



Increased detection of *Dirofilaria immitis* antigen in cats after heat pretreatment of samples

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Abstract

Objectives To determine whether pretreating diagnostic samples with heat increases the detection of *Dirofilaria immitis* antigen in adult cats, we evaluated feline serum and plasma samples collected in heartworm-endemic areas of the southern United States.

Methods Commercial microtiter well assays for detection of *D immitis* antigen were used to evaluate serum or plasma samples from 385 shelter and free-roaming cats from the southcentral and southeastern United States before and after heat treatment; commercial antibody tests were performed on a subset of samples.

Results Prior to sample heat treatment, 1/220 (0.5%) shelter cats and 4/165 (2.4%) free-roaming cats had detectable *D immitis* antigen. After heat pretreatment, the detection rate increased to 13/220 (5.9%) and 13/165 (7.9%), respectively. Antibody reactive to *D immitis* was significantly more common ($P < 0.001$) in the serum of cats that were antigen positive after heat treatment (10/13; 76.9%) than serum from cats that remained antigen negative after heat treatment (22/163; 13.5%).

Conclusions and relevance Heat pretreatment of feline samples increased antigen detection by commercial assays for *D immitis* and improved overall concordance of antigen and antibody test results in antigen-positive samples in this population. Although further work to investigate the specificity of *D immitis* antigen assays when using pretreated samples is warranted, this approach may be useful in the diagnosis of heartworm infection in individual cats and may increase the accuracy of surveys based on antigen detection.

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Introduction

Feline infection with *Dirofilaria immitis*, the canine heartworm, has been documented in a majority of states in the United States based on presence of antigen, detection of antibody, identification of heartworms at necropsy or reported clinical cases.^{1–6} Ante-mortem identification of *D immitis* infection in cats can be challenging and often requires careful clinical review of results from a variety of tests including serologic assays, thoracic radiography and echocardiography. Microtiter well-based ELISAs for *D immitis* antigen are considered the most sensitive and specific means of identifying heartworm infection in dogs, but these assays can be unreliable for detecting active infection in cats, presumably due to a low number of worms, presence of only male worms or low concentrations of circulating antigen in many cats.^{7,8} These factors, together with the presence of only immature worms in some cats, appear to result in a high frequency of false negative antigen

test results and subsequent underestimation of the true prevalence of *D immitis* in cats.^{9,10}

Pretreatment of canine and feline serum with heat improves detection of *D immitis* antigen on commercial assays.^{8,11,12} Historically, pretreatment of sera was recommended as a precautionary step to disrupt immune

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Table 1 Detection of *Dirofilaria immitis* antigen in feline serum and plasma samples before and after heat treatment

Source of samples	Before heat treatment positive/tested (%; 95% CI)	After heat treatment positive/tested (%; 95% CI)
Shelter cats*	1/220 (0.5%; <0.0001–2.8%)	13/220 (5.9%; 3.4–9.9%)
Free-roaming cats†	4/165 (2.4%; 0.7–6.3%)	13/165 (7.9%; 4.6–13.1%)
Total	5/385 (1.3%; 0.5–3.1%)	26/385 (6.8%; 4.6–9.8%)

*Adult (>1 year old) domestic cats surrendered to animal shelters in northeastern Oklahoma and throughout the southeastern United States

†Adult (>1 year old) free-roaming cats presented to a trap–neuter–return service in northcentral Oklahoma

complexes which were presumed to block detection of *D immitis* antigen in some samples; however, this process is not included in currently available commercial assays.^{6,13–17} Pretreatment of sera from a small number of experimentally infected cats harboring 1–6 young adult *D immitis* resulted in improved detection of antigen in the majority of samples.⁸ To determine the prevalence of blocked antigen in serum and plasma samples from cats in the southern United States, we tested samples from 385 shelter and free-roaming cats both before and after heat treatment.

Materials and methods

Samples

The feline serum (n = 281) and plasma (n = 104) samples available for the present study were originally collected as part of the routine management protocol for evaluating adult cats (estimated age >1 year) at animal shelters in the southeastern or southcentral United States or in a free-roaming cat trap–neuter–return (TNR) program at Oklahoma State University. In total, 116 serum samples from domestic cats at animal shelters in northeastern Oklahoma, 104 plasma samples from domestic cats at animal shelters throughout the southeastern United States and 165 randomly collected serum samples from free-roaming cats presented to a TNR service in northcentral Oklahoma were available for this study. Serum or plasma was separated from cellular components at the time of collection and then stored at –20°C until use in the present study. All samples were originally obtained under standard protocols for management of cats at collaborating animal shelters or TNR programs and approved by the applicable Institutional Animal Care and Use Committees.

