Original research paper

Serum Protein Electrophoresis in *Dirofilaria immitis* naturally infected dogs: latest news and a systematic literature review

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Abstract

According to the main Guidelines on canine heartworm disease (HWD), a correct diagnosis of *Dirofilaria immitis* infection should include both parasitological and serological assays. So far, scant data are available on laboratory abnormalities in dogs affected by HWD, although techniques including serum protein electrophoresis (SPEP) have proved to be useful for the diagnosis and monitoring of other vector-borne diseases, such as the canine leishmaniosis. Therefore, this study aims to evaluate the SPEP pattern in dogs naturally infected by *D. immitis*. Furthermore, a systematic review of the literature on this topic was carried out. Medical records from heartworm-positive dogs, of any sex, age, and breed and with available clinical examination and laboratory test results (i.e., complete blood count, serum biochemical profile, and SPEP) were retrospectively collected. If available, laboratory results obtained after treatment of dogs for HWD were also acquired and evaluated. Moreover, a systematic literature search was conducted to identify all publications on SPEP in dogs infected by *D. immitis.* When compared with the reference intervals, out of 30 enrolled dogs, 63.3% (n=19) had a lower percentage of albumin, and 80.0% (n=24) had higher percentages of beta globulins, with beta-2, and especially beta-3 globulins the most frequently altered fractions. In terms of absolute values (g/dL), the proportion of dogs with hypoalbuminemia, and increased total globulin, alpha, beta- and gamma globulins were 4/30 (13.3%), 6/30 (20.0%), 2/30 (6.7%), 16/30 (56.3%) and 8/30 (26.2%), respectively. For 7 dogs, SPEP results evaluated three and six months after the HWD treatment with doxycycline (10 mg/kg BID for 4 weeks) were available. In these dogs a significant post-treatment increase in the percentage of albumin, alpha 2-globulin, and albumin/globulins ratio was observed, as well as a significant decrease both in the percentage and in the absolute value of total-, beta-, and beta-3 globulins. The systematic review of literature databases yielded a total of three studies that were considered eligible and included in the qualitative synthesis.

This study provides novel information on SPEP alterations in dogs naturally infected by *D. immitis*. The evaluation of serum proteins and their electrophoretic pattern may represent an important diagnostic tool for a prompt and accurate diagnosis (e.g., differentiating infections in dogs sharing similar clinical signs and endemic in the same geographical area) and monitoring of HWD.

Keywords: albumin, doxycycline, globulins, heartworm disease, SPEP

1. Introduction

Canine heartworm disease (HWD) caused by the filarial nematode *Dirofilaria immitis* is a mosquito-borne disease endemic throughout Europe, the Americas, and the Southeast Asia regions, and increasingly reported in Africa (Mendoza-Roldan et al., 2020; Dantas-Torres and Otranto, 2020; Genchi and Kramer, 2020; Colella et al., 2020). Dogs act as definitive hosts harbouring the thread-like adult female and male parasites in the pulmonary artery and its branches while *D. immitis* can also be found in the right heart when worm burden is high (Deplazes et al., 2016). After mating, mature viviparous females release microfilariae (mfs) into the bloodstream where they can be picked up by mosquito vectors during the blood meal (Deplazes et al., 2016).

The presence of *D. immitis* in the cardiovascular system as well as the shedding of metabolic products and antigens by mfs and adult heartworms are responsible for the wide variety of pathological changes observed in the pulmonary vascular bed and lung parenchyma (McCall et al., 2008). The major vaso-occlusive effects are due to chronic endarteritis and endothelial proliferation of the peripheral pulmonary branches. Long-term infections result in a chronic cardiorespiratory disease that can be fatal if left untreated (McCall et al., 2008). Clinical signs usually include chronic cough, moderate to severe dyspnea, weakness, and sometimes lipothymia after exercise or excitement, while in severe cases, pulmonary hypertension, heartworm thromboembolism, and heart failure may also occur (McCall et al., 2008).

