International Journal for Parasitology 50 (2020) 555–559

Contents lists available at ScienceDirect

International Journal for Parasitology

journal homepage: www.elsevier.com/locate/ijpara

Succinctus

Hyperendemic *Dirofilaria immitis* infection in a sheltered dog population: an expanding threat in the Mediterranean region

Rossella Panarese [a], Roberta Iatta [a], Maria Stefania Latrofa [a], Andrea Zatelli [a], Aleksandra Ignjatović Ćupina [b], Fabrizio Montarsi [c], Marco Pombi [d], Jairo Alfonso Mendoza-Roldan [a], Frederic Beugnet [e], Domenico Otranto [a,f,*]

[a] *Dipartimento di Medicina Veterinaria, Università degli Studi di Bari, Bari, Italy*
[b] *Faculty of Agriculture, University of Novi Sad, Novi Sad, Serbia*
[c] *Istituto Zooprofilattico Sperimentale delle Venezie, Legnaro PD, Italy*
[d] *Dipartimento di Sanità Pubblica e Malattie Infettive, Università di Roma "Sapienza", Roma, Italy*
[e] *Boehringer Ingelheim Animal Health, Lyon, France*
[f] *Faculty of Veterinary Sciences, Bu-Ali Sina University, Hamedan, Iran*

ARTICLE INFO

Article history:
Received 14 February 2020
Received in revised form 27 March 2020
Accepted 3 April 2020
Available online 30 May 2020

Keywords:
Dirofilaria immitis
Dirofilaria repens
Emerging disease
Vector
Culiseta annulata

ABSTRACT

A study on the occurrence of *Dirofilaria immitis* and its vectors was carried out in order to assess the prevalence of the disease in dogs in previously non-endemic areas of southern Italy. Blood samples (n = 385) and mosquitoes (n = 1540) were collected in two dog shelters and analysed by Knott's test and duplex real-time PCR, respectively. *Dirofilaria immitis* was the most prevalent filarioid (44.2%), while *Culex pipiens* was the most prevalent mosquito species (68.8%). This high prevalence of *D. immitis* infection confirms this location as one of the most hyperendemic foci of dirofilariosis in Europe.

Dirofilaria immitis (Leidy, 1856) and *Dirofilaria repens,* Railliet and Henry, 1911, are parasitic filarioids (Spirurida, Onchocercidae) distributed worldwide and transmitted by mosquitoes to a wide range of animal species including humans (Otranto et al., 2009; Dantas-Torres and Otranto, 2013, 2020). In particular, *D. immitis*, the causative agent of canine and feline heartworm disease (HWD) is distributed in tropical and temperate regions (Otranto et al., 2009), whereas *D. repens*, the agent of subcutaneous dirofilariosis (SCD), in continental and eastern European countries (Genchi et al., 2009; Otranto et al., 2009; Capelli et al., 2018). In humans, these filarial species mainly cause pulmonary, subcutaneous and ocular dirofilarioses (Otranto and Eberhard, 2011; Simón et al., 2012). The majority of human cases of infection by *Dirofilaria* spp. is associated with *D. repens* in Europe and *D. immitis* in the Americas (Dantas-Torres and Otranto, 2013). A strong relationship has been demonstrated between human cases and a high prevalence of SCD in dogs, indicating the strong zoonotic boundaries of this infection (Otranto et al., 2011). Up to 70 mosquito species included in the genera *Aedes, Ochlerotatus*, *Anopheles* and *Culex* are putative or competent vectors of *Dirofilaria* spp. (Eldridge and Edman, 2000). However, the main vectors of *D. immitis* are *Culex pipiens* and *Aedes albopictus* (Genchi et al., 2009; Otranto et al., 2009). In Italy, differing from *D. repens*, which is distributed throughout the whole country (Otranto et al., 2009), *D. immitis* has long been regarded as endemic mostly in the northern Po Valley regions, with a prevalence of up to 80% in dogs (Otranto et al., 2009). However, from 1999 to 2009, an inverted trend has been reported with a reduced number of cases of HWD in northern Italy and new cases in dogs from southern Italy (Otranto et al., 2009). This trend could have been due to the effect of chemoprophylaxis in endemic areas, which is not routinely carried out in southern regions (Mendoza-Roldan et al., 2020). Although some cases of HWD have been reported in southern Italy (Piantedosi et al., 2017), comprehensive epidemiological data are not yet available. Therefore, a study on the epidemiology of *D. immitis* and its vectors is timely, both to avoid spread of the disease through animal populations and to reduce zoonotic risk.

