum*

In the 68 kennels, 537 blood samples (from 5 to 10 per each kennel) were collected. In each kennel we selected the dogs that were hosted for 2 years at least. The samples were transported to the laboratory and centrifuged at 3000 rpm for 10 min to obtain sera, then stored at -20°C until testing for seroprevalence of *D. immitis* using DiroCHEK® ELISA (Synbiotics, San Diego, USA) according to the manufacturer's instructions (sensitivity = 85-100% and specificity = 100%) (Courtney et al., 2001).
After the analysis with DiroCHEK® ELISA, the dog sera resulted positive to *D. immitis* were also tested with the antigenic test AngioDetect® (IDEXX Laboratories, Westbrook, Maine, USA) (sensitivity = 84.6%; specificity = 100%) (Schnyder et al., 2014). The blood samples were collected by the permission of the dog shelters. The animals used in the present study were sampled following approval by the animal ethics and welfare committee of the University of Naples Federico II (protocol number 0093412).

3.2.3   Fecal sampling and analysis for *A. vasorum*

In each of the 68 studied kennels, the box (hosting a different number of dogs, i.e. min 1, max 4; mean = 2.4 dogs) was considered as the epidemiological unit of the study for practical reasons as reported in Rinaldi et al. (2015). In each kennel, 20 boxes were sampled by systematic sampling (total number of boxes = 1,360). In each box, the fecal samples were collected directly from the ground (pooled samples), placed in a container and transported to the laboratory (within 5 hours from sampling). The feces were fixed in 5% formalin (dilution ratio 1:4). Copromicroscopic analyses were performed using the FLOTAC technique, having an analytic sensitivity

of 2 larvae per gram (LPG) of faeces. A zinc sulphate-based solution (specific gravity = 1.20) was used for diagnosis of *A. vasorum* (Schnyder et al., 2011).

3.3   Results and discussion

Antigens of *D. immitis* were detected in dogs from 6 out of the 68 kennels (8.8%; 95% CI = 3.6-18.9). Specifically, 24/537 (4.4%; 95% CI= 3.0-6.7) dogs of 6 kennels were positive to *D. immitis* (Tab.3.1). The 24 sera resulted positive to *D. immitis* were tested also with AngioDetect® in order to detect possible cross-reactions (Schnyder et al., 2012). Only 1 sample (4.2%; 95% CI = 0.2- 23.1) resulted positive for *A. vasorum* antigens. This dog did not show any clinical sign and *A. vasorum* L1 were detected in the corresponding box.

A. vasorum (L1) were detected in dogs from 9 out of the 68 kennels (13.2%; 95% CI = 21.8-44.9; LPG min = 4; LPG max = 106; LPG mean = 25.1) (Fig.3.1). Pooled fecal samples from 25 boxes out of the 1,360 analyzed resulted positive to *A. vasorum* L1 (1.8%; 95% CI = 1.2-2.7). The mean number of *A. vasorum* positive boxes/kennel was 2.8 (min 1- max 7).

The strength of the present study was the contemporaneous detection of *D. immitis* antigens (8.8%) and *A. vasorum* L1 (13.2%) in kennels conducted by a cross-sectional survey at regional-scale.

However, also some limits emerged from our study. First, the lack of knowledge of the history of the kenneled dogs; we did not know if they were all natives of the Campania Region, even if autochthonous foci of canine *D. immitis* infection were recently reported in owned dogs from the same region (Rinaldi et al., 2015). Second, the impossibility to collect the blood from all the dogs examined by coprological techniques due to the aggressiveness of the kennel animals. Third, the using of pooled fecal samples; the percentage of positivity for *A. vasorum* found in this study (13.2%) cannot directly be compared with prevalence of other study based on individual samples.

The main findings of the present survey confirm that polyparasitism is the rule in the studied kennels in southern Italy as already described by Rinaldi et al. (2015) concerning other endoparasites. Different considerations emerged from this study related to: (i) epidemiology and (ii) diagnosis and control of cardiopulmonary nematodes infecting dogs.

D. immitis has spread in the last years in Europe due to climate change, the spreading of mosquito *Aedes albopictus* and the introduction of new vector *Aedes koreicus* (Montarsi et al., 2015). These factors caused new autochthonous foci of heartworm disease that have recently been reported in previously non-endemic areas of southern Italy (Genchi et al., 2009; Otranto et al., 2010). While the distribution and prevalence of *D. immitis* infection has been widely studied in dogs from central and northern Italy, epidemiological data on the occurrence of *D. immitis* infection in southern Italy are scant and limited to sporadic case reports. The prevalence of *D. immitis* reported in this study was 8.8%. This prevalence is higher than the previous study conducted in the Vesuvius area (0.6%) of the same region confirming the spread of this parasite (Rinaldi et al., 2015). Similarly, also in Apulia and Calabria regions *D. immitis* was found in autochthonous dogs (Otranto et al 2009).

Different predictive studies, conducted using Geographical Information System (GIS) tools (Genchi et al., 2005, 2009), confirmed a correlation between the actual trend of *D. immitis* spread and the temperature increase into previously infection free areas as demonstrated also in a questionnaire survey by Genchi et al., (2014) where an increasing number of positive cases to *D. immitis* was detected in non- endemic area (10%) and in endemic- area (12%) of Europe.

A. vasorum infection is considered endemic in certain areas of Europe, including regions of Denmark, Germany, Hungary, Finland, France, Ireland, Italy, the Netherlands, Poland, Portugal, Slovakia, Spain, Sweden, Switzerland, Turkey and the United Kingdom. The country list is growing as the *A. vasorum* geographic distribution continues to evolve in several countries of Europe (Elsheika et al., 2014).

Our present study expands on that one recently published by Rinaldi et al (2014) which described a case of fatal disseminated *A. vasorum* infection in the same region. Also in the same region a prevalence of 33.3% was found in red foxes at post-mortem examination (Santoro et al., 2015) and the authors indicated the potential infection risk for dogs living in the same area. The most important and best-studied wildlife reservoir for *A. vasorum* infection is the red fox (*Vulpes vulpes*) (Elsheika et al., 2014; Taylor et al., 2015). A possible explanation for increased transmission of infection between red fox and dog populations is an increasing density of foxes. It can be assumed that the growing density of foxes in an area populated with dogs

is likely to increase the number of fox-dog interactions, and hence increase the opportunity for transmission of infection. Direct wild canid-dog interaction is not necessary for (and does not lead to) transmission of this parasite from one to the other because transmission occurs via ingestion of L3 (in gastropods or frogs, or possibly free in the environment) (Elsheika et al., 2014).

