



Case Report

Incidental Finding of *Dirofilaria immitis* (Spirurida: Onchocercidae) Microfilariae in the Bone Marrow of a Dog with Mixed *Leishmania infantum*-*Dirofilaria immitis* Infection

Iliaria Lensi ¹, George Lubas ^{1,2} and Roberto Amerigo Papini ^{3,*}

¹ Clinica Veterinaria Colombo, VetPartners Italia, Lido di Camaiore, 55041 Lucca, Italy; lensi.ilaria@gmail.com (I.L.); george.lubas.vet@gmail.com (G.L.)

² Canine Leishmaniasis Working Group, Seravezza, 55047 Lucca, Italy

³ Department of Veterinary Sciences, University of Pisa, c/o Veterinary Teaching Hospital “Mario Modenato”, Via Livornese (Lato Monte) 1, San Piero a Grado, 56122 Pisa, Italy

* Correspondence: roberto.amerigo.papini@unipi.it

Simple Summary: Canine leishmaniasis and cardiopulmonary dirofilariasis are common parasitoses in dogs and are endemic in various regions of Italy. Although *Leishmania infantum* and *Dirofilaria immitis* have commonly been reported as single infections in dogs in Italy, mixed infection with both parasites is seldom reported. This case report describes a very rarely reported localization of *Dirofilaria immitis* microfilariae in the bone marrow of an adult dog, which was serologically and PCR-positive for *Leishmania infantum*. This is the second reported case of the localization of *D. immitis* microfilariae in the bone marrow of a *Leishmania*-infected dog.

Abstract: We report a rare and interesting case of mixed infection with *Leishmania infantum* and *Dirofilaria immitis* associated with the incidental finding of microfilariae in the bone marrow of a 9-year-old, intact, male Bullmastiff which was seropositive to *L. infantum*. Clinical signs showed progressive weakness, pale mucosae membranes, and a very low body condition score. Laboratory abnormalities included moderate, normocytic, normochromic, non-regenerative anemia; mild leukocytosis, neutrophilia, monocytosis, and eosinopenia; low platelet count; elevated C reactive protein; mild hyperkalemia, hypoalbuminemia, and hyperbeta-2-globulinemia; and a low A/G ratio. Hypoadrenocorticism, euthyroid sick syndrome, and alteration in the fibrinolytic phase of hemostasis were also detected. Microfilariae were incidentally found in bone marrow cytology aspirate in the absence of clinical features indicative of co-infection with *D. immitis*. PCR confirmed the identification of the *Dirofilaria* species. It is assumed that the microfilariae may have left the microcirculation and migrated to bone marrow tissues by crossing the vessel wall. To the best of our knowledge, only one such case has been previously reported in dogs.

Keywords: *Leishmania infantum*; *Dirofilaria immitis*; co-infection; microfilariae; dog; bone marrow



Citation: Lensi, I.; Lubas, G.; Papini, R.A. Incidental Finding of *Dirofilaria immitis* (Spirurida: Onchocercidae) Microfilariae in the Bone Marrow of a Dog with Mixed *Leishmania infantum*-*Dirofilaria immitis* Infection. *Zoonotic Dis.* **2023**, *3*, 162–175. <https://doi.org/10.3390/zoonoticdis3020013>

Academic Editor: Stephen Kenneth Wikel

Received: 9 March 2023

Revised: 2 May 2023

Accepted: 4 May 2023

Published: 9 May 2023



Copyright: © 2023 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<https://creativecommons.org/licenses/by/4.0/>).

1. Introduction

Leishmania infantum (Kinetoplastida: Trypanosomatidae) is a vector-borne flagellate protozoa. Amastigotes (the aflagellate form of the parasite) live and multiply within macrophages and other cells of the reticuloendothelial system of mammals as vertebrate hosts. Dogs are the main reservoir of *L. infantum*; however other species can be infected, such as cats [1], ferrets [2], and humans [3]. Livestock (sheep, goats, cattle, and donkeys) reared in outbreak areas [4], as well as wildlife [5], can be important sources of human leishmaniasis. Female sand flies (genera *Phlebotomus*, *Sergentomyia*, *Lutzomyia*) ingest amastigotes during the blood meal on an infected vertebrate host. In the intestinal tract of sand flies, amastigotes transform into promastigotes (the flagellate form of the parasites) and start to multiply. Promastigotes then move to the buccal apparatus of the insect vector

and are transmitted to a new vertebrate host with the next blood meal. Canine leishmaniasis (CanL) is generally considered to be a typical parasitosis of Mediterranean countries.

Heartworm disease (HWD) or cardiopulmonary dirofilariasis is a vector-borne disease in dogs caused by the filarial nematode *Dirofilaria immitis* (Spirurida: Onchocercidae) and transmitted by female mosquitoes (genera *Ochlerotatus*, *Culex*, *Aedes*, *Anopheles*, *Culiseta*) as intermediate hosts [6]. Adult heartworms typically live in the right ventricle and pulmonary arteries of the definitive hosts, including dogs, foxes, coyotes, jackals, and wolves [7–9]. Although definitive hosts are primarily canids, *D. immitis* does not show strict host specificity and patent infections can occasionally be found in other species such as domestic cats [10], ferrets [11], and even pinnipeds [12]. Wildlife can be a potential source of human infection with *D. immitis* [5]. The females and males are 25–31 and 12–20 cm in length with a diameter of 1–1.3 and 0.7–0.9 mm, respectively. The females are viviparous, and release circulating L1 larvae (so-called microfilariae) in the bloodstream of infected hosts. When uninfected mosquitoes bite an infected definitive host, they ingest microfilariae via the blood meal. In the insect vector, microfilariae develop by undergoing two molts up to the third larval stage (infective stage). When infected mosquitoes bite an uninfected definitive host for a blood meal, they inoculate L3 larvae. After inoculation, L3 larvae reach the right ventricle and pulmonary arteries of the new host via the bloodstream, where they develop and become adult and sexually mature heartworms. Accidentally, *D. immitis* L3 larvae can be transmitted to humans via the bite of infected mosquitoes, causing zoonotic infections [13–19].

Unusual localizations of *D. immitis* adult worms (i.e., other than the right ventricle and pulmonary arteries) have occasionally been reported in dogs. These include the brain [20], abdominal cavity [21], abdominal aorta [22], cardiac pulmonary lobes, blood vessels of the lung, bronchial tree, pericardiac sac [23], femoral arteries [24], left and right external and internal iliac arteries, left and right popliteal arteries, and testicular arteries [25]. In addition, rarely unusual localizations of *D. immitis* adult worms have also been reported in humans [13–17]. Similarly, the incidental finding of *D. immitis* microfilariae in uncommon sites (i.e., outside of the bloodstream) has also been reported in dogs, although to a very little extent [26–28].

D. immitis and *L. infantum* have commonly been detected as unassociated infections in surveys carried out in different canine populations, where their prevalence has been simultaneously investigated [29–33]. Mixed infection by both of these parasites has thus seldom been found in dogs [26,30,34].

In this report, we present a rare and interesting case of mixed infection with *L. infantum* and *D. immitis* associated with the incidental finding of *D. immitis* microfilariae in the bone marrow of an adult dog. To the best of our knowledge, only one such case has been previously reported [26].

2. Case Report

In April 2021, Kyle, a 9-year-old, intact, male Bullmastiff was referred to two receiving veterinarians (IL and GL) due to progressive weakness, pale mucosae membranes, and a body condition score of 2/9. The referring veterinarian provided the receiving veterinarians with all the appropriate information pertinent to the case before the time of the referral. The dog had been adopted from a shelter in Campania (Southern Italy) one year prior to presentation, and had since moved to Tuscany (Central Italy) where he had been in the same owner's possession, and where he was currently living. Nothing was known about the dog's lifestyle before arriving at the shelter. Upon arrival, the dog received core vaccines and deworming treatment with febantel/pyrantel/praziquantel. The prevention of vector-borne diseases such as CanL and HWD via the use of a permethrin-based spot-on product against sand fly and mosquito bites had rarely been administered. Similarly, HWD-specific prevention with oral administration of ivermectin at the standard dosage of 6 mcg/kg once a month had been patchy. Before referral to the receiving veterinarians, the referring veterinarian had performed extensive blood and urinary analyses, and the results of which

were as follows: The complete blood count (CBC) was performed using an automated hematology analyzer (Idexx ProCyte[®] Dx laser cell counter, Idexx Laboratories, Westbrook, ME, USA) and revealed moderate, normocytic, normochromic, non-regenerative anemia. The white blood cell differential count showed mild leukocytosis with neutrophilia, monocytosis, the occurrence of bands, and eosinopenia; the platelet count was low. The results of the CBC therefore suggested bicytopenia (Table 1).