According to the American Heartworm Society, the European Society of Dirofilariosis and Angiostrongylosis, and the European Scientific Counsel Companion Animal Parasites Guidelines (ESDA, 2017; AHS, 2018; ESCCAP, 2019), a correct diagnosis of *D. immitis* infection in dogs should include the demonstration of the presence of circulating mfs or adult antigens in serum or plasma samples. Among methods to concentrate mfs in dog peripheral blood, the direct detection of *D. immitis* mfs through the modified Knott's method is considered the most applied parasitological diagnostic test for HWD (AHS, 2018). However, the results of this technique as well as others (e.g., blood smear, filtration, molecular testing) relying on the isolation and identification of mfs may be impaired by occult infections (i.e., presence of adult worms but no circulating mfs), the long pre-patent period of the parasite (i.e., up to 7 months), the low mfs concentration in blood samples, and the individual exposure to the vectors (e.g., mosquito density and seasonality). Furthermore, Knott's method requires expertise to morphologically discriminate mfs of other filarial species (e.g., *Dirofilaria repens*, *Acanthocheilonema reconditum*) based on the evaluation of cephalic and caudal morphologies and the measurement of the mfs length (Magnis et al., 2013). On the other hand, immunochromatography-based assays, SNAP test and enzyme-linked immunosorbent assay (ELISA) for the detection of circulating heartworm antigens of the adult nematodes are considered highly specific and sensitive, and less time-consuming although cross-reaction may occur with antigens of other nematodes (e.g., *D. repens*, *Angiostrongylus vasorum,* and *Spirocerca lupi*). Therefore, the results obtained combining the mfs and antigen detection should be interpreted along with those from the clinical examination, other laboratory tests, as well as of thoracic imaging, especially to decide on the treatment protocol.

So far, scant data are available on serum protein electrophoresis (SPEP) in dogs affected by HWD (de Caprariis et al., 2009; Milanović et al., 2017; Asawakarn et al., 2021), although SPEP has proved to be extremely useful for the diagnosis and monitoring of other vector-borne diseases such as canine leishmaniosis (CanL) (Paltrinieri et al 2016). Indeed, the typical electrophoretogram of dogs with active CanL displays hypoalbuminemia, a moderate increase of alpha-2 globulins, which include

most of the positive acute-phase proteins, and a marked increase of gamma globulins, due to the high titers of circulating antibodies, immune complexes, and other molecules with γ-globulin-like mass and charge (Paltrinieri et al., 2016).

Thus, considering the almost overlapping endemicity of *Leishmania infantum* and *D. immitis* infections in canine population from some regions in the Mediterranean basin (Mendoza-Roldan et al., 2020), a more in-depth study on laboratory findings is useful for optimizing the diagnostic process and possible therapeutic and monitoring decisions of HWD. Therefore, this study aims to evaluate the SPEP pattern in dogs naturally infected by *D. immitis* and to provide a systematic review of the literature on this topic.

2. Material and methods

2.1. Study design and availability of data

Medical records of dogs, of any sex, age, and breed which were clinically evaluated in recently published clinico-parasitological trials (Panarese et al., 2020) or still ongoing (data unpublished), were retrospectively collected. Dogs were considered eligible for this study if heartworm-positive by modified Knott's test and SNAP 4Dx Plus test (IDEXX Laboratories, Inc., Westbrook, ME, USA), and with data on clinical examination and laboratory test results (i.e., complete blood count [CBC], serum biochemical profile, and SPEP). As regards SPEP, all the sera were processed in a single laboratory, using a capillary zone electrophoresis (CZE) system (suppongo Capillarys oppure Minicap – verificare con il lab, Sebia Italia S.r.l., Florence, Italy), on which proteins migrate through a silica capillary for at high voltage (9000 V) in an alkaline buffer, pH 9.9, and absorbances generated by the flow of proteins through a spectrophotometer set at 240nm are recorded by the instrument software (Phoresis, Sebia Italia S.r.l.) that produces typical electrophoretic peaks. The electrophoretograms were visually inspected to assess the correctness of the automated separation of