The positive percentage found in our present study (13.2%), even if is not comparable with the prevalence of the other study because of the different epidemiological unit considered, is very high if compared other (Tieri et al., 2011; Traversa et al., 2013). The scenario of *A. vasorum* infection is alarming if we consider the clinical signs can be severe: respiratory dysfunctions, bleeding, neurological, ocular, cardiovascular and gastrointestinal symptomatology, skin lesions (Koch et al., 2009; Tieri et al., 2011). Even if current data of this disease are referred to symptomatic dogs, *A. vasorum* infection may be also asymptomatic, especially in the early stages and so affected dogs can develop clinical signs after months to years from the infection (Elsheika et al., 2014).

Kennel dogs are at the highest risk of infection with helminths, for this reason anthelmintics or combinations of anthelmintics with a broad spectrum activity, as suggested in a previous study (Rinaldi et al., 2014), are required to treat the polyparasitism currently encountered in this situations. Unfortunately, the anthelmintics used still now do not work as preventives of *D. immitis*. The reasons of the lack of a prophylaxis for *D. immitis* are: the unawareness of the severity of this parasite, the economic issue and the difficulties of managing and monitoring of the kenneled dogs.

Tab. 3.1 Prevalence of Dirofilaria immitis *antigen at individual level in the positive kennels, n = 6*

Kennel ID	No. of dogs examined	No. of dogs positive to *Dirofilaria immitis* antigen	Prevalence (%)	95 % confidence interval
1	20	5	25.0	9.6–49.4
2	93	5	5.3	2.0–12.7
3	46	3	6.5	1.7–18.9
4	94	6	4.2	2.6–13.9
5	94	4	4.2	1.4–11.2
6	9	1	11.0	0.6–49.3
Total	356	24		

3.4 Conclusion

In conclusion, the present study indicates that the cardiopulmonary nematodes *D. immitis* and *A. vasorum* circulate in the kennels of the Campania region of southern Italy. Therefore, regular parasitological surveillance, appropriate diagnostic tools, treatment strategies and high-quality standard of hygiene are required to guarantee the health and welfare of kennel dogs as recommended by the European Scientific Counsel for Companion Animals Parasites (www.esccap.org).
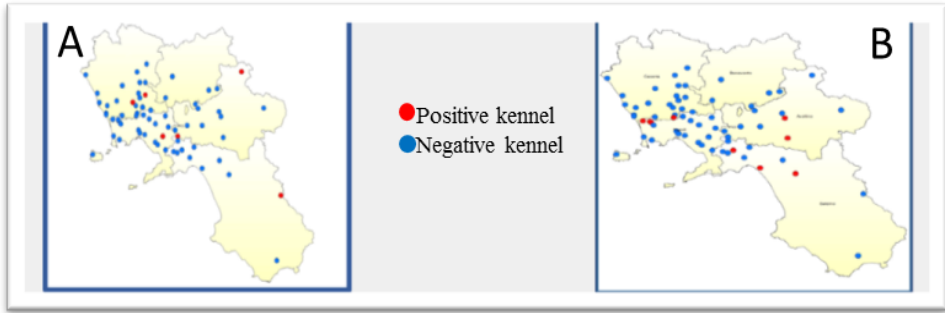
Fig3.1 Geographical distribution of A) D. immitis *and B)* A. vasorum *in Campania region, southern Italy.*

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Chapter 4

Angiostrongylus vasorum in stray and owned dogs in southern Italy: prevalence and clinical findings

4.1 Aim

Angiostrongylus vasorum resides in the heart and pulmonary arterial circulation of domestic dogs and wild canids and, occasionally, other animals (Levine 1980). There is also evidence of ectopic canine angiostrongylosis (Oliveira-Junior et al., 2004; Colella el at., 2016; Manning et al., 2007). As a disease with a substantial animal health impact, canine angiostrongylosis remains a high priority for clinicians and researchers (Helm et al., 2010). Infected dogs usually exhibit signs of respiratory and/or cardiovascular disease, and occasionally coagulopathies and neurological signs, with fatal consequences in severe cases (Elsheika et al., 2014; Rinaldi et al., 2014).

Canine angiostrongylosis is considered endemic in certain areas of Europe, including regions of Denmark, Germany, Hungary, Finland, France, Ireland, Italy, the Netherlands, Poland, Portugal, Slovakia, Spain, Sweden, Switzerland, Turkey and the United Kingdom, in Canada, in South America (Brazil and Colombia), and in Uganda in Africa. The country list is growing as the *A. vasorum* geographical distribution continues to evolve in several countries of Europe with an estimated prevalence at 0.3-56% both in dogs and foxes (Koch et al., 2009). In Italy, *A. vasorum* is being diagnosed in dogs with an increasing frequency in central regions (Traversa et al., 2008; Di Cesare et al., 2010, 2011, 2012), but the current distribution of this lungworm included new areas considered free of infection so far (Guardone et al., 2013; Traversa et al., 2013; Guardone et al., 2016). The presence of *A. vasorum* in southern Italy was confirmed by different authors using histopathological, serological and coprological methods (Rinaldi et al., 2014; Pipia et al., 2014; Del Prete et al., 2015). However, occurrence of angiostrongylosis in symptomatic and asymptomatic dogs are lacking (Traversa et al., 2013). Therefore, the present study aimed to evaluate the extent and clinical relevance of *A. vasorum* infection in owned and stray dogs from southern Italy using the FLOTAC technique.

4.2 Methods

4.2.1   Study area and sampling

The survey was conducted between 2015 and 2016 in the Campania region, southern Italy which has an area of about 13500 km^2, and a coastline along the Tyrrhenian Sea (Dragoni and Sukhja, 2008).

A total of 1499 faecal samples, 656 from owned dogs (Veterinary Hospital, University of Naples Federico II) and 843 from stray dogs (Veterinary Hospital Frullone, Naples) were collected. Dogs with different ages (6 months-120 months), sexes (723 males, 776 females) and breeds were randomly enrolled in the study. A minimum of 2 g of faeces was collected from each animal and examined in the same day at the Laboratories of Parasitology located at the Department of Veterinary Medicine and Animal Production, and at the Veterinary Hospital Frullone, Naples.

All samples used in this study were collected under standard protocols for management of dogs of participating animal shelter (Veterinary Hospital Frullone) and by "Institutional Animal Care and Use" committees at University of Naples, Federico II, Italy.

The data of the dogs were provided based on a written consent of the owners in order to participate to the study.

4.2.2   Parasitological analysis for *Angiostrongylus vasorum*

All the 1499 faecal samples were examined in order to identify the presence of first stage larvae (L1) of *A. vasorum.* Copromicroscopic analyses were performed using the FLOTAC basic technique (Schnyder et al., 2011) with a zinc sulphate-based solution (specific gravity = 1.20). The analytic sensitivity of the FLOTAC technique was 2 larvae per gram (LPG) of faeces. L1 of *A. vasorum* were detected and counted, using a light microscope (×10 or ×40 magnification).

4.2.3   Statistical analysis

The statistical analysis regarding the prevalence of *A. vasorum* in stray and owned dogs were performed using Chi-Square test (SPSS 20 software). The differences were considered significant at $p<0.05$.

