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

L. Ciuca [a,b], A. Vismarra [c], W. Lebon [d], F. Beugnet [d], R. Morchon [e], L. Rinaldi [b,*], G. Cringoli [b], L. Kramer [c], M. Genchi [c]

[a] *Ion Ionescu de la Brad, University of Agricultural Sciences and Veterinary Medicine Iasi, Faculty of Veterinary Medicine, M. Sadoveanu Alley No. 8, 700489, Iasi, Romania*
[b] *Department of Veterinary Medicine and Animal Productions, University of Naples Federico II, Via della Veterinaria, 1, Naples, Italy*
[c] *Dipartimento di Scienze Medico-Veterinarie, Università di Parma, via del Taglio 10, 43126, Parma, Italy*
[d] *Boehringer Ingelheim Animal (BI-AH) Health Research Centre, France*
[e] *Group of Animal and Human Dirofilariosis, Parasitology Area, Faculty of Pharmacy, University of Salamanca, Salamanca, Spain*

ARTICLE INFO

ABSTRACT

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

1. Introduction

Dirofilaria repens (Spirurida, Onchocercidae) is among the most widespread vector-borne helminths in dogs and is an emerging zoonosis in Europe (Otranto et al., 2013; Genchi and Kramer, 2020). However, despite its emergence and zoonotic impact, *D. repens* continues to be a neglected parasite, when compared to others like *D. immitis,* the cause of

a serious and potentially fatal canine heartworm disease (Genchi and Kramer, 2017), due to the development of pulmonary and cardiac pathologies (Venco, 2007). Subcutaneous dirofilariosis caused by *D. repens* is commonly associated with the presence of the adults in subcutaneous tissues and/or subcutaneous nodules. The infection usually progresses asymptomatically (Grandi et al., 2007). Therefore, the clinical relevance of *D. repens* infections in dogs is relatively minor compared with the one

induced by *D. immitis*. Even though there is evidence of geographical spread of *D. repens*, there is still a need to further evaluate pathogenicity, effective treatment options and better diagnostic options (Capelli et al., 2018).

The geographic distribution of *D. repens* is changing rapidly, as testified by the increasing prevalence in endemic areas (e.g. Italy, France, Spain) and the spreading into previously unaffected areas (e.g. Genchi et al., 2011; Iglódyová et al., 2012; Ionică et al., 2015; Joke-lainen et al., 2016; Kartashev et al., 2015; Miterpáková et al., 2010; Simón et al., 2012; Şuleşco et al., 2016; Tasić-Otašević et al., 2015; Simón et al., 2017). A recent review on *D. repens* (Capelli et al., 2018), including analysis of current geographical distribution, epidemiology, and zoonotic impact, highlights the increased prevalence and the spread of *D. repens* from endemic areas of Southern Europe towards countries in Central Europe. Several factors are likely responsible for the spread of infection into new areas, including the movement of infected dogs from endemic areas, climate change, and the lack of diagnostic tools that do not rely on mff identification. Indeed, the asymptomatic nature of canine subcutaneous dirofilariasis may lead to under-diagnosis and consequent risk of infected dogs, the main reservoir for both canine and human infections, to go unobserved and untreated. (Genchi and Kramer, 2017; Simón et al., 2017; Capelli et al., 2018; Genchi et al., 2019).

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

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

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

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

2. Materials and methods

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

2.1. Animals

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

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

2.2. Dirofilaria repens experimental infection

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

2.3. Blood sampling

Starting from Day 58, blood samples were collected on a monthly basis for *D. repens* antibody testing. Blood was placed in plain tubes and serum was obtained and stocked at -20 °C until testing. Additional samples were collected on Days 220, 245 and 281 for the detection of mff using the modified Knott's test and biomolecular analysis. Samples were collected and placed in EDTA tubes. Sampling was always performed in the morning before 10:00 am. The experimental design (study days and laboratory techniques used) is reported in Table 1.

