Evaluation of urine for Dirofilaria immitis antigen detection in dogs

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Research Article

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Abstract

Background

Infection by the canine heartworm, *Dirofilaria immitis*, causes a potentially fatal, multisystemic disease in dogs. Diagnosis of *D. immitis* relies on serologic antigen detection coupled with microfilariae identification. It has been shown that immune-complex dissociation (ICD) of serum/plasma by heat treatment increases detection of infection, especially in dogs from endemic areas and with inconsistent use of heartworm prevention. The aim of this study was to assess the use of urine for heartworm antigen detection in dogs using a commercial ELISA (DiroCHEK®).

Methods

Matching serum/plasma and urine of 29 experimentally infected dogs at different time points post-infection were tested to assess the feasibility of antigen detection in urine. Matching serum and urine samples of 267 dogs admitted to a spay/neuter clinic with unknown infection status were also evaluated for antigen detection.

Results

In the experimental samples, antigenemia was detected in 86.2% and 90.9% of samples pre- and post-ICD, respectively, while antigenuria was detected in 75.9% and 72.4% of samples pre- and post-ICD, respectively. In the clinic samples, antigenemia was detected in 7.9% of dogs pre-ICD and 12.4% post-ICD, while antigenuria was detected in 11.6% and 6.4% of dogs pre- and post-ICD, respectively. In experimental samples, sensitivity (SEN), specificity (SPE), and positive predictive value (PPV) of antigenuria were high (85%, 100%, 100%, respectively). In clinic samples, SPE and negative predictive value (NPV) were high (95% and 92%, respectively). In contrast, NPV in experimental samples (40%), and SEN and PPV in clinic samples (58% and 61%, respectively) were low. Overall kappa agreement between antigenemia and antigenuria was fair to moderate.

Conclusion

These data suggest that urine might be a useful and less invasive biofluid for heartworm antigen detection in dogs.

Background

Infection with the canine heartworm, *Dirofilaria immitis*, causes a multisystemic and potentially fatal disease in dogs and wild canids, with some degree of infection seen in cats, as well [1]. Infections with this mosquito-borne filarial nematode have been identified in dogs worldwide, especially in warmer,
tropical regions [2]. In the United States, heartworm infection in dogs has been diagnosed in all 50 states, with an increased occurrence in the warmer southern and southeastern regions [3]. Clinical signs in the canine definitive host are absent for months after infection. Chronically affected dogs may eventually present with cough, dyspnea, exercise intolerance, or weakness, with potential progression to signs of right-sided congestive heart failure, abdominal effusion, anorexia, or caval syndrome [1].

The current recommendation to detect canine heartworm infections is to perform a sensitive and specific antigen test of the blood or blood products coupled with a microfilariae detection test [3, 4]. Circulating antigen is primarily secreted by adult female worms 6–9 months post-infection and the soonest it is most commonly detected is between 5–7 months post-infection. [4]. Heartworm infections have been detected as early as 98 days post-infection using immune complex dissociation (ICD) methods [5]. Heat treatment of either serum or plasma for ICD has been shown to free bound heartworm antigen, decreasing the likelihood of a false negative result [6]. However, this is not recommended for routine screening for heartworm disease due to the necessity of equipment not commonly kept in companion animal practices; it should be performed only when a negative result is obtained with clinician suspicion of heartworm disease [7]. Microfilariae can be detected as early as 6 months post-infection in whole blood but may take longer to appear in some cases [4]. The modified Knott’s test is more sensitive than other microfilariae detection methods as it is a concentration technique and can be used to quantify and identify species of microfilariae [4]. The American Heartworm Society recommends screening dogs at 7 months of age and annually thereafter, or 7 months after a missed dose of preventative for the presence of antigen and microfilariae [4].

The multisystemic effects of heartworm infection have been well documented, including pulmonary and cardiac effects [8, 9] and renal effects [10, 11, 12, 13, 14, 15].