Serologic assays

Each sample (n = 385) was tested by commercial microtiter plate assay (DiroCHEK; Zoetis) according to the manufacturer's directions without any pretreatment of sample, and again following heat pretreatment of samples as previously described.⁸ Briefly, an aliquot of 300–400 µl of serum or plasma was placed in a 1.5 ml snap-cap microcentrifuge tube and held at 104°C for 10 mins in a dry heat block, the coagulum centrifuged

for 10 mins at 16,000 × g, and the resulting supernatant used to perform the antigen assay. Both untreated and heat-treated samples from each cat were analyzed together on the same plate along with positive and negative kit controls provided by the manufacturer and a well-characterized low antigen-positive control sample from an experimentally infected dog. All positive results were confirmed using a second antigen-detecting microtiter assay (PetChek Heartworm PF; IDEXX) both before and after heat pretreatment of samples as previously described.⁸ A commercial in-clinic antibody assay (Solo Step FH; Heska) was used according to the manufacturer's instructions to test unheated serum from a subset of samples (n = 176) which were selected on the basis of adequate sample volume.

Statistical analysis

For all prevalence values, 95% confidence intervals were calculated using the modified Wald method.¹⁸ A two-tailed Fisher's exact test was used to compare prevalence of antibody with antigen results before and after heat treatment (QuickCalc; GraphPad.com).

Results

Antigen of *D immitis* was detected in 1/220 (0.5%) cats from animal shelters and 4/165 (2.4%) free-roaming cats prior to heat treatment of samples. After heat treatment, antigen was detected in 13/220 (5.9%) shelter and 13/165 (7.9%) free-roaming cat samples (Table 1). All five samples positive prior to heat treatment remained positive following heat treatment. Retesting of antigen-positive sera before and after heating on a second, different commercial microtiter well assay yielded identical results.

Antibody to *D immitis* was detected in 32/176 (18.2%; 95% CI 12.8–24.7%) samples. Of the antibody-positive samples, 1/32 (3.1%; 95% CI <0.0001–17.1%) was antigen positive prior to heat treatment. No association was found between antigen results prior to heat treatment and antibody status ($P = 0.18$). Antibodies to *D immitis* were more commonly detected in feline samples that were antigen positive after heat treatment (10/13; 76.9%; 95% CI 49.1–92.5%) than in samples from shelter cats that remained antigen negative after heat treatment (22/163; 13.5%; 95% CI 9.0–19.7%; $P < 0.001$).

Discussion

Surveys for *D immitis* infection in cats by conventional antigen testing suggest a prevalence of 0.9% in the US, ranging between 0.5% and 1.4% depending on the year considered.³ Antibody tests are more likely to be positive, with prevalence estimates of 5.1–32.7%.⁵ However, in the present study, pretreatment of feline serum and plasma resulted in a more than five-fold increase in antigen detection in samples from shelter and free-roaming cats from the southern United States on two different commercial assays. While dramatic, this increase was not altogether surprising given that an earlier study revealed that serum samples from only 1 of 6 experimentally infected cats confirmed at necropsy to harbor *D immitis* were antigen positive prior to heat treatment, but 5 of 6 became antigen positive after heat treatment.⁸ Antigen was not detected in samples from 20 purpose-bred cats raised in confinement and thus free of heartworm infection either before or after heat treatment (data not shown). Although additional research on the mechanism responsible for blocking antigen from detection is needed, when antigen tests were first developed, pretreatment of samples was routinely recommended to disrupt immune complexes.^{6,13–17,19} Cats develop profound inflammation in response to *D immitis* infection,^{20,21} suggesting immune complexes may play a role in blocking antigen detection in some feline infections.

False positive results on *D immitis* antigen tests, while considered rare in North America, can occur. Infection of dogs with other nematodes, including *Angiostrongylus vasorum* and *Spirocerca lupi*, has been shown to yield false positive results on some assays for *D immitis* antigen.^{22,23} To investigate the possibility that some of the reversals seen in the present study were due to the presence of antigen to other nematodes, we also tested a subset of field samples for antibody to *D immitis*. A majority (76.9%) of the samples with antigen detected after heat treatment were also positive for antibody to *D immitis*. Indeed, significant association was found between antibody-positive results and antigen-positive results only following heat treatment. If some of the positive antigen results in the present study do represent detection of antigen of nematodes other than *D immitis*, then those nematodes also appear likely to induce false positive results on heartworm antibody tests. Finally, some of our positive results may be from cats that have cleared a *D immitis* infection but residual antigen remains; antigen of *D immitis* can be detected for several months after worms are eliminated.²⁴

Pretreatment of samples is readily conducted in a diagnostic laboratory with access to high-speed centrifugation and increases the sensitivity of *D immitis* antigen assays.⁸ The antigen is heat stable and the loss in sample volume that occurs may concentrate antigen further, supporting detection. However, given uncertainty about the exact performance characteristics of *D immitis*

antigen tests on feline serum or plasma using heated samples, this approach should only be considered when there is a strong clinical suspicion of heartworm infection, and results considered together with other diagnostic information, including history of preventive use, geographic location, physical examination findings, imaging studies and antibody status. Antibody tests remain a valuable tool for identification of past or current heartworm infection in cats. Indeed, for 22 cats in the present study, antibody was the only indication of a history of heartworm infection, presumably because the heartworm and any associated antigen had been cleared by the immune reaction in these individual cats.

Adulticide treatment is not available for cats infected with *D immitis*, and a confirmed positive antigen test result after heat treatment may not change the management plan for an individual patient, but this information could provide justification for more intense vigilance on the part of the client. A majority of cats infected with heartworm harbor at least one adult female worm, suggesting microtiter well-based antigen tests should be able to detect many of these infections.^{8,25} Concomitant use of both antibody and antigen tests increases accuracy of diagnosis,²⁶ but pretreatment to disrupt immune complexes (eg, heat treatment) destroys antibody, limiting the use of the treated sample for other assays. When combination antigen/antibody tests are requested, separate aliquots of samples must be made prior to any heat treatment and larger volume samples are required. In addition, samples from multiple animals should not be pooled prior to testing; blocking factors present in one sample may mask antigen in all samples, and combining could dilute antigen below detectable limits.^{11,27}

Conclusions

The present study suggests many cats in areas where heartworm is endemic may test negative on routine antigen tests for heartworm but convert to positive when pre-treated serum or plasma samples are used in the assay, suggesting they could have a current or recent *D immitis* infection. Although additional research is needed to determine sensitivity, specificity and predictive value of the assays when used on samples that have been heat-treated, pretreatment of samples could provide a valuable adjunct for heartworm testing in cats, particularly those with other evidence of heartworm infection or disease.

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Conflict of interest JT, MR, BB and SL have accepted research grants, honoraria or travel funds from manufacturers of feline heartworm preventives and diagnostic assays. CA is

currently employed by Zoetis, a company that manufactures feline heartworm preventives and diagnostic assays, although he was not employed by Zoetis at the time of sample collection. JG has no conflicts of interest to report.