4.3.   Results

4.3.1   Prevalence of *A. vasorum*

A. vasorum L1 were detected in 29 of 1499 (1.9%; 95% Confidence Interval, C.I. = 1.3-2.8%) samples. Out of 656 owned dogs examined, seven (4 females, 3 males) were positive for *A. vasorum* (1.1%; 95% C.I.= 0.5-2.3 %); age ranged between 18-48 months; the LPG values detected varied between 10-560 (mean value = 216 LPG).

Out of 843 stray dogs investigated, 22 (14 females, 8 males) were positive for *A. vasorum* (2.6%; 95% C.I.= 1.7- 4.0%).

Age ranged between 12-84 months. The LPG varied between 10-668 (mean value = 254.8 LPG). The value of Chi-square revealed a statistically significant difference for the prevalence in stray and owned dogs (P = 0.0315). There was no statistically significant difference for age or gender between infected and not infected dogs for *A. vasorum*.

4.3.2   Dogs and clinical pictures

Seven dogs resulted positive to faecal examination for *A. vasorum* showed clinical evidences compatible with the lungworm infection, which are described below.

Case 1
A 3-year-old of Boxer male weighing 30 kg, was referred to the hospital for several symptoms including tachypnea, weight loss and dysorexia. The dog lived outdoor and never left the Campania region. The haematological exams showed leukocytosis (14.17 x1000/uL), the plasma protein framework highlighted a hypoalbuminemia and a hyperglobulinemia (total

protein 8.3 gr/dl). Radiological exam showed no pulmonary lesions. The FLOTAC technique showed the presence of *A. vasorum* larvae (48 LPG).

Case 2

A 18-month-old male of Shiba inu living in the Campania region was referred to the hospital for various symptoms including weight loss, exercise intollerance and other signs directly related to the respiratory system, such as coughing, gagging, dyspnoea. The haematological exam showed leukocytosis (16.79 x1000/uL) and thrombocytopenia (122 x1000/uL), the prothrombine time (PT) was >60. The radiographical exam showed a bronchial pattern. At first, the clinical signs were compatible with bronchopaty, nonetheless, several larvae of *A.vasorum* were found by the FLOTAC thechnique (226 LPG).

Case 3

A young unsterilized female hunting dog (Poenter breed) living outdoors, was referred to the hospital with a very important haematological value. The animal showed a serious leukocytosis (26.55 x1000/uL) and a significant increase of granulocytis (91.1%). The dog was lethargic, exercise intolerant and presented hyperthermia. The radiographical exam showed a diffuse interstitial to alveolar pattern, accentuated in the periphery. Also a coprological exam with FLOTAC found larvae of *A.vasorum* (560 LPG).

Case 4

A 1-year-old female Golden Retriever living in an urban area of the Campania region was referred to the hospital for a clinical evaluation due to the dyspnea. The dog was thin but no other apparent abnormalities have been showed during the physical examination. The haematological exam showed leukocytosis (19.74 x1000/uL) and the prothrombine time (PT) was >35. In this case thoracic radiograph was not been carried out because the FLOTAC technique gave immediatly, in the same day of the clinical evaluation, the diagnosis of angiostrongylosis (68 LPG).

Case 5

A 3- years-old female Cocker Charles Espaniel was referred for respiratory distress. At clinical examination the dog showed severe dyspnoea and

profuse hemoptysis. Radiographic examination of the thorax revealed a generalised interstitial and alveolar pattern. The haematological exam showed a leukocytosis (20.20 x1000/uL). Coprological exame with FLOTAC technique showed the presence of *A. vasorum* (L1) (220 LPG).

Case 6
A 10 years old male of English Setter living with another hunting dog in an -urban area of the Campania region was referred with a severe respiratory distress that did not respond to antimicrobial therapy. Haematological abnormalities included anemia and leukocytosis. Thoracic radiographs showed a mixed alveolar/interstrital pattern and a perihilar bronchial pattern (Fig.4.1). First stage larvae of *A. vasorum* was detected with FLOTAC technique (380 LPG).

Case 7
Three days after the diagnosis of angiostrongylosis also the other hunting dog was referred by the owner, even though it was asymptomatic. Therefore, the FLOTAC thechnique was performed and few L1 of *A. vasorum* were detected (10 LPG).
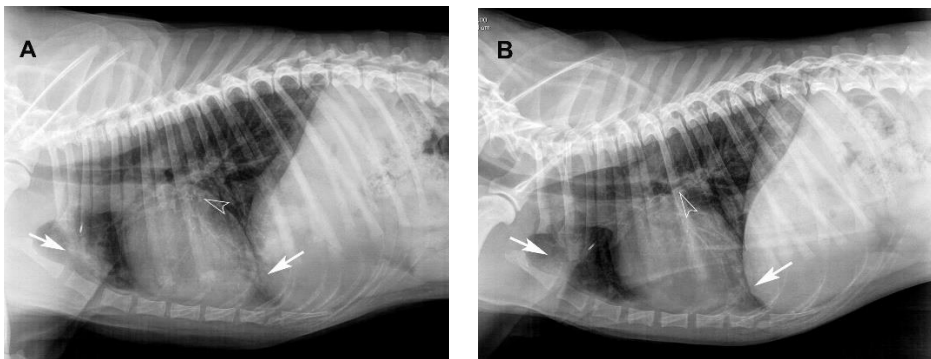

Fig. 4.1. Lateral radiograph of the thorax of dog n.6: A) Right lateral recumbency; B) Left lateral recumbency. There are multiple marginal areas in the cranial and caudal lobes (white arrows) with a mixed alveolar/interstitial pattern and a perihilar bronchial pattern (empty arrowheads). (Ph.courtesy of professor Meomartino Leonardo)

4.4 Discussion and conclusion

The results of this study confirm, as reported by Traversa et al. (2013), that canine angiostrongylosis is endemic in Italy and not confined to central regions.

The prevalence of *A. vasorum* found in this study in owned dogs (1.1%) was slightly lower than in the prevalence detected in stray dogs (2.6%) of the same area. The results of statistical analysis confirmed that the infection risk among the stray dogs is higher than in owned dogs (p< 0.05%). Therefore, the awareness of canine angiostronylosis by vet practioners is very important especially for dogs living outdoor that should be screened for infection in order to prevent fatal cases of angiostrongylosis (Traversa et al., 2008; Rinaldi et al., 2014).

In the present study, sex and age do not seem to represent risk factors for canine angiostrongylosis as previously reported by Traversa et al. (2013).