The purpose of this study was to assess the reliability of heartworm antigen detection in urine using a commercially available microtiter plate enzyme linked immunosorbent assay (ELISA). Our objectives were: 1) to assess the feasibility of heartworm antigen detection in urine from experimentally infected dogs at different times post-infection, and 2) to assess the use of heartworm antigen detection in urine versus serum from dogs with unknown infection status in Texas, USA. We hypothesized that due to the filtration by the kidneys and concentration in the urinary bladder, antigen will be reliably detected in the urine of dogs that have detectable antigen in serum or plasma.

Materials And Methods

Sample acquisition

Experimental samples

Matching samples of whole blood, serum, and urine were obtained from 13 dogs experimentally infected with third stage *D. immitis* larvae as previously described [5]. Samples were obtained at different time
points post-infection, ranging from 3–37 months. Urine was collected via catheterization.

An additional 7 archived samples obtained from experimentally infected dogs 5 months post-infection at necropsy (IACUC 2019 – 0200 CA) were also included in the study. Antigen testing was performed on serum or heparinized plasma (archived samples) and urine from these samples as described below.

**Clinical samples**

Matching samples of whole blood, serum, and urine were obtained from 267 dogs with unknown heartworm infection status admitted for sterilization surgery at a high-volume spay and neuter clinic in southwest Texas. Samples were acquired from any dog admitted to the clinic for sterilization surgery that was estimated to be 6 months of age or older during the study period. Serum, urine, and whole blood samples were refrigerated after collection for a maximum of 2 days prior to processing. Whole blood from each animal was collected in a vacutainer K2-EDTA tube (BD, Franklin Lakes, NJ, USA) and a vacutainer clot activator tube (BD, Franklin Lakes, NJ, USA). The clotted blood was centrifuged at room temperature for 10 minutes (1700 X g), and serum was transferred to a second red top tube. The bladder of dogs anesthetized for sterilization surgery was manually expressed, and mid-stream urine was collected in a sample collection cup.

This study was approved by the Texas A&M University Institutional Animal Care and Use Committee (IACUC 2019 – 0200 CA).

**Modified Knott’s Test**

Modified Knott’s testing was performed for evaluation of microfilariaemia in K2-EDTA blood as previously described [16] with modifications. Briefly, 0.5mL of blood was added to 4.5mL 2% formalin, centrifuged (3000 X g, for 5 minutes), supernatant was removed, and the remaining blood solution was mixed with 0.035mL methylene blue. Samples were examined under 100x magnification for quantification and 400x as needed to identify morphology of microfilariae. Remaining whole blood was stored at -20°C.

**Antigen Testing**

Serum or plasma, and urine were tested using the DiroCHEK® Canine Heartworm Antigen Test Kit (Zoetis, Florham Park, NJ). This test kit has been validated for antigen detection in serum and plasma with a reported sensitivity and specificity of 100% (Zoetis, 2021). Testing was first performed following manufacturer protocols followed by the addition of heat treatment as previously described in literature [17]. For heat treatment, both serum/plasma and urine samples were heated at 104°C for 10 minutes, centrifuged (16,000 X g, for 10 minutes), then tested with the DiroCHEK® following manufacturer protocols with non-heat-treated samples. Positive or negative results were determined visually per the manufacturer’s instructions. A color change from colorless to blue was considered positive. In addition, optical density (OD) was determined by spectrophotometry (BioTek Synergy H1 microplate reader) at 590nm 5 minutes after the addition of the final solution and following determination of the color change. Optical density values of ≥ 0.069 were considered “positive” [18]. Cut-off values for positive and negative
status were determined as previously described [19]. Remaining serum, plasma, and urine were stored at -20°C.

**Urine Specific Gravity**

Urine specific gravity was obtained for the experimentally infected samples via the Texas A&M Veterinary Medical Diagnostic Laboratory (TVMDL) as part of a complete urinalysis.