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References

- Ryan WG, Newcomb KM, Soll MD, et al. **Prevalence of feline heartworm disease – a global review.** In: Soll M and Knight D (eds). Proc Am Heartworm Symp; 1995 March 31–April 2; Auburn, USA. Batavia: American Heartworm Society, pp 79–86.
- Bowman DD, Torre CJ and Mannella C. **Survey of 11 western states for heartworm (*Dirofilaria immitis*) infection, heartworm diagnostic and prevention protocols, and fecal examination protocols for gastrointestinal parasites.** *Vet Ther* 2006; 8: 293–304.
- Lorentzen L and Caola AE. **Incidence of positive heartworm antibody and antigen tests at IDEXX Laboratories: trends and potential impact on feline heartworm awareness and prevention.** *Vet Parasitol* 2008; 158: 183–190.
- Fox JC, Wagner R and Crotty C. **Development of a PCR test and an IFA test for *Dirofilaria immitis* in cats and their use along with an ELISA heartworm antigen test to determine the prevalence of infections in the Tulsa, Oklahoma area.** In: Seward L (ed). Proc Am Heartworm Symp; 1998 May 1–3; Tampa, USA. Batavia: American Heartworm Society, pp 167–171.
- Miller MW, Atkins CE, Stemme K, et al. **Prevalence of exposure to *Dirofilaria immitis* in cats in multiple areas of the United States.** *Vet Ther* 2000; 1: 169–175.
- Watkins BF, Toro M and Toro G. **Prevalence of heartworm antibody and antigen-positive sera among submissions to a commercial laboratory in the USA.** In: Seward L (ed). Proc Am Heartworm Symp; 1998 May 1–3; Tampa, USA. Batavia: American Heartworm Society, pp 145–152.
- Litster AL and Atwell RB. **Feline heartworm disease: a clinical review.** *J Feline Med Surg* 2008; 10: 137–144.
- Little SE, Raymond MR, Thomas JE, et al. **Heat treatment prior to testing allows detection of antigen of *Dirofilaria immitis* in feline serum.** *Parasit Vectors* 2014; 7: 1–4.
- Simón F, Siles-Lucas M, Morchón R, et al. **Human and animal dirofilariasis: the emergence of a zoonotic mosaic.** *Clin Microbiol Rev* 2012; 25: 507–544.
- Berdoulay P, Levy JK, Snyder PS, et al. **Comparison of serological tests for the detection of natural heartworm infection in cats.** *J Am Anim Hosp Assoc* 2004; 40: 376–384.
- Velasquez L, Blagburn BL, Duncan-Decoq R, et al. **Increased prevalence of *Dirofilaria immitis* antigen in canine samples after heat treatment.** *Vet Parasitol* 2014; 206: 67–70.
- Drake J, Gruntmeir J, Merritt H, et al. **False negative antigen tests in dogs infected with heartworm and placed on macrocyclic lactone preventives.** *Parasit Vectors* 2015; 8: 68.
- Weil GJ, Malane MS, Powers KG, et al. **Monoclonal antibodies to parasite antigens found in the serum of *Dirofilaria immitis*-infected dogs.** *J Immunol* 1985; 134: 1185–1191.
- Vodian MA, Bean ES, Cowan K, et al. **Evaluation of a new enzyme linked immunosorbent assay for the detection of circulating *Dirofilaria immitis* antigen in the dog.** In: G Otto (ed). Proc Am Heartworm Symp; 1986 March 21–23; New Orleans, USA. Washington DC: American Heartworm Society, pp 91–94.
- Ely ML and Courtney CH. **Sensitivity and specificity of Filarcohek heartworm antigen test and Dirotest heartworm antibody test for immunodiagnosis of canine dirofilariasis.** *J Am Anim Hosp Assoc* 1987; 23: 367–371.
- Tonelli QJ and Quentin AB. **Factors affecting the accuracy of enzyme immunoassays for *Dirofilaria immitis* adult antigen.** In: Otto G (ed). Proc Am Heartworm Symp; 1989 March 17–19; Charleston, USA. Washington DC: American Heartworm Society, pp 161–165.
- Piché CA, Cavanaugh MT, Donoghue AR, et al. **Results of antibody and antigen testing for feline heartworm infection at Heska Veterinary Diagnostic Laboratories.** In: Seward L (ed). Proc Am Heartworm Symp; 1998 May 1–3; Tampa, USA. Batavia: American Heartworm Society, pp 139–143.
- Agresti A and Coull BA. **Approximate is better than ‘exact’ for interval estimation of binomial proportions.** *Amer Statistician* 1998; 52: 119–126.
- Hamilton RG and Scott AL. **Immunoradiometric assay for quantitation of *Dirofilaria immitis* antigen in dogs with heartworm infections.** *Amer J Vet Res* 1984; 45: 2055–2061.
- Dillon AR, Warner AE, Brawner, et al. **Activity of pulmonary intravascular macrophages in cats and dogs with and without adult *Dirofilaria immitis*.** *Vet Parasitol* 2008; 158: 171–176.
- Blagburn BL and Dillon AR. **Feline heartworm disease: solving the puzzle.** *Vet Med* 2007; 102: 7–14.
- Aroch I, Rojas A, Slon P, et al. **Serological cross-reactivity of three commercial in-house immunoassays for detection of *Dirofilaria immitis* antigens with *Spirocerca lupi* in dogs with benign esophageal spirocercosis.** *Vet Parasitol* 2015; 211: 303–305.
- Schnyder M and Deplazes P. **Cross-reactions of sera from dogs infected with *Angiostrongylus vasorum* in commercially available *Dirofilaria immitis* test kits.** *Parasit Vectors* 2012; 5: 258.
- Levy JK, Snyder PS, Taveres LM, et al. **Prevalence and risk factors for heartworm infection in cats from northern Florida.** *J Am Anim Hosp Assoc* 2003; 39: 533–537.
- McCall JW, Dzimianski MT, McTier TL, et al. **Biology of experimental heartworm infections in cats.** In: Soll M (ed). Proc Am Heartworm Symp; 1992 March 27–29; Austin, USA. Batavia: American Heartworm Society, pp 71–79.
- Snyder PS, Levy JK, Salute ME, et al. **Performance of serologic tests used to detect heartworm infection in cats.** *J Am Vet Med Assoc* 2000; 216: 693–700.
- Carleton RE and Tolbert MK. **Prevalence of *Dirofilaria immitis* and gastrointestinal helminths in cats euthanized at animal control agencies in northwest Georgia.** *Vet Parasitol* 2004; 119: 319–326.