fractions, according to the recommendations previously reported for CZE fractioning (citaz). The percentage of each fraction generated by the software was then recorded, as well as the absolute value in g/dL of each fraction, calculated based on the concentration of total protein (immagino, verificare con il lab) determined using the biuret method on an automated spectrophotometer (chiedere marca e modello al lab). Results were then compared with the reference intervals generated in the laboratory. Dogs were excluded if affected by other vector-borne pathogens (VBPs), such as *L. infantum*, *Ehrlichia canis,* and *Anaplasma phagocytophilum* tested by indirect immunofluorescent antibody test (IFAT), and the snail-borne pathogen *Angiostrongylus vasorum* diagnosed by enzyme-linked immunosorbent assay (ELISA) techniques (Schnyder et al., 2011; Schucan et al., 2012). Moreover, dogs were excluded if, based on physical examination, they were suspected or known to be affected by diseases able to influence the immune response and to increase the levels of inflammatory markers (e.g., neoplastic, auto-immune and heart diseases, diabetes mellitus and insipidus, hypo-, and hyperadrenocorticism or hyper- and hypothyroidism, suppurative dermatopathies). All animals that had been administered glucocorticoids in the previous 3 months were also excluded. If available, laboratory results obtained after treatment of dogs for HWD with AHS-Recommended Treatment Protocol (i.e., doxycycline and a macrocyclic lactone prior to the three-dose regimen of melarsomine dihydrochloride) (AHS, 2018), or only with microfilaricidal (e.g., doxycycline) or adulticidal (e.g., melarsomine dihydrochloride) drugs were acquired and evaluated.

2.2. Literature search, inclusion and exclusion criteria

On the 30th of January 2022, a systematic literature search was conducted according to the Preferred Reporting Items for Systematic reviews and Meta-Analyses (PRISMA) 2020 statement (Page et al., 2021) to identify all publications on SPEP in dogs naturally infected by *D. immitis*. Three online literature databases (i.e., PubMed, Scopus, and BASE) were screened for publications. The keywords "canine", "dog", "*Dirofilaria immitis*", "dirofilariasis", "electrophoresis", "electrophoretic",

156 “heartworm”, “serum protein electrophoresis”, and “SPEP” were used. The literature search on
157 PubMed and BASE was performed using the Boolean Operators AND and OR as follows: [(dog OR
158 canine) AND (Dirofilaria OR dirofilariasis OR heartworm) AND immitis AND (electrophoretic OR
159 electrophoresis OR SPEP)]. To be considered, articles were required to meet the following inclusion
160 criteria: i) should be in the Italian, English, Spanish, Portuguese, or French languages; ii) should be
161 observational studies such as cross-sectional, longitudinal, case report, case series; iii) should have
162 accessible abstract and full text. Literature that did not satisfy the aforementioned criteria, such as
163 review, articles with no original data, letters, editorials, heartworm-not related articles or regarding
164 co-infections, and articles without data on SPEP were excluded. Articles reporting *D. immitis* in
165 humans, and animals other than dogs were also excluded. The systematic literature search and the
166 consequent data assessment were performed by two authors (AZ and MAC) independently. All
167 disagreements were discussed and resolved by consensus. A third author (RI) was also involved in
168 the search for full-text papers to ensure that all relevant publications were included. Using a Microsoft
169 Excel® spreadsheet, the following information was extracted from the included articles: title, first
170 author name, year of publication, journals, sample size, and type of SPEP alterations.
171
172 *2.3. Statistical analysis*
173 The proportion of dogs with abnormal percentage or absolute value of each electrophoretic fraction
174 was calculated on samples collected at admission. Additionally, electrophoretograms were visually
175 inspected by two independent observers (S.P., A.G.), since visual analysis of the electrophoretograms,
176 that is considered a complementary approach to the quantification of single fractions, since, through
177 the analysis of the shape of peaks, it may provide important information about the prevalent pattern,
178 with particular emphasis on the presence and type of an acute phase response or of gammopathies
179 (citaz). Also in this case the number and proportion of dogs with abnormal electrophoretic profiles
180 was recorded.