**Statistical Analysis**

Antigen presence pre- and post-ICD as determined by color change, and OD values pre- and post-ICD were recorded for all serum, plasma, and urine samples. Results of modified Knott’s testing were recorded for all whole blood samples. Urine specific gravity (USG) was recorded for all urine samples from experimentally infected animals. All data were compiled into spreadsheets, and test performance characteristics were calculated using Excel (Microsoft Office 365 version 18.2104.12721.0).

Sensitivity (SEN), specificity (SPE), positive predictive value (PPV), and negative predictive value (NPV) were calculated for the detection of antigen in urine in comparison to serum/plasma both pre- and post-ICD.

Cohen’s Kappa (κ) was calculated to assess agreement between the following categories of results: pre-ICD serum/plasma (ICD-SP) and pre-ICD urine (ICD-U), pre-ICD-SP and post-ICD-U, post-ICD-SP and pre-ICD-U, and post-ICD-SP and post-ICD-U. Kappa agreement between serum/plasma and urine was considered “fair” if 0.21–0.40, “moderate” if 0.41–0.60, “substantial” if 0.61–0.80, and “almost perfect” if 0.81–1.00 [20]. The percent change in OD$_{590}$ between pre- and post-ICD was calculated for each population for both serum/plasma and urine, and average percent change was calculated. Mean and median USG were calculated for all samples in the experimental population. The mean USG for positive urine samples and negative urine samples in the experimental population was then compared using an unpaired t-test, with 0.05 as the threshold for significance.

**Results**

**Experimental Population**

Pre-ICD, 86.2% (25/29) of samples were antigen positive in serum/plasma, and 90.9% (20/22) tested positive post-ICD. Due to inadequate sample volume, 7 plasma samples were unavailable for post-ICD testing. In urine, antigen was detected in 75.9% (22/29) of samples pre-ICD and 72.4% (21/29) post-ICD. In samples that were antigen positive in serum/plasma pre-ICD, 84% (21/25) of corresponding urine samples had antigenuria. Compared to all positive serum/plasma samples (both pre- and post-ICD) antigen was detected in the urine in 93.1% (27/29) of samples. Agreement between serum/plasma and urine positivity status in the experimental population was variable (Table 1). Agreement between serum/plasma and urine was fair to moderate when serum/plasma samples were not heat-treated, but agreement improved when serum/plasma were heat-treated regardless of urine treatment. Overall
agreement of antigenuria and antigenemia regardless of heat-treatment status of all samples was moderate (0.38).

Sensitivity, specificity, and positive predictive value of urine for heartworm antigen were all high, with 100% specificity and PPV regardless of heat treatment. However, NPV of urine was low (29%), although sensitivity and NPV of urine for heartworm antigen increased slightly when compared to heat treatment of serum/plasma (Table 2). Of the twelve samples that were collected 7 months post-infection or longer, all were positive for antigen in both biofluids, pre- and post-ICD. SEN, SPE, PPV, and NPV were all 100%, and agreement was “almost perfect“ at 1.00.

The percent change in optical density of serum samples was positive after heat treatment (22.3%), while in urine, the percent change between pre- and post-ICD samples was negative (-6.4; Fig. 1A).

The mean USG in the experimental population was 1.033 (median: 1.034, range: 1.012 to > 1.060). The average USG of samples with antigenuria was 1.031, and those without antigenuria had an average USG of 1.039. There was no significant difference between USG of antigen positive and antigen negative samples (p = 0.1025, df = 18, t-value = 1.7203).

Fifteen (15) of 22 (68.1%) of samples with adequate volume of whole blood were microfilaric. All 15 samples were antigenemic pre- and post-ICD, and 14/15 were antigenuric pre- and post-ICD. Seven (7) of 22 (31.8%) samples were amicrofilaric. Of the amicrofilaric samples, 3 were antigenuric and antigenemic, 2 were antigenemic only, and 2 were negative for antigen in both biofluids. Seven samples did not have adequate whole blood volume for microfilariae testing to be performed.