2.4. Serology

All the sera samples were analyzed with a non-commercial ELISA to detect the IgG antibody response using adult *D. repens* somatic antigens and rWSP as described by Kramer et al. (2005, 2007) with some modifications. For ELISA IgG anti *D. repens* analysis, 96-well microplates (Corning® 3369, 96 Well EIA/RIA Assay Microplate) were incubated overnight at 4 °C with 0.8 μg of an extract of *D. repens* adult worms

Table 1

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

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

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

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

For ELISA IgG anti-WSPr analysis, 96-well microplates (Corning® 3369, 96 Well EIA/RIA Assay Microplate) were incubated overnight at 4 °C with 0.3 μg of rWSP, (100 μl/well) which was produced in recombinant form and purified as described by Diosdado et al. (2017). All serum samples were analyzed at a 1:20 dilution with dilution buffer and incubated at 37 °C for 1 h. Anti-human peroxidase-conjugated IgG was diluted 1:5,000 with dilution buffer (100 μl/well) and incubated at 37 °C for 2 h. The reaction was revealed with substrate solution (100 μl/well) for 10 min. After each incubation step, the wells were washed with washing buffer (PBS pH 7; Tween$_{20}$ 0.05 %) for 3 times (200 μl/well). The cut-off (OD = 0.5) was established, using the mean value ± 3 SD of 45 serum samples from clinically healthy blood donors (negative controls) living in a *D. repens*-free area. Optical densities were measured at 492 nm with iMark™ Microplate Absorbance Reader for both ELISAs.

The serum samples of naturally infected dogs (diagnosed by the presence of adults and mff of *D. repens*) were used as positive controls and serum samples from healthy dogs living in a *D. repens*-free area as negative controls, for both ELISAs (*D. repens* and *Wolbachia* antibodies),

2.5. Modified Knott's test and Multiplex PCR

A modified Knott's test was used for the detection of circulating mff of *D. repens* (Knott, 1939; Magnis et al., 2013) as follows. One mL of EDTA blood was mixed with 9 mL of formalin 2% and centrifuged for 3–5 minutes at approximately 1500 rpm. The supernatant was removed from the tube and the content was stained with 1–2 drops of 1% methylene blue. A drop was placed on a microscope slide covered with a cover slip and observed under an optical microscope at 100 ×. For each positive sample, the level of microfilariaemia was quantified using the full content of the tube (mff/mL).

For molecular determination of microfilaria species, genomic DNA was extracted from 200 microliters of each blood sample using the DNeasy® Blood and Tissue kit (Qiagen, Germany), following the

manufacturer's instructions. Molecular analyses were performed following two different protocols of multiplex PCR for simultaneous detection of the different Dirofilaria species, i.e. the protocol described by Gioia et al. (2010; protocol A) and the protocol described by Rishniw et al. (2006; protocol B). For the latter, the PCR reactions were increased to 25 μL of total volume, containing 5 μL of genomic DNA for each sample amplification.

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

2.6. Statistical analysis

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

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

3. Results

3.1. Modified Knott's test, PCR and serological analyses

The results of Knott's test, multiplex PCRs and serology are reported in Tables 2 and 3.

Knott's tests were negative for all the dogs on day 220, positive on day 245 in thirteen dogs (65.0 %) and positive on day 288 in sixteen dogs (80.0 %). Four dogs remained negative for the entire study period.

Molecular analyses performed on day -5 were negative for all 20 dogs included in the study. On day 220, 3 samples (15.0 %) were positive with protocol A and 12 samples (60.0 %) gave positive results with protocol B. On day 245, 10 samples (50.0 %) were positive with protocol A and 15 samples (75.0 %) were positive with protocol B. Finally,12 samples (60.0 %) were positive with protocol A and 17 (85.0 %) were positive with protocol B by day 281.

Table 3 reports the results of anti-*D. repens* antibody response in experimentally infected dogs. Beginning at day 58, an increasing number of dogs had antibodies against somatic antigens of *D. repens* and, from day 148, a total of eighteen out of twenty dogs were positive and remained so until the end of the study (day 281). Two dogs maintained OD values under the cut-off (0.8) during the entire study.