Table 1. Complete blood count results found in an intact 9-year-old male Bullmastiff. The results refer to the history (Dh) and the admission to the referral unit (Drv).

| Parameters and Units | Reference Interval | Dh * | Drv ** |
|----------------------|--------------------|--------------|--|
| RBC M/mL | 5.65–8.87 | <u>3.84</u> | <u>3.64</u> |
| HCT % | 37.3–61.7 | <u>23.5</u> | <u>23.2</u> |
| HGB g/dL | 13.1–20.5 | <u>8.7</u> | <u>8.6</u> |
| MCV fL | 61.6–73.5 | 61.6 | 63.7 |
| MCH pg | 21.2–25.9 | 22.7 | 23.6 |
| MCHC g/dL | 32.0–37.9 | 37.0 | 37.1 |
| RDW % | 13.6–21.7 | 17.3 | 20.0 |
| Retics K/mL | 10.0–110.0 | 49.9 | 32.0 ^ |
| Retic-HGB pg | 22.3–29.6 | 28.8 | 17.5 |
| Notes on RBCs | | None | <u>Howell–Jolly bodies ++</u> |
| WBC K/mL | 5.05–16.76 | <u>28.58</u> | 14.8 |
| NEU seg K/mL | 3.7–11.9 | <u>24.73</u> | <u>12.28</u> |
| NEU band K/mL | 0.0–0.3 | <i>Flag</i> | 1.33 |
| EOS K/mL | 0.1–1.35 | <u>0.01</u> | 0.15 |
| BAS K/mL | 0.0–0.1 | 0.05 | 0.0 |
| LYM K/mL | 0.7–5.1 | 1.44 | <u>0.15</u> |
| MON K/mL | 0.2–1.5 | <u>2.35</u> | 0.89 |
| Notes on WBCs | None | None | <u>Toxic neutrophils +/-, activated monocytes ++</u> |
| PLT K/mL | 148–484 | <u>72</u> | <u>79</u> |
| MPV fL | 8.7–13.2 | 12.6 | <u>15.1</u> |
| PDW fL | 9.1–19.4 | ND | ND |
| PCT % | 0.14–0.46 | <u>0.09</u> | <u>0.12</u> |
| PLT estimate | Adequate | ND | <u>Inadequate</u> |
| Other notes | | None | One microfilaria in each of the two smears examined |

* Values only obtained via cell blood counter; ** values obtained via the blood cell counter and via examination of stained blood smears; the differential count of white blood cells was performed under a light microscope; ND—not determined; ^ the reticulocyte count estimation was also determined using light microscopy and was 25.48 K/mL. Underlining and italic are used to distinguish some results from others in Table 1 because the italicized and underlined results are outside the reference range. +/- and ++ are subjective frequency estimates of the hematological alterations that were observed in the blood smear, on a scale ranging from +/- to ++++.

The serum biochemical profile (Idexx Catalist One, Idexx Laboratories, Westbrook, ME, USA) was unremarkable and showed only a mild increase in urea (30 mg/dL, RI 7–27) and alkaline phosphatase (250 U/L, RI 23–212). In addition, elevated C reactive protein (39.9 mg/L, RI 0.0–1.5) was assessed and the electrolyte pattern (AU 5800, Beckman Coulter, Inc., Brea, CA, USA, with dedicated reagent kits) evidenced mild hyperkalemia (6.1 mEq/L, 3.5–5.5) with a Na/K ratio of 24.2 (RI > 27). The finding of an elevated C reactive protein (39.9 mg/L; RI 0–1.5) was in agreement with the results of the leukogram, showing the presence of an acute inflammatory disorder. Urinalysis of a catheter urine specimen was unremarkable with a specific gravity of 1.035, positivity for blood (50 RBC/mcL) in the reagent strip, and few erythrocytes in the urine sediment (VetLab UA and SediVue DX Idexx Laboratories, Westbrook, ME, USA). Serum protein electrophoresis (Capillarys Tera, Sebia, Evry Cedex, France) showed hypoalbuminemia (32.1%, RI 51–65), hyperalpha-2-globulinemia (20.6%, RI 7–15), and a low A/G ratio (0.47, RI 0.6–1.3) (Figure 1).

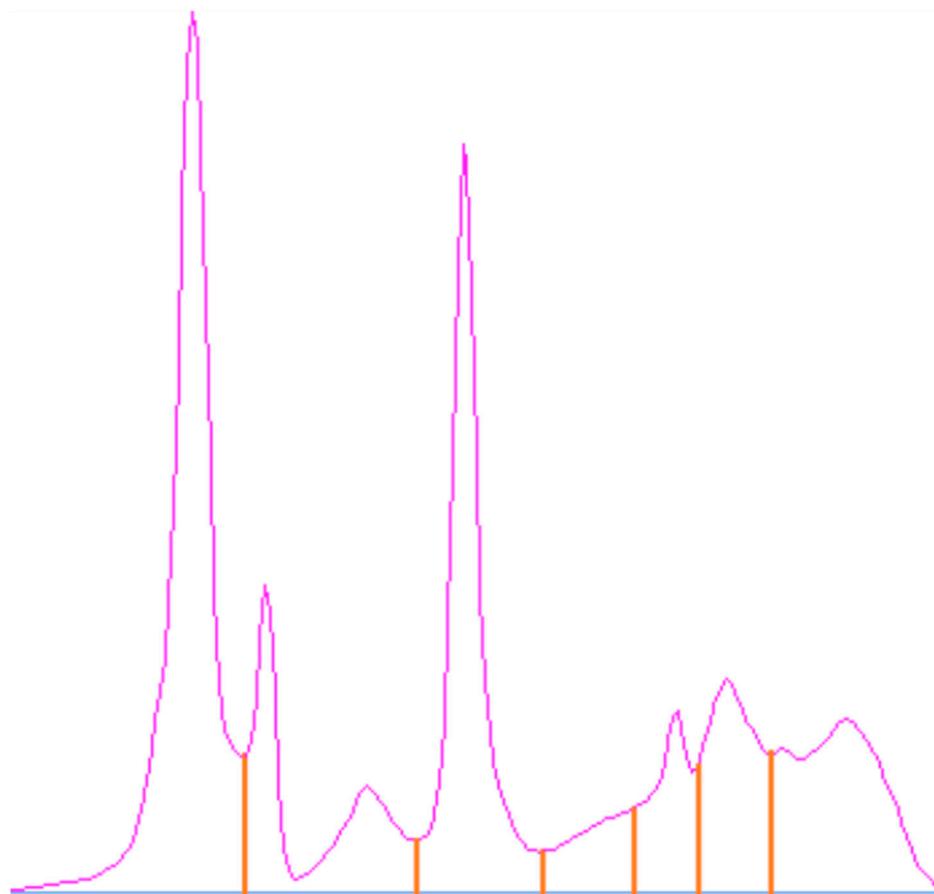


Figure 1. Serum protein electrophoretic graph in an intact 9-year-old male Bullmastiff with mixed *Leishmania infantum*-*Dirofilaria immitis* infection.

These additional data confirmed the presence of an acute inflammatory disorder. The ACTH stimulation test (Immunolyte 2000 xpi with dedicated reagent kit, Siemens Healthcare s.r.l, Milano, Italy) was performed due to clinical signs and electrolyte imbalance. The results showed a low baseline cortisol level ($<1.0 \mu\text{g/dL}$, RI 1–5) associated with a low post-ACTH cortisol level ($6.76 \mu\text{g/dL}$, RI 6–18), which therefore suggested hypoadrenocorticism. The thyroid profile was also investigated which included measurements of TSH, tT4, and fT4 (Immunolyte 2000 xpi with dedicated reagent kit, Siemens Healthcare s.r.l, Milano, Italy). The results evidenced only a slight decrease in fT4 (11.8 pmol/l , $11.9\text{--}24.5$), suggesting a condition characterized by euthyroid sick syndrome. A serum sample showed seropositivity for antibodies against *L. infantum* with an immunofluorescence antibody test (IFAT) titer of 1:320. The coagulation profile (BCS XP, Siemens Healthcare Diagnostics, Marburg, Germany, with dedicated reagent kits) showed a slight increase in aPTT (18.6s , RI $12.2\text{--}15.5$), a marked increase in FDPs ($24.4 \mu\text{g/mL}$, RI $0.01\text{--}2.7$), and a marked reduction in antithrombin (63% , RI $99\text{--}143$). These results therefore suggested an alteration in the fibrinolytic phase of hemostasis.

At the time of referral, all clinical signs reported by the referring veterinarian were confirmed, with a body temperature of $38.4 \text{ }^\circ\text{C}$, pulse of 78 bpm, respiratory rate of 18 pm, no heart murmur audible on auscultation, and no evidence of pain on abdominal palpation. As prior to the referral, automated CBC had been performed but stained blood smears were not examined and evaluated by a trained clinical pathologist despite the detection of bicytopenia (Table 1), the two receiving veterinarians (IL and GL) decided to perform a visual microscopic examination of blood smears and bone marrow aspirate to obtain a clearer picture of the blood cell abnormalities. The diagnostic plan was organized as follows.

