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Research paper

Comparison of six commercial antigen kits for detection of *Dirofilaria immitis* infections in canines with necropsy-confirmed heartworm status

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ABSTRACT

Patient-side test kits for detecting antigenemia in dogs associated with sexually mature female heartworms (*Dirofilaria immitis*) have been available for three decades, and these tests are continually updated and improved. To define the sensitivity (Se) and specificity (Sp) of contemporary antigen detection tests against cardiopulmonary *D. immitis* burden, we evaluated five patient-side kits—Anigen Rapid One Step® (Bio note), SNAP® 4Dx Plus Test Kit (IDEXX), WITNESS® Heartworm Canine Heartworm Antigen Test Kit (Zoetis), VetScan® Canine Heartworm Rapid Test (Abaxis), and Solo Step® CH Canine Heartworm Antigen Test (Heska), and one microplate ELISA (DiroCHEK®; Zoetis), using archived canine sera divided into five subclasses of female worms (0, 1–5, 6–20, 21–40, and > 40). The patient-side tests were performed once, side-by-side according to each manufacturer's protocol by personnel blinded to the *D. immitis* status of each dog. The overall Se and Sp of the patient-side kits was ≥97.5 and =94.0%, respectively. For samples from dogs with 1–5, 6–20, and 21–40 *D. immitis*, the Se was between 96 and 100%, with a slight increase in Se in dogs with ≥41 worms. The agreement between tests for all subclasses of *D. immitis* burden was between 99 and 100%. The Se and Sp for the ELISA compared with the necropsy results of dogs was 99 and 96%, respectively. Agreement between each patient-side test and the ELISA was between 97 and 100%. All commercially available tests can give practitioners excellent patient-side information, allowing them to make informed decisions on the need for additional diagnostic work-up before instituting new or continuing *D. immitis* prophylaxis.

1. Introduction

Dirofilaria immitis infection in canines is a chronic disease affecting the pulmonary vasculature, lungs, and heart (Jackson et al., 1966; Jackson et al., 1962; Knight, 1977, 1987). In antemortem testing, diagnosis of *D. immitis* infection relies upon the detection of antigen several months after infection when the female *D. immitis* matures (Weil et al., 1984; Weil et al., 1985; Weil, 1987). Antigenemia, mainly contributed by the sexually mature female worm, generally precedes the appearance of microfilariae in the blood; thus, antigen-based testing of whole blood or serum has become the primary tool for diagnostic screening (Goodwin, 1998). Antigen-based enzyme-linked immunosorbent assay (ELISA) testing has been available for 35 years, and many studies have been performed investigating the sensitivity (Se) and specificity (Sp) of tests using necropsy examination to verify disease status (Atkins, 2003; Brunner et al., 1988; Courtney and Cornell, 1990; Courtney and Zeng, 2001a; Courtney and Zeng 2001b; Lee et al., 2011). Many of these screening tests have been compared with each other and available ELISA microwell formats. Recently, a meta-analysis was published which included the aforementioned studies (and others) and found that the overall Se and Sp of testing for *D. immitis* antigen was 78.2% and 97.3%, respectively (Rohrbach and Patton, 2013). Importantly, this study demonstrated that the use of *D. immitis* antigen detection tests is affected by disease prevalence and *D. immitis* prophylaxis use in the patient. Furthermore, test accuracy in practice is affected by sample quality, *D. immitis* burden, and the tester (Martini et al., 1996; Rohrbach et al., 2011; Rohrbach and Patton, 2013; Vezzani et al., 2008). The studies re-analyzed in the meta-analysis of Rohrbach and Patton (2013) spanned two decades. Recent work has demonstrated limitations of commercial antigen detection assay specificity to *D. immitis* due to cross-reactivity with sera from dogs infected with vascular filarids *D. repens* and *Angiostrongylus vasorum* (Schnyder and Deplazes,

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2012; Venco et al., 2017), and the nematode *Spirocerca lupi* (Aroch et al., 2015). Direct testing of secretory/excretory antigens secreted from live nonfilarid parasites *Toxocara canis*, *T. catis*, *Toxocara spp.*, *Dipylidium caninum*, *Taenia teaniaeformis* can react when tested via certain kits (Venco et al., 2017). These parasites, except Toxocara, generally are not known to develop antigenemia in the serum of infected canines.