The anti-WSP antibody responses started on Day 58, with one positive dog, and increased until the last day of the study with sixteen positive dogs.

The overall findings revealed that all the positive dogs had an increase of OD values for the antibodies against *D. repens* somatic antigens and against *Wolbachia* endosymbiont with peaks on Days 220, 245 and 281 (Fig. 1).

Overall, the outcome of the study revealed that out of the 20 dogs experimentally infected with *D. repens*, 16 (80.0 %) were microfilaraemic, 17 (85.0 %) were positive at DNA detection in the blood, 18 (90.0 %) had *D. repens* antibodies and 16 (80.0 %) had *Wolbachia* antibodies on the last day of the study.

Table 2
Results of Knott' test and multiplex PCR on different study days in the 20 dogs experimentally infected with *Dirofilaria repens*.

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

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

The overall k agreement between Knott's and PCR protocol B was 0.442 (P = 0.0001) and increased throughout the study, reaching 0.828 (P = 0.0001) on Day 281. Analyses were not carried out comparing Knott's with protocol A, given that this protocol resulted in a lower number of positive samples.

The results of the univariate statistical analysis are reported in Tables 4 and 5. Briefly, a statistical significant association (p < 0.05) was observed between the number of positive dogs with the Knott's test on

Day 245 and the outcome of protocol B multiplex PCR as well as with the number of the positive dogs at *D. repens* IgG-ELISA (p < 0.005). Similarly, on Day 281, the Knott's test outcome was statistically associated (p < 0.05) with both molecular and *D. repens* IgG-ELISA tests. Instead, there was no statistically significant association (p < 0.05) between the results of the Knott's test and the results of the *D. repens* IgG and IgG anti-WSPr ELISAs on either study day (245, 281).

Table 3
Results of serological diagnosis on *Dirofilaria repens* and its *Wolbachia* on different study days (-5 to 281) in the 20 dogs experimentally infected with *Dirofilaria repens*.

Dog ID	Non-commercial IgG-ELISA results (+/-) *Dirofilaria repens* (Dr) and recombinant *Wolbachia* surface (rWSP)								
	Study Days								
	−5 Dr/rWSP (+/-)	58	91	121	148	178	220	245	281
1	-/-	-/-	-/-	+/-	+/-	+/-	+/-	+/+	+/+
2	-/-	-/-	-/-	-/+	-/+	-/+	-/+	-/+	-/+
3	-/-	-/+	-/-	+/-	+/-	+/-	+/-	+/-	+/+
4	-/-	-/-	-/-	+/-	+/-	+/-	+/-	+/-	+/+
5	-/-	-/-	+/-	+/-	+/-	+/-	+/+	+/+	+/-
6	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/+
7	-/-	-/-	+/-	+/-	+/+	+/-	+/+	+/+	+/+
8	-/-	-/-	-/-	+/-	+/-	+/-	+/-	+/-	+/-
9	-/-	+/-	+/-	+/+	+/-	+/+	+/+	+/+	+/+
10	-/-	-/-	-/-	+/+	+/+	+/-	+/-	+/-	+/-
11	-/-	-/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-
12	-/-	-/-	+/-	+/-	+/+	+/+	+/+	+/+	+/+
13	-/-	+/-	-/+	-/+	+/+	+/+	+/+	+/+	+/+
14	-/-	-/-	+/-	+/-	+/-	+/+	+/-	+/-	+/+
15	-/-	-/-	-/-	+/+	+/+	+/+	+/+	+/+	+/+
16	-/-	-/-	-/-	+/-	+/-	+/-	+/-	+/-	+/+
17	-/-	-/-	+/-	+/-	+/-	+/+	+/+	+/-	+/+
18	-/-	-/-	-/-	+/-	+/-	+/-	+/-	+/-	+/+
19	-/-	-/-	+/+	+/+	+/+	+/+	+/+	+/+	+/+
20	-/-	-/-	+/-	+/-	+/-	+/+	+/+	+/+	+/+
Total positive Dr (%)/ rWSP (%)	0 (0%)/ 0 (0%)	2 (10.0 %)/ 1 (5.0 %)	9 (45.0 %)/ 2 (10.0 %)	17(85.0 %)/ 6(30.0 %)	18 (90.0 %)/ 7 (35.0 %)	18 (90.0 %)/ 9 (45.0 %)	18(90.0 %)/ 10(50.0 %)	18(90.0 %)/ 10(50.0 %)	18(90.0 %)/ 16(80.0 %)