A peripheral blood sample was collected in a K3-EDTA tube and used for initial blood count evaluation using an automated hematology analyzer (Idexx ProCyte[®] Dx laser cell counter, Idexx Laboratories, Westbrook, ME, USA). Cell counter parameters showed bicytopenia, as previously highlighted. A bone marrow harvest was then arranged. Bone marrow aspiration was performed at two sites. Initial attempts were made to aspirate bone marrow at the costochondral junction due to the large size of the dog breed. Some slides of the aspirate from this site were stained with May–Gründwald–Giemsa and scanned under a microscope at a low power magnification; however, the smears were highly hemodiluted with almost no hematopoietic spicules. Another bone marrow sample was then taken from the left iliac crest. Slides of the aspirate from this site were stained with May–Gründwald–Giemsa, scanned under a microscope at low power magnification, and yielded a fairly rich sample of hematopoietic cells and spicules. During the initial smear evaluation, performed to assess the quality of the bone marrow harvest, two microfilariae were unexpectedly observed on each slide, out of a total of eight slides. The remaining bone marrow aspirate was stored in a K3-EDTA vial for further testing. After bone marrow harvesting, the complete examination of a blood smear stained with May–Gründwald–Giemsa and New methylene blue (for the evaluation of reticulocytes) was performed. The blood smear examination revealed moderate, normocytic, normochromic, non-regenerative anemia, acute inflammatory leukogram with mildly toxic neutrophils and activated monocytes, and mild thrombocytopenia with few macro platelets. In addition, a microfilaria, showing morphological compatibility with *D. immitis* microfilariae, was observed on the feathered edge of each of two blood smears (Figure 2).

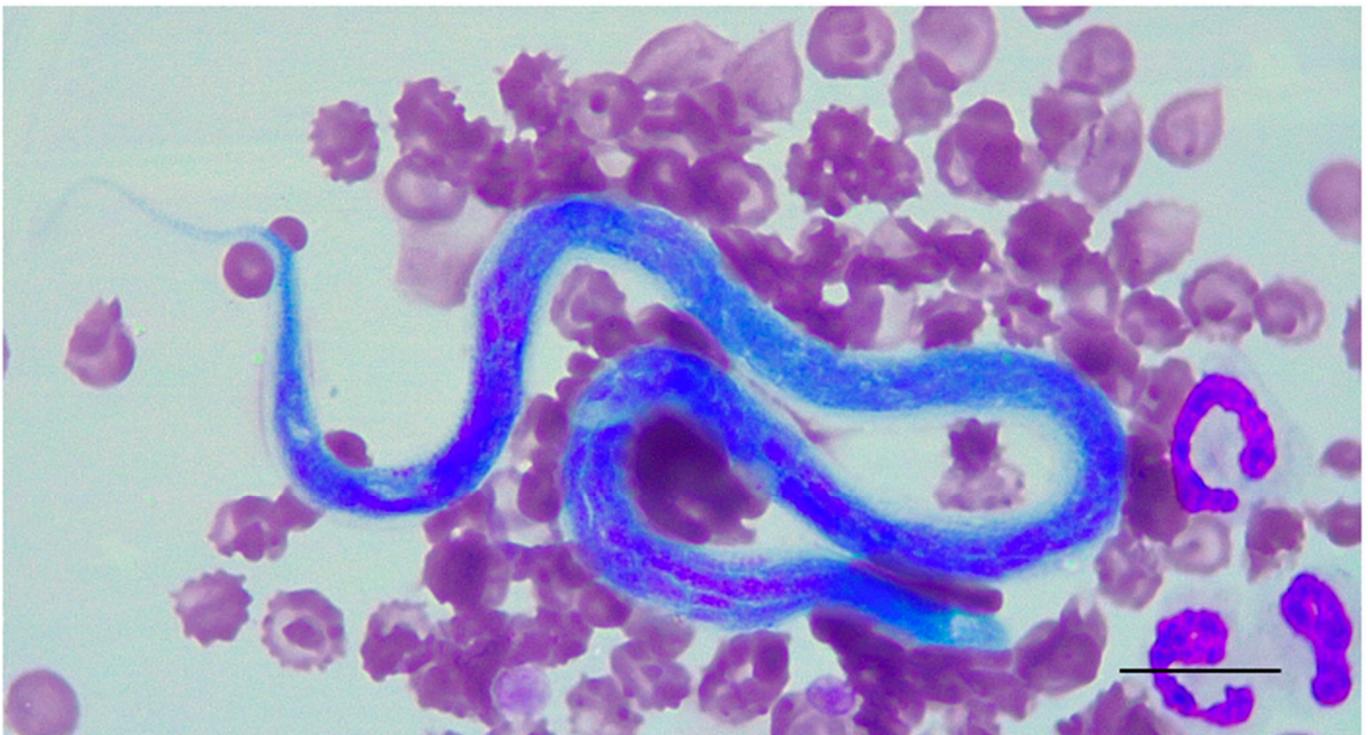


Figure 2. Microfilaria of *D. immitis* in a stained blood smear from an intact 9-year-old male Bullmastiff with mixed *Leishmania infantum*-*D. immitis* infection (May–Gründwald–Giemsa, 1000 \times , scale bar: 25 μ m).

A complete evaluation of the bone marrow aspirate slides was then arranged. Bone marrow aspirates, used for morphological evaluation in dogs, are usually obtained from the collection of some spicules, which are clusters of the various hematopoietic cells of the bone marrow, and of the blood flowing in the vascular network of the marrow. For this reason, samples inevitably contain at least a small amount of blood. However, high quality samples show only minimal hemodilution. It is of the utmost importance to evaluate

the quality of the specimens prior to cytological evaluation, with younger dogs having higher cellularity (hematopoietic cell to fat ratio > 75:25) and older dogs having lower cellularity (50:50 or smaller ratio) [35,36]. In our clinical experience (GL), the assessment of hemodilution is performed as a routine daily practice for accurate interpretation of bone marrow sample aspirates. In particular, in this case report, the bone marrow was successfully accessed, aspiration was performed correctly, and a thorough evaluation of the bone marrow aspirates was performed by a highly qualified and experienced clinical pathologist (GL). Based on his expertise, a high-quality sample was collected showing many bone marrow particles in the slides and adequate cellularity, estimated with respect to the patient's age. Overall, at low magnification (200×), an excessive presence of dispersed adipose tissue was promptly observed on all of the slides. The megakaryocytes were adequate and with euplasia. The myeloid and erythroid series showed normal proliferative sequences, normal mature sequences, and normal storage pools. The myeloid/erythroid ratio was around one. The other cells were represented by a striking infiltration of several macrophages, phagocytosing melanin-like or bilirubin-like bodies, and several plasma cells. An osteoblast was also observed. Very few microfilariae, similar to those previously seen in the initial bone marrow smear and in the stained blood smears, were detected again (Figure 3).

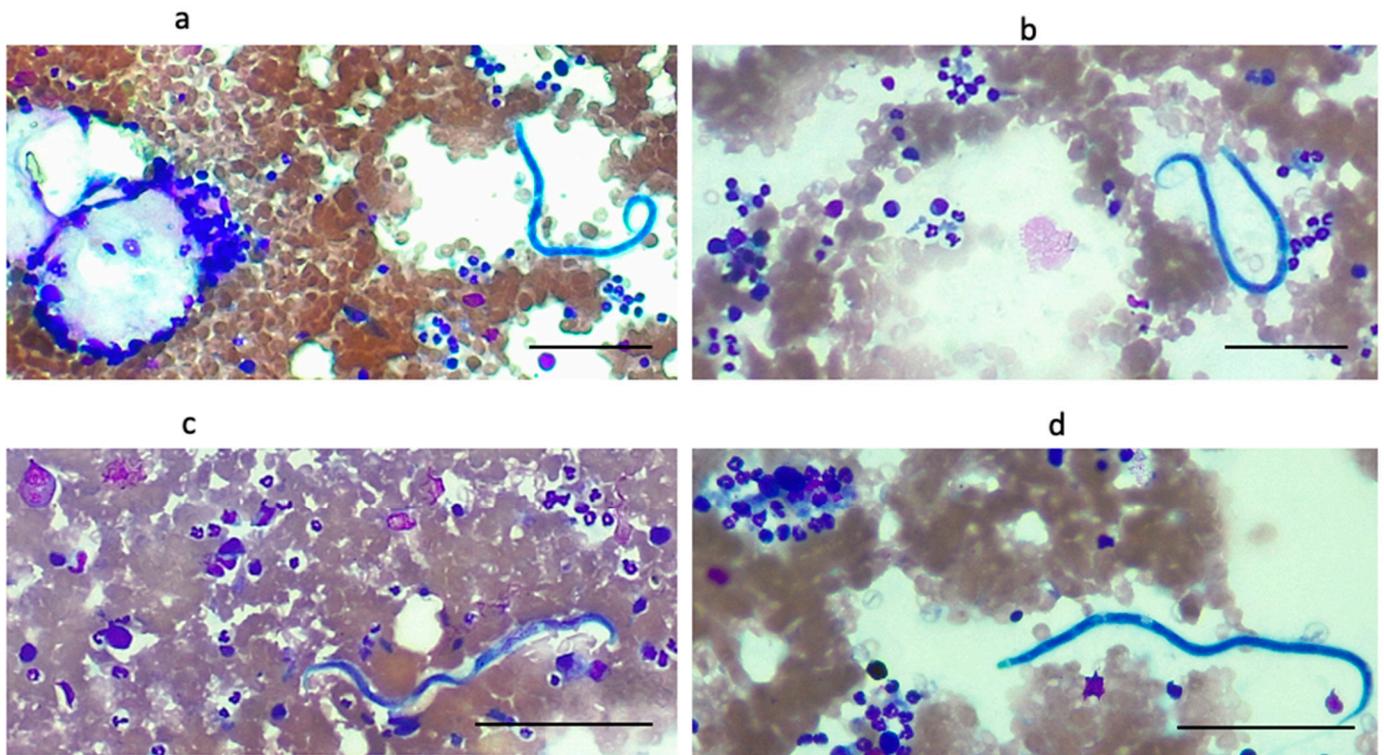


Figure 3. Microfilariae of *D. immitis* in bone marrow smears from an intact 9-year-old male Bullmastiff with mixed *L. infantum*-*D. immitis* infection. Microfilariae were detected in microscopic fields with fewer spicules and higher hemodilution (200×: (a,b); scale bar: 70 μm; 400×: (c,d); scale bar: 100 μm).