The percentage and absolute values of each fractions recorded during the follow-up from animals receiving HWD treatments were statistically compared using a non-parametric ANOVA test for paired samples (Friedmann test), followed by a Wilcoxon signed-rank test to compare the results of samples collected at T1 and T2 with those recorded before treatment (T0). The level of significance was set at $P < 0.05$. Statistical analysis was performed using an Excel spreadsheet and specific software (Analyse-it, Analyse-it Software Ltd, Leeds, UK)

3. Results

Out of 330 dogs, records of 30 animals (i.e14 female and 16 male) aged from 2 up to 15 years (median 7.4 years) accomplished all the criteria and were enrolled in this study. The majority of dogs did not present any clinical signs related to HWD (n=22, 73.3%) (McCall et al., 2008), whereas the remaining 8 animals showed cough (n=4), weakness (n=2), cough and weakness (n=8).

3.1. Electrophoretic findings

When compared with the reference intervals, a high proportion of dogs had lower percentages of albumin (n=19, 63.3%), and higher percentages of beta globulins (n=24, 80.0%), while the proportion of dogs with an increased percentage of gamma globulins and alpha globulins was moderate (n=9, 30.0%) and scarce (n=2, 6.7%), respectively (Table 1). In terms of absolute values (g/dL), the proportion of dogs with hypoalbuminemia or hyperglobulinemia (i.e., n=4, 13.3% and n=6, 20.0%, respectively) as well as the proportion with increased beta- and gamma-globulins (i.e., n=16, 56.3% and n=8, 26. 2%, respectively) were lower than that recorded in terms of percentage, whereas the proportion of dogs with increased alpha globulins (n=2, 6.7%) was similar (Table 1). Among beta globulin fractions, beta-2, and especially beta-3 globulins were the most frequently altered fractions, being beta-3 globulins also increased in terms of absolute values in the majority of dogs (n=22,

73.3%) (Table 1). In all the cases above, results classified as abnormal exceeded the intrinsic variability of the electrophoretic method that, according to a previous study were generally lower than 2%, except for alpha-1 globulin, whose intra- and inter-assay imprecision accounted for 4% and 5%, respectively (citaz).

Moreover, the visual analysis of the electrophoretograms revealed in 7 out of 30 dogs (23.3%) the presence of a moderate to very severe polyclonal gammopathy, often including visually abnormal peaks on the right side of the beta fraction (Fig. 1). In the remaining 23 dogs, the electrophoretograms were classified by at least two out of three observers as normal (n=12, whereas a third observer indicated a slight increase in beta-3 globulins) or characterized by slight increases of beta-3 globulins (n=11, whereas a third observer classified as normal the electrophoretograms).

For 7 dogs, SPEP results evaluated three (T1) and six (T2) months after the HWD treatment with doxycycline (10 mg/kg body weight p.o. BID for 4 weeks) were available. No other treatments (e.g., corticosteroids) were administered during the follow-up period. The comparison of SPEP results recorded before treatment (T0) with those recorded at the two different time points after treatment (T1 and T2) did not reveal a significant change in total proteins. Conversely, the comparison of electrophoretograms recorded over time revealed a reduction trend of beta peaks in all the dogs evaluated (Fig. 2), associated with a significant increase in the percentage of albumin ($P = 0.011$) , alpha-2 globulins ($P = 0.034$), and albumin/globulins ratio ($P = 0.012$) (Fig. 3), as well as a significant decrease both in the percentage and in the absolute value of total- ($P = 0.049$ and $P = 0.028$, respectively), beta- ($P = 0.012$ for both values), and beta-3 globulins ($P = 0.049$ and $P = 0.028$, respectively) (Fig. 4). The electrophoretic profile became visually normal in 4 dogs, while in the remaining three dogs, which had particularly evident peaks at T0, residual peaks were still evident at T2, although strongly decreased in height (Fig. 2).