(+/-)= (positive/negative).
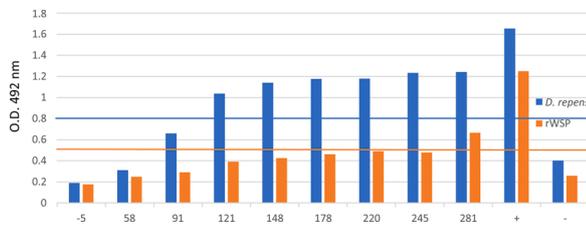
Fig. 1. Immunological responses of anti-*Dirofilaria repens* and anti-*Wolbachia* in 20 dogs experimentally infected with *Dirofilaria repens*. Cut-off for ELISA IgG anti-*D. repens* (OD = 0.8); cut-off for ELISA IgG anti-WSPr (OD = 0.5); "+"=positive control; "-"=negative control.

Table 4
Results of molecular and serological analyses compared to Knott's test outcomes (mff/mL) on study day 245 (onset of patency).

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

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

Table 5
Results of molecular and serological analyses compared to Knott's test outcomes (mff/mL) on study day 281 (final day of study).

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

3.2. Clinical features

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

4. Discussion

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

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

This wide variation in pre-patency renders the Knott's test, currently the most commonly used diagnostic method for *D. repens* infection, highly prone to false-negative results. In order to find a more sensitive alternative, and to assist in the differentiation between *D. repens* and *D. immitis*, several molecular methods have been developed in recent years.

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

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

To the authors knowledge, this is only the second study reporting antibody response to *D. repens* somatic antigen in experimentally infected dogs. Joekel et al. (2017) evaluated the antibody response in dogs naturally infected with several different filarial species. Three *D. repens*-experimentally infected dogs were also analyzed. Response to crude somatic antigen from adult *D. repens* showed dogs becoming antibody positive as early as 24 days post-infection, much earlier than in the present study. The increasing antibody titres were observed until

approximately 5–6 months post-infection, following the same trend as seen in the present study. Cancrini et al. (2000) also looked at antibody responses in naturally infected dogs, using an adult somatic antigen. The authors reported positive serology for all PCR/Knott positive dogs and also for a portion of PCR/Knott negative dogs. The authors conclude that a combination of parasitological, biomolecular and serological techniques might increase the diagnostic reliability for naturally infected dogs.

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

In the present study, this was not a concern given that the dogs were experimentally infected with *D. repens* and were free from any other filarial infections. However, it would have been interesting to explore the cross-reactions and the specificity of the serological test to better evaluate its suitability for diagnostic purposes. The lack of such analyses represents a limitation of the study that warrants further investigation.

The most problematic issue regarding the use of serology is the potential cross-reactivity between the somatic antigens of *D. immitis/D. repens* and those of other parasites that may be present in a dog population (e.g. ascarids, hookworms). It has been reported that most individuals exposed to *D. immitis* or *D. repens* infection produce anti-WSP antibodies (Grandi et al., 2008; Cabrera et al., 2018). Since *Wolbachia* is present only in filarial nematodes and not in other helminths (e.g. *Toxocara, Ascaris* or hookworms, etc), the presence of anti-WSP antibodies would be highly suggestive of exposure of *Dirofilaria* spp. in humans living in endemic areas. A further challenge will be the development of a serological assay that can discriminate between *D. immitis* and *D. repens*. This problem is already noted with the *D. immitis* antigen test which has been reported as cross reacting when used in dogs with mono-infections with *D. repens* (Ciuca et al., 2018).