Comparing the results herein obtained with the previous data reported in owned dogs in Southern Italy, the overall prevalence found in the Campania region is comparable with the prevalence found in the Sardinia region (3.4%) (Pipia et al., 2014), being the only area of southern Italy screened for *A. vasorum* in owned dogs. Instead, a recent study conducted in the Campania region showed a high prevalence of this parasite in red foxes (Santoro et al., 2016), therefore further study on the prevalence in dogs is necessary to add more information about the epidemiological scenario of angiostrongylosis in this area. Other previous studies showed 1.04% prevalence of *A. vasorum* in stray dogs in the Campania Region (Rinaldi et al., 2014) and 13.2% in kenneled dogs in the same area (Del Prete et al., 2015). However, the percentage of positivity found in the study performed on kenneled dogs cannot be compared with the prevalence of the present study because pooled faecal samples were used and the epidemiological unit was the kennel rather than the individual dog. However, the current and other recent reports provide important evidence that *A. vasorum* infection has spread in southern Italy more than expected, probably due to the fact that this infection is still neglected in canine clinical practice.

Under a clinical point of view, as expected, clinical signs of infected animals herein examined were the most common symptoms that are in general associated with angiostrongylosis such as: gagging, coughing, anorexia, weight loss, exercise intolerance (Chapman et al., 2004; Ferdushy et al., 2010). The respiratory syndrome and the pulmonary lesions found in our dogs are in accordance with previous studies (Patteson et al., 1993; Chapman et al., 2004; Helm et al., 2010). The most consistent findings were: generalized interstitial and alveolar pattern and enlargement of tracheobronchial lymph nodes. The infected dogs from the present study with hematological abnormalities inlcuding anemia and leukocitosis and no pulmonary lesions (case1) as well as the seventh case with no clinical signs, underline the fact that the clinical manifestations, associated with this disease may vary greatly from subclinical state (with no or minor signs) to fatal condition (Lepri et al., 2011; Rinaldi et al., 2014).

The presence of *A. vasorum* detected in dog's faeces with FLOTAC technique has been justified by the clinical signs compatible with the infection found in the owned dogs of the present study. Instead, a similar report revealed that the detection of *A. vasorum* in symptomatic dogs was underestimated when performing Baermann test (Traversa et al., 2013). Thus, the faecal Baermann examination can produce false negative results due to the intermittent shedding of larvae and the long pre-patent period of the lungworm (Denk et al., 2009). Hence, veterinarians should consider the necessity of three consecutive daily faecal samples (Morgan et al., 2010).

The strength of the present study was the use of the FLOTAC technique that can be utilized successfully for diagnosis of *A. vasorum* infection (Schnyder et al., 2011), as already demonstrated for other lungworms as *Crenosoma vulpis* (Rinaldi et al., 2007) in dogs and *Aelurostrongylus abstrusus* (Gaglio et al., 2008) in cats. Furthermore, given the lack of specificity of clinical signs, this infection is often not included in differential diagnosis, and animals remain infected and untreated (Rinaldi et al., 2014). The FLOTAC has also the advantage to be multivalent and therefore also other parasites can be detected. The routinary use of copromicroscopic exams with high sensitivity and specificity could allow more possibilities for clinicians to identify the extent of canine angiostrongylosis in owned and stray dogs.

In conclusion, the spread of *A. vasorum* in several European areas including Italy demands the necessity of stimulating concern on this infection among vet practitioners, which should always include angiostrongylosis on differential diagnosis when signs are consistent.

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Chapter 5

Preliminary investigations of the heat treatment of stray dogs serum samples naturally exposed to *Dirofilaria immitis* and *Dirofilaria repens* in Romania.

5.1 Aim

Diagnosis of Heartworm disease (HWD) is based on the presence of circulating microfilariae and/or circulating antigens derived from adult female worms. The accuracy of the serologic tests is influenced by the numbers of adult *Dirofilaria immitis* present and is not affected by the circulating *D. immitis* microfilariae (Brunner et al., 1988). Most of the original heartworm tests used a method of antigen retrieval to minimize the effects of immune complexing on assay performance (Little et al, 2014).

Pre-treatment of serum samples with heat before antigen testing has been reported as being able to reverse false negatives due to antigen-antibody complexes in both experimentally and naturally *D. immitis*-infected hosts (Little et al., 2014a; Little et al., 2014b; Velasquez et al., 2014). Pre-treatment was also done with chemicals. It is not only heat treatment which is used to remove the inhibitors of antigen detection. Diagnostic laboratory commonly uses pepsin treatment, and acid treatment was used historically (Rodriguez, et al. 1992).

Heat treatment disrupt antigen-antibody complexes and releases antigen that then becomes available for detection. This may have important consequences for diagnosis of clinical disease, but also for epidemiological studies, in particular in areas where infection prevalence is not well known. Furthermore, there have been no studies on the possible effects of heat treatment on the cross reactivity with *D. repens*.

The aim of the present study was to determine infection prevalence for *D. immitis*, through antigen testing before and after heat treatment, in several areas of Romania where both *D. immitis* and *D. repens* are endemic.

5.2 Materials

5.2.1 Animals

A total of 194 stray dogs (aged 2 to 8 years; 70 males and 124 females) were sampled from five shelters in four counties situated in the eastern region of Romania: Galaţi, Iaşi (Probota and Paşcani), Vaslui and Bacău. Dogs were housed outdoors and were not subjected to antiparasitic treatment with

macrocyclic lactones. All blood samples were conserved in tubes without anticoagulant agents and transported to the laboratory in few hours.

In addition, blood samples in EDTA were collected from a subsample of 108 dogs in the kennels of Galaţi and Iași.

All samples used in this study were collected under standard protocols for management of dogs at participating animal shelters and by the Institutional Animal Care and Use Committees at the University of Iasi (Romania).

5.2.2 *D. immitis* antigen testing before and after heat treatment

Serum was obtained from all 194 whole blood samples and kept at -20^0C until further analysis. Antigen testing was carried out using a commercial microtiter well-based assay (DiroCHECK®, Zoetis), according to manufacturer's instructions. Serum samples were then treated with heat, according to Drake et al (2015). Briefly, approximately 1.-1.5 ml of serum was placed in an eppendorf and heated to 104°C for 10 minutes in a dry heat block. Heating caused the formation of a coagulum which was then centrifuged at $16,000 \times$ g for 5 minutes. After centrifugation, the supernatant was tested with the same commercial test (DiroCHEK®, Zoetis) according to manufacturer's instructions. The supernatant from a heated serum sample is not an approved sample type for the licensed test kit.

5.2.3. Knott test

Modified Knott tests were performed as previously described (Knott, 1939; Magnis et al., 2013) on 108 blood samples in EDTA. Briefly, 9 mL of 2% formalin was added to 1 mL of whole blood and agitated to allow cells to lyse. The sample was then centrifuged at $1,200 \times$ g, and the pellet stained with 1% methylene blue. The circulating microfilariae were identified based on their morphology and morphometry (Magnis et al., 2013) and counted (mf/ml of blood) in 20µl of blood. The study area is potentially endemic for *D. repens* and *D. immitis* (prevalence of *D. immitis* = 60% in Galati city, (unpublished data), therefore species identification of microfilariae was confirmed by morphology and by multiplex PCR. Morphometric analyses

of the mf were performed with standard microscope equipped with calibrated measuring eyepieces at final magnification of 200-400 x. Body length and diameter of ten randomly selected mf were determined.