* Corresponding author at: Dipartimento di Medicina Veterinaria, Università di Bari, 70010, Valenzano, Bari, Italy.
E-mail address: domenico.otranto@uniba.it (D. Otranto).

https://doi.org/10.1016/j.ijpara.2020.04.002
0020-7519/

The aims of this study were to assess: (i) the prevalence of canine dirofilariosis in southern Italy where the infection has never been regarded as endemic, (ii) the mosquito vector population composition in two foci of infection, and (iii) the occurrence of *Dirofilaria* spp. in mosquito populations, in an effort to define their role in the epidemiology of the infection.

The protocol of this study was approved by the ethical committee of the Department of Veterinary Medicine of the University of Bari, Italy (Prot. Uniba 8/19). From April to June 2019, blood samples were collected in two dog shelters in southern Italy. Dog shelters differed in the buildings' structure and environment (i.e. irrigation channels, rich in reed bed and vegetation, presence of other animals such as birds, reptiles and amphibians) as well as the number of dogs kept. The first shelter (n = 316 dogs), site 1 (40° 36′ 30.3″N, 17° 59′ 40.0″E, Brindisi), was built in a wetland area, 3.0 km from the nearest coastal area. The second shelter (n = 220 dogs), site 2 (40° 25′ 09.6″N, 18° 09′ 56.1″E, Lecce), is located in a dry and windy area, 8.0 km from the nearest coastal area.

An overall estimated minimum sample size of 385 shelter dogs was enrolled based on the following assumptions: population of shelter dogs (n = 536), a confidence limit of 95%; an expected prevalence of 20% and confidence interval of 2.88%. All dogs which were a minimum of 7 months of age were included in the study (i.e. n = 6 dogs, 1.6%). A clinical examination was conducted for each animal, in order to establish their health status. Anamnestic data (e.g. age, sex, breed, clinical signs such as weight loss, fever, etc.) and previous treatments were recorded in each animal's file together with their individual microchip number.

Whole blood (2 mL) was collected in vacuum containers with EDTA from each dog and processed by a modified Knott's test for the detection of microfilariae (mfs) as previously described (Knott, 1939). Two aliquots of the sediment were transferred onto two slides (i.e. 2 × 50 μL) and covered with two cover slips. The count of the mfs was based on the average of the counts of the two slides. Dogs with a microfilaremic load greater than or equal to 3000 mfs/100 μL were considered highly microfilaremic. Five mfs for each sample were identified using morphometrical keys (Kelly, 1973).

From May to November 2019, mosquito specimens were collected from both sites. Sampling was performed between 17:00 h and 08:00 h at weekly and biweekly intervals at sites 1 and 2 respectively, with greater frequency during the warmer part of the season. Active adult mosquitoes were collected by CDC light traps (Centers for Disease Control and Prevention, Atlanta, USA) (n = 8) and BG sentinel-2 mosquito traps (Biogents, Regensburg, Germany) (n = 4), baited with dry ice and BG lure, respectively. Furthermore, fed mosquitoes were collected from each doghouse at site 1, once each week between 08:00 h and 11:00 h, using electric mosquito aspirators (i.e. InsectaVac Aspirator, BioQuip Products, California, USA). During the entire sampling period, traps were switched within each building. CDC light traps were placed outdoors, on the perimeter of the dog shelters, while the BG sentinel-2 mosquito traps were placed indoors or next to the dog cages. After each sampling, the collection bags were kept at −4 °C, until transfer to the laboratory. All the specimens were identified using morphological keys (Severini et al., 2009). Fed female mosquitoes were kept in cages for 21 days under temperature-controlled conditions (28 ± 1 °C), 90% relative humidity and fed with 10% sucrose solution until their dissection in saline solution for the detection of *Dirofilaria* spp. larvae (Severini et al., 2009). Females that naturally died during the rearing were immediately dissected. All dissected mosquitoes were stored in 70% alcohol in plastic tubes (1.5 mL) for further molecular analyses. Five *Ae. albopictus* specimens were raised from eggs and used as negative controls for the molecular analysis. The minimum infection rate (MIR) was calculated by the standard formula: number of positive pools/total number of mosquitoes in pools tested × 1000 (Ferreira et al., 2015). The estimated rate of infection (ERI), which is adjusted for pooled samples, was calculated by the formula: $ERI = 1 - (1 - x/m)^{1/k}$ where x is the number of positive pools; m the number of examined pools and k the average number of specimens in each pool (Cowling et al., 1999). All parameters such as temperature, relative humidity, rainfall and wind speed were recorded for each mosquito collection site (see below).