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

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

5. Conclusions

Results from both ELISAs, anti-*D. repens* and anti-WSPr, confirm that the development of serological tests for *D. repens* infection could be a starting point for application in epidemiological studies and as an aid in the diagnosis of infection in dogs, in particular for early stage infections

and in absence of mff. Indeed, only 1 dog in the present study was negative for all tests carried out.

The identification of specific immune-active proteins for *D. repens* infection could be useful in diagnosing, for example, the presence of adult parasites in the absence of both clinical signs and circulating mff. They could also be useful in monitoring the efficacy of adulticide treatment. It would also be necessary to evaluate the present tests in terms of specificity in the field, given the risk of cross reactivity with other filarial nematodes.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Ethical standards

The study was performed in compliance with current national laws and regulations.

CRediT authorship contribution statement

L. Ciuca: Conceptualization, Investigation, Methodology, Data curation, Formal analysis, Writing - original draft. **A. Vismarra:** Investigation, Methodology, Data curation, Writing - original draft. **W. Lebon:** Investigation, Methodology, Data curation, Formal analysis, Writing - original draft. **F. Beugnet:** Conceptualization, Methodology, Validation, Resources, Writing - review & editing. **R. Morchon:** Investigation, Formal analysis, Resources, Writing - original draft. **L. Rinaldi:** Conceptualization, Methodology, Validation, Resources, Writing - review & editing. **G. Cringoli:** Supervision, Resources. **L. Kramer:** Conceptualization, Methodology, Validation, Resources, Writing - review & editing. **M. Genchi:** Conceptualization, Investigation, Methodology, Formal analysis, Resources, Writing - review & editing.

Acknowledgments

References

Albanese, F., Abramo, F., Braglia, C., Caporali, C., Venco, L., Vercelli, A., Ghibaudo, G., Leone, F., Carrani, F., Giannelli, A., Otranto, D., 2013. Nodular lesions due to infestation by *Dirofilaria repens* in dogs from Italy. Vet. Dermatol. 24, 255–256.

Altman, D.G. (Ed.), 1991. Practical Statistics for Medical Research. Chapman & Hall, London, pp. 277–321.

Bazzocchi, C., Ceciliani, F., McCall, J.W., Ricci, I., Genchi, C., Bandi, C., 2000. Antigenic role of the endosymbionts of filarial nematodes: IgG response against the *Wolbachia* surface protein in cats infected with *Dirofilaria immitis*. Proc. Biol. Sci. 267, 2511–2516.

Cabrera, E.D., Carretón, E., Morchón, R., Falcón-Cordón, Y., Falcón-Cordón, S., Simón, F., Montoya-Alonso, J.A., 2018. The Canary Islands as a model of risk of pulmonary dirofilariasis in a hyperendemic area. Parasitol. Res. 117, 933–936.

Cancrini, G., Tassi, P., Coluzzi, M., 1989. Ivermectin against larval stages of *Dirofilaria repens* in dogs. Parassitologia 31, 177–182.

Cancrini, G., Favia, G., Giannetto, S., Tringali, R., Pietrobelli, M., Simón, F., 1999. Serological assays on eight cases of human dirofilariasis identified by morphology and DNA diagnostics. Ann. Trop. Med. Parasitol. 93, 147–152.

Cancrini, G., Allende, E., Favia, G., Bornay, F., Antón, F., Simón, F., 2000. Canine dirofilariosis in two cities of southeastern Spain. Vet. Parasitol. 92, 81–86.

Capelli, G., Genchi, C., Baneth, G., Bourdeau, P., Brianti, E., Cardoso, L., Danesi, P., Fuehrer, H.P., Giannelli, A., Ionică, A.M., Maia, C., Modrý, D., Montarsi, F., Krücken, J., Papadopoulos, E., Petrić, D., Pfeffer, M., Savić, S., Otranto, D., Poppert, S., Silaghi, C., 2018. Recent advances on *Dirofilaria repens* in dogs and humans in Europe. Review. Parasit. Vectors 11, 663.