3.2. Literature search results

233 The PRISMA flow diagram indicating the selection process for eligible studies is presented in Fig. 5.
234 Overall, 142 articles on SPEP in dogs naturally infected by *D. immitis* were retrieved from the
235 electronic databases mentioned above in the time period from 1976 to 2021. After excluding 53
236 articles because of duplication, the remaining articles were screened by checking the title and abstract.
237 During the screening process, 83 articles were excluded, and then the rest of the studies (n=6) were
238 sought for retrieval. Finally, four full-text articles were chosen for eligibility assessment, and three of
239 them were found eligible and included in the qualitative synthesis (Fig. 5, Table 2).
240
241 **4. Discussion**
242
243 The present study describes the SPEP pattern in dogs naturally infected by *D. immitis* as characterized
244 by a significant decrease in the albumin percentage and by an increase in beta globulin percentages
245 compared with the normal range, being beta-3 globulin the most frequently altered fraction. The
246 analysis of results expressed in g/dL reveals that the percentage changes reported above are mostly
247 due to the beta-3 globulin increase, inducing secondary changes in the percentage of the other
248 fractions. Furthermore, results also suggest that these peculiar electrophoretic alterations may be
249 solved after treatment of HWD with doxycycline, demonstrating to be potentially associated with *D.*
250 *immitis* infection. Moreover, this study offers a systematic literature review on the topic, showing
251 only three articles eligible for a qualitative synthesis (Davoust et al., 1991; De Caprariis et al., 2009;
252 Asawakarn et al., 2021).
253 Among the electrophoretic findings, the reduced level of albumin has already been reported as a SPEP
254 (De Caprariis et al., 2009; Asawakarn et al., 2021) but also as a biochemical finding (Heshem and
255 Badawy, 2007) finding in dogs infected with *D. immitis*. Considering that an acute-phase response in
256 animals affected by HWD has been described (Méndez et al., 2014), albumin likely acted as a
257 negative acute-phase protein especially because the hypoalbuminemia was also associated with an
258 increase in the beta globulin fraction, being both normalized after treatment with doxycycline as

already reported (De Caprariis et al., 2009; Méndez et al., 2015). At least in two cases, the hypothesis that inflammation is the main mechanism responsible for the decrease of albumin is also supported by the simultaneous increase of alpha-2 globulins. Indeed, acute phase proteins have been shown to increase in some dogs with dirofilariasis, especially in the presence of clinical signs (Venco et al., 2014; Méndez et al., 2015). However, in the other dogs presenting hypoalbuminemia, the lack of a severe increase of alpha-2 globulins does not exclude the existence of a chronic inflammation that is known to reduce the concentration of albumin due to the decreased production of new albumin coupled with the progressive catabolism of pre-existing albumin at the end of their normal half-life (Gershwin, 2008). Furthermore, chronic inflammation may also contribute to the increase of beta- and gamma-globulin fractions.

Hyperglobulinemia, due to antigenic stimulation, may be considered an expected feature of HWD, being observed as a monoclonal (Asawakarn et al., 2021) or polyclonal (Davoust et al., 1991; De Caprariis et al., 2009**)** gammopathy. In our study, the electrophoretograms of about a quarter of the enrolled dogs (23.3%) visually show a moderate to very severe polyclonal gammopathy, often with abnormal peaks on the right side of the beta fraction. In an even higher proportion of cases, though the gammopathy was not identified at a glance by the visual analysis of the electropherograms, the presence of an increased beta- or gamma- globulin fraction was detectable in terms of percentage area, being beta-2 and especially beta-3 globulins the most frequently altered fractions. This SPEP alteration has been already identified (Asawakarn et al., 2021), probably due to chronic antigenic stimulation, as previously mentioned. Increased beta globulins levels, possibly associated with a beta-gamma bridging similar to the ones observed in this study, have been reported in chronic inflammation, inducing an increase of proteins with beta motility such as complement fractions or immunoglobulin subclasses, among which IgM is the most abundant.

