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Short Communication

Pre-treatment with heat facilitates detection of antigen of *Dirofilaria immitis* in canine samples

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ABSTRACT

Diagnosis of *Dirofilaria immitis* infection in dogs is largely dependent on detection of antigen in canine serum, plasma, or whole blood, but antigen may be bound in immune complexes and thus not detected. To develop a model for antigen blocking, we mixed serum from a microfilaremic, antigen-positive dog with that of a hypergammaglobulinemic dog not currently infected with *D. immitis* and converted the positive sample to antigen-negative; detection of antigen was restored when the mixed sample was heat-treated, presumably due to disruption of antigen/antibody complexes. A blood sample was also evaluated from a dog that was microfilaremic and for which microfilariae were identified as *D. immitis* by morphologic examination. Antigen of *D. immitis* was not detected in this sample prior to heating but the sample was strongly positive after heat treatment of whole blood. Taken together, our results indicate that blood samples from some dogs may contain factors that inhibit detection of antigen of *D. immitis*, and that heat treatment of these samples prior to testing could improve the sensitivity of these assays in some patients.

Veterinarians rely on detection of antigen of *Dirofilaria immitis* as a sensitive and specific means of diagnosing heartworm infection in dogs (Atkins, 2003; McCall et al., 2008). A number of different commercial assays are available, all of which detect antigen of *D. immitis* in canine serum, plasma, or whole blood (Courtney and Zeng, 2001; McCall et al., 2001; Atkins, 2003). When antigen-based assays were first developed, serum samples were pre-treated with EDTA and/or heat in order to destroy immune complexes and release antigen for detection (Weil et al., 1985; Brunner et al., 1988; Tonelli and Quentin, 1989). In recent decades, this step has been dropped from protocols for all commercial diagnostic assays.

Filarial infections, including *D. immitis*, result in persistent parasite antigenemia in which antigen is free or trapped in immune complexes; the latter situation can prevent detection on commercial assays, leading to false negative results (Weil et al., 1984, 1985). In other antigen detection systems, treatment of samples to disrupt immune complexes prior to testing facilitates assay performance (Wheat and Walsh, 2008; Swartzentruber et al., 2009). For example, as many as two-thirds of serum samples from patients with probable histoplasmosis but negative initial antigen test results became positive after heat treatment of serum (Swartzentruber et al., 2009). Recently, we showed that heat treatment of feline samples greatly improves sensitivity of detection of *D. immitis* antigen (Little et al., 2014). Here we report an in vitro model for blocking detection of *D. immitis* antigen, heat treatment to reverse the block, and

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then application of that approach to confirm heartworm infection by detection of antigen in a clinical case.

Serum samples. To develop our in vitro model, whole blood samples were collected into vacuum tubes containing either EDTA or no additive from a dog infected with *D. immitis* as confirmed by consistent detection of antigen of *D. immitis* on multiple commercial assays as described below and morphologic identification of microfilariae. Anti-coagulated whole blood was assayed for microfilariae by modified Knott's test and the identity of microfilariae confirmed by morphology (Zajac and Conboy, 2012). Whole blood was also collected from a hypergammaglobulinemic dog with no known history of *D. immitis* infection and which was microfilaria negative and antigen negative on multiple assays. For tubes without additive, blood was allowed to clot, the serum separated by centrifugation, placed into aliquots, and stored at −20 °C until further use. Additional *D. immitis* antigen-positive canine serum samples ($n = 10$) were identified through the Oklahoma Animal Disease Diagnostic Laboratory (OADDL) clinical parasitology service and held at −20 °C until further use.

All antigen testing was conducted using commercial assays according to manufacturers' instructions and prior to the printed expiration dates. Test kits evaluated included enzyme linked immunosorbent assay (ELISA) in microtiter plate formats (DiroCHEK®, Synbiotics Corporation, Zoetis; PetChek® Heartworm PF Antigen Test, IDEXX Laboratories, Inc.), membrane bound ELISAs (SNAP® 4Dx® Test, IDEXX Laboratories, Inc.), and lateral flow immunochromatographic tests (SoloStep® CH Heartworm Test, Heska Corporation; WITNESS® HW, Synbiotics Corporation, Zoetis). Serum from the heartworm-negative dog was admixed with that of the principal heartworm positive dog in two-fold serial dilutions from 1:1 to 1:64, and all dilutions retested on each assay in triplicate. Serum from the additional antigen-positive dogs ($n = 10$) were admixed 1:1 with the heartworm negative dog and retested once on each assay.