Ciuca, L., Genchi, M., Kramer, L., Mangia, C., Miron, L.D., Prete, L.D., Maurelli, M.P., Cringoli, G., Rinald, I.L., 2016. Heat treatment of serum samples from stray dogs naturally exposed to *Dirofilaria immitis* and *Dirofilaria repens* in Romania. Vet. Parasitol. 225, 81–85.

Ciuca, L., Simòn, F., Rinaldi, L., Kramer, L., Genchi, M., Cringoli, G., Acatrinei, D., Miron, L., Morchon, R., 2018. Seroepidemiological survey of human exposure to *Dirofilaria* spp. in Romania and Moldova. Acta Trop. 187, 169–174.

Diosdado, A., Gómez, P.J., Morchón, R., Simón, F., González-Miguel, J., 2017. Interaction between *Wolbachia* and the fibrinolytic system as a possible pathological mechanism in cardiopulmonary dirofilariosis. Vet. Parasitol. 247, 64–69.

ESDA-Guidelines, 2017. Guidelines for Clinical Management of Subcutaneous Dirofilariosis in Dogs and Cats (accessed on January 2020). https://www.esda.vet/guide-lines-tutorials.

Genchi, C., Kramer, L., 2017. Subcutaneous dirofilariosis (*Dirofilaria repens*): an infection spreading throughout the old world. Parasit. Vectors 10, 517.

Genchi, C., Kramer, L., 2020. The prevalence of *Dirofilaria immitis* and *D. repens* in the Old World. Vet. Parasitol. 21, 280–108995.

Genchi, M., Pengo, G., Genchi, C., 2010. Efficacy of moxidectin microsphere sustained release formulation for the prevention of subcutaneous filarial (*Dirofilaria repens*) infection in dogs. Vet. Parasitol. 170, 167–169.

Genchi, C., Mortarino, M., Rinaldi, L., Cringoli, G., Traldi, G., Genchi, M., 2011. Changing climate and changing vector-borne disease distribution: the example of *Dirofilaria* in Europe. Vet. Parasitol. 176, 295–299.

Genchi, C., Genchi, M., Petry, G., Kruedewagen, E.M., Schaper, R., 2013. Evaluation of the efficacy of imidacloprid 10%/moxidectin 2.5 % (Advocate®, Advantage® multi, Bayer) for the prevention of *Dirofilaria repens* infection in dogs. Parasitol. Res. 112, 81–89.

Genchi, M., Rinaldi, L., Venco, L., Cringoli, G., Vismarra, A., Kramer, L., 2019. *Dirofilaria immitis* and *D. repens* in dog and cat: A questionnaire study in Italy. Vet. Parasitol. 267, 26–31.

Gioia, G., Lecová, L., Genchi, M., Ferri, E., Genchi, C., Mortarino, M., 2010. Highly sensitive multiplex PCR for simultaneous detection and discrimination of *Dirofilaria immitis* and *Dirofilaria repens* in canine peripheral blood. Vet. Parasitol. 172, 160–163.

Giori, L., Garbagnoli, V., Venco, L., Genchi, M., Bazzocchi, C., Bertazzolo, W., 2010. What is your diagnosis? Fine-needle aspirate from a subcutaneous mass in a dog. Mixed neutrophilic-eosinophilic inflammation with Dirofilaria fragments. Vet. Clin. Pathol. 39, 255–256.

Grandi, G., Živičnjak, T., Beck, R., 2007. Pathogenesis of *Dirofilaria* spp. infection. In: Cringoli, G. (Ed.), Mappe Parasitologiche, Vol 8. Università degli Studi di Napoli Federico II, Naples, Italy, pp. 59–66.

Grandi, G., Morchon, R., Kramer, L., Kartashev, V., Simon, F., 2008. *Wolbachia* in *Dirofilaria repens*, an Agent Causing Human Subcutaneous Dirofilariasis. J. Parasitol. 94, 1421–1423.

Iglódyová, A., Miterpáková, M., Hurníková, Z., Antolová, D., Dubinský, P., Letková, V., 2012. Canine dirofilariosis under specific environmental conditions of the Eastern Slovak Lowland. Ann. Agric. Environ. Med. 19, 57–60.

