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Increased prevalence of *Dirofilaria immitis* antigen in canine samples after heat treatment

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ABSTRACT

Canine serum samples may contain factors that prevent detection of antigen of *Dirofilaria immitis* on commercial assays, precluding accurate diagnosis. To determine the degree to which the presence of blocking antibodies or other inhibitors of antigen detection may interfere with our ability to detect circulating antigen in canine samples, archived plasma and serum samples ($n = 165$) collected from dogs in animal shelters were tested for *D. immitis* antigen before and after heat treatment. Negative samples were also evaluated for their ability to block detection of *D. immitis* antigen in a sample from a positive dog. All 165 samples were negative prior to heating, but 11/154 (7.1%) became positive after heat treatment, a conversion that was documented and quantified on spectrophotometric plate assays, and 7/165 (4.2%) samples decreased detection of antigen when mixed with a known positive sample, suggesting some blocking ability was present. An additional 103 plasma and serum samples that tested positive prior to heating also were evaluated; the optical density of 14/101 (13.9%) increased by ≥50%, and one sample by as much as 15-fold, after heat treatment. Our results suggest that canine serum and plasma samples from dogs in the southeastern United States can contain inhibitors of *D. immitis* antigen detection, and that prevalence estimates of heartworm infection based on these assays would benefit from heat treatment of samples prior to testing.

1. Introduction

Routine diagnosis of *Dirofilaria immitis* infection in dogs is principally made by detection of circulating antigen in canine serum, plasma, or whole blood (Graham et al., 2012). Commercially available *D. immitis* antigen tests are consistently highly specific, while sensitivity is associated with the number of adult female heartworms present in a dog (Courtney and Zeng, 2001; Atkins, 2003; Lee et al., 2011). Microtiter plate enzyme linked immunosorbent assays (ELISAs) are considered the most sensitive antigen assays available, and have been confirmed to detect the presence of a single female worm, but membrane-bound ELISAs and lateral flow immunochromatographic tests are also very sensitive even when as few as 1–3 adult female worms are present (McCall et al., 2001; Courtney and Zeng, 2001; Atkins, 2003). Pre-treatment of samples to destroy immune complexes was recommended when *D. immitis*

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antigen detection assays were first introduced (Weil et al., 1985; Brunner et al., 1988; Tonelli, 1989). However, pretreatment is no longer included in the instructions for any currently available, commercial antigen test for *D. immitis*.

Despite the excellent sensitivity of antigen-based assays, false negatives occasionally occur in naturally infected canine patients. False negative reactions are considered to be due to levels of antigen that are below the limit of detection (Tonelli, 1989; Graham et al., 2012). Low levels of antigen may be the result of infection with immature worms or low numbers of adult female worms, but could also be due to enhanced clearance of adult antigen by the host immune system or the presence of antibodies with high affinity for adult antigen (Tonelli, 1989). Occasionally, dogs may be highly microfilaremic for *D. immitis* but still test antigen negative regardless of the assay used (Little et al., 2014a). To determine the prevalence of blocking antibodies or other inhibitors of antigen detection that can interfere with our ability to detect circulating antigen in canine samples, we measured antigen of *D. immitis* in plasma samples before and after heat treatment from dogs from animal shelters.

2. Materials and methods

2.1. Samples

All samples used in this project were tested for antigen of *D. immitis* by commercial assay prior to selection for inclusion in this research. A national survey of dogs in animal shelters in the United States conducted by researchers at Auburn University provided 106 heartworm-negative plasma samples and 99 heartworm-positive plasma samples; heartworm status had been determined by testing EDTA-anticoagulated whole blood samples with a commercial membrane bound ELISA (SNAP® 4Dx® Test, IDEXX Laboratories Inc.) according to manufacturer's instructions. Remaining plasma was held frozen at −20 °C until used in this research. An additional 59 heartworm-negative samples and four heartworm-positive samples were collected from an animal shelter in northcentral Oklahoma as part of routine wellness care provided to dogs at intake; heartworm status had been determined by testing serum samples with a commercial microtiter plate ELISA (DiroCHEK®, Synbiotics Corporation, Zoetis) according to manufacturer's instructions. Remaining serum was held frozen at −20 °C until used in this research.

In addition, serum from a dog known to be antigenemic for *D. immitis* and serum from a dog antigen negative for *D. immitis*, but which had been shown capable of blocking detection of antigen when admixed with positive serum, were included as controls throughout the studies. The performance of these two samples on commercial assays to detect antigen of *D. immitis* are described in detail elsewhere (Little et al., 2014a). All samples used in this research were collected under standard protocols for management of dogs at participating animal shelters and by the Institutional Animal Care and Use Committees at the collaborating institutions (Auburn University, Auburn, Alabama, and Oklahoma State University, Stillwater, Oklahoma).

2.2. Antigen testing

All samples were tested with a commercial microtiter plate ELISA (PetChek® Heartworm PF Antigen Test, kindly donated by IDEXX Laboratories Inc.) according to manufacturer's instructions using the laboratory protocol for spectrophotometric interpretation described in the package insert. Each sample was tested before and after heat treatment as previously described (Little et al., 2014a). Briefly, an aliquot (0.7–1.5 mL) of each serum sample was placed in a dry heat block at 103 °C for 10 min, the resultant coagulum centrifuged, and the supernatant used in the microtiter plate assay. The optical density (OD) of each individual sample, before and after heat treatment, was determined on the same microtiter plate using the same positive and negative controls and identical calculated cut-off values.