With the expressed permission of the dog owner, further diagnostic tests were performed. A rapid screening test (SNAP 4Dx Plus test, Idexx Laboratories, Westbrook, ME, USA) was performed for the detection of the *D. immitis* antigen and antibodies to *Anaplasma phagocytophilum*, *Anaplasma platys*, *Borrelia burgdorferi*, *Ehrlichia canis*, and *Ehrlichia ewingii*. The in-clinic assay tested positive for *D. immitis*. Molecular investigations for vector-borne diseases and filarial nematodes were also recommended. For this purpose, the residual sample of bone marrow, collected in a K3-EDTA vial, was sent to an external laboratory (Genefast srl, Forli, Italy). Two different sets of molecular investigations were conducted at the laboratory. The vector-borne disease set included the DNA detection of *Rickettsia* spp.

and *Ehrlichia canis* and real-time PCR for *Leishmania*. The filarial nematode set included DNA research for *D. immitis* and *Dirofilaria repens*. Real-time PCR for *Leishmania* was positive at the rate of 485.42 parasite/mL and *D. immitis* DNA was detected. A final diagnosis of mixed infection with *L. infantum* and *D. immitis* was given. The following etiological treatments were suggested: (I) leishmanicidal therapy with meglumine antimoniate at a dosage of approximately 95–100 mg/kg (subcutaneously) and allopurinol 15 mg/kg per os subdivided into 2 times a day; and (II) microfilaricide therapy with ivermectin at a dosage of approximately 10 mcg/kg PO every day for 6–8 months and treatment with doxycycline at 10 mg/kg PO once a day for 4 weeks. Supportive treatment was also suggested (initial treatment with fluid therapy, immunomodulatory therapy, and complex B vitamins).

Once the referral had been completed, the receiving veterinarians promptly informed the referring veterinarian about the diagnosis and proposed treatments with a detailed and complete written report. As the referring veterinarian communicated regularly and collaborated closely with the receiving veterinarians about the progression and decisions of the case, we were informed that unfortunately the owner did not agree to the treatments due to the high cost involved and the case was lost.

3. Discussion

Leishmania infantum and *D. immitis* are transmitted from dog to dog via the blood meal of two different groups of insect vectors. *Leishmania infantum* is spread by the bites of infected phlebotomine sand flies, while *D. immitis* is spread by the bites of infected mosquitoes. Both sand flies and mosquitoes are nocturnal biting insects and behave differently according to the season. However, for their development and life cycle, they need different habitats with very different environmental conditions. Phlebotomines require areas with cool, moist soil that is rich in organic matter for larval growth and with many hiding places to rest in the dark during the day, such as rock crevices, caves, rodent burrows, corners of animal shelters, or human dwellings [37]. Conversely, mosquitoes require warm, humid environments such as sheds, bathrooms, and areas with tall grass or plant shade during the day. In addition, any place with standing water enables the larvae to grow, such as lakes, ponds, swamps, puddles, water-filled holes, and hollows in tree trunks and branches, as well as artificial water containers such as buckets, flowerpots, tires, or any other abandoned object that holds water. The profoundly different characteristics of the habitats that are suitable for the life cycle of the insect hosts may explain why the two vector-borne parasitoses have rarely been associated in dogs [26,30,34]. This is because dogs may be exposed to the risk of bites from infected sand flies or from infected mosquitoes depending on the environmental conditions of the place where they live. It is likely that dogs with mixed infections lived for a period of time in an area endemic for *L. infantum* and for another period in an area endemic for *D. immitis*, thus moving from one to the other. For example, in Campania and Tuscany, both *L. infantum* [38,39] and *D. immitis* [40,41] have been reported in dog populations from different areas and with different levels of endemicity. Mixed infections with the two parasites are therefore more likely to occur in dogs living in these regions or moving from one to the other. *Leishmania infantum* and *D. immitis* are cosmopolitan parasites and their geographical distribution is linked to habitat conditions that are suitable for the development of a wide range of sand fly or mosquito species that are responsible for their transmission, respectively. In Italy, prevalence values of 21.6–29.6% for *L. infantum* [31] and 2.83–7.75% [31] or 44.2% for *D. immitis* [42] have been reported in different dog populations. In the last few decades, due to climate change and global warming, the geographical ranges of some sand fly [43–47] and mosquito species [48–51] have expanded and, as a consequence, *L. infantum* [43–47] and *D. immitis* [51–53] have, in turn, progressively spread to new non-endemic areas.

CanL can be asymptomatic or show a wide range of unspecific clinical signs and laboratory abnormalities. These include weight loss, exercise intolerance, lethargy, epistaxis, lymphadenomegaly, splenomegaly, onychogryphosis, localized or generalized exfoliative dermatitis, cutaneous ulcers, hyperkeratosis, keratoconjunctivitis, uveitis, lameness due to

joint injuries, and polyuria/polydipsia due to kidney involvement [37,45]. If left untreated, it can lead to death mostly because of severe kidney damage [37]. Similarly, many cases of HWD in dogs are asymptomatic; however, the disease can cause serious clinical signs including cough, dyspnea, weight loss, poor exercise tolerance, weakness, hemoptysis, and cyanosis, as well as right congestive heart failure and caval syndrome [54,55]. In this case report, the co-infected dog had non-specific clinical signs that were neither indicative of CanL nor HWD. The laboratory abnormalities observed were moderate, normocytic, normochromic, non-regenerative anemia, acute inflammatory leukogram, mild thrombocytopenia, and clear signs of inflammation (as demonstrated by the C-reactive protein level and serum protein electrophoresis). All of these abnormalities are described in both HWD and CanL [37,45,56]. Electrolyte imbalance with a Na/K ratio less than 27 prompted us to carry out the ACTH stimulation test and the results indicated secondary hypoadrenocorticism, while the thyroid panel results indicated euthyroid sick syndrome. Neither secondary endocrine disorder was attributed to co-infection with *Dirofilaria* and *Leishmania*. To the authors' knowledge, only infection with the intestinal nematode *Trichuris vulpis* has been reported as a possible parasitic cause of pseudohypoadrenocorticism [57]. In a retrospective study evaluating thyroid function before and during treatment in 36 dogs with different stages of CanL, hypothyroidism did not appear to be an important predisposing factor or a frequent complication of the disease [58]. Conversely, to our knowledge, the associations between hypoadrenocorticism and CanL or HWD as well as between thyroid function and HWD in dogs have not yet been studied. Co-infection with *Toxoplasma gondii*, *Ehrlichia* spp., and *Anaplasma* spp. was not shown to complicate clinical signs in *L. infantum*-infected dogs [32]. However, increased severity of clinical signs has been reported in dogs when co-infection with *L. infantum* and filarial nematodes, including *D. immitis*, was detected [59]. No treatment for the diagnosed secondary endocrine disorders was prescribed as further specialist investigations would have been required. This was beyond the particular condition we were asked to diagnose and treat in the received referral request. Therefore, we let the attending veterinarian propose a diagnostic plan and treatment for the two identified endocrine disorders.

The diagnosis of CanL mainly relies on several serological techniques that determine antibody levels, PCR-based tests to detect and amplify *Leishmania* DNA in blood and tissues, and the direct visualization of amastigotes via bone marrow cytology [60]. In veterinary medicine, the collection of bone marrow samples is advisable for diagnosing and monitoring CanL [61] and mostly for the diagnosis of hematologic disorders [62]. It is therefore not recommended as an adequate diagnostic tool for HWD in dogs. The diagnosis of HWD commonly relies on the detection of circulating microfilariae in blood smears. Thereafter, *D. immitis* microfilariae should be morphologically differentiated from those of other filarial nematodes, such as *D. repens* (inhabiting subcutaneous tissues) and *Achantocheilonema* spp. (inhabiting subcutaneous tissues and visceral organs of the peritoneal and thoracic cavities), via Knott's test based on the size and shape of the microfilariae [63]. Nonetheless, the detection of circulating microfilariae can be complicated since some cases of occult HWD (amicrofilaremic form) may occur [64]. Other diagnostic procedures include circulating antigen detection via ELISA or rapid in-clinic tests [42,65] and molecular techniques [66]. In this case report, CanL was promptly diagnosed via serology using the IFAT but co-infection with *D. immitis* was not suspected and remained undiagnosed until the first bone marrow aspirate cytology was performed. Dirofilariasis was then confirmed via the detection of a circulating *D. immitis* antigen using a commercial kit and PCR. Molecular tools should be considered as the preferred diagnostic techniques for the diagnosis of dirofilariasis, as they allow for direct identification at the species level [66].