In contrast to the publications from the systematic review reporting a severe increase in gamma globulins as a common finding in HWD (Davoust et al., 1991; De Caprariis et al., 2009; Asawakarn et al., 2021), our results show a severe gammopathy only in the 23% of dogs mentioned above, while

285 in the other dogs, gamma-globulins were normal or show only a moderate increase in this fraction.
286 However, a potential limitation of this study could be represented by the comparison of data from
287 articles with different SPEP techniques used (i.e., capillary zone electrophoresis [CZE] and agarose
288 gel electrophoresis [AGE]), making the results not completely comparable. However, in order to
289 assess whether the features recorded in this study using CZE were method-dependent, 8 samples
290 randomly selected from the caseload were processed also with AGE. Since AGE does not differentiate
291 beta-3 from beta-2 globulins having a lower resolution compared with CZE, results of beta-2 and
292 beta-3 globulins obtained by CZE were compared with those of beta-2 obtained in AGE. This
293 approach revealed that all the samples that had results higher than the reference interval with CZE
294 were abnormal also with AGE (data not shown). This suggests that in our caseload severe increases
295 of gamma globulins were less frequent than in previous studies (Davoust et al., 1991; De Caprariis et
296 al., 2009; Asawakarn et al., 2021) and that increases of proteins with gamma motility (e.g., IgG,
297 cytokines and acute phase proteins) are not common.
298 The main limitations of the present study consisted of the small number of medical records available
299 from dogs infected only by *D. immitis* and negative for other vector- or snail-borne diseases as well
300 as free from illness which could potentially influence the SPEP pattern. Under the above
301 circumstances, future studies would be needed to assess the SPEP alterations in a higher caseload and
302 after their treatment with the complete protocol used according to the major guidelines (e.g., including
303 adulticide drugs) (ESDA, 2017; AHS, 2018)
304 Furthermore, the lack of urinalysis did not allow the identification of proteinuria resulting from *D.*
305 *immitis*-induced glomerulonephritis, which could also cause hypoalbuminemia and increases in beta
306 globulin fractions, as in the dogs evaluated in the current study. However, the normalization of the
307 SPEP alterations after the treatment with doxycycline suggests that these changes were mostly due to
308 inflammatory processes and that inflammation has been likely mitigated (but not completely
309 removed, as demonstrated by the simultaneous increase of alpha globulins) by the antibiotic
310 treatments.

5. Conclusions

This study provides novel information on SPEP alterations in dogs naturally infected by *D. immitis*. The electrophoretic changes in the serum protein pattern herein described are slightly different from those reported in the systematic literature review performed on the topic (e.g., reduced albumin level, increased gamma globulin level, monoclonal peak in the gamma region). Indeed, only a few dogs had severe gammopathy or signs consistent with acute inflammation, whereas the majority of them had a moderate increase in beta fractions, mostly located in the beta-3 or beta-gamma regions. Though the present study design did not allow us to definitively identify which protein is mostly responsible for the recorded increase of beta fractions, the presence of proteins involved in chronic inflammation is very likely, also based on the reduction of beta fractions after doxycycline treatment.

The evaluation of serum proteins and their electrophoretic pattern may represent an important diagnostic tool for a prompt and accurate diagnosis (e.g., differentiating infections sharing similar clinical signs and endemic in the same geographical area) and monitoring of HWD.

List of abbreviations

CanL: canine leishmaniosis

CBC: complete blood count

ELISA: enzyme-linked immunosorbent assay

HWD: canine heartworm disease

IFAT: indirect immunofluorescent antibody test

SPEP: serum protein electrophoresis

Ethics approval

The protocol of this study was approved by the Ethics Committee of the Department of Veterinary Medicine of the University of Bari (Prot. Uniba 12/20)

Availability of data and materials

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declaration of Competing Interests

The authors declare no conflict of interest.

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CRediT authorship contribution statement

Maria Alfonsa Cavalera: Writing - original draft, Formal analysis, Data curation. **Saverio Paltrinieri**: Methodology, Investigation, Formal analysis, Writing - Review & Editing. **Alessia Giordano:** Investigation, Writing - Review & Editing. **Roberta Iatta**: Methodology, Writing – Review & Editing. **Floriana Gernone**: Writing - Review & Editing. **Jairo Alfonso Mendoza-Roldan**: Writing - Review & Editing. **Oana Gusatoaia**: Investigation. **Domenico Otranto**: Methodology, Writing - Review & Editing. **Andrea Zatelli:** Conceptualization, Methodology, Supervision, Writing - Review & Editing.