To disrupt immune complexes or remove other inhibitory factors which may be present, individual serum samples from the hypergammaglobulinemic heartworm negative dog, the principal heartworm positive dog, and mixed (1:1) samples were heated to 104 °C for 10 min (Swartzentruber et al., 2009), the resulting coagulum centrifuged, and the supernatant tested with all five commercial assays according to manufacturers' instructions. In addition, optical density readings were obtained by spectrophotometry before and after heat treatment for one of the microtiter plate assays (PetChek® Heartworm PF Antigen Test, IDEXX Laboratories, Inc.) according to manufacturer's directions.

An additional EDTA anticoagulated whole blood sample from a dog suspected to be infected with heartworm was identified through the OADDL clinical parasitology service and evaluated by modified Knott's test and for antigen of *D. immitis* on a single assay (SoloStep® CH Heartworm Test, Heska Corporation), as previously described, before and after heat treatment.

Microfilariae of *D. immitis* were confirmed by characteristic morphology on modified Knott's test of our principal heartworm positive dog but not in our hypergammaglobulinemic heartworm negative dog. Modified Knott's test was not conducted on the additional antigen-positive canine samples as only serum was available. Mixing serum from the negative dog and the principal heartworm positive dog resulted in consistent blocking of antigen detection in each of the five commercial assays evaluated out to a 1:16 dilution. Mixing serum from the negative dog with each of the additional antigen-positive canine serum samples also resulted in blocking on all assays evaluated. Heat treatment of mixed samples resulted in significant coagulum formation, necessitating use of excess serum, but also resulted in consistent detection of antigen in coagulum supernatant from all blocked samples on all five commercial assays.

Microfilariae of *D. immitis* were confirmed by characteristic morphology on modified Knott's test from the additional EDTA anticoagulated patient sample, but antigen was not detected by testing at the referral practice (SNAP® 4Dx® Test, IDEXX Laboratories, Inc.) or through the OADDL clinical parasitology laboratory service (SoloStep® CH Heartworm Test, Heska Corporation). Heating this whole blood sample and retesting the supernatant resulted in detection of antigen on commercial assay (SoloStep® CH Heartworm Test, Heska Corporation).

In the present study, heat pre-treatment of serum samples containing antigen reversed the block, allowing detection. In addition, although only a limited number of samples were evaluated, heat treatment did not appear to interfere with detection of antigen in positive samples. Indeed, spectrophotometry revealed increased optical density (OD) of most antigen positive samples following heat pre-treatment, although the OD of our negative sample and negative kit controls remained unchanged (data not shown). However, this approach requires excess serum due to coagulum formation in the sample and is not consistent with current manufacturers' instructions for use of the assays. Additional research into this phenomenon is ongoing, but at present, routinely heating all samples prior to testing is not recommended.

The mechanism by which heat treatment enhances detection of antigen has not been identified, but disruption of immune complexes is considered likely (Swartzentruber et al., 2009). Development of antigen–antibody complexes is likely common in *D. immitis* infection, but apparently does not interfere with detection of antigen in most canine patients. The hypergammaglobulinemic dog, the serum of which consistently blocked antigen detection of antigen when mixed with positive samples in the present study, was known to be infected with *Hepatozoon americanum* and had previously been identified to harbor microfilariae of *Acanthochielonema reconditum* (data not shown), conditions which may have contributed to development of excessive antigen-antibody complex formation.

The extent to which this phenomenon interferes with accurate diagnosis of *D. immitis* infection in dogs is not fully understood. A related study from our research group suggests heating samples prior to testing can greatly improve sensitivity of *D. immitis* antigen tests in cats infected with a low number of adult worms (Little et al., 2014). Dogs with microfilariae of *D. immitis* but which test antigen-negative are occasionally reported by veterinarians

(S. Little, pers. commun.) and have been documented in the literature both with and without concomitant detection of adult worms at necropsy (Courtney, 1986; Dzimianski and McCall, 1986; Brunner et al., 1988). In the present paper, we describe using heat treatment to reveal antigen in a microfilaremic but initially antigen-negative dog. The 2012 American Heartworm Society guidelines state that less than 1% of dogs infected with heartworm are microfilaremic but antigen negative, and use that as a basis for the recommendation that dogs be screened for infection by antigen testing alone (Graham et al., 2012). In light of the data in the present study, and because pretreatment of samples may have been included as part of the standard protocol when original evaluations of antigen tests were conducted, this concept warrants revisiting (Courtney, 1986; Dzimanski and McCall, 1986). Evaluating dogs for microfilariae by examination of a wet mount of whole blood or by modified Knott's test is readily achieved and may enhance detection of some infections in practice.

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