**Clinical Population**

Heartworm antigen was detected in 7.9% of serum samples pre-ICD (21/267) and in 12.4% (33/267) of serum samples post-ICD. In urine, antigen was detected in 11.6% (31/267) of samples pre-ICD, and 6.4% (17/267) post-ICD. In all serum samples that were positive both pre- and post-ICD (n = 33), antigen was detected in the urine in 57.6% (19/33) of samples. In samples that were negative for heartworm antigen detection both pre- and post-ICD (n = 234), antigen was detected in the urine in 5.1% (12/234) of samples.

Agreement between antigen positivity in serum and urine in the clinic population was variable (Table 3). Agreement between serum and urine was moderate to almost perfect when serum was not heat-treated, and moderate to substantial when serum was heat-treated. Heat-treatment of urine samples improved agreement to substantial to almost perfect. Overall agreement between antigenuria and antigenemia regardless of heat-treatment status was moderate (0.50).

Mean specificity and NPV of urine for antigen detection in the clinic population were high, while mean sensitivity and PPV were lower. When neither sample type was heat-treated, specificity and NPV of urine were high, while sensitivity was moderate and PPV was low. When only urine was heat-treated, specificity, PPV, and NPV of urine were high while sensitivity was moderate. When only serum was heat-treated, specificity and NPV of urine were high, while sensitivity and PPV were low. When both serum and urine
samples were heat-treated, specificity, PPV, and NPV of urine were high, while sensitivity was low (Table 4).

In the clinic population, the percent change in OD$_{590}$ post-ICD compared with pre-ICD demonstrated similar trends as the experimental population, with an average percent increase for serum (1.88%) and decrease for urine (-3.80; Fig. 1B). Of 15 dogs that were antigenuric but not antigenemic pre-ICD, 3 became antigenemic post-ICD.

Three samples (1.14%) were microfilaremic and the remaining 264 samples were amicrofilaremic. All 3 microfilaremic samples had antigen detected in serum and urine, both pre- and post-ICD. Of the amicrofilaremic samples, 34 had antigen detected in serum and 18 had antigen detected in urine.

**Discussion**

In experimental infections, antigen detection in urine was consistent with blood products for infections 7 months in length and greater. As a screening test, urine was specific with a high negative predictive value, and was positive in some cases where antigenemia was not detected. In both experimental and clinical populations, percent change in OD after heat treatment was positive in blood products and negative in urine.

Antigenuria following antigenemia is a common result of many infectious processes in humans and animals [21, 22]. Therefore, urine has been used as a diagnostic template for antigen detection in other infectious diseases in dogs and humans, including histoplasmosis and blastomycosis [23]. Parasitic nematode infections of the urinary tract can be diagnosed via detection of eggs with sedimentation and urinalysis [24, 25, 26], but urine has not been extensively explored for detection of nematode infections present outside the urinary tract.

The potential use of urine as a diagnostic sample for heartworm antigen detection is based on the presence of adult *D. immitis* and microfilariae in the cardiovascular system and the role of the kidneys in filtering the blood. Previous studies have demonstrated glomerular changes secondary to heartworm disease, including thickening and vacuolation of the glomerular basement membrane, deposition of immune complexes in the glomerular basement membrane leading to immune complex-mediated glomerulonephritis, proliferation of mesangial cells and matrix and occasionally the presence of microfilariae lodged in the glomeruli [10, 11, 12]. Rarely, microfilariae have been detected on urine sedimentation examinations, and can be associated with hematuria, proteinuria, or other signs of renal disease [27].

To date, only a few studies have investigated heartworm-associated antigenuria in dogs, but our knowledge on the use of urine as an alternative diagnostic template for routine heartworm screening is limited. In experimental heartworm studies, *D. immitis* antigenuria was seen in infected dogs by day 398 post-infection [28], and in infected cats by day 240 post-infection [9]. However, our results show that
antigenuria may be detected as early as 4 months post-infection as seen in samples of experimentally infected dogs assessed in the present study.