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References

American Heartworm Society. Current Canine Guidelines for the Prevention, Diagnosis, and Management of Heartworm Infection in Dogs. Revised 2018. https://d3ft8sckhnqim2.cloudfront.net/images/pdf/AHS_Canine_Guidelines_11_13_20.pdf?1605556516 Accessed 4 February 2022

Asawakarn, S., Sirisawadi, S., Kunnasut, N., Kamkong, P., Taweethavonsawat, P., 2021. Serum protein profiles and C-reactive protein in natural canine filariasis. Vet. World 14, 860–864.

Barsanti, J.A., Kristensen, F., Drumheller, F.B., 1977. Analysis of serum proteins, using agarose electrophoresis in normal dogs and in dogs naturally infected with *Dirofilaria immitis*. Am. J. Vet. Res. 38, 1055–1058.

Colella, V., Nguyen, V.L., Tan, D.Y., Lu, N., Fang, F., Zhijuan, Y., Wang, J, Liu, X., Chen, X., Dong, J., Nurcahyo, W., Hadi, U. K., Venturina, V., Tong, K., Tsai, Y. L., Taweethavonsawat, P., Tiwananthagorn, S., Le, T. Q., Bui, K. L., Watanabe, M., Rani, P.A.M.A., Annoscia, G., Beugnet, F., Otranto, D., Halos, L., 2020. Zoonotic vector borne pathogens and ectoparasites of dogs and cats in Eastern and Southeast Asia. Emerg. Infect. Dis. 26, 1221–1233.

de Caprariis, D., Sasanelli, M., Paradies, P., Otranto, D., Lia, R., 2009. Monoclonal gammopathy associated with heartworm disease in a dog. J Am Anim Hosp Assoc 45, 296–300.

Dantas-Torres, F., Otranto, D., 2020. Overview on *Dirofilaria immitis* in the Americas, with notes on other filarial worms infecting dogs. Vet Parasitol 109113

Davoust, B., Foulon, T., Louboutin-Croc, J.P., Ducos de Lahitte, J., Groslambert, P., Groulade, P., 1991. Modifications de l'électrophorèse des protéines sériques dans la dirofilariose canine à *Dirofilaria immitis*, *Dirofilaria repens* et à infestation mixte. Bull. Acad. Vét. De France 64, 453-458.

Deplazes, P., Eckert, J., Mathis, A., von Samson-Himmelstjerna, G., Zahner, H. (Eds), 2016. Parasitology in Veterinary Medicine. Wageningen Academic Publishers, Wageningen. 653 pp.

European Scientific Counsel Companion Animal Parasites (ESCCAP). Control pf Vector-Borne Diseases in Dogs and Cats. ESCCAP Guideline 05 Third Edtion – March 2019. https://www.esccap.org/uploads/docs/yhwdhifq_0775_ESCCAP_Guideline_GL5_v10_1p.pdf Accessed 4 Feb 2022.

European Society of Dirofilariosis and Angiostrongylosis (ESDA). Guidelines for clinical management of canine heartworm. 2017. https://www.esda.vet/media/attachments/2021/08/19/canine-heartworm-disease.pdf Accessed 4 Feb 2022.

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

Gershwin, L.J., 2008. Chapter 5 - Proteins, Proteomics, and the Dysproteinemias. In: Kaneko, J.J., Harvey, J.W., Bruss, M.L. (Eds), Clinical Biochemistry of Domestic Animals. Academic Press, San Diego, pp. 117-155.

Heshem, M., Badawy, A., 2007. Hematological and biochemical studies on filariasis of dogs. Internet J. Vet. Med. 4, 1-7.

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.

McCall, J.W., Genchi, C., Kramer, L.H., Guerrero, J., Venco, L., 2008. Heartworm disease in animals and humans. Adv. Parasitol. 66, 193–285.

Méndez, J.C., Carretón, E., Martínez, S., Tvarijonaviciute, A., Cerón, J.J., Montoya-Alonso, J.A., 2014. Acute phase response in dogs with *Dirofilaria immitis*. Vet. Parasitol. 204, 420–425.