5.2.4   Multiplex PCR

Molecular studies were performed on twentyfour blood samples resulted positive at the Knott test. Genomic DNA was extracted from one hundred microliters of each mf-positive sample using the DNeasy Blood and Tissue kit (Qiagen, Germany), following the manufacturer's instructions. An equimolar combination of general and specific primers (12SF/12SRdeg/12SF2B/12SR2) to amplify a portion of the small subunit ribosomal RNA gene of the mitochondrion (12S rDNA) were used to perform a multiplex PCR, according to Gioia et al. (2010). The PCR products were detected on a 2.5% ethidium bromide- stained low melting agarose gel (BIO- RAD, Spain). The amplification of the conserved region was 500bp (12SF/12SRdeg) for canine filarial samples, and for the simultaneous amplification of *D. immitis* and/or *D. repens* the specific fragment was 204 bp (12SF2B/12SRdeg) and 327bp (12SF/12SR2) respectively. The final PCR volume used was: 20ul (19ul mix+1ul DNA) for each sample. The thermal profile used was: 95^0C for 10 min; 40 cycles at 92^0C for 30s, 49^0C for 45 s, 72^0C for 1 min and final elongation step at 72^0C for 10 min.

5.3 Results

Out of 194 dogs sampled from four cities in Romania, *D. immitis* circulating antigens were found in 16 (8.2%) non-heated samples and in 52 (26.8%) heated samples (Tab. 5.1, 5.2).
Out of 108 dogs examined by Knott test and multiplex PCR, a total of 24 dogs (22.2%) were positive for circulating mf. Six dogs had *D. immitis* mf only, 12 dogs had only *D. repens* mf, and 5 were positive for both *D. immitis* and *D. repens*. Only one dog was positive to *Acanthocheilonema reconditum* mf; this sample tested by PCR was positive for *D. immitis* and *D. repens*.

The number of mf/ml varied from 100-2460 mf/ml for *D. immitis*, 5-885 mf for *D. repens*; only 3 mf/ml of *A. reconditum* were found.

The mean length and width of *D. immitis* mf were 305.51±7.3 and 5.20±1.79 μm; Those of *D. repens*: 364.87±2.73 μm and 7.90±1.2 μm. The length and width of *A. reconditum* mf from the only positive dog were: 274.11± 3.56 and 5.01± 0.24 μm.

Three of the six dogs with solely circulating *D. immitis* mf had positive antigen tests before and after heating. The other three mf+ dogs were antigen negative before heating and became positive only after heat treatment. Three of the 5 dogs with mixed *D. immitis/D. repens* infection were antigen positive before and after heating. The other two were antigen negative and then converted to positive after heating. These results would confirm that false negative antigen tests for *D. immitis* infection do occur in naturally infected dogs.

Regarding the antigen testing for *D. immitis* in the 12 dogs with *D. repens* mf, only two dogs were antigen negative both before and after heat treatment. Six dogs (50%) were antigen negative before heat treatment, but became antigen positive after heating. Finally, four *D. repens* mf+ dogs were antigen positive both before and after heat treatment.

5.4 Discussion

The results from the present study would confirm previous reports that dogs infected with *D. immitis* can have negative antigen tests and that these false negatives can revert to positive following heat treatment (Little et al., 2014a; Little et al., 2014b; Velasquez et al., 2014; Drake et al., 2015). The fact that this was also shown in dogs with a confirmed infection status (mf+ with species identification in PCR) gives even greater support to this result. A recent study by Ionica et al (2015) reported nine (24.3%) samples antigen negative in animals but positive for *D. immitis* at PCR. These results probably are correlated with a low number of adult worms or due to a delayed antigenaemia. This is important because most epidemiological studies depend on antigen test results, which likely underestimate true prevalence. However, PCR testing on blood clots revealed that antigen testing was missing many infected dogs. The present study reports an overall prevalence of 28.6% for *D. immitis* infection when samples were treated

with heat (vs. 8.2% untreated). In some areas, the increase of positive samples was over 7-fold. The same trend was confirmed in dogs with co-infections by *D. immitis* and *D. repens*.

It is likely therefore that *D. immitis* prevalence based solely on antigen testing without pre-treatment with heat is greatly underestimating prevalence in areas like south eastern Romania (e.g. the prevalence of *D. immitis* in Galati county = 25% before heat treatment, 45% after heat treatment). Recent data suggest that dogs placed on monthly macrocyclic lactones and doxycycline may develop false negative results on the heartworm antigen tests, suggesting that antigen test is not an efficacy indicator for the dogs with *D. immitis* infection and managed with slow kill (SK) treatment (Drake et al., 2015). After the heat treatment of the serum samples 8/15 dogs (53.3%) converted to positive to the antigen of *D. immitis*, suggesting that post-heating treatment is leading to a destruction of antigen-antibody complexes. However, the dogs tested in the present study had not received macrocyclic lactones.

The presence of *Dirofilaria* species in the north-eastern part of the country indicates that the parasites are spreading from the south, southwest and southeast parts of the country (Mircean et al.,2012). Prevalence values are likely skewed due to the fact that stray dogs in animal shelters that are more likely to acquire heartworm than privately-owned dogs (Miller et al., 2011). Perhaps the most interesting and unexpected result from the present study, however, was the cross reactivity of the antigen test towards *D. repens.* Ionica et al. (2014) revealed a potentially cross-reaction between *D. repens* and *D. immitis*, given the fact that nine samples were antigen positive for *D. immitis* but positive for *D. repens* at PCR. They concluded as a co-infection with an occult infection of *D. immitis* or an inhibition of *D. immitis* microfilariae by the presence of *D. repens* microfilariae. In addition Pantchev et al. (2009b, 2011) have exluded the potential cross-reaction between *D. repens* and *D. immitis.* We have revealed with this current study a reactivity of the antigen test towards *D. repens.* There is no data regarding a cross-reaction between *D. repens* and *D. immitis* after the heat treatment of serum samples infected with both agents.

Cross reactivity has recently been reported for *Angiostrongylus vasorum* (Schnyder et al., 2012) and for *Spirocerca lupi* (Aroch et al., 2015). In earlier studies carried out during the development of the first antigen tests, serum

from dogs infected with other common helminths failed to cross-react with the *D. immitis* antigen, with the exception of *Dipetalonema reconditum* (Gillis et al, 1984). So it is known that filarial species can have antigens in common. Instead Brunner et al. (1988) didn't find cross-reactions with intestinal parasites and with *D. reconditum.* However, the dogs used in the present study were evaluated for other such as *A. vasorum* and *D. reconditum* against a potential cross-reaction with *D. immitis* antigen test. Coprological exams showed the presence of intestinal parasites like: *Toxocara canis*, *Isospora* spp., *Capillaria* spp., *Trichuris vulpis* and Ancylostomidae (data unpublished). So according the published literature there is no cross-reaction between these intestinal parasites and *D. immitis* antigen test.