The localization of *D. immitis* microfilariae in the bone marrow is not a specific manifestation of HWD in dogs and appears to be extremely rare. Only one isolated case of this atypical localization has been previously reported to date. Fifty-five years ago, Bossie [26] described the incidental finding of *D. immitis* microfilaria in a May-Grünwald-Giemsastained smear from the bone marrow of an apparently healthy wolfhound, detected during

an epidemiological study on canine leishmaniasis in areas located along the Danube river in Romania. Our case report is strikingly similar to the one reported in 1968. In fact, both in the previous case and in our case, microfilariae were picked up by chance and cardiopulmonary dirofilariasis was diagnosed based on the occurrence of microfilariae in the bone marrow aspirate. In our case, *D. immitis* was subsequently also highlighted via a stained blood smear and PCR on a bone marrow sample. Similarly, in a previous case of co-infection with *L. infantum* and *D. repens* reported in Italy, circulating microfilariae were first identified via a qualitatively modified Knott test and subsequently subcutaneous dirofilariasis was confirmed via PCR of blood and skin biopsy of an ear lesion [67]. Interestingly, some cases of infection with *Wuchereria bancrofti* (a filarial nematode inhabiting the lymphatic system in humans) report that microfilariae were present in the bone marrow aspirates but not in the peripheral blood [68,69]. In our case report, as previously mentioned, the presence of circulating *D. immitis* microfilariae was also detected via examining stained blood smears, although Knott's test, used for the concentration and identification of microfilariae, was not performed. We can therefore assume that the level of microfilaremia was quite high.

The number of published reports on uncommon localizations of *D. immitis* microfilariae in dogs is limited. *D. immitis* microfilariae were found during urine analysis in the urine sediment of a 6-year-old, mixed-breed, male dog in Thailand, showing typical features of HWD in clinical, radiographic, and laboratory examinations [27]. In the latter case, microfilaruria was attributed to inflammation or hemorrhage of the lower urinary tract and/or to glomerulonephritis. In another case of microfilaruria, *D. immitis* microfilariae were found via cytology of the abdominal fluid and urine analysis of a symptomatic 6-year-old intact female Labrador in Brazil [28]. Microfilariae were also detected in the bronchial and bronchiolar lumen, interalveolar region of the lungs, alveoli, and bile ducts at histopathological examination in one of four dogs with serious HWD which died spontaneously during hospitalization and showed ectopic localizations of adult worms in necropsy [23]. Therefore, in contrast to previous reports on the uncommon localization of *D. immitis* microfilariae in dogs, neither the present case nor the previously published case focusing on bone marrow localization had classical clinical manifestations of HWD. Unlike the microfilariae of *D. immitis*, the microfilariae of *W. bancrofti* have frequently been reported in cytologic smears. These have not only included bone marrow aspirates [68–70] but also diagnostic biopsies from lymph nodes, subcutis, soft tissues, thyroid, salivary glands, breast, pleural or pericardial fluid, lung, ascitic fluid, liver, hernial sac, testicles, scrotum, vaginal fluid, uterine cervix, ovaries, and tibia performed via fine needle aspiration cytology [71–76]. This suggests that *D. immitis* microfilariae have a much lower ability than *W. bancrofti* microfilariae to reach the extravascular space. However, any definitive conclusion is precluded.

Therapy options for CanL should be based on the disease stage and may include allopurinol alone or in combination with antimonials and miltefosine [60]. For the treatment of serious HWD cases, first the dog's clinical conditions need to be stabilized, if necessary, with appropriate therapy before the specific therapeutic regimen is started. Melarsomine (an arsenical compound) is the only approved drug commercially available against adult heartworms in dogs. According to the guidelines of the American Heartworm Society, the treatment requires the administration of a series of different drugs given at subsequent times. These include macrocyclic lactone as microfilaricidal, doxycycline as antibiotic against *Wolbachia* bacteria (endosymbionts of *D. immitis*), multiple injections of melarsomine as adulticidal, and prednisone (or prednisolone) to reduce the risk of pulmonary thromboembolism and pulmonary hypertension induced by heartworms dying after the administration of melarsomine [77]. Either mechanical or surgical heartworm removal may be indicated in severe cases [77]. Therefore, the specific treatments of both CanL and HWD are complex, time-consuming, and expensive. Both require tremendous compliance on the part of the dog's owner. In this case report, daily oral administration of allopurinol alone for the treatment of CanL and ivermectin as microfilaricide plus doxycycline for the treatment of HWD were prescribed for several months. However, because of the high costs

and time involved, unfortunately the owner did not agree to the two treatments and the case was lost.

Preventive measures against CanL and HWD include limiting exposure to sand flies and mosquitoes as well as the regular and appropriate use of topical repellents/insecticides (impregnated collars, spot-on and spray formulations), thus blocking transmission from infected insect vectors to dogs. To date, the most effective way to control CanL consists of the application of topical pyrethroids in association with a commercially approved vaccine that does not interfere with the most widely used serological diagnostic tests [78]. On the other hand, the most effective preventive measure against HWD consists of the topical application of a repellent/insecticide combined with the oral, topical, or parenteral administration of a macrocyclic lactone (ivermectin, milbemycin oxime, moxidectin, or selamectin) at scheduled time intervals, blocking the development from the larval stages to adult worms [77]. Asymptomatic infection with *L. infantum* or *D. immitis* in dogs can often go undiagnosed for a long time. Infected dogs, as long as they are left untreated, represent a potential source of infection to other dogs and other susceptible hosts such as cats [1,10] and ferrets [2,11]. Moreover, both CanL and HWD are potentially zoonotic parasitoses. *L. infantum* is a causative agent of visceral leishmaniasis in humans with clinical manifestations of fever, anemia, weight loss, splenomegaly, hepatomegaly, and even death, if left untreated; however, a large number of cases may be asymptomatic [3]. Humans are not specific hosts for *D. immitis* to develop to the adult stage. As a consequence, infective larvae usually die after inoculation in humans and dead larvae tend to embolize in pulmonary vessels. This results in scattered pulmonary nodules (so-called “coin lesions”), mimicking other inflammatory or neoplastic pathologies during imaging workup [18]. In some cases, a diagnostic thoracotomy is required [19]. Despite this, the incidental finding of adult worms in the heart and large vessels as well as in deep inner organs, such as the liver, spleen, uterus, abdominal cavity, and spermatic cord, has occasionally been reported in humans [13–17].

It is of the utmost importance to underline that, according to the integrated One Health approach, human health, animal health, and safe ecosystems are closely connected and interdependent, especially with reference to zoonoses. In this regard, the routine analysis of clinical case data in hospitalized animals can provide a lot of information for the creation of communicable disease surveillance systems, as highlighted in some articles [79,80]. Furthermore, new technologies can be of great help in understanding the spread of zoonotic diseases and the importance of this type of prevention for public health [81].

4. Conclusions

The present case report shows that *D. immitis* microfilariae can be found in the bone marrow of dogs, in particular in dogs with mixed *L. infantum*-*D. immitis* infection. To the best of our knowledge, this is an extremely rare finding in canine HWD. At present, very little is known about the mechanism by which *D. immitis* microfilariae reach the bone marrow. In an experimental study on the relationship between the number of circulating microfilariae and the total population of microfilariae in a host, Pacheco [82] speculated that a major proportion of *D. immitis* microfilariae are sequestered in capillaries, or possibly in the tissues in extravascular sites, and have ready access to the circulation. Microfilariae have been observed in the glomerular capillaries and the medullary vessels of Beagle dogs experimentally inoculated with 200 3rd-stage larvae of *D. immitis* [83]. Therefore, there is some evidence that *D. immitis* microfilariae can reach the microcirculation in organs and tissues moving from the peripheral circulation in dogs. Based on cases of bone marrow localization of *W. bancrofti* microfilariae in humans [68–70], it is likely that microfilariae have the ability to cross the unbroken wall of blood capillaries located in the extravascular space, probably by piercing the vascular wall. As a consequence, they can then be picked up accidentally during aspiration from various sites, for example, from the bone marrow, as in this case. Bone marrow fat has been negatively correlated with heartworm burden

in free-ranging coyotes (*Canis latrans*) naturally infected by *D. immitis* [84]. Cases of bone marrow hypoplasia associated with the finding of *W. bancrofti* microfilariae in the marrow have been reported in humans [85]. Further investigations would therefore be of interest to determine whether chronic bone marrow localization of *D. immitis* microfilariae could affect blood cell production and fat storage in the bone marrow of heartworm-infected dogs. It would also be interesting to investigate whether dogs with mixed *L. infantum*-*D. immitis* infection are more likely to have a localization of microfilariae in the bone marrow.

Author Contributions: Conceptualization, I.L. and G.L.; methodology, I.L. and G.L.; investigation, I.L. and G.L.; resources, I.L. and G.L.; data curation, G.L.; writing—original draft preparation, R.A.P. and G.L.; writing—review and editing, R.A.P.; visualization, G.L.; supervision, R.A.P. and G.L. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Ethical review and approval were waived for this study because this is not an experimental study but the clinical report of a spontaneously occurring mixed infection. The client-owned dog did not receive any treatment with drugs during the clinical and laboratory examinations. Therefore, there were no inherent risks that the owners needed to be aware of.

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The raw data from this case report are available upon request to George Lubas.