For several years, the University of Florida, College of Veterinary Medicine has been archiving sera and *D. immitis* collected from euthanized canines that were deemed unadoptable from various Florida shelters (Courtney and Cornell, 1990; Courtney and Zeng, 2001a; Courtney and Zeng, 2001b). This archive includes an extensive assemblage of data that includes *D. immitis* cardiopulmonary burden, sex of each *D. immitis* obtained, and microfilaria status from each dog. This archive is a valuable resource for provision of industry and research reference standards (Atkins, 2003; Courtney and Cornell, 1990; Courtney and Zeng, 2001a; Courtney and Zeng, 2001b). During the last 10 years of archiving serum, anecdotally, we observed a high level of sensitivity and specificity beyond what was reported by previous studies of various tests and proposed a side-by-side comparison of several marketed tests. More recent publications have demonstrated a higher Se for the SNAP® Heartworm RT Test (SNP, IDEXX Laboratories) than was reported eight years earlier (Lee et al., 2011). Another test now designated the WITNESS® Heartworm test (WIT, Zoetis LLC) has been relicensed twice, first in 2013 and then in 2015 with major technological improvements. Evaluations of the earlier iteration of the WIT test kit indicated a wide range of Se (71%–95%), yet high Sp (100%; Courtney and Zeng, 2001a; McCall et al., 2004). In particular a recent study examining canine plasma from diagnostic samples and research dogs, comparative side-by-side testing demonstrated 95% and 90.0% Se while Sp was 96.4% and 98.8%, for WIT and SNP respectively (Starkey et al., 2017). Currently, there is limited data on the field accuracy of this newly re-configured test and others that may have been modified without required relicensing using these sera defined by D. immitis cardiopulmonary burden.

The goal of this study was to evaluate the diagnostic Se (estimated ability to detect a reference-positive sample) and Sp (estimated ability of a test to detect a reference-negative sample) of five commercially available rapid patient-side tests using canine sera defined by adult female *D. immitis* burden (Altman and Bland, 1994a,b; Sackett, 1969). Additionally, Se and Sp for the rapid microplate ELISA DiroCHEK® (DRK, Zoetis LLC) were determined using the same samples.

2. Materials and methods

2.1. Animals and sampling

All work was performed under the approval of the University of Florida Institutional Animal Care and Use Committee Study #200902501 with federal compliance monitored by the United States Department of Agriculture-Animal and Plant Health Inspection Service (USDA-APHIS; Washington, DC) and Office of Laboratory Animal Welfare (OLAW; National Institutes of Health, Bethesda MD). The institutional Animal Care Services program is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC International, Frederick MD). Individual serum samples were collected from 250 shelter dogs that were deemed unadoptable were humanely euthanized by the shelter veterinarian. Serum and *D. immitis* were collected from each cadaver. Prior to necropsy, a 10–15 mL sample of heart blood was collected within 1 h of euthanasia, serum was separated from blood solids, archived at −80 °C and, for this study, sera were organized according to female *D. immitis* burden and samples were randomly chosen from 2004 to 2011. *Dirofilaria immitis* status of the dogs had been determined by necropsy of the cardiopulmonary cavity by a veterinary parasitologist or pathologist. The left side of the heart was dissected following the blood flow, starting at the vena cava. The right atrium and the right ventricle were dissected along the septum into the pulmonary artery. Then, the pulmonary artery and the continuing arterial vasculature extending into the pulmonary parenchyma were dissected following major arterial branches (Courtney and Cornell, 1990). Upon collection, *D. immitis* were immediately washed with saline to remove blood clots, whole blood, and tissue and the HWs were sexed, separated, and counted. A 200 µL aliquot of a non-coagulated blood sample from each dog was examined for microfilariae. If a dog was found to be *D. immitis*-positive but negative for microfilariae on blood smear, a Knott's test was performed (Courtney and Zeng, 2001b).

2.2. Study design

Five in-clinic patient-side *D. immitis* tests were compared in this study: Anigen Rapid One Step® (ANG; Bionote Co, Republic of Korea obtained from Modern Veterinary Therapeutics, Miami, FL), SNAP® 4Dx Plus Test Kit (SNP; IDEXX, Westbrook, ME), WITNESS® Heartworm Canine Heartworm Antigen Test Kit (WIT; Zoetis LLC, Parsippany, NJ), VetScan® Canine Heartworm Rapid Test (VSN; Abaxis, Union City, CA), and Solo Step® CH Canine Heartworm Antigen Test (SLO; Heska, Loveland, CO). All tests were performed according to each manufacturer's protocol. All samples were also tested by a commercial microwell ELISA format (DRