parasitologia

MDPI

Article

Improved Antigen Detection of Male-Only *Dirofilaria immitis* Infections in Canine Serum after Heat Treatment for Immune Complex Dissociation

Jeff Gruntmeir [1,2,3,*], Maureen Long [4], Byron Blagburn [5] and Heather Walden [4]

1 Emerging Pathogens Institute, University of Florida, Gainesville, FL 32611, USA
2 Department of Infectious Diseases & Immunology, College of Veterinary Medicine, University of Florida, Gainesville, FL 32611, USA
3 Southeastern Center of Excellence in Vector Borne Diseases, Gainesville, FL 32611, USA
4 Department of Comparative, Diagnostic and Population Medicine, College of Veterinary Medicine, University of Florida, Gainesville, FL 32611, USA
5 Department of Pathobiology, College of Veterinary Medicine, Auburn University, Auburn, AL 36849, USA
* Correspondence: jgruntmeir@ufl.edu

Abstract: Since the mid-1990s, male-only heartworm infections have been considered undetectable using antigen tests based on experimental studies. Results from those studies are in contrast to reports in the decade prior showing variable male heartworm antigen detection using naturally infected animals and antigen tests using chemical and/or heat immune complex dissociating steps. Several recent studies utilizing heat treatment for immune complex dissociation (Heat ICD) demonstrated increased antigen sensitivity for necropsy verified male-only infections and a higher-than-expected frequency of this type of infection. This study utilized archived canine serum with verified male-only heartworm infections to evaluate detection of the heartworm antigen using the DiroCHEK® (Zoetis LLC, Parsippany, NJ, USA), Witness® (Zoetis LLC, Parsippany, NJ, USA), and SNAP® Heartworm RT (IDEXX Laboratories, Inc., Westbrook, ME, USA) antigen tests. Results showed significant increases in sensitivity for the heartworm antigen following heat treatment for DiroCHEK® (+42.1%, $p < 0.0001$) and Witness® (+26.3%, $p = 0.0020$), but not the SNAP® Heartworm RT (+10.5%, $p = 0.1250$). Prior to heat treatment, false negative results were obtained in 76.3–83.0% of mature infections. Infections with only immature male worms were never detected using any heartworm test used. Heat treatment of serum allows improved detection of mature male-only heartworm infections, which may occur more frequently than previously recognized, and like all heartworm infections pose a risk of chronic and cumulative pathology as well as thromboembolic disease regardless of infection intensity.

Keywords: heartworm antigen; *Dirofilaria immitis*; canine heartworm; male heartworms; heat treatment; false-negative antigen tests; immune complex dissociation; heat ICD

Citation: Gruntmeir, J.; Long, M.; Blagburn, B.; Walden, H. Improved Antigen Detection of Male-Only *Dirofilaria immitis* Infections in Canine Serum after Heat Treatment for Immune Complex Dissociation. *Parasitologia* **2023**, *3*, 79–86. https://doi.org/10.3390/parasitologia3010010

Academic Editor: Geoff Hide

Received: 12 January 2023
Revised: 23 February 2023
Accepted: 27 February 2023
Published: 2 March 2023

1. Introduction

Male *Dirofilaria immitis*, or heartworms, are considered undetectable using antigen tests; their contribution to antigenemia levels is assumed to be negligible or only detectable following the death of the worm [1]. Early commercial antigen tests utilizing immune complex dissociating (ICD) steps in their protocols were extensively evaluated using serum from naturally infected dogs with necropsy verified heartworm infections. Results showed antigen detection was most strongly associated with the increasing number of mature female worms, and there was variable detection (8–75%) of male-only infections [2–9]. The variable detection of male heartworms reported in those studies agreed with findings published by Weil et al. in 1987, who demonstrated two major targeted antigen glycoproteins primarily from female heartworms, and only one of the two major antigen glycoproteins released at lower levels by males [10]. However, testing of samples from dogs experimentally infected by transplantation of mature worms [1,11–13] or subcutaneous injection of

infective third stage (L3) larvae [14] reported no detection of male-only infections. Those experimental studies, except for that by Chandrashekar et al. [14], used the same laboratory heartworm "strain" historically used in preventive and adulticidal efficacy studies: a single macrocyclic lactone susceptible "strain" passaged from 1960 to 2000 [15,16]. Interestingly, a recent experimental study using subcutaneous injected infective L3s of a recently isolated macrocyclic lactone resistant strain reported a single male heartworm detected 195 days post-infection only after heat ICD [17].

The longstanding conclusion of the experimental studies above was that the target glycoprotein antigen is only produced by mature female worms and that mature male worms are undetectable [13–18]. Serodiagnosis of *D. immitis* by antigen detection was primarily developed to address the problem of heartworm infection without microfilariae (occult infection), occurring in 10–67% of heartworm infected dogs and comprising single sex, immature, mixed sex but prepatent, and immune mediated or drug induced amicrofilaremia [19–21]. Although heartworm tests are considered highly sensitive, a recent study using the most sensitive format, a microtiter well-based antigen test and manufacturer protocols, reported 31% of heartworm infected dogs had no antigen detected. Occult infections represented 90.9% of these negative samples that tested antigen negative initially but seroconverted following heat ICD [22].

It is believed that in high prevalence areas, the female to male heartworm ratio skews to favor females at low infection intensities, thus male-only infections are uncommon and therefore an extremely unlikely cause of false negative antigen results [23]. However, a study conducted in the same northcentral Florida area showed a high necropsy prevalence of heartworm of 35.6% among shelter dogs, but similar ratios of female to male single sex infections with 13.7% females (8/58), 12.1% males (7/58), and 12.1% (7/58, 3 female and 4 male) for immature heartworms [22]. Two studies using serum from verified natural infections showed antigen sensitivity for female-only infections, improving from 78.3% to 100% and 62.5% to 100% in both studies, and male-only infections increasing from 22.2% to 77.8% and from 28.5% to 85.8%, following heat ICD [22,24]. In these studies, it is likely that immune complex formation resulting from a high ratio of specific antibodies to target antigens prevented the initial detection of the heartworm antigen released by the mature male heartworms [25–28]. The ability of antigen tests to detect male heartworms has not been thoroughly evaluated since the mid-1990s.

The detection of mature male-only heartworms using commercial heartworm antigen tests should be re-evaluated due to conflicting results between studies using samples from naturally acquired infections and those of experimentally transplanted infections. A re-evaluation is further supported by the recently observed high proportion of male infections and similar frequency of female- and male-only infections in a high prevalence area and reported improved detection following heat ICD. This study evaluated the extent to which male-only heartworm infections are detectable, before and after heat ICD, using two patient side antigen tests and a microtiter well-based commercial antigen test using archived canine sera of necropsy verified male-only heartworm infections.

2. Results

A total of 38 archived canine serum samples had enough serum for evaluation on all three tests. Sensitivity for the heartworm antigen from mature male-only heartworm infections before and after heat ICD improved from 8/38 (21.1%) to 24/38 (63.2%) for DCK HW, from 9/38 (23.7%) to 19/38 (50.0%) for WIT HW, and from 7/38 (18.4%) to 11/38 (28.9%) using SNP HW. Nine additional samples were tested only using the DCK HW due to the available serum volume. Considering these additional samples for the DCK HW, sensitivity for the heartworm antigen before and after heat ICD was 8/47 (17.0%) and 24/38 (61.7%), respectively. Infections with immature male heartworms were not detected by any of the tests. Significant increases in sensitivity for male heartworm infection following heat ICD (n = 38) were demonstrated for DCK HW (+42.1%, $p < 0.0001$) and WIT HW (+26.3%, $p = 0.0020$), but not for the SNP HW (+10.5%, $p = 0.1250$) (Table 1). Considering the nine

additional samples tested using the DCK HW, a significant increase in sensitivity for the heartworm antigen following heat ICD (n = 47) was observed (+44.7%, $p < 0.0001$) (Table 1). None of the samples evaluated had microfilariae of *D. immitis* and three dogs had detectable microfilariae of *Acanthocheilonema reconditum*; all three seroconverted from negative to positive following heat ICD. The age of samples, number of mature male heartworms, or combined effects showed no significant effect on heartworm antigen detection assessed using multiple logistic regression before or after heat ICD for all the antigen tests.

Table 1. Test sensitivity for heartworm antigen detection pre- and post-heat ICD of serum from dogs with mature male-only heartworm infections.

	DCK HW		WIT HW		SNP HW	
	Non-Heat ICD	**Heat ICD**	**Non-Heat ICD**	**Heat ICD**	**Non-Heat ICD**	**Heat ICD**
Mature Males #Positive/#Tested	8/47	29/47				
% Sensitivity	17.0%	61.7%				
Change in Sensitivity, p = value	+44.7%, $p < 0.0001$					
Mature Males #Positive/#Tested	8/38	24/38	9/38	19/38	7/38	11/38
% Sensitivity	21.1%	63.2%	23.7%	50.0%	18.4%	28.9%
Change in Sensitivity, p = value	+42.1%, $p < 0.0001$		+26.3%, $p = 0.002$		+10.5%, $p = 0.1250$	

DCK HW = DiroCHEK® Heartworm Antigen Test Kit; WIT HW = Witness® Canine Heartworm Antigen Assay; SNP HW = SNAP® Heartworm RT.

3. Discussion

The results of this study demonstrate improved detection of mature male-only heartworm infections following heat ICD of canine serum. Prior to heat ICD, the tests evaluated here initially detected 17.0–23.7% of mature male-only infections, increasing to 28.9–63.2% following heat ICD (Table 1). This increase in detection of mature male heartworms is due to the dissociation of immune complexes through the heat denaturation of antibodies [25–28]. Currently, there is no way to differentiate or estimate the number or sex of the worms present in a dog with a positive antigen test, and no assumptions should be made based on weak versus strong antigen intensity pre- or post-heat ICD, as both low and high intensity heartworm infections can have a low detectable antigen, which is a factor of both the age, potential prophylaxis, and/or treatment history of infections [24,27,29]. These data of detectable male-only infections, generally characterized by low antigen levels, may provide additional context for interpretation of previous reports indicating short treatment duration to achieve no antigen detected, seen for some but not all dogs, during non-arsenical adulticidal studies using naturally infected dogs [22,30,31]. Additionally, detection of these occult, single sex heartworm infections post-ICD may also explain scenarios of suspected cross-reactivity to other parasites due to post-heat ICD positive antigen detection, in the absence of knowing the true *D. immitis* infection status in amicrofilaremic asymptomatic dogs from *D. immitis* endemic areas [22,32].

The presence of *Acanthocheilonema reconditum* or intestinal helminths in these dogs is unlikely to have contributed to the positive post-heat ICD antigen results observed based on previously reported results [22]. In that study, the potential pre- and post-heat ICD cross-reactivity of these parasites was evaluated and found 100% specificity using the DCK HW for 105 dogs verified free of pulmonary heartworm but parasitized with other helminth parasites. Parasites recovered in those 105 dogs noninfected with heartworm and which remained heartworm antigen negative post-heat ICD included *A. reconditum* (8 dogs), *Ancylostoma* spp. (72 dogs), *Dipylidium caninum* (44 dogs), *Toxocara canis* (4 dogs), *Trichuris vulpis* (15 dogs), *Spirometra mansonoides* (1 dog), and *Macracanthorynchus ingens* (1 dog) [22].

While the aim of this study was not to compare the performance of the three diagnostic tests evaluated here, it is interesting to observe the difference in pre- and post-heat ICD results between the two test manufacturers. While purely speculative, these differences are likely due to the preparation or source of the heartworm antigen used to generate the antibody reagents incorporated into these commercial tests. It is currently unknown what variability (if any) is present in the heartworm antigen targeted by tests across geographically distinct populations or between different sexes or ages of heartworms.

While the clinical significance of male-only infections in dogs is unknown, the authors' subjective observations of evident gross pathology of the pulmonary vasculature were comparable between male-only infections and low intensity mixed sex (<5 heartworms) or female-only infections for infected dogs opportunistically necropsied in 2017–2021 [22]. While purely speculative, this observation of comparable pathology may be due to the age of infections, activity level of the dogs, or potentially the low population density and lower infection pressure in geographic areas of origin, despite the high prevalence. Presumably in areas of high prevalence and high population density, infection pressure would be greater, and dogs would acquire heartworms more rapidly; thus, in necropsy studies, the dogs from these areas with low intensity or female- or male-only infections may show less pathology due to the tendency to be younger infections. Regrettably, pulmonary pathology by histology was not evaluated in samples obtained by the authors and these statements again are purely speculative and warrant additional research. The results of this study are important clinically for cases with a high index of suspicion for HW infection in symptomatic dogs with initially negative antigen and microfilaria tests as well as for identifying infected cats for supportive care, which are more likely to have single sex infections and unlikely to test antigen positive without the use of heat ICD [26,27,33]. Regardless, the presence of a few heartworms in dogs should not be disregarded and assumed irrelevant due to the chronic and cumulative pathology and potential for possible thromboembolic disease [21].

In the mid-1990s, test manufacturers transitioned away from the inclusion of ICD steps in protocols, presumably to make the antigen assays faster and easier to use in the veterinary clinic [12,34]. Initial studies reporting variable detection of male heartworms in dogs occurred during the years 1986–1994, when ICD steps were still included in antigen testing protocols. Meanwhile, most of the experimental infection studies reporting antigen results for male-only infections occurred during or after transitions of antigen test protocols to exclude ICD steps. It should be acknowledged that the temperature used for heat ICD in this study of 104 °C is much higher than the 60–70 °C historically used in some manufacturer protocols and reference lab protocols. It is unknown what effect lower temperatures for ICD may have on male heartworm detection with the currently available tests. Interestingly, a recent study suggested changes to the F_{ab} epitope binding region of canine IgG antibody occurs at temperatures between 65 °C and 75 °C and showed lower heat ICD temperatures restored heartworm antigen detection to levels similar to that observed at 104 °C [28].

The majority of experimental studies assessing male-only heartworm detection used a continually passaged laboratory heartworm strain and transplanted mature heartworms to evaluate diagnostic potential and limits of the antigen tests. Transplanted heartworms were used primarily to overcome inherent difficulties in assessing the age of infections and maturity associated with the biological variability in the length of worms as well as problematic ectopic migrations [1]. Both scenarios were demonstrated experimentally (subcutaneous inoculation of 50–500 infective L3) using the same lab strain [35]. These types of experimental infection also effectively bypass the host immune response and the parasites' immune evading mechanisms associated with the tissue phase of the lifecycle, a time interval prior to *D. immitis* arriving in the pulmonary vasculature 67–120 days post-infection as previously shown [35–37]. It is unclear why induced male infections were not detected in those experiments; based on the reporting, post-transplantation antigen testing occurred months after, potentially allowing for an antibody response to

overcome the low level of antigen released by males, but not that for female worms, which were all detected regardless of number [1]. Alternatively, male heartworms of the continually passaged laboratory strain may not have produced a detectable antigen, or the current generation of antigen tests is inherently more sensitive to the target antigen due to proprietary modifications. Either of these scenarios is plausible and supported by the recent report of a male-only detection following only heat ICD in a subcutaneously infected dog, using a second passage (2010 original isolate) macrocyclic lactone resistant strain [17].

Limitations of this study include the extended age of some of the samples since acquisition, unknown freeze/thaw cycles, unknown history of the dogs, estimation of heartworm age/maturity based on variable length measurements, as well as the potential for female worms missed at necropsy or in an ectopic location [5,35]. While male heartworm intensity showed no significant influence on antigen detection, increasing the sample size for those samples with higher infection intensities may have shown a different result. Since samples were collected between 1985 and 2019, in older samples multiple freeze thaw cycles or long-term freezing may have somehow negatively affected the antigen, antibody, or immune complex integrity or solubility, possibly resulting in a reduction of positive antigen results. This possibility may explain why post-heat ICD antigen test sensitivities for mature male-only heartworm infections observed in this study (28.9–63.2%) are lower than those observed (78.8–85.8%) in recent studies using samples from 2011 to 2019 [22,24].

4. Materials and Methods

4.1. Animals and Sampling

All work involving animals to obtain biological samples for this project was approved by the Institutional Animal Care and Use Committee (IACUC# 201810115) at the University of Florida. Serum samples were collected opportunistically post-mortem from canine cadavers humanely euthanized for reasons not related to any study and according to procedures at various Florida county sheltering agencies during the years 1984–2020. Blood was collected from the heart and processed for archive at −80 °C. A limited necropsy was performed, dissecting the heart, lungs, and associated vasculature to determine *D. immitis* infection status. Recovered heartworms were rinsed in deionized water and saline before determination of live/dead status, maturity, and enumeration by sex. Whole blood samples were examined for microfilariae by direct smear and/or the modified Knott's technique (MKT), and microfilariae identified morphologically to species where applicable [5,22–24,28,38]

4.2. Study Design

For this study, 54 archived canine serum samples (47 mature and 7 immature male worms) from naturally infected dogs, determined as male-only heartworm infections by necropsy, were included for testing using the DiroCHEK® Heartworm Antigen Test Kit (Zoetis LLC, Parsippany, NJ, USA), (hereafter referred to as DCK HW) before and after heat ICD. Due to the limited volume for 9 samples, separate testing was performed on 44 of the 54 samples originally identified (38 mature and 6 immature male worms) to also evaluate the Witness® Canine Heartworm Antigen Assay (Zoetis LLC, Parsippany, NJ, USA) (WIT HW) and SNAP® Heartworm RT (IDEXX Laboratories, Inc., Westbrook, ME, USA) (SNP HW) antigen tests before and after heat ICD. All samples were thawed overnight in the refrigerator and allowed to equilibrate to room temperature prior to analysis. All samples were mixed by vortex and 400–600 µL sera transferred to 1.5 mL micro-centrifuge tubes, heated at 104 °C for 10 min in a dry heat block and immediately centrifuged at 16,000× *g* (rcf) for 10 min in a micro-centrifuge and the resulting supernatant was used for antigen testing [24,25]. Each sample heated and non-heated was run in parallel using the DCK HW and additionally on the WIT HW and SNP HW according to the manufacturer's instructions. The DCK HW antigen test was chosen for this study since most previously published data using heat ICD has used this test. The WIT HW and SNP HW antigen tests were chosen due to their frequent use in veterinary clinics for heartworm antigen detection and not based on any preference of the authors. Limited sample volumes excluded a

meaningful inclusion of other commercial antigen tests marketed in the United States. All tests used in this study were purchased.

4.3. Statistical Analysis

For each test evaluated, the sensitivity for male heartworm antigen pre- and post-heat ICD was determined using a diagnostic test 2 × 2 contingency table. The McNemar paired X^2 test was used to compare the change in sensitivity between pre- and post-heat ICD results. (MedCalc Software bvba 18.2.1, Ostend, Belgium) (Courtney and Cornell, 1990). Multiple logistic regression was performed using GraphPad Prism (GraphPad version 9.0.0 for Windows, GraphPad Software, San Diego, CA, USA).

5. Conclusions

Conclusions of this study confirm the low sensitivity of commercial heartworm antigen tests for mature male-only heartworm infections using standard test protocols and a statistically significant increase in sensitivity for mature male heartworms by two of the three evaluated antigen tests, following heat ICD. The prevalence of male-only heartworms among heartworm infected dogs may occur more frequently than previously recognized, even in areas of higher prevalence, although detailed data of necropsy studies of naturally infected animals are rare. Approximately 76.3% to 83% of mature male-only infections went undetected using manufacturer protocols for the three antigen tests evaluated here; however, increases in sensitivity ranged from +10.5%, +26.3%, and +42.1% (or +44.7%) for the three commercial tests evaluated, following heat denaturation of antibodies. While not specifically investigated in this study, the observed pulmonary pathology due to the male-only and low intensity (<5) heartworm infections observed by the author during the years 2017–2019 reiterates the importance of the chronic and cumulative nature of this disease. Heat ICD should be considered an important tool for detection of heartworm in high-risk dogs and cats, particularly where clinical suspicion or unknown history warrants further testing despite negative antigen and microfilaria test results. Heat ICD may also be a useful diagnostic tool for the earlier detection of heartworm infection in high-risk animals prior to exportation to other geographic areas and prior to the onset of symptoms, allowing for earlier supportive care or treatment.

Author Contributions: J.G.: conceptualization, methodology, sample and data acquisition and analysis, writing, and funding acquisition; M.L.: sample and data acquisition and writing; B.B.: analysis and writing; H.W.: conceptualization, methodology, sample and data acquisition and analysis, writing, funding acquisition, and project supervision. All authors have read and agreed to the published version of the manuscript.

Funding: Funding received to support this research was provided in part by a joint grant from the National Center for Veterinary Parasitology and the American Heartworm Society received on 12 January 2017. Other monetary funds supporting this research originated from Heather Walden's internal lab funds not associated with any outside commercial, private, or public grant.

Institutional Review Board Statement: All work involving animals to obtain biological samples for this work was approved by the Institutional Animal Care and Use Committee (IACUC# 201810115) at the University of Florida.

Informed Consent Statement: Not Applicable.

Data Availability Statement: All data are reported in the article.

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

References

1. McCall, J.W.; McTier, T.L.; Supakorndej, N.; Ricketts, R.P.; Dzimianski, M.T. Further characterization of the sensitivity of several commercially available heartworm antigen test kits. In Proceedings of the North American Veterinary Conference, Orlando, FL, USA, 15–20 January 1994; Volume 8, pp. 461–463.
2. Courtney, C.H. Comparison of tests for immunodiagnosis of canine dirofilariasis. In Proceedings of the Heartworm Symposium'86, New Orleans, LA, USA, 21–23 March 1986; Otto, G.F., Ed.; American Heartworm Society: Washington, DC, USA, 1986; pp. 77–82.
3. Ely, M.L.; Courtney, C.H. Sensitivity and specificity of Filarochek heartworm antigen test and Dirotect heartworm antibody test for immunodiagnosis of canine dirofilariasis. *J. Am. Anim. Hosp. Assoc.* **1987**, *23*, 367–371.
4. Courtney, C.H.; Zeng, Q.Y.; Bean, E.S. Sensitivity and specificity of the DiroChek heartworm antigen test for immunodiagnosis of canine dirofilariasis and a comparison with other immunodiagnostic tests. *J. Am. Anim. Hosp. Assoc.* **1988**, *24*, 27–32.
5. Courtney, C.H.; Zeng, Q.Y. The structure of heartworm populations in dogs and cats in Florida. In Proceedings of the Heartworm Symposium '89, Charleston, SC, USA, 17–19 March 1989; Otto, G.F., Ed.; American Heartworm Society: Washington, DC, USA, 1989; pp. 1–6.
6. Courtney, C.H.; Zeng, Q.Y.; Tonelli, Q. Sensitivity and specificity of the CITE heartworm antigen test and a comparison with the DiroChek heartworm antigen test. *J. Am. Anim. Hosp. Assoc.* **1990**, *26*, 623–628.
7. Courtney, C.H.; Cornell, J.A. Evaluation of heartworm immunodiagnostic tests. *J. Am. Vet. Med. Assoc.* **1990**, *197*, 724–729. [PubMed]
8. Tseggai, T.; Zeng, Q.Y.; Cochran, D.; Courtney, C.; Mackowiak, M. Comparisons of currently used canine heartworm antigen detection kits with VetRed. In Proceedings of the 39th Annual American Association of Veterinary Parasitologists Meeting (AAVP), San Francisco, CA, USA, 9–12 July 1994; Volume 91, p. 62.
9. Martini, M.; Capelli, G.; Poglayen, G.; Bertotti, F.; Turilli, C. The validity of some haematological and ELISA methods for the diagnosis of canine heartworm disease. *Vet. Res. Commun.* **1996**, *20*, 331–339. [CrossRef]
10. Weil, G.J. *Dirofilaria immitis*: Identification and partial characterization of parasite antigens in the serum of infected dogs. *Exp. Parasitol.* **1987**, *64*, 244–251. [CrossRef]
11. McCall, J.W.; Supakorndej, N.; McTier, T.L.; Dzimianski, M.T.; Ricketts, R.P. Commercial heartworm test kits detect infections with a single adult female worm but not those with numerous adult male worms only. In Proceedings of the American Association of Veterinary Parasitologists, Minneapolis, MN, USA, 17–20 July 1993. #44.
12. McCall, J.W.; Supakorndej, N.; Donoghue, A.R.; Turnbull, R.K.; Radecki, S.V. Evaluation of the performance of canine heartworm antigen test kits licensed for use by veterinarians and canine heartworm antigen tests conducted by diagnostic laboratories, and canine heartworm antigen tests conducted by diagnostic laboratories. In Proceedings of the Recent Advances in Heartworm Disease Symposium'01, San Antonio, TX, USA, 20–22 April 2001; Seward, L.E., Knight, D.H., Eds.; American Heartworm Society: Batavia, IL, USA, 2001; pp. 135–140.
13. McTier, T.L.; McCall, J.W.; Supakorndej, N. Comparative Evaluation of the sensitivity of adult heartworm antigen test kits using whole blood and/or Serum. In Proceedings of the 39th Annual American Association of Veterinary Parasitologists Meeting (AAVP), San Francisco, CA, USA, 9–12 July 1994; Volume 92, p. 62.
14. Chandrashekar, R.; Beall, M.J.; Saucier, J.; O'Connor, T.; McCall, J.W.; McCall, S.D. Experimental *Dirofilaria immitis* infection in dogs: Effects of doxycycline and advantage multi®administration on immature adult parasites. *Vet. Parasitol.* **2014**, *206*, 93–98. [CrossRef]
15. McCall, J.W. Heartworm strains and testing preventive drugs. *Am. Heartworm Soc. Bull.* **2011**, *38*, 12–13.
16. Vidyashankar, A.N.; Castro, P.D.; Kaplan, R.M. A statistical approach for evaluating the effectiveness of heartworm preventive drugs: What does 100% efficacy really mean? *Parasites Vectors* **2017**, *10*, 97–108. [CrossRef]
17. McTier, T.L.; Holzmer, S.; Kryda, K.; Mahabir, S.; McCall, J.W.; Trombley, J.; Maeder, S.J. Comparative preventive efficacy of ProHeart® 12, Heartgard® Plus and Interceptor® Plus against a macrocyclic lactone-resistant strain (JYD-34) of heartworm (*Dirofilaria immitis*) in dogs. *Parasit. Vectors* **2021**, *14*, 226. [CrossRef]
18. Nelson, C.T.; McCall, J.W.; Jones, S.; Moorhead, A. (Eds.) Current Canine Guidelines for the Prevention, Diagnosis and Management of Heartworm (*Dirofilaria immitis*) Infection in Dogs. 2020. Available online: https://www.heartwormsociety.org/veterinary-resources/american-heartworm-society-guidlines (accessed on 26 April 2021).
19. Otto, G.F. The significance of microfilaremia in the diagnosis of heartworm infection. In Proceedings of the Heartworm Symposium '77, Atlanta, GA, USA, 18–20 March 1977; Morgan, H.C., Otto, G.F., Eds.; Veterinary Medicine Publishing Co.: Bonner Springs, KS, USA, 1978; pp. 77–82.
20. Grieve, R. Advances in the immunologic diagnosis of *Dirofilaria immitis* infection. *Sem. Vet. Med. Surg.* **1987**, *2*, 4–14.
21. Rawlings, C.A.; Dawe, D.L.; McCall, J.W.; Keith, J.C.; Prestwood, A.K. Four types of occult *Dirofilaria immitis* infection in dogs. *J. Am. Vet. Med. Assoc.* **1982**, *180*, 1323–1326.
22. Gruntmeir, J.M.; Thompson, N.M.; Long, M.T.; Blagburn, B.L.; Walden, H.D. Detection of heartworm antigen without cross-reactivity to helminths and protozoa following heat treatment of canine serum. *Parasit. Vectors* **2021**, *14*, 71. [CrossRef] [PubMed]
23. Rishniw, M.; Schukken, Y.; Greiner, E. Sex ratios of *Dirofilaria immitis* in naturally infected dogs show female bias at low worm intensities. *Res. Vet. Sci.* **2012**, *93*, 1324–1328. [CrossRef] [PubMed]

24. Gruntmeir, J.M.; Long, M.T.; Blagburn, B.L.; Walden, H.S. Canine heartworm and heat treatment: An evaluation using a well based enzyme-linked immunosorbent assay (ELISA) and canine sera with confirmed heartworm infection status. *Vet. Parasitol.* **2020**, *283*, 109169. [CrossRef] [PubMed]
25. Little, S.E.; Munzing, C.; Heise, S.R.; Allen, K.E.; Starkey, L.A.; Johnson, E.M.; Meinkoth, J.; Reichard, M.V. Pre-treatment with heat facilitates detection of antigen of *Dirofilaria immitis* in canine samples. *Vet. Parasitol.* **2014**, *203*, 250–252. [CrossRef]
26. Little, S.E.; Raymond, M.R.; Thomas, J.E.; Gruntmeir, J.; Hostetler, J.A.; Meinkoth, J.H.; Blagburn, B.L. Heat treatment prior to testing allows detection of antigen of *Dirofilaria immitis* in feline serum. *Parasit. Vectors* **2014**, *7*, 1. [CrossRef]
27. Little, S.; Saleh, M.; Wohltjen, M.; Nagamori, Y. Prime detection of *Dirofilaria immitis*: Understanding the influence of blocked antigen on heartworm test performance. *Parasites Vectors* **2018**, *11*, 186. [CrossRef]
28. Gruntmeir, J.M.; Abbott, J.R.; Kima, P.E.; Long, M.T.; Blagburn, B.L.; Walden, H.D. Increasing temperature denatures canine IgG reducing its ability to inhibit heartworm antigen detection. *Parasit. Vectors*, 2023; *in press*.
29. Drake, J.; Gruntmeir, J.; Merritt, H.; Allen, L.; Little, S.E. False negative antigen tests in dogs infected with heartworm and placed on macrocyclic lactone preventives. *Parasites Vectors* **2015**, *8*, 68. [CrossRef]
30. Ames, M.K.; VanVranken, P.; Evans, C.; Atkins, C.E. Non-Arsenical heartworm adulticidal therapy using topical moxidectin-imidacloprid and doxycycline: A prospective case series. *Vet. Parasitol.* **2020**, *282*, 109099. [CrossRef]
31. Paterson, T.; Fernandez, C.; Burnett, P.J.; Lessey, L.; Hockley, T.; Hagen, R.; Coomansingh, C.; Sharma, B.; Chandrashekar, R.; Schaper, R. Heartworm control in Grenada, West Indies: Results of a field study using imidacloprid 10%+ moxidectin 2.5% and doxycycline for naturally-acquired *Dirofilaria immitis* infections. *Vet. Parasitol.* **2020**, *284*, 109194. [CrossRef] [PubMed]
32. Szatmári, V.; van Leeuwen, M.W.; Piek, C.J.; Venco, L. False positive antigen test for *Dirofilaria immitis* after heat treatment of the blood sample in a microfilaremic dog infected with *Acanthocheilonema dracunculoides*. *Parasit. Vectors* **2020**, *13*, 501. [CrossRef] [PubMed]
33. Gruntmeir, J.M.; Adolph, C.B.; Thomas, J.E.; Reichard, M.V.; Blagburn, B.L.; Little, S.E. Increased detection of *Dirofilaria immitis* antigen in cats after heat pretreatment of samples. *J. Fel. Med. Surg.* **2017**, *19*, 1013–1016. [CrossRef]
34. McTier, T.L.; McCall, J.W.; Supakorndej, N. Features of adult heartworm antigen test kits. In Proceedings of the Heartworm Symposium '95, Auburn, AL, USA, 31 March–2 April 1995; Soll, M.D., Knight, D.S., Eds.; American Heartworm Society: Batavia, IL, USA, 1995; pp. 115–120.
35. Kotani, T.; Powers, K.G. Developmental stages of *Dirofilaria immitis* in the dog. *Am. J. Vet. Res.* **1982**, *43*, 2199–2206.
36. Kume, S.; Itagaki, S. On the life-cycle of *Dirofilaria immtis* in the dog as the final host. *Br. Vet. J.* **1955**, *111*, 16–24. [CrossRef]
37. Orihel, T.C. Morphology of the larval stages of *Dirofilaria immitis* in the dog. *J. Parasitol.* **1961**, *147*, 251–262. [CrossRef]
38. Henry, L.; Brunson, K.; Walden, H.; Wenzlow, N.; Beachboard, S.; Barr, K.; Long, M. 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] [PubMed]