Conflicts of Interest: The authors declare no conflict of interest.

References

- Pereira, A.; Maia, C. *Leishmania* infection in cats and feline leishmaniosis: An updated review with a proposal of a diagnosis algorithm and prevention guidelines. *Curr. Res. Parasitol. Vector Borne Dis.* **2021**, *1*, 100035. [[CrossRef](#)] [[PubMed](#)]
- Alcover, M.M.; Giner, J.; Rabasedas, J.; Roca-Geronés, X.; Verde, M.; Fernández, A.; Riera, C.; Fisa, R.; Villanueva-Saz, S. First epidemiological survey of *Leishmania infantum* in the domestic ferret (*Mustela putorius furo*) in a canine leishmaniosis endemic area using serology and PCR. *Parasit. Vectors* **2022**, *15*, 372. [[CrossRef](#)] [[PubMed](#)]
- Picard, M.; Soundaramourty, C.; Silvestre, R.; Estaquier, J.; André, S. *Leishmania infantum* infection of primary human myeloid cells. *Microorganisms* **2022**, *10*, 1243. [[CrossRef](#)] [[PubMed](#)]
- Gao, C.H.; Wang, J.Y.; Zhang, S.; Yang, Y.T.; Wang, Y. Survey of wild and domestic mammals for infection with *Leishmania infantum* following an outbreak of desert zoonotic visceral leishmaniasis in Jiashi, People's Republic of China. *PLoS ONE* **2015**, *10*, e0132493. [[CrossRef](#)]
- Polley, L. Navigating parasite webs and parasite flow: Emerging and re-emerging parasitic zoonoses of wildlife origin. *Int. J. Parasitol.* **2005**, *35*, 1279–1294. [[CrossRef](#)]
- Demirci, B.; Bedir, H.; Taskin Tasci, G.; Vatansver, Z. Potential mosquito vectors of *Dirofilaria immitis* and *Dirofilaira repens* (Spirurida: Onchocercidae) in Aras Valley, Turkey. *J. Med. Entomol.* **2021**, *58*, 906–912. [[CrossRef](#)]
- Tolnai, Z.; Széll, Z.; Sproch, Á.; Szeredi, L.; Sréter, T. *Dirofilaria immitis*: An emerging parasite in dogs, red foxes and golden jackals in Hungary. *Vet. Parasitol.* **2014**, *203*, 339–342. [[CrossRef](#)]
- Gomes-de-Sá, S.; Santos-Silva, S.; Moreira, A.S.; Barradas, P.F.; Amorim, I.; Cardoso, L.; Mesquita, J.R. *Dirofilaria immitis* antigenemia and microfilaremia in Iberian wolves and red foxes from Portugal. *Parasit. Vectors* **2022**, *15*, 119. [[CrossRef](#)]
- Sobotyk, C.; Nguyen, N.; Negrón, V.; Varner, A.; Saleh, M.N.; Hilton, C.; Tomeček, J.M.; Esteve-Gasent, M.D.; Verocai, G.G. Detection of *Dirofilaria immitis* via integrated serological and molecular analyses in coyotes from Texas, United States. *Int. J. Parasitol. Parasites Wildl.* **2022**, *18*, 20–24. [[CrossRef](#)]
- Tonev, A.S.; Kirkova, Z.; Iliev, P.T.; Roussenov, A.; Chaprazov, T.; Roydev, R.; Pirovski, N. Clinical case of life-threatening co-infection due to *Dirofilaria immitis* and *Aelurostrongylus abstrusus* in a cat: First report of feline heartworm disease in Bulgaria. *Helminthologia* **2021**, *58*, 106–114. [[CrossRef](#)]
- Molnár, V.; Pazár, P.; Rigó, D.; Máthé, D.; Fok, E.; Glávits, R.; Vajdovich, P.; Jacsó, O.; Balogh, L.; Sós, E. Autochthonous *Dirofilaria immitis* infection in a ferret with aberrant larval migration in Europe. *J. Small Anim. Pract.* **2010**, *51*, 393–396. [[CrossRef](#)] [[PubMed](#)]
- Alho, A.M.; Marcelino, I.; Colella, V.; Flanagan, C.; Silva, N.; Correia, J.J.; Latrofa, M.S.; Otranto, D.; Madeira de Carvalho, L. *Dirofilaria immitis* in pinnipeds and a new host record. *Parasit. Vectors* **2017**, *10*, 142. [[CrossRef](#)] [[PubMed](#)]
- Selvachandran, A.; Foley, R.J. Subcutaneous and pulmonary dirofilariasis with evidence of splenic involvement. *Case Rep. Pulmonol.* **2016**, *2016*, 8212387. [[CrossRef](#)] [[PubMed](#)]
- Kim, M.K.; Kim, C.H.; Yeom, B.W.; Park, S.H.; Choi, S.Y.; Choi, J.S. The first human case of hepatic dirofilariasis. *J. Korean Med. Sci.* **2002**, *17*, 686–690. [[CrossRef](#)] [[PubMed](#)]
- Akao, N. Human dirofilariasis in Japan. *Trop. Med. Health* **2011**, *39*, 65–71.