Ionică, A.M., Matei, I.A., Mircean, V., Dumitrache, M.O., D'Amico, G., Győrke, A., Pantchev, N., Annoscia, G., Albrechtová, K., Otranto, D., Modrý, D., Mihalca, A.D., 2015. Current surveys on the prevalence and distribution of *Dirofilaria* spp. and *Acanthocheilonema reconditum* infections in dogs in Romania. Parasit. Res. 114, 975–982.

Joekel, D., Maier, S., Huggel, K., Schaper, R., Deplazes, Peter, 2017. Specific antibody detection in dogs with filarial infections. Parasitol. Res. 116, 81–90.

Jokelainen, P., Mõtsküla, P.F., Heikkinen, P., Ülevaino, E., Oksanen, A., Lassen, B., 2016. *Dirofilaria repens* Microfilaremia in Three Dogs in Estonia. Vector Borne Zoonotic Dis. 16, 136–138.

Kartashev, V., Tverdokhlebova, T., Korzan, A., Vedenkov, A., Simón, L., González-Miguel, J., Morchón, R., Siles-Lucas, M., Simón, F., 2015. Human subcutaneous/ocular dirofilariasis in the Russian Federation and Belarus, 1997–2013. Int. J. Infect. Dis. 33, 209–211.

Knott, J., 1939. A method for making microfilarial surveys on day blood. Trans. Royal Soc. Trop. Med. Hyg. 33, 191–196.

Kramer, L.H., Tamarozzi, F., Morchón, R., López-Belmonte, J., Marcos-Atxutegi, C., Martín-Pacho, R., Simón, F., 2005. Immune response to and tissue localization of the *Wolbachia* surface protein (WSP) in dogs with natural heartworm (*Dirofilaria immitis*) infection. Vet. Immunol. Immunopathol. 106, 303–308.

Kramer, L.H., Kartashev, V.V., Grandi, G., Morchón, R., Nagornii, S.A., Karanis, P., Simón, F., 2007. Human subcutaneous dirofilariasis. Russia. Emerg Infect Dis. 13, 150–152.

Latrofa, M.S., Weigl, S., Dantas-Torres, F., Annoscia, G., Traversa, D., Brianti, E., Otranto, D., 2012. A multiplex PCR for the simultaneous detection of species of filarioids infesting dogs. Acta Trop. 122, 150–154.

Magnis, J., Lorentz, S., Guardone, L., Grimm, F., Magi, M., Naucke, T.J., Deplazes, P., 2013. Morphometric analyses of canine blood microfilariae isolated by the Knott's test enables *Dirofilaria immitis* and *D. repens* species-specific and *Acanthocheilonema* (syn. *Dipetalonema*) genus-specific diagnosis. Parasit. Vectors 6, 48.

Manzocchi, S., Venco, L., Piseddu, E., Pantchev, N., Masserdotti, C., Bonfanti, U., Albanese, F., Kramer, L.H., Bertazzolo, W., 2017. Positive PCR alone should not be considered sufficient to establish *Dirofilaria repens* as the cause of subcutaneous nodular lesions in the absence of a clear cytologic picture. Vet. Clin. Pathol. 46, 389–390.

Marcos-Atxutegi, C., Kramer, L.H., Fernandez, I., Simoncini, L., Genchi, M., Prieto, G., Simon, F., 2003. Th1 response in BALB/c mice immunized with *Dirofilaria immitis* soluble antigens: A possible role for Wolbachia? Vet. Parasitol. 112, 117–130.

McCall, J.W., 1981. The role of arthropods in the development of animal models for filariasis research. J. Georgia Entomol. Soc. 16, 283–293.

Miterpáková, M., Antolová, D., Hurníková, Z., Dubinský, P., Pavlacka, A., Németh, J., 2010. *Dirofilaria* infections in working dogs in Slovakia. J. Helminthol. 84, 173–176.