During the 5-6-month post-infection period, antigen detection in blood or blood products has been described as “inconsistent” [5]. Previous studies have also demonstrated the variability in detection of serum/plasma heartworm antigen during this period [29]. One explanation is that during this time there are not many adult worms present, so the amount of antigen available for detection is low [30]. Given that the soonest antigen is commonly detected is between 5–7 months post-infection [4], or as early as 3 months post-infection with ICD methods [5], discrepancies in results are possible earlier in infection. These discrepancies are potentially responsible for the “moderate” agreement demonstrated in this study.

In the present study, samples of all biofluids from infections fewer than 4 months in duration were antigen negative, pre- and post-ICD. All experimental serum/plasma samples tested for antigen at 5 months post-infection (n = 11) and 6 months post-infection (n = 3) were positive for antigen, regardless of antigenuria status. During the same time, 7 urine samples tested positive at 5-months post-infection (n = 11), and 2 urine samples tested positive at 6-months post-infection (n = 3). Urine samples from one dog (Dog #3) in this population were negative for antigen at both 5- and 6-months post-infection, but positive at 7-months both pre- and post-heat-treatment. The change in this sample’s positivity from 6 to 7-months post-infection highlights the variability during this period as the developing larvae finish maturing into adults and establishing infection in the pulmonary arteries (Table 5). All serum/plasma and urine samples were positive from 7 months onward, supporting the use of urine for antigen detection when following the AHS recommendations. Data from this population also demonstrates that samples ≥ 5 months post infection typically contained adequate antigen levels for detection in the urine.

Seven urine samples in the experimental population were negative. Infection duration of these samples ranged from 3–6 months post-infection. Two of the negative samples were 3- and 4-months post-infection and the associated serum was also negative for antigen with or without the addition of heat treatment. Both samples were collected before the expected time for antigen to be in high enough concentrations for detection. The other 5 negative urine samples were either 5-months post-infection (4/5) or 6-months post-infection (1/5), where antigen was detected in serum in all instances, 4 without heat treatment, and 1 only after the addition of heat treatment.

In the clinical population, urine had high specificity and low sensitivity for detecting heartworm antigen, which may limit its use as a clinical screening test but might still be useful in a research setting, as few false positive results would be expected. The low sensitivity was a result of 18 urine samples testing negative for antigen pre- and post-ICD, with matching serum samples testing positive pre- or post-ICD. The low PPV in this population was a result of 12 urine samples testing positive for antigen while matching serum samples were negative for antigen, pre- and post-ICD. While these results are difficult to interpret in a diagnostic and clinical context and further investigations are warranted, there might be plausible biological explanations for such results. If these results are true negatives, the urine positivity could have been due to cross-reactivity of antigens associated with other parasites (Spirocerca lupi, Dirofilaria repens, etc.) that may have been altered during the heat treatment protocol as seen in serum and plasma of dogs.
infected with other nematodes [31, 32], even if matching sera tested negative. Alternatively, in the case that all or some of these were true positives, heartworm antigen present in urine could have been detectable because of an increased urine concentration due to time of collection or dehydration, while antigen levels in serum were still present at low concentrations. Adequate urine volume was not available to measure USG on all samples; however, in the experimental population, samples had an adequate sample volume, and no significant difference was detected in the urine of animals with positive or negative serum/plasma samples. According to the manufacturer, false positives in serum and plasma may occur in animals infected with 3 or fewer adult females. This, coupled with the detection of heartworm antigenuria in experimental samples as early as 4 months post-infection could suggest that these are true positive results.

Our combined results of antigen detection in serum pre- and post-ICD was comparable to that found in previous studies in a similar population from the same geographic area [33], and higher than the expected prevalence for client-owned dogs in Texas [3]. While heat treatment of urine did not prove to be beneficial for antigen detection, our study further supports the value of heat-treatment of serum or plasma samples from dogs in endemic areas, or with an unknown history of heartworm prevention [6]. However, it is possible that in a different biofluid, such as urine, heartworm antigens are less stable and more prone to degradation, rendering them undetectable post-heat treatment. This was demonstrated in both populations in the present study when urine positivity changed from positive to negative post-heat treatment. This change in urine antigen positivity was more evident in the clinical samples compared with the experimental samples.

In the experimental sample population, urine was positive when serum/plasma was positive in all but 4 samples. The percentage of urine antigen-positive samples (i.e., pre- or post-ICD) out of all serum/plasma positive samples was 81.4% (22/27) in this population.

In the clinic sample population, urine was most often negative when serum was negative, except for the 12 samples discussed previously. The percentage of urine positive samples out of all serum positive samples was 51.4% (19/37).

Regarding the agreement between antigen detection in the serum/plasma and the presence of antigen in the urine, the results were variable depending on the population: experimental or clinical, and whether urine had been subjected to ICD via heat treatment. In both populations, the average optical density of serum samples increased with the addition of heat, indicating the presence of more antigen for detection, and decreased in urine after the addition of heat, indicating less antigen available for detection. In experimental samples, agreement was higher between post-ICD serum and pre-ICD urine (kappa = 0.77) when each fluid should have maximum antigen available for detection, vs pre-ICD serum and pre-ICD urine (kappa = 0.45). Agreement was lowest between pre-ICD serum samples and post-ICD urine (k = 0.39), when antigen for detection in each fluid should theoretically be lowest. In the clinic population, agreement was similar between post-ICD serum and pre-ICD urine (kappa = 0.54) and pre-ICD serum and urine (kappa = 0.58). In both of these groups, antigen in urine should be highest, but antigen in serum
would be expected to be higher after heat treatment. Therefore, the finding of only moderate agreement between post-ICD serum and pre-ICD urine was unexpected and is due to a higher number of positive urine samples compared with serum.

**Conclusion**

The current AHS recommendations are that antigen testing should be performed annually on dogs 7 months of age and older, and on any dog 7 months after a lapse in administration of macrocyclic lactone-based preventive products [4]. All urine samples collected 7-months post-infection or greater in the experimental population tested positive both pre- and post-ICD, suggesting that urine may be a useful alternative biological fluid for *D. immitis* antigen detection in dogs. As a screening test in the clinical population, urine was positive for antigen more often than serum, but often tested negative for antigen when serum was positive. Heat-treatment for ICD might be detrimental to antigen detection in urine samples. Heat-treatment and other ICD methods are not common in clinic settings, so these results would not impact the use of urine as a routine sample for heartworm antigen testing. Further studies are needed to determine if antigenuria in the absence of antigenemia is an indicator of early infection, a false positive result, or results from dogs that are 5-6-months post-infection during the variable period discussed previously.

**Abbreviations**

ICD

immune complex dissociation

ICD-SP

immune complex dissociation of serum/plasma

ICD-U

immune complex dissociation of urine

OD

optical density

USG

urine specific gravity

SEN

sensitivity

SPE

specificity

PPV

positive predictive value

NPV

negative predictive value
Declarations

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Availability of data and materials

All data is presented in this manuscript, figures, and supplemental data. Further inquiries may be directed to the corresponding author.

Authors’ contributions

A.C.B, M.N.S., M.B.N. and G.G.V designed the study. A.C.B, and J.M.F. collected samples. A.C.B and M.N.S processed samples and drafted the manuscript. M.N.S, M.B.N., and G.G.V. supervised study. All authors contributed to data analysis, read, and approved the final manuscript.

Ethics approval and consent to participate

Collection of samples from this study were approved by the Texas A&M University IACUC (2019-0200 CA).

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

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Tables

Tables 1 to 5 are available in the Supplementary Files section.

Figures

![Figure 1](image)

**Figure 1**

Average percent change in optical density (OD \(_{590}\)) using the DiroCHEK® Canine Heartworm Antigen Test Kit after immune complex dissociation (ICD) via heat treatment in urine and serum from dogs in the experimental group (A) and the clinical group (B).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Table1.pdf
- Table2.pdf
- Table3.pdf
- Table4.pdf
- Table5.pdf