In the present study, only mono-detection of *D. repens* microfilaria was found. Most of the dogs with Knott test-confirmed on *D. repens* were either positive before and after or became positive after heat treatment.

It is not easy to explain these results and further study is necessary. However, it may be that the antigen test used here is coated with a polyclonal antibody for antigen capture and this may decrease specificity. Or, as reported above, antigens among filarial species are similar and this test is indeed able to capture antigens from the tissue-dwelling *D. repens*. Five out of seven dogs that were negative before heat treatment reverted to positive after heating. Could this imply that *D. repens* induces immune complex formation in the same way or even better than *D. immitis* and that destruction of the immune complex releases antigens that cross react with current tests? Another explanation for these results could be that the dogs are co-infected with both agents *D. immitis* and *D. repens* and heat-treatment is simply revealing the occult *D. immitis* infection, given the fact that the population of dogs used in this study is originating from a highly endemic region for *D. immitis*.

It would also be interesting to evaluate serum protein levels and albumin: globulin ratios in dogs which seroconvert after heating in order to determine if indeed immune complex formation is due to a hyperglobulinemic state. According to Little et al. (2014), the serum of a hyper-gammaglobulinemic dog infected with other parasites can block the antigen detection of *D. immitis* with commercial assays. The authors showed that heating the serum reversed the blockage, allowing detection. Therefore, it is possible that the

dogs from the present study infected with *D. immitis* which were negative before heat treatment, but became antigen positive after heating, might been co-infected with other pathogens. Indeed, if concomitant infections with other pathogens that induce hyper-gammaglobulinemia (i.e. *Leishmania infantum, Ehrlichia canis*, *Babesia* spp., etc) can affect antigen test results, this would have important implications for prevalence studies in areas where these infections co-exist. There is a little evidence regarding leishmaniosis in Romania. A recent study revealed four out of 138 dogs with *Leishmania* spp. infection by IFAT (Hamel et al., 2012). The first clinical case of autochthonous canine leishmaniasis in the last 80 years was reported by Mircean et al. (2014). Furthermore, recent studies also reported the spread of other vector-borne infections (e.g. *Babesia canis, B. gibsoni, Ehrlichia canis* and *Hepatozoon canis*) in dogs in Romania (Ioniță et al., 2012; Hamel et al., 2012; Mircean et al., 2012; Imre et al., 2013; Morar et al., 2015).

Although the research has reached its aims, there were some unavoidable limitations. First, because of the test limit, the blood samples positive on *D. immitis* with ELISA was conducted without spectrophotometry, therefore the optical density (OD) of the antigen test couldn't showed a correlation between the infection and the titer of antigen. Second the dogs might be co-infected with other pathogens. Indeed, if concomitant infections with other pathogens that induce hyper-gammglobulinemia (i.e. *Leishmania infantum, Ehrlichia canis*, *Babesia* spp., etc) can affect antigen test results, this would have important implications for prevalence studies in areas where these infections co-exist.

5.5 Conclusions

The data obtained from this study highlights the risk of the eastern part of Romania of becoming an endemic area for *Dirofilaria* spp. both in animals and in humans. Given the fact that transmission of dirofilarial infections by mosquitoes is strictly dependent on a suitable climate which enables development of the larvae in the vectors (Kalluri et al., 2007; Medlock et al., 2007), the areas included in our study were characterized by a temperate-continental climate. Periodic epidemiological studies are necessary in order to assess changes in the prevalence of *Dirofilaria* spp. infections.

In geographical areas where the two parasites overlap, which are increasing due to the spread of both (Genchi et al., 2009, 2011), the performance of commercial antigen tests, and the recommendation to heat serum, must be carefully evaluated and studied much further, including studies with sera from experimentally-infected animals.

Table.5.1 Experimental design. (* positive for Knott)

Number of dogs	Test
194	DiroCheck® before/after heat
108	Knott
24*	Multiplex PCR

Table 5.2 Results of antigen testing (DiroCHECK®) before and after heat treatment in 194 dogs from 5 cities in Romania.

City	Total samples	Positive samples before heat treatment (%)	Positive samples after heat treatment (%)
Galati	31	8	14
Iasi –Probota	26	4	10
Iasi-Pascani	51	0	5
Vaslui – Barlad	64	3	22
Bacau	22	1	1
TOTAL	**194**	**16**	**52**

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Chapter 6

Evaluation of single or concomitant pathogen infections (*Dirofilaria*, *Leishmania*, *Ehrlichia*) in dogs by *Dirofilaria immitis* antigen test, following the heat treatment process

6.1 Aim

Vector-borne diseases (VBDs) represent a major concern due to the increasing prevalence in dog populations and their zoonotic potential. Recent studies reported the spread of some VBDs (*Dirofilaria*, *Leishmania*, *Ehrlichia*) in Italy (Foglia-Manzillo et al., 2006; Otranto et al., 2013; Del Prete et al., 2016) as well as in Spain (Mirò et al., 2013; Montoya- Alonso et al., 2017) and other European countries. Laboratory diagnosis of heartworm disease by *D. immitis* is based on the detection of microfilariae in the blood of infected dogs and/or the circulating antigens derived from adult female worms. However, the commercial assays currently available for testing *D. immitis* antigen can give false negative results due to the presence of only male worms (Rishniw et al., 2012), or due to the use of macrocyclic lactones (Rawlings et al.,1982). Previous studies showed that dogs infected with *D. immitis* can have negative antigen tests and that these false negatives can revert to positive following the heat treatment (Velasquez et al., 2014; Drake et al., 2015; Ciuca et al., 2016). Furthermore, the serum of a hyper-gammaglobulinemic dog infected with other pathogens (e.g. *Ehrlichia canis*, *Leishmania infantum, Babesia* spp.) can block the antigen detection of *D. immitis* with commercial assays (Little et al., 2014).

The findings from a recent study by Ciuca et al. (2016) hypothesized that the dogs co-infected with other pathogens may induce false negative results on *D. immitis* antigen test. In order to further verify this latter hypothesis, the aim of the present study was to verify the false negative results to *D. immitis* antigen of single and concomitant pathogen infections (*Dirofilaria*, *Leishmania*, *Ehrlichia*) using heat treatment of serum samples from randomly sampled dogs (positive and negative to any VBDs).