16. Požgain, Z.; Dulić, G.; Segó, K.; Blažeković, R. Live *Dirofilaria immitis* found during coronary artery bypass grafting procedure. *Eur. J. Cardiothorac. Surg.* **2014**, *46*, 134–136. [[CrossRef](#)] [[PubMed](#)]
17. Theis, J.H.; Gilson, A.; Simon, G.E.; Bradshaw, B.; Clark, D. Case report: Unusual location of *Dirofilaria immitis* in a 28-year-old man necessitates orchiectomy. *Am. J. Trop. Med. Hyg.* **2001**, *64*, 317–322. [[CrossRef](#)]
18. Malik, D.; Amaraneni, A.; Singh, S.; Roach, R. Man's best friend: How humans can develop *Dirofilaria immitis* infections. *IDCases* **2016**, *4*, 43–45. [[CrossRef](#)]
19. Merrill, J.R.; Otis, J.; Logan, W.D., Jr.; Davis, M.B. The dog heartworm (*Dirofilaria immitis*) in man. An epidemic pending or in progress? *JAMA* **1980**, *243*, 1066–1068. [[CrossRef](#)]
20. Hamir, A.N. Heartworm (*Dirofilaria immitis*) in the brain of a dog. *Vet. Rec.* **1987**, *120*, 207–208. [[CrossRef](#)]
21. Oh, H.W.; Jun, H.K.; You, M.J.; Hayasaki, M.; Song, K.H. Ectopic migration of an adult heartworm in a dog with dirofilariasis (case report). *Korean J. Parasitol.* **2008**, *46*, 171–173. [[CrossRef](#)] [[PubMed](#)]
22. Grimes, J.A.; Scott, K.D.; Edwards, J.F. Aberrant heartworm migration to the abdominal aorta and systemic arteriolitis in a dog presenting with vomiting and hemorrhagic diarrhea. *Can. Vet. J.* **2016**, *57*, 76–79. [[PubMed](#)]
23. Sevimli, F.K.; Kozan, E.; Bülbül, A.; Birdane, F.M.; Köse, M.; Sevimli, A. *Dirofilaria immitis* infection in dogs: Unusually located and unusual findings. *Parasitol. Res.* **2007**, *101*, 1487–1494. [[CrossRef](#)] [[PubMed](#)]
24. Upchurch, D.A.; Ogden, D.M.; Baker, D.G. Bilateral femoral arterial dirofilariasis caused by *Dirofilaria immitis* in a dog. *Vet. Rec. Case Rep.* **2015**, *3*, e000184. [[CrossRef](#)]
25. Liu, S.K.; Das, K.M.; Tashjian, R.J. Adult *Dirofilaria immitis* in the arterial system of a dog. *J. Am. Vet. Med. Assoc.* **1966**, *148*, 1501–1507.
26. Bossie, A. Microfilarie de *Dirofilaria immitis* dans la moelle osseuse d'un chien. *Arch. Roum. Pathol. Exp. Microbiol.* **1968**, *27*, 651–654.
27. Kaewthamasorn, M.; Assarasakorn, S.; Niwetpathomwat, A. Microfilaruria caused by canine dirofilariasis (*Dirofilaria immitis*): An unusual clinical presence. *Comp. Clin. Pathol.* **2008**, *17*, 61–65. [[CrossRef](#)]
28. Monobe, M.M.; da Silva, R.C.; Araujo Junior, J.P.; Takahira, R.K. Microfilaruria by *Dirofilaria immitis* in a dog: A rare clinical pathological finding. *J. Parasit. Dis.* **2017**, *41*, 805–808. [[CrossRef](#)]
29. Savic, S.; Vidic, B.; Grgic, Z.; Petrovic, T.; Potkonjak, A.; Cupina, A.; Vaselek, S.; Petric, D. Dirofilariosis and leishmaniasis in the Northern region of Serbia. In *An Overview of Tropical Diseases*; Amidou, S., Ed.; IntechOpen: Rijeka, Croatia, 2015; Chapter 6, pp. 107–125. [[CrossRef](#)]
30. Ntais, P.; Christodoulou, V.; Dokianakis, E.; Antoniou, M. *Leishmania infantum* and *Dirofilaria immitis* coinfection in dogs in Greece. *Parasitol. Open* **2016**, *2*, e17. [[CrossRef](#)]
31. Mendoza-Roldan, J.; Benelli, G.; Panarese, R.; Iatta, R.; Furlanello, T.; Beugnet, F.; Zatelli, A.; Otranto, D. *Leishmania infantum* and *Dirofilaria immitis* infections in Italy, 2009–2019: Changing distribution patterns. *Parasit. Vectors* **2020**, *13*, 193. [[CrossRef](#)]
32. Oliveira, V.D.C.; Junior, A.A.V.M.; Ferreira, L.C.; Calvet, T.M.Q.; Dos Santos, S.A.; Figueiredo, F.B.; Campos, M.P.; Rodrigues, F.D.C.C.; de Oliveira, R.V.C.; de Lemos, E.R.S.; et al. Frequency of co-seropositivities for certain pathogens and their relationship with clinical and histopathological changes and parasite load in dogs infected with *Leishmania infantum*. *PLoS ONE* **2021**, *16*, e0247560. [[CrossRef](#)]
33. Tasić-Otašević, S.; Savić, S.; Jurhar-Pavlova, M.; Stefanovska, J.; Stalević, M.; Ignjatović, A.; Randelović, M.; Gajić, B.; Cvetković, A.; Gabrielli, S. Molecular survey of *Dirofilaria* and *Leishmania* species in dogs from Central Balkan. *Animals* **2022**, *12*, 911. [[CrossRef](#)]
34. Alves Rodrigues, R.T.G.; Moreira Borges, O.M.; Pereira Dantas, A.K.F.; Tôres, L.M.; Lucena, R.D.S.; Souza, A.P. Presença de *Leishmania* sp. e *Dirofilaria immitis* em tumor venéreo transmissível canino cutâneo. *Acta Sci. Vet.* **2019**, *47*, 399. [[CrossRef](#)]
35. Harvey, J.W. Bone marrow examination. In *Veterinary Hematology. A Diagnostic Guide and Color Atlas*, 1st ed.; Harvey, J.W., Ed.; Elsevier Saunders: St. Louis, MO, USA, 2012; pp. 234–259. [[CrossRef](#)]
36. Bauer, N.B.; Harr, K.E. Bone marrow evaluation. In *Schalm's Veterinary Hematology*, 7th ed.; Brooks, M.B., Harr, K.E., Seelig, D.M., Wardrop, K.J., Weiss, D.J., Eds.; Wiley-Blackwell: Hoboken, NJ, USA, 2022; pp. 1285–1294. [[CrossRef](#)]
37. Solano-Gallego, L.; Miró, G.; Koutinas, A.; Cardoso, L.; Pennisi, M.G.; Ferrer, L.; Bourdeau, P.; Oliva, G.; Baneth, G. LeishVet guidelines for the practical management of canine leishmaniosis. *Parasit. Vectors* **2011**, *4*, 86. [[CrossRef](#)]
38. Gradoni, L.; Pozio, E.; Bettini, S.; Gramiccia, M. Leishmaniasis in Tuscany (Italy). (III) The prevalence of canine leishmaniasis in two foci of Grosseto Province. *Trans. R. Soc. Trop. Med. Hyg.* **1980**, *74*, 421–422. [[CrossRef](#)]
39. Piantedosi, D.; Veneziano, V.; Di Muccio, T.; Manzillo, V.F.; Fiorentino, E.; Scalone, A.; Neola, B.; Di Prisco, F.; D'Alessio, N.; Gradoni, L.; et al. Epidemiological survey on *Leishmania* infection in red foxes (*Vulpes vulpes*) and hunting dogs sharing the same rural area in Southern Italy. *Acta Parasitol.* **2016**, *61*, 769–775. [[CrossRef](#)] [[PubMed](#)]
40. Santoro, M.; Miletti, G.; Vangone, L.; Spadari, L.; Reccia, S.; Fusco, G. Heartworm disease (*Dirofilaria immitis*) in two roaming dogs from the urban area of Castel Volturno, Southern Italy. *Front. Vet. Sci.* **2019**, *6*, 270. [[CrossRef](#)] [[PubMed](#)]
41. Macchioni, F.; Sed, G.; Cecchi, F. Canine filarial infections in an area of Central Italy (Tuscany-Latium border) historically free from the disease. *Vet. Parasitol. Reg. Stud. Rep.* **2020**, *20*, 100404. [[CrossRef](#)]
42. Panarese, R.; Iatta, R.; Latrofa, M.S.; Zatelli, A.; Čupina, A.I.; Montarsi, F.; Pombi, M.; Mendoza-Roldan, J.A.; Beugnet, F.; Otranto, D. Hyperendemic *Dirofilaria immitis* infection in a sheltered dog population: An expanding threat in the Mediterranean region. *Int. J. Parasitol.* **2020**, *50*, 555–559. [[CrossRef](#)] [[PubMed](#)]

43. Naucke, T.J.; Menn, B.; Massberg, D.; Lorentz, S. Sandflies and leishmaniasis in Germany. *Parasitol. Res.* **2008**, *103*, 65–68. [[CrossRef](#)] [[PubMed](#)]
44. Salomón, O.D.; Quintana, M.G.; Mastrángelo, A.V.; Fernández, M.S. Leishmaniasis and climate change-case study: Argentina. *J. Trop. Med.* **2012**, *2012*, 601242. [[CrossRef](#)]
45. Táncczos, B.; Balogh, N.; Király, L.; Biksi, I.; Szeredi, L.; Gyurkovsky, M.; Scalone, A.; Fiorentino, E.; Gramiccia, M.; Farkas, R. First record of autochthonous canine leishmaniasis in Hungary. *Vector Borne Zoonotic Dis.* **2012**, *12*, 588–594. [[CrossRef](#)] [[PubMed](#)]
46. Kholoud, K.; Denis, S.; Lahouari, B.; El Hidan, M.A.; Souad, B. Management of leishmaniasis in the era of climate change in Morocco. *Int. J. Environ. Res. Public Health* **2018**, *15*, 1542. [[CrossRef](#)]
47. Morosetti, G.; Toson, M.; Trevisiol, K.; Idrizi, I.; Natale, A.; Lucchese, L.; Michelutti, A.; Ceschi, P.; Lorenzi, G.; Piffer, C.; et al. Canine leishmaniasis in the Italian northeastern Alps: A survey to assess serological prevalence in dogs and distribution of phlebotomine sand flies in the Autonomous Province of Bolzano-South Tyrol, Italy. *Vet. Parasitol. Reg. Stud. Rep.* **2020**, *21*, 100432. [[CrossRef](#)]
48. Rochlin, I.; Ninivaggi, D.V.; Hutchinson, M.L.; Farajollahi, A. Climate change and range expansion of the Asian tiger mosquito (*Aedes albopictus*) in Northeastern USA: Implications for public health practitioners. *PLoS ONE* **2013**, *8*, e60874. [[CrossRef](#)] [[PubMed](#)]
49. Petrucci, T.A.; McMillan, J.R.; Shepard, J.J.; Andreadis, T.G.; Armstrong, P.M. Increased mosquito abundance and species richness in Connecticut, United States 2001–2019. *Sci. Rep.* **2020**, *10*, 19287. [[CrossRef](#)] [[PubMed](#)]
50. Gorris, M.E.; Bartlow, A.W.; Temple, S.D.; Romero-Alvarez, D.; Shutt, D.P.; Fair, J.M.; Kaufeld, K.A.; Del Valle, S.Y.; Manore, C.A. Updated distribution maps of predominant *Culex* mosquitoes across the Americas. *Parasit. Vectors* **2021**, *14*, 547. [[CrossRef](#)]
51. Morchón, R.; Carretón, E.; González-Miguel, J.; Mellado-Hernández, I. Heartworm disease (*Dirofilaria immitis*) and their vectors in Europe—New Distribution Trends. *Front. Physiol.* **2012**, *3*, 196. [[CrossRef](#)]
52. Montoya-Alonso, J.A.; Carretón, E.; Morchón, R.; Silveira-Viera, L.; Falcón, Y.; Simón, F. The impact of the climate on the epidemiology of *Dirofilaria immitis* in the pet population of the Canary Islands. *Vet. Parasitol.* **2016**, *216*, 66–71. [[CrossRef](#)]
53. Drake, J.; Wiseman, S. Increasing incidence of *Dirofilaria immitis* in dogs in USA with focus on the southeast region 2013–2016. *Parasit. Vectors* **2018**, *11*, 39. [[CrossRef](#)]
54. Polizopoulou, Z.S.; Koutinas, A.F.; Saridomichelakis, M.N.; Patsikas, M.N.; Leontidis, L.S.; Roubies, N.A.; Desiris, A.K. Clinical and laboratory observations in 91 dogs infected with *Dirofilaria immitis* in northern Greece. *Vet. Rec.* **2000**, *146*, 466–469. [[CrossRef](#)]
55. Vieira, A.L.; Vieira, M.J.; Oliveira, J.M.; Simões, A.R.; Diez-Baños, P.; Gestal, J. Prevalence of canine heartworm (*Dirofilaria immitis*) disease in dogs of central Portugal. *Parasite* **2014**, *21*, 5. [[CrossRef](#)] [[PubMed](#)]
56. Falcón-Cordón, Y.; Tvarijonaviciute, A.; Montoya-Alonso, J.A.; Muñoz-Prieto, A.; Caro-Vadillo, A.; Carretón, E. Evaluation of acute phase proteins, adiponectin and endothelin-1 to determine vascular damage in dogs with heartworm disease (*Dirofilaria immitis*), before and after adulticide treatment. *Vet. Parasitol.* **2022**, *309*, 109759. [[CrossRef](#)]
57. Car, S.; Croton, C.; Haworth, M. Pseudohypoadrenocorticism in a Siberian Husky with *Trichuris vulpis* infection. *Case Rep. Vet. Med.* **2019**, *2019*, 3759683. [[CrossRef](#)]
58. Saridomichelakis, M.N.; Xenoulis, P.G.; Chatzis, M.K.; Kasabalis, D.; Steiner, J.M.; Suchodolski, J.S.; Petanides, T. Thyroid function in 36 dogs with leishmaniasis due to *Leishmania infantum* before and during treatment with allopurinol with or without meglumine antimonate. *Vet. Parasitol.* **2013**, *197*, 22–28. [[CrossRef](#)] [[PubMed](#)]
59. Tabar, M.D.; Altlet, L.; Martínez, V.; Roura, X. *Wolbachia*, filariae, and *Leishmania* coinfection in dogs from a Mediterranean area. *J. Small Anim. Pract.* **2013**, *54*, 174–178. [[CrossRef](#)]
60. Morales-Yuste, M.; Martín-Sánchez, J.; Corpas-Lopez, V. Canine leishmaniasis: Update on epidemiology, diagnosis, treatment, and prevention. *Vet. Sci.* **2022**, *9*, 387. [[CrossRef](#)]
61. Paparcone, R.; Fiorentino, E.; Cappiello, S.; Gizzarelli, M.; Gradoni, L.; Oliva, G.; Foglia Manzillo, V. Sternal aspiration of bone marrow in dogs: A practical approach for canine leishmaniasis diagnosis and monitoring. *J. Vet. Med.* **2013**, *2013*, 217314. [[CrossRef](#)] [[PubMed](#)]
62. Marks, S.L.; Kendall, A. Anemia Caused by Primary Bone Marrow Diseases in Animals. Available online: <https://www.msddvetmanual.com/circulatory-system/anemia/anemia-caused-by-primary-bone-marrow-diseases-in-animals> (accessed on 6 March 2023).
63. Magnis, J.; Lorentz, S.; Guardone, L.; Grimm, F.; Magi, M.; Naucke, T.J.; Deplazes, P. 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* **2013**, *6*, 48. [[CrossRef](#)]
64. Borthakur, S.K.; Deka, D.K.; Islam, S.; Sarmah, P.C. Occult dirofilariosis in dogs of North Eastern region in India. *J. Arthropod Borne Dis.* **2015**, *10*, 92–97.
65. Henry, L.G.; Brunson, K.J.; Walden, H.S.; Wenzlow, N.; Beachboard, S.E.; Barr, K.L.; Long, M.T. Comparison of six commercial antigen kits for detection of *Dirofilaria immitis* infections in canines with necropsy-confirmed heartworm status. *Vet. Parasitol.* **2018**, *254*, 178–182. [[CrossRef](#)]
66. Albonico, F.; Loiacono, M.; Gioia, G.; Genchi, C.; Genchi, M.; Mortarino, M. Rapid differentiation of *Dirofilaria immitis* and *Dirofilaria repens* in canine peripheral blood by real-time PCR coupled to high resolution melting analysis. *Vet. Parasitol.* **2014**, *200*, 128–132. [[CrossRef](#)] [[PubMed](#)]