Single mosquito specimens were removed from 70% ethanol and freeze-thawed in 50 μL of DNA extraction buffer (20 mM Tris-HCl pH = 8; 100 mM ethylenediaminetetraacetic acid and 1% sodium dodecyl sulphate), cycled twice for 15 min between 100 °C and −80 °C. Next, specimens were homogenised, without separating the different anatomical parts, and single specimen aliquots (10 μL) were pooled until a maximum of 10 specimens per pool. Female mosquito pools were made based on the specific criteria: species, site of collection from the dog shelter and collection date. Genomic DNA was extracted from the pools using an in-house method (Latrofa et al., 2017) and tested by duplex real-time PCR (qPCR) as described in Latrofa et al. (2012). All DNA samples were tested in duplicate, and positive and negative controls were included in each qPCR run. The specificity of the qPCR assay was established by melting curve analysis as described in Latrofa et al. (2012).

Meteorological data [i.e. temperature (°C), relative humidity (%), monthly rainfall (mm), wind speed (m/s)] were acquired from the climatological database of the "Agenzia Regionale per la Prevenzione e la Protezione dell'Ambiente" of Apulia, Italy.

Data regarding the prevalence of *Dirofilaria* spp. in dogs and mosquito populations were recorded in a Microsoft Excel spreadsheet and analysed by Quantitative Parasitology 3.0 software for the subsequent statistical analyses (Rozsa et al., 2000). The association between the category variables in dog population (i.e. sex, age, weight, time in the dog shelter), in mosquitoes collected and the positive results for *Dirofilaria* spp. were analysed using contingency tables and χ^2 values were calculated. Odds ratio (OR) values were calculated at a 95% confidence interval (CI). P values <0.05 were considered significant.

Of 385 dogs, 189 (49.1%; 95% CI: 0.44–0.54) tested positive for *Dirofilaria* spp. and, specifically, 109 (46.2%; 95% CI: 0.39–0.52) and 80 (53.7%; 95% CI: 0.46–0.62) from sites 1 and 2, respectively, with an overall *D. immitis* positivity rate of 44.2%. Conversely, the overall prevalence of *D. repens* was 7%. The prevalence of each *Dirofilaria* spp. based on the collection site, together with the average length of circulating mfs, is reported in Table 1. An overall number of 83 dogs (43.9%) were highly microfilaremic (i.e. ≥3000 mfs/100 μL of blood), while 106 dogs (56.1%) showed a lower microfilaremia. In 73 of 106 dogs (38.6%) the mfs count was lower or equal to 100 mfs/100 μL of blood. Age (OR = 0.36, χ^2 = 11.32; P < 0.001) and time in the dog shelter (OR = 0.38, χ^2 = 11.10; P < 0.001) were statistically significant as risk factors for dirofilarial infection. In particular, adult dogs over 3 years of age as well as dogs kept in the shelter for more than 2 years were at a higher risk of *Dirofilaria* spp. infection than young dogs (≤3 years) or dogs living in the shelter for less than 2 years. Conversely, sex (OR = 0.68, χ^2 = 3.56; P > 0.05), weight (OR = 0.71, χ^2 = 1.09; P > 0.05) and sampling site (OR = 0.75, χ^2 = 1.83; P > 0.05) were not statistically significant risk factors. Of the 1564 mosquitoes collected from both sites, 1150 were females, of which 190 (12.2%) were engorged. Eleven species belonging to five different genera were identified (Table 2), with *Cx. pipiens* the most prevalent one (n = 791, 68.8%). During dissection of the live specimens, mfs were found in one specimen of *Culiseta annulata* (Fig. 1A–C), but no filarial larvae were found when dissecting mosquitoes kept for 21 days under laboratory conditions (n = 60). Out of 216 mosquito pools

Table 1
Prevalence and confidence interval (95% CI) of *Dirofilaria* spp. infection in dog populations from site 1 and site 2, and the average length of the microfilariae detected in dogs' blood samples using a modified Knott's test.