6.2 Materials

6.2.1 Study area and animal sampling

The study was conducted between May and July 2017, in Spain (Madrid) and central Italy (Molise region). A total of 46 blood samples (23 from Madrid and 23 from Italy) were randomly collected from stray, hunting and owned dogs. From each dog, a total of 1.5 ml of blood in EDTA and 3 ml of serum was collected. All samples used in this study were collected under standard protocols for management of dogs at participating animal shelters and owners and by the Institutional Animal Care and Use Committees at the University of Madrid (Spain) and University of Naples (Italy).

6.2.2 Laboratory techniques

Knott test

Modified Knott test was performed as previously described (Magnis et al., 2013). The circulating microfilariae were identified based on their morphology and morphometry (Magnis et al., 2013). Morphometric analyses of the mf were performed with standard microscope equipped with calibrated measuring eyepieces at final magnification of (200-400 X).

D. immitis *antigen testing before and after heat treatment*

Antigen testing was carried out using a commercial microtiter well-based assay (Petcheck®, Idexx), according to manufacturer's instructions. Serum samples were then treated with heat, according to Drake et al. (2015). Briefly, approximately 1-1.5 ml of serum was heated to 104°C for 10 minutes in a dry heat block. Heating caused the formation of a coagulum which was then centrifuged at $16,000 \times g$ for 5 minutes. After centrifugation, the supernatant was tested with the same commercial test (Petcheck®, Idexx) according to manufacturer's instructions. The supernatant from a heated serum sample is not an approved sample type for the licensed test kit.

Measurements of the optical density (OD) were done spectrophotometrically at 650 nm. For the assay to be valid the OD positive control value minus the OD negative control value (P-N) was set to be greater than 0.150 with the negative control OD value ≤0.150.

Immunofluorescence antibody test (IFAT) for Leishmania infantum

An immunofluorescence antibody test (IFAT; provided by the National Reference Center for Leishmaniosis, Palermo, Italy) was used to detect anti-*Leishmania infantum* antibodies in the serum samples. The cut-off point chosen for positivity was 1:160.

Antibody test for Ehrlichia canis

The SNAP 4Dx Plus Test-detection was used to detect antibodies of *Ehrlichia canis.*

6.3   Results and Discussion

Out of 46 samples examined, 16 were positive for *L. infantum* (34.7%), 8 were positive for *E. canis* (17.4%), three were positive for *D. immitis* (6.5%) and four were positive for *D. repens* (8.6%).

Specifically, out of 23 dogs from Spain, 8 were positive for *L. infantum* (34.7%), 7 were positive for *E. canis* (30.4%) and two were positive for *D. immitis* (8.6%). None of the dogs tested positive for *D. repens*. Five dogs were co-infected with both pathogens *L. infantum* and *E. canis* and two dogs were co-infected with *D. immitis* and *L. infantum*.

Out of 23 dogs from central Italy, 8 were positive for *L. infantum* (34.7%), only one dog was positive for *E. canis* (4.3%), one dog was positive to *D. immitis* (4.3%) and four were positive for *D. repens* (17.4%). No co-infection was found.

The results of OD (Optical density) of *D. immitis* antigen test showed a range of 0.031-1.561. (Tab. 6.1).

Despite the ELISA antigenic test used in the present study was different from the one reported in previous studies by Ciuca et al. (2016) and Velasquez et al. (2014) (PetCHECK vs DiroCheck), the OD of the three heartworm positive samples increased after heating as described by other authors. The rising of the OD confirms that canine serum and plasma of some dogs may contain inhibitors of *D. immitis* antigen detection and the heat treatment of these samples prior to testing could improve the sensitivity of these assay in some patients (Little et al., 2014).

The results showed also that in the samples analysed there was no occult *D. immitis* infection. The possible explanation is that the pathogens *Leishmania* and *Ehrlichia*, even if they induce hyper-gammaglobulinemia, not always can affect antigen test results as expected. Since there were only two positive dogs both coinfected by *Leishmania* and *D. immitis* we cannot deny the occurrence of possible cross-reactions between *D. immitis* antigen and other pathogens.

One limit of the study was the small number samples, for this reason further studies are needed by using a higher number of samples with single or multiple VBD infection in order to i) confirm the increase of the OD after heating in heartworm positive samples and ii) evaluate the possibility of false negatives to *D. immitis* antigen in blood samples positive to *D. repens* and/or other canine vector borne diseases.

Table 6.1 Results of the screening for vector-borne pathogens (L. infantum, E. canis, D. immitis, D. repens*) and OD of* D. immitis *antigen before and after heat treatment of serum samples.*

ID samples	*Leishmania* (IFAT)	*Ehrlichia* (SNAP)	*D. immitis* (Knott)	*D.repens* (Knott)	*D. immitis* (Petcheck OD Pre-heat)	*D. immitis* (Petcheck OD Post-heat)
1 I	Negative	Negative	**Positive**	Negative	**0.734**	**1.100**
2 I	Negative	Negative	**Positive**	Negative	**0.873**	**1.561**
3 I	**Positive**	Positive	Negative	Negative	0.034	0.145
4 I	Negative	Negative	Negative	Negative	0.047	0.065
5 I	Negative	Negative	Negative	Negative	0.047	0.050
6 I	**Positive**	Negative	Negative	**Positive**	0.048	0.053
7 I	Negative	Negative	Negative	Negative	0.072	0.056
8 I	Negative	Negative	Negative	Negative	0.078	0.053
14 I	Negative	Negative	Negative	**Positive**	0.047	0.048
17 I	**Positive**	Negative	Negative	Negative	0.044	0.047
16 I	Negative	Negative	Negative	**Positive**	0.072	0.055
9 I	Negative	Negative	Negative	Negative	0.084	0.046
10 I	**Positive**	Negative	Negative	Negative	0.052	0.059
11 I	Negative	Negative	Negative	Negative	0.047	0.039
12 I	**Positive**	Negative	Negative	Negative	0.041	0.045
13 I	Negative	Negative	Negative	Negative	0.058	0.049
15 I	**Positive**	Negative	Negative	Negative	0.044	0.047
18 I	Negative	Negative	Negative	Negative	0.064	0.042
19 I	**Positive**	Negative	Negative	Negative	0.057	0.081
20 I	Negative	Negative	Negative	Negative	0.036	0.047
21 I	**Positive**	Negative	Negative	Negative	0.041	0.062
22 I	Negative	Negative	Negative	**Positive**	0.048	0.056
23 I	Negative	Negative	Negative	Negative	0.042	0.080
24 S	Negative	Negative	Negative	Negative	0.031	0.053
25 S	**Positive**	Negative	Negative	Negative	0.035	0.055
26 S	Negative	**Positive**	Negative	Negative	0.045	0.062
27 S	**Positive**	**Positive**	Negative	Negative	0.046	0.083
28 S	**Positive**	**Positive**	Negative	Negative	0.038	0.052
29 S	**Positive**	**Positive**	Negative	Negative	0.039	0.046
30 S	Negative	**Positive**	Negative	Negative	0.040	0.063
31 S	**Positive**	**Positive**	Negative	Negative	0.040	0.047
32 S	Negative	**Positive**	Negative	Negative	0.046	0.048
33 S	**Positive**	**Positive**	Negative	Negative	0.043	0.047
34 S	Negative	**Positive**	Negative	Negative	0.047	0.047
35 S	Negative	Negative	Negative	Negative	0.041	0.046
36 S	Negative	Negative	Negative	Negative	0.040	0.082
37 S	Negative	Negative	Negative	Negative	0.046	0.050
38 S	Negative	Negative	Negative	Negative	0.044	0.054
39 S	Negative	Negative	Negative	Negative	0.039	0.046
40 S	**Positive**	Negative	Negative	Negative	0.050	0.046
41 S	**Positive**	Negative	**Positive**	Negative	**0.217**	**1.054**
42 S	Negative	Negative	Negative	Negative	0.036	0.048
43 S	Negative	Negative	Negative	Negative	0.047	0.054