67. Cornegliani, L.; Di Cesare, A.; Aste, G.; Traversa, D.; Di Giulio, E.; Santori, D.; Vercelli, A.; Pampurini, F.; Boari, A. Simultaneous infection by *Dirofilaria repens* and *Leishmania infantum* in a dog. *Rev. Veterinaire Clin.* **2015**, *50*, 59–63. [[CrossRef](#)]
68. Pradhan, S.; Lahiri, V.L.; Elhence, B.R.; Singh, K.N. The microfilariae of *Wuchereria bancrofti* in bone marrow smears. *Am. J. Trop. Med. Hyg.* **1976**, *25*, 199–200. [[CrossRef](#)] [[PubMed](#)]
69. Tummidi, S.; Patro, M.K.; Bal, A.K.; Choudhury, A. Microfilariae in a bone marrow aspirate. *BMC Res. Notes* **2016**, *9*, 256. [[CrossRef](#)] [[PubMed](#)]
70. Rahman, K.; Harankhedkar, S.; Gupta, R.; Gupta, T.; Sharma, S.; Nityanand, S. Microfilariae in bone marrow aspirate of a case of myelofibrosis: A cause or coincidence? *J. Cancer Res. Ther.* **2020**, *16*, 164–166. [[CrossRef](#)]
71. Ahluwalia, C.; Choudhury, M.; Bajaj, P. Incidental detection of microfilariae in aspirates from Ewing's sarcoma of bone. *Diagn. Cytopathol.* **2003**, *29*, 31–32. [[CrossRef](#)]
72. Andola, S.K.; Naik, A.A. Microfilaria and filarial granulomas from fine needle aspirates: A study of 25 cases. *Southeast Asian J. Trop. Med. Public Health* **2011**, *42*, 1351–1358.
73. Chakraborty, S.; Saha, M.; Pradhan, S.G.; Biswas, S. Microfilaria infection in metastatic node in a case of breast carcinoma. *J. Midlife Health* **2019**, *10*, 153–155. [[CrossRef](#)]
74. Dhanya, C.S.; Jayaprakash, H.T. Microfilariae, a common parasite in an unusual site: A case report with literature review. *J. Clin. Diagn. Res.* **2016**, *10*, ED08–ED09. [[CrossRef](#)]
75. Pal, S.; Mondal, S.; Pradhan, R.; Bose, K.; Chakrabarti, S.; Sikder, M. Cytological findings of microfilariae in different sites: A retrospective review of 22 cases from endemic region. *Trop. Parasitol.* **2018**, *8*, 24–28. [[CrossRef](#)]
76. Vasantham, V.; Yadav, S.K.; Sarin, N.; Singh, S.; Pruthi, S.K. Incidental detection of microfilaria in cyst fluid of mucinous cystadenocarcinoma of ovary: A rare case report. *Int. J. Surg. Case Rep.* **2020**, *70*, 56–59. [[CrossRef](#)]
77. American Heartworm Society. Current Canine Guidelines for the Prevention, Diagnosis, and Management of Heartworm (*Dirofilaria immitis*) Infection in Dogs. Available online: https://d3ft8sckhnqim2.cloudfront.net/images/pdf/2020_AHS_Canine_Guidelines.pdf?1580934824 (accessed on 7 March 2023).
78. Velez, R.; Gállego, M. Commercially approved vaccines for canine leishmaniosis: A review of available data on their safety and efficacy. *Trop. Med. Int. Health* **2020**, *25*, 540–557. [[CrossRef](#)]
79. Chalifoux, N.V.; Parker, S.E.; Cosford, K.L. Prognostic indicators at presentation for canine parvoviral enteritis: 322 cases (2001–2018). *J. Vet. Emerg. Crit. Care (San Antonio)* **2021**, *31*, 402–413. [[CrossRef](#)] [[PubMed](#)]
80. Machado, I.C.; Nunes, T.; Maximino, M.; Malato, J.; Tavares, L.; Almeida, V.; Sepúlveda, N.; Gil, S. Epidemiologic factors supporting triage of infected dog patients admitted to a veterinary hospital biological isolation and containment unit. *Vet. Sci.* **2023**, *10*, 186. [[CrossRef](#)] [[PubMed](#)]
81. Orusa, T.; Orusa, R.; Viani, A.; Carella, E.; Enrico, B.M. Geomatics and EO data to support wildlife diseases assessment at landscape level: A pilot experience to map infectious keratoconjunctivitis in chamois and phenological trends in Aosta Valley (NW Italy). *Remote Sens.* **2020**, *12*, 3542. [[CrossRef](#)]
82. Pacheco, G. Relationship between the number of circulating microfilariae and the total population of microfilariae in a host. *J. Parasitol.* **1974**, *60*, 814–818. [[CrossRef](#)] [[PubMed](#)]
83. Ludders, J.W.; Grauer, G.F.; Dubielzig, R.R.; Ribble, G.A.; Wilson, J.W. Renal microcirculatory and correlated histologic changes associated with dirofilariasis in dogs. *Am. J. Vet. Res.* **1988**, *49*, 826–830.
84. Sacks, B.N.; Blejwas, K.M. Effects of canine heartworm (*Dirofilaria immitis*) on body condition and activity of free-ranging coyotes (*Canis latrans*). *Can. J. Zool.* **2000**, *78*, 1042–1051. [[CrossRef](#)]
85. Sharma, S.; Rawat, A.; Chowhan, A. Microfilariae in bone marrow aspiration smears, correlation with marrow hypoplasia: A report of six cases. *Indian J. Pathol. Microbiol.* **2006**, *49*, 566–568.

Disclaimer/Publisher's Note: The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.