Sampling site (number of animals)	*Dirofilaria immitis*			*Dirofilaria repens*			Co-infection	
	n (%)	95% CI	Average length (μm) ± σ	*n* (%)	95% CI	Average length (μm) ± σ	*n* (%)	95% CI
Site 1 (*n* = 236)	91 (38.6)	0.32–0.45	311.96 ± 28.79	14 (5.9)	0.35–0.96	364.53 ± 16.85	4 (1.7)	0.01–0.04
Site 2 (*n* = 149)	71 (47.7)	0.40–0.56	294.97 ± 13.93	5 (3.4)	0.01–0.07	354.93 ± 8.71	4 (2.7)	0.01–0.6

Table 2
Number of female mosquito specimens and positive pools molecularly tested for *Dirofilaria* spp. together with the average number of mosquitoes per pool, minimal infection rate, percentage of estimated rate of infection, confidence interval (95% CI) and positivity to *Dirofilaria immitis* and *Dirofilaria repens*.

Mosquito species	No. mosquitoes	No. pools positive/tested	Mean no. mosquitoes per pool	MIR (/1000)	ERI (%)	95% CI	*D. immitis*	*D. repens*
Culex pipiens	791	13/106	7.46	16.4	1.74	0.009–0.028	+	–
Aedes caspius	154	5/29	5.31	32.5	3.49	0.012–0.073	+	–
Coquillettidia richiardii	86	5/24	3.58	58.1	6.30	0.023–0.131	+	–
Aedes albopictus	40	1/8	5.0	25.0	2.63	0.001–0.133	+	–
Culiseta annulata	27	2/13	2.10	74.1	7.50	0.013–0.237	+	–
Aedes detritus	6	1/6	1.0	166.7	16.00	0.086–0.589	–	+
Other species[a]	46	0/30	1.53	–	–	–	–	–
Total	1150	27/216	5.39	23.5	2.4	0.016–0.034	+	+

[a] Includes unidentifiable mosquito specimens and other mosquito species (i.e. *Culiseta longiaerolata, Anopheles maculipennis* sensu lato, *Anopheles claviger, Aedes mariae and Aedes vexans*) scored negative for *Dirofilaria* spp.

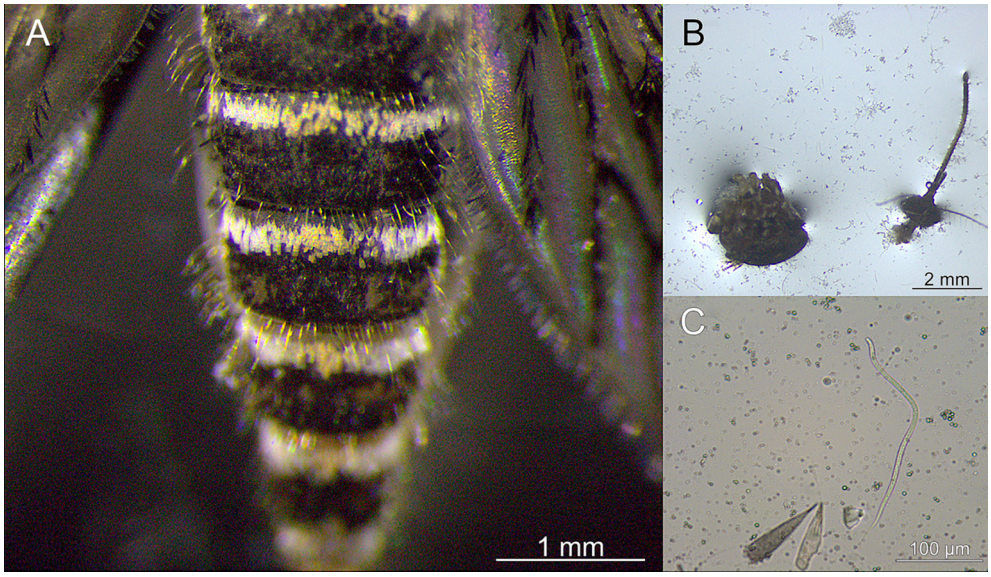

Fig. 1. Dissection of a live specimen of *Culiseta annulata*, collected from a doghouse in site 1 and observed to be positive for *Dirofilaria immitis*. (A) Morphological details of the specimen's abdomen and wings; (B) head and thorax of *Cs. annulata* after the dissection; (C) microfilaria of *D. immitis* detected after dissection of the specimen.

tested by qPCR, 26 scored positive for *D. immitis* and one for *D. repens* with an overall MIR of 23.47 (95% CI: 0.016–0.034). The overall ERI (i.e. the probability of a single positive mosquito specimen) was 2.4%. The MIR and the ERI are reported for each mosquito species in Table 2. The mean meteorological values obtained monthly at site 1 and site 2, respectively, were as follows: 26 ± 3 °C and 27 ± 2 °C of mean environmental temperature, 66% and 70% of mean relative humidity, 0.005 mm and 0 mm of mean rainfall and 1.48 m/s and 1.89 m/s of mean wind speed.