Méndez, J.C., Carretón, E., Martínez-Subiela, S., Tvarijonaviciute, A., Cerón, J.J., Montoya-Alonso, J.A., 2015. Acute phase protein response in heartworm-infected dogs after adulticide treatment. Vet Parasitol 209, 197–201.

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.

Milanović, Z., Ilić, A., Andrić, J.F., Radonjić, V., Beletić, A., Filipović, M.K., 2017. Acute-phase response in Babesia canis and *Dirofilaria immitis* co-infections in dogs. Ticks Tick Borne Dis 8, 907–914.

Page, M. J., McKenzie, J. E., Bossuyt, P. M., Boutron, I., Hoffmann, T. C., Mulrow, C. D., Shamseer, L., Tetzlaff, J. M., Akl, E. A., Brennan, S. E., Chou, R., Glanville, J., Grimshaw, J. M., Hróbjartsson, A., Lalu, M. M., Li, T., Loder, E. W., Mayo-Wilson, E., McDonald, S., McGuinness, L. A., Stewart, L.A., Thomas, J., Tricco, A.C., Welch, V.A., Whiting, P., Moher, D., 2021. The PRISMA 2020 statement: an updated guideline for reporting systematic reviews. Syst. Rev. 10, 89.

Paltrinieri S, Gradoni L, Roura X, Zatelli A, Zini, E. Laboratory tests for diagnosing and monitoring canine leishmaniasis. Vet. Clin. Pathol. 2016;45:552–78.

Panarese, R., Iatta, R., Beugnet, F., Otranto, D., 2021. Incidence of *Dirofilaria immitis* and *Leishmania infantum* infections in sheltered dogs from Southern Italy. Transbound Emerg Dis. 69, 891-894

Schnyder, M., Tanner, I., Webster, P., Barutzki, D., Deplazes, P., 2011. An ELISA for sensitive and specific detection of circulating antigen of *Angiostrongylus vasorum* in serum samples of naturally and experimentally infected dogs. Vet. Parasitol. 179, 152–158.

Schucan, A., Schnyder, M., Tanner, I., Barutzki, D., Traversa, D., Deplazes, P., 2012. Detection of specific antibodies in dogs infected with *Angiostrongylus vasorum*. Vet. Parasitol. 185, 216–224.

Venco, L., Bertazzolo, W., Giordano, G., Paltrinieri, S., 2014. Evaluation of C-reactive protein as a clinical biomarker in naturally heartworm-infected dogs: a field study. Vet Parasitol. 206, 48-54.

Figure legends

Fig. 1 Representative electrophoretograms characterizing the differences in serum protein fractions in dogs naturally infected by *Dirofilaria immitis*. A. Normal electrophoretic profile (total serum protein concentration 6.2 g/dL); B. Electrophoretic profile characterized by a mild increase of the beta-3 peak, which represents 15.1 % (1.04 g/dL) of the total serum protein concentration (6.9 g/dL); C. Electrophoretic profile characterized by a polyclonal gammopathy involving beta-3 and gamma globulins.

Fig. 2 Sequential serum protein electrophoresis patterns in a dog naturally infected by *Dirofilaria immitis* before treatment (T0), and three (T1) and six (T2) months after treatment with doxycycline. Note the progressive reduction trend of the height of beta-gamma peaks in the electrophoretic profiles from T0 to T2.

Fig. 3 Box-plot graph of albumin percentage, alpha-2 globulin percentage, and albumin/globulins (A/G) ratio obtained by serum protein electrophoresis in dogs naturally infected with *Dirofilaria immitis* before treatment (T0), and three (T1) and six (T2) months after treatment with doxycycline. Values were tested for significance by the Friedman test.

Fig. 4 Box-plot graph of percentage and the absolute value of total-, beta-, and beta-3 globulins obtained by serum protein electrophoresis in dogs naturally infected with *Dirofilaria immitis* before treatment (T0), and three (T1) and six (T2) months after treatment with doxycycline. Values were tested for significance by the Friedman test.

Fig. 5 PRISMA 2020 flow diagram illustrating the process of study selection on serum protein electrophoresis (SPEP) in dogs naturally infected with *Dirofilaria immitis.*

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