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Chapter 7
Overall discussion

7.1   Discussion

The present thesis provides important insights into two cardio-pulmonary nematodes in dogs: *Dirofilaria immitis* and *Angiostrongylus vasorum*, with particular emphasis on the epidemiological and diagnostic challenges of these parasites.

Significant findings emerged regarding: i) the contemporaneous detection of *D. immitis* and *A. vasorum* in kennels of southern Italy; ii) the prevalence of *A. vasorum* in owned and stray dogs with clinical findings; iii) the evaluation of false negatives to *D. immitis* antigens and of single or concomitant pathogen infections (*Dirofilaria*, *Leishmania*, *Ehrlichia*) in dogs tested by the *D. immitis* antigen test, following the heat treatment process.

The results obtained on the prevalence of *D. immitis* and *A. vasorum* in kennels (chapter 3) revealed the spread of *D. immitis* toward southern Italy, probably due to climate changes and to the spreading of vector mosquitoes (*Aedes*, *Culex*, *Anopheles*) (Genchi et al., 2005). These factors caused new autochthonous foci of heartworm disease that have been already reported in previously non-endemic areas of southern Italy (Genchi et al., 2009). Similarly, *A. vasorum* has extended its geographical distribution in southern Italy. A possible explanation for increased transmission could be the rising disease awareness, the availability of better diagnostic tools and the presence of the red fox that acts as a reservoir of the parasite. Specifically, an increased density of foxes in an area populated with dogs is likely to increase the number of fox-dog interactions, these interactions allow the opportunity to transmit infections (Elsheika et al., 2014; Santoro et al., 2016). The prevalence of *D. immitis* and *A. vasorum* observed in kennels of the Campania region in southern Italy (Del Prete et al., 2015) confirmed that regular parasitological surveillance, appropriate diagnostic tools, treatment strategies and high-quality hygiene standards are required to guarantee the health and welfare of kennel dogs as also recommended by the European Scientific Counsel for Companion Animals Parasites (www.esccap.org).

The findings of the study presented in chapter 4 highlighted some considerations regarding *A. vasorum* in stray and owned dogs: the current and other recent reports provide important evidence that in southern Italy *A. vasorum* infection is spreading more than expected, probably because of the

neglection of this infection in canine clinical practice. Therefore, the description of clinical signs of angiostrongylosis in the present thesis added new information on the clinical relevance of the lungworm infection (Del Prete et al., submitted). Despite of the availability of scattered information on the prevalence of *A. vasorum* in dogs in Italy, obtained by different diagnostic methods (e.g. Baermann, FLOTAC, serological or molecular methods), a standard protocol for active monitoring and surveillance of this parasite is needed in order to assess the real extent and the clinical importance of this nematode infection.

The results obtained by the study on dog serum samples naturally exposed to *D. immitis* (chapter 5) and on the evaluation of single or concomitant pathogen infections (*Dirofilaria*, *Leishmania*, *Ehrlichia*) in dogs by *D. immitis* antigen test, following the heat treatment process (chapter 6) showed that: i) dogs infected with *D. immitis* can have negative antigen tests that can be reverted to positive if following heat treatment (Little et al., 2014; Velasquez et al., 2014); ii) the optical density of heartworm positive samples increased after heating as described by other authors (Ciuca et al., 2016). As concerns the positive reversion in previously negative samples probably there is correlation with a low number of adult worms or with a delayed antigenemia of the serum samples. The increase of the OD after heat treatment process, also in positive *D. immitis* serum samples confirms that canine serum and plasma of some dogs may contain inhibitors of *D. immitis* antigen detection.

Both results in the latter two chapters confirmed that the heat treatment of the samples prior to testing could improve the sensitivity of these assay in some patients (Little et al., 2014). These results are very important for the diagnosis of *D. immitis* because most epidemiological studies depend on antigens test results, which likely underestimate true prevalence.

7.2   Conclusion and recommendations

Some clinical issues on canine heartworm disease by *D. immitis* and angiostrongylosis by *A. vasorum* may impair their appropriate diagnosis. Both diseases may present a wide spectrum of clinical manifestations that can lead clinicians to overlook infections with these two pathogens, inducing to treatments for other conditions that are generally considered to

be more prevalent. Additionally, some clinicians tend to perform therapeutic treatments without attempting a definitive diagnosis, disregarding important evidence on local epidemiological risk and consequent data on treatment and prevention regimens (Elsheikha et al., 2014). The standardization of diagnostic techniques such as Knott's test, serology, heat treatment process, Baermann or FLOTAC examination and the use of these techniques should be more routinely employed in endemic and non-endemic areas, to increase the active surveillance of both infections and facilitate diagnosis and control. Keeping in mind the impact that *D. immitis* and *A. vasorum* may have on animal health, the zoonotic potential of *D. immitis* and the geographical range trend of both infections, it is vital to adopt effective prophylactic and adequate vector control measures, as also suggested by the American Heartworm Society (AHS, https://www.heartwormsociety.org) and the European Society of Dirofilariosis and Angiostrongylosis (ESDA, https://www.esda.vet). In particular, the mission and the aims of of ESDA are to: (i) further scientific progress in the study of heartworm and *Angiostrongylus* infections in Europe; (iii) inform the membership and medical and veterinary practitioners of new developments; (iii) harmonize procedures for the diagnosis, prevention and treatment of *Dirofilaria* and *Angiostrongylus* infections throughout Europe by writing official Guidelines; (iv) inform animal owners and inhabitants of the importance of these worms and of their prevention.

Considering the ongoing changes in climate and ecosystems, dog exposure to infection seems likely to increase in the future, in endemic areas as well as in non-endemic ones. Therefore, promoting awareness among practitioners and dog owners is also one of the priority purpose for an integrated parasite control in pets as recommended by the European Scientific Counsel Companion Animal Parasites (www.esccap.org).

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