The high prevalence of *D. immitis* infection in shelter dogs (i.e. 44.2%) indicates that the examined area is one of the most hyperendemic for dirofilariosis recorded in Europe. Indeed, using a modified Knott's test and/or molecular analysis, prevalence rates ranging from 2.6% (i.e. 5/191) to 36% (i.e. 9/25) have been detected in dog populations from Spain (Diosdado et al., 2018) and Slovakia (Miterpáková et al., 2018), respectively. This data could be due to both the increase in the presence of competent vectors in a suitable environment and to the absence of chemoprophylaxis treatments in an area where practitioners are not aware of HWD. Accordingly, the invasive species *Ae. albopictus* and the common species *Cx. pipens*, both of which are known as competent vectors for *D. immitis* (Genchi et al., 2009), were present in both shelters, with *Cx. pipiens* the most prevalent mosquito species collected during the study period. On the other hand, historically, the high prevalence of infection in hyperendemic areas (e.g. 58.9% in Spain, Montoya et al., 1998; 22-80% in northern Italy, Otranto et al., 2009) decreased after the regular use of preventative treatments (Diosdado et al., 2018). Thus, the absence of chemoprophylaxis treatment could be a major driver for the spread of infection, mostly in a confined animal population such as that of shelters. In fact, in the absence of any preventive treatment, shelter dogs represent a suitable source for the spread of parasites (Otranto

et al., 2017). This is also demonstrated by the higher risk of infection in adult dogs (>3 years) and those kept in the shelter for more than 2 years. Nonetheless, the overall prevalence of *D. immitis* recorded (i.e. 44.2%) is even higher than that reported in other dog populations living in shelters (i.e. 11.8% in France; Laidoudi et al., 2019) or in breeding facilities (i.e. 36% in Slovakia; Miterpáková et al., 2018). Conversely, the prevalence of *D. repens* infection (i.e. 7%) is in the range of that previously recorded in southern Italy (i.e. 1.5–12%; Capelli et al., 2018). Considering that the diagnosis of dirofilarial infection has been herein performed by Knott's test, the prevalence data presented may be underestimated as suggested in previous studies (Miterpáková et al., 2018), where higher prevalences of HWDs were recorded by the detection of heartworm antigens (i.e. 64% in Slovakia, Miterpáková et al., 2018).