In addition, mixed samples were tested as previously described (Little et al., 2014a). To determine whether serum or plasma samples contained factors that could block detection of antigen, samples from dogs that initially tested negative for *D. immitis* antigen (regardless of whether they were later shown to be positive after heating) were mixed (1:1) with serum from a single known heartworm-positive dog (described above) and retested. To determine whether a heartworm-negative dog that had previously been shown able to block antigen detection on all assays considered and which was known to be hypergammaglobulinemic, infected with *Hepatozoon americanum*, and had a history of infection with *Acanthocheilonema reconditum*, could consistently block detection of antigen in all positive samples, serum from this dog was mixed (1:1) with serum or plasma samples from dogs which initially tested positive for *D. immitis* antigen and retested.

3. Results

Of the 165 dogs that were heartworm negative when first tested, 154 had sufficient volume to heat treat and then retest the sample before and after heat treatment. Of these, 11/154 (7.1%) converted to positive after heat treatment (Table 1). A total of 7/165 (4.2%) heartworm-negative samples, when mixed with a known heartworm-positive sample, reduced the OD of the known heartworm-positive sample by ≥50% ($n = 3$) or converted it to negative ($n = 4$).

Of the 103 dogs that were heartworm positive when first tested, 101 had sufficient volume to heat the sample and then retest the sample before and after heat treatment. The OD of 14/101 (13.8%) heartworm-positive samples increased by ≥50% after heating (Table 1); the OD of one sample increased by more than 15-fold. When mixed with serum from the dog that had been shown previously to consistently block detection of heartworm antigen, 95/103 (92.2%) samples converted to negative ($n = 91$) or reduced in OD by >50% ($n = 4$); the OD of 8/103 (7.8%) samples was not diminished.

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Table 1
Effect of heat treatment of canine serum and plasma samples on detection of circulating antigen of *Dirofilaria immitis*.

Before treatment (*n*)	After heat-treatment (result/number tested)			
Negative	Negative	Positive		Insufficient volume
165	143/154 (92.9%)	11/154 (7.1%)		11
Before treatment (*n*)	After heat-treatment (result/number tested)			
Positive	Negative	Positive		Insufficient volume
		OD unchanged	OD increased[a]	
103	0/101 (0)	87/101 (86.1%)	14 (13.9%)	2

[a] Increase of ≥50% in optical density.

4. Discussion

Our results support the growing awareness that serum and plasma samples from some dogs contain factors that can inhibit detection of *D. immitis* antigen on standard commercial assays. This observation is not new (Weil et al., 1985; Brunner et al., 1988; Tonelli, 1989), and our research group recently described a method to both mimic this interference *in vitro* and reverse the block, restoring detection of antigen (Little et al., 2014a). In addition, we recently reported that almost all serum samples collected from cats early in heartworm infection contain detectable antigen after, but not before, heat treatment (Little et al., 2014b). However, the data from the present study are important because they reveal that antigen blocking, which has been shown to occur on all commercial testing platforms in the United States evaluated to date (Little et al., 2014a), is a fairly common phenomenon in samples collected from dogs in animal shelters in the southern United States. Further investigation is necessary to definitively identify what is responsible for the antigen blocking phenomenon. Several sources suggest antigen–antibody complexes, including antibodies generated against nematodes other than *D. immitis*, but additional research will be needed to confirm this supposition (Weil et al., 1985; Brunner et al., 1988; Tonelli, 1989; Swartzentruber et al., 2009).

Although our finding that more than 7% of the samples that initially tested negative in this study were actually antigen positive was surprising, we suspect the percentage may be even higher in areas of the southeastern United State where *D. immitis* infection is more common. Almost one-fourth of the samples in the present study came from an area of Oklahoma where prevalence rates in shelter dogs are relatively low (5–10%, data not shown); we would be very interested to learn what proportion of samples from dogs in areas where heartworm is more prevalent, such as coastal Texas, coastal Louisiana, or Florida (Bowman et al., 2009), may convert to positive with heat treatment. Our results from mixing the negative samples with a known positive sample also showed that several additional plasma and serum samples were capable of decreasing or masking detection of antigen on the assay, suggesting if these dogs became infected with *D. immitis* they may not develop detectable antigenemia. We also suspect the overall impact of heating on the results in the present study may have been decreased by the young age of the dogs from which the samples were drawn; dogs in animal shelters tend to be younger, on average, than pet dogs, and thus have had less time to acquire heartworm infection (Little et al., 2009).

Our results from heating the antigen-positive plasma and serum samples support the interpretation that antigen–antibody complexes likely form in many dogs infected with *D. immitis*. Indeed, more than 15% of the samples with detectable antigen of *D. immitis* prior to heating had much higher OD readings following heat treatment, and the great majority of these positive samples could be converted to negative by the addition of our heartworm-negative serum that had been shown previously to block antigen detection. Antigen–antibody complex formation likely occurs, to some extent, in many dogs infected with heartworm (Weil et al., 1985), but this process would be expected to interfere with detection of *D. immitis* antigen only when antibody levels are high and the amount of circulating antigen is low. Previous research has repeatedly shown that antigen levels are lowest in dogs infected with very few heartworms (Courtney and Zeng, 2001; Atkins, 2003; Lee et al., 2011; Graham et al., 2012), making sensitivity of detection of paramount importance. Heating samples prior to testing could help improve diagnosis of *D. immitis* in some dogs, particularly when antigen blocking is occurring. This approach is particularly important to generate accurate data in prevalence surveys relying on antigen testing alone, but requires a larger volume of serum and thus may not be practical in all situations.

Conflict of interest

In the past five years, BB and SL have received honoraria payments and/or research support from several manufacturers of heartworm diagnostic tests and heartworm preventives. All other authors have no conflicts of interest to report.

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