Morchón, R., Ferreira, A.C., Martín-Pacho, J.R., Montoya, A., Mortarino, M., Genchi, C., Simón, F., 2004. Specific IgG antibody response against antigens of *Dirofilaria immitis* and its *Wolbachia* endosymbiont bacterium in cats with natural and experimental infections. Vet. Parasitol. 125, 313–321.

Morchón, R., Carretón, E., Grandi, G., González-Miguel, J., Montoya-Alonso, J.A., Simón, F., Genchi, C., Kramer, L.H., 2012. Anti-*Wolbachia* Surface Protein antibodies are present in the urine of dogs naturally infected with *Dirofilaria immitis* with circulating microfilariae but not in dogs with occult infections. Vector Borne Zoonotic Dis. 12, 17–20.

Otranto, D., Dantas-Torres, F., Brianti, E., Traversa, D., Petrić, D., Genchi, C., Capelli, G., 2013. Vector-borne helminths of dogs and humans in Europe. Parasit. Vectors 16, 6–16.

Petry, G., Genchi, M., Schmidt, H., Schaper, R., Lawrenz, B., Genchi, C., 2015. Evaluation of the adulticidal efficacy of imidacloprid 10 %/moxidectin 2.5 % (w/v) spot-on (Advocate®, Advantage® Multi) against *Dirofilaria repens* in experimentally infected dogs. Parasitol. Res. 114, 131–144.

Punkosdy, G.A., Dennis, V.A., Lasater, B.L., Tzertzinis, G., Foster, J.M., Lammie, P.J., 2001. Detection of serum IgG antibodies specific for *Wolbachia* surface protein in Rhesus monkeys infected with Brugia malayi. J. Infec. Dis. 184, 385–389.

Rishniw, M., Barr, S.C., Simpson, K.W., Frongillo, M.F., Franz, M., Dominguez Alpizar, J. L., 2006. Discrimination between six species of canine microfilariae by a single polymerase chain reaction. Vet. Parasitol. 135, 303–314.

Simón, F., Prieto, G., Muro, A., Cancrini, G., Cordero, M., Genchi, C., 1997. Human humoral immune response to *Dirofilaria* species. Parassitologia 39, 397–400.

Simón, F., Prieto, G., Morchón, R., Bazzocchi, C., Bandi, C., Genchi, C., 2003. Immunoglobulin G antibodies against the endosymbionts of filarial nematodes (*Wolbachia*) in patients with pulmonary dirofilariasis. Clin. Diagn. Lab. Immunol. 10, 180–181.

Simón, F., Siles-Lucas, M., Morchón, R., González-Miguel, J., Mellado, I., Carretón, E., Montoya-Alonso, J.A., 2012. Human and animal dirofilariasis: the emergence of a zoonotic mosaic. Clin. Microbiol. Rev. 25, 507–544.

Simón, F., González-Miguel, J., Diosdado, A., Gómez, P.J., Morchón, R., Kartashev, V., 2017. The complexity of zoonotic filariasis episystem and its consequences: a multidisciplinary view. Biomed Res. Int. 2017, 6436130.

Şuleşco, T., von Thien, H., Toderaş, L., Toderaş, I., Lühken, R., Tannich, E., 2016. Circulation of *Dirofilaria repens* and *Dirofilaria immitis* in Moldova. Parasit. Vectors 9 (1), 627.

Tasić-Otašević, S.A., Trenkić Božinović, M.S., Gabrielli, S.V., Genchi, C., 2015. Canine and human Dirofilaria infections in the Balkan Peninsula. Vet. Parasitol. 209, 151–156.

Venco, L., 2007. Heartworm (*Dirofilaria immitis*) disease in dogs. In: Genchi, C., Rinaldi, L., Cringoli, G. (Eds.), Dirofilaria immitis and D. repens in Dog and Cat and Human Infections. Rolando Editore, Naples, Italy, pp. 117–125.

Webber, W., Hawking, F., 1995. Experimental maintenance of *Dirofilaria repens* and *D. immitis* in dogs. Exp. Parasitol. 4, 143–164.