Although the highest prevalence of dirofilariosis infection (i.e. 53.7%) was found at site 2, the largest mosquito population, in terms of the number of collected specimens and species composition, was registered at site 1. This suggested that more favourable environmental conditions occurred in the latter site for the development of *Aedes* spp., competent vectors of the parasite. The overall MIR (i.e. 23.47/1000) recorded is lower than that reported in Portugal (i.e. 31/1000, Ferreira et al., 2015), whereas the MIR based on the positive pools of each species and, in particular, of *Aedes detritus* (i.e. MIR = 166.7/1000), is higher than that previously recorded for the same species (i.e. 43.5/1000, Ferreira et al., 2015). Although this infection rate is used for large numbers of samples (>1000 caught mosquitoes), the low average number of mosquitoes per pool (i.e. up to 7.46 specimens) used in this study still provides an accurate index of the rate of infection in the examined mosquito population (Gu et al., 2003). On the other hand, the overall ERI, representing an estimation of infected mosquito specimens collected from both sites (i.e. 2.4%), indicates a higher rate of infection than that previously reported in northern Italy (0.057%; Latrofa et al., 2012), but lower than in Portugal (3.21%; Ferreira et al., 2015). *Aedes detritus* (i.e. ERI = 16%), followed by *Cs. annulata* (i.e. ERI = 7.5%) and *Coquillettidia richiardii* (i.e. ERI = 6.3%) were the most infected species by *D. immitis* in the study area of South-eastern Italy, although only a few specimens were collected for each species. On the contrary, *Cx. pipiens* (i.e. ERI = 1.74%) and *Aedes caspius* (i.e. ERI = 3.49%) confirmed their roles as vectors of *D. immitis*, as previously recorded in North-eastern Italy (Latrofa et al., 2012). Indeed, these mosquito species were the most represented at sites 1 and 2 suggesting their primary role in the transmission of *D. immitis* in both the examined areas. In contrast to previous reports, *Anopheles maculipennis* sensu lato and *Aedes vexans* scored negative for *D. immitis* (Ferreira et al., 2015). This data, together with the absence of L3 at the dissection of the reared mosquitoes, may depend also on the large number of highly microfilaremic dogs (i.e. 43.9%) herein detected with up to 3000 mfs/ 100 μL of blood. This condition could be not compatible with the survival of the majority of the mosquito species, representing a limiting factor for the vectorial capacity (Coluzzi, 1964). This phenomenon represents a biological paradox in which in a limited hyperendemic area for dirofilariosis, such as the shelter, the transmission of the parasite is reduced due to the hyper-infection of the vectors. *Dirofilaria repens* DNA was detected in one pool of *Ae. detritus* (i.e. ERI = 0.1%), confirming its role as a competent vector of this species (Capelli et al., 2018). During the dissection of live specimens, *D. immitis* mfs were found in one *Cs. annulata*, however further studies are needed to investigate its vector competence and potential role in the transmission of this filarial species (Otranto et al., 2009; Şuleşco et al., 2016).

In conclusion, based on the above data, dirofilariosis should no longer be considered an emerging, but rather an endemic, disease in southern Mediterranean regions. Given the zoonotic potential of this parasite, strategic chemoprophylaxis treatments against canine dirofilariosis are needed to minimise the risk of infection for humans and animals in southern Mediterranean regions, which had long been considered non-endemic areas.

Acknowledgements

The authors thank Roberta Catanzariti (Istituto Zooprofilattico Sperimentale della Puglia e della Basilicata, Sez. Taranto, Italy), Giovanni Tortorella (Direzione ASL, Lecce, Italy), Oana Gusatoaia (Veterinario Responsabile, Canile "Aura", Lecce, Italy) and Denise Giordano (Canile "Liberi tutti", Brindisi, Italy) for their fruitful discussions and support during the field activities. The authors also thank Viet Linh Nguyen, Maria Alfonsa Cavalera, Thomas Matthew Arme and Giada Annoscia (Dipartimento di Medicina Veterinaria, Università degli Studi di Bari, Italy) for their support throughout blood sampling and laboratory work. In addition, the authors thank all staff of MediLabSecure Project (N° 2018/402-247) for support during mosquito collection. This work was supported by Boehringer Ingelheim Animal Health (France). The funders had no role in data collection, data analysis, decision to publish, or preparation of the manuscript.

References

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. Parasit. Vectors 11 (1), 663.

Coluzzi, M., 1964. Morphological divergences in the *Anopheles gambiae* complex. Rivista di Malariol. 43, 197–232.

Cowling, D.W., Gardner, I.A., Johnson, W.O., 1999. Comparison of methods for estimation of individual-level prevalence based on pooled samples. Prev. Vet. Med. 39, 211–225.

Dantas-Torres, F., Otranto, D., 2020. Overview on *Dirofilaria immitis* in the Americas, with notes on other filarial worms infecting dogs. Vet. Parasitol. (in press).

Dantas-Torres, F., Otranto, D., 2013. Dirofilariosis in the Americas: a more virulent *Dirofilaria immitis*?. Parasit. Vectors 6 (1), 288.

Diosdado, A., Gómez, P., González-Miguel, J., Simón, F., Morchón, R., 2018. Current status of canine dirofilariosis in an endemic area of western Spain. J. Helminthol. 92 (4), 520–523.

Eldridge, B.F., Edman, J.D., 2000. Introduction to medical entomology. In: Eldridge, B.F., Edman, J.D. (Eds.), Medical Entomology. Springer, Dordrecht.

Ferreira, C.A., de Pinho Mixão, V., Novo, M.T., Calado, M.M., Gonçalves, L.A., Belo, S. M., de Almeida, A.P., 2015. First molecular identification of mosquito vectors of *Dirofilaria immitis* in continental Portugal. Parasit. Vectors 8, 39.

Genchi, C., Rinaldi, L., Mortarino, M., Genchi, M., Cringoli, G., 2009. Climate and *Dirofilaria* infection in Europe. Vet. Parasitol. 163, 286–292.

Gu, W., Lampman, R., Novak, R.J., 2003. Problems in estimating mosquito infection rates using minimum infection rate. J. Med. Entomol. 40 (5), 595–596.

Kelly, J.D., 1973. Detection and differentiation of microfilariae in canine blood. Aust. Vet. J. 49 (1), 23–27.

Knott, J., 1939. A method for making microfilarial survey on day blood. Med. Hyg. 33, 191–196.

Laidoudi, Y., Ringot, D., Watier-Grillot, S., Davoust, B., Mediannikov, O., 2019. A cardiac and subcutaneous canine dirofilariosis outbreak in a kennel in central France. Un foyer de dirofilariose canine cardiaque et sous-cutanée dans un chenil du centre de la. France Parasite 26, 72.

Latrofa, M.S., Montarsi, F., Ciocchetta, S., Annoscia, G., Dantas-Torres, F., Ravagnan, S., Capelli, G., Otranto, D., 2012. Molecular xenomonitoring of *Dirofilaria immitis* and *Dirofilaria repens* in mosquitoes from north-eastern Italy by real-time PCR coupled with melting curve analysis. Parasit. Vectors 20, 5–76.

Latrofa, M.S., Angelou, A., Giannelli, A., Annoscia, G., Ravagnan, S., Dantas-Torres, F., Capelli, G., Halos, L., Beugnet, F., Papadopoulos, E., Otranto, D., 2017. Ticks and associated pathogens in dogs from Greece. Parasit. Vectors 10, 301.

Mendoza-Roldan, J., Benelli, G., Panarese, R., Iatta, R., Furlanello, T., Beugnet, F., Zatelli, A., Otranto, D., 2020. *Leishmania infantum* and *Dirofilaria immitis* infections in Italy, 2009-2019: changing distribution patterns. Parasit. Vectors 13, 193.

Miterpáková, M., Valentová, D., Čabanová, V., Berešíková, Ľ., 2018. Heartworm on the rise-new insights into *Dirofilaria immitis* epidemiology. Parasitol. Res. 117, 2347–2350.

Montoya, J.A., Morales, M., Ferrer, O., Molina, J.M., Corbera, J.A., 1998. The prevalence of *Dirofilaria immitis* in Gran Canaria, Canary Islands, Spain (1994–1996). Vet. Parasitol. 75 (2–3), 221–226.

Otranto, D., Capelli, G., Genchi, C., 2009. Changing distribution patterns of canine vector borne diseases in Italy: leishmaniosis vs. dirofilariosis. Parasit. Vectors 26 (2), 1–S2.

Otranto, D., Brianti, E., Gaglio, G., Dantas-Torres, F., Azzaro, S., Giannetto, S., 2011. Human ocular infection with *Dirofilaria repens* (Railliet and Henry, 1911) in an area endemic for canine dirofilariasis. Am. J. Trop. Med. Hyg. 84 (6), 1002–1004.

Otranto, D., Eberhard, M.L., 2011. Zoonotic helminths affecting the human eye. Parasit. Vectors 4, 41.

Otranto, D., Dantas-Torres, F., Mihalca, A.D., Traub, R.J., Lappin, M., Baneth, G., 2017. Zoonotic parasites of sheltered and stray dogs in the era of the global economic and political crisis. Trends Parasitol. 33 (10), 813–825.

Piantedosi, D., Neola, B., D'Alessio, N., Di Prisco, F., Santoro, M., Pacifico, L., Sgroi, G., Auletta, L., Buch, J., Chandrashekar, R., Breitschwerdt, E.B., Veneziano, V., 2017. Seroprevalence and risk factors associated with *Ehrlichia canis*, *Anaplasma* spp. *Borrelia burgdorferi* sensu lato, and *D. immitis* in hunting dogs from southern Italy. Parasitol. Res. 116, 2651–2660.

Rozsa, L., Reiczigel, J., Majoros, G., 2000. Quantifying parasites in samples of hosts. J. Parasitol. 86, 228–232.

Severini, F., Toma, L., Di Luca, M., Romi, R., 2009. Le zanzare italiane: generalità e identificazione e gli adulti (Diptera, Culicidae). Fragmenta Entomol. 41, 213–372.

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 (3), 507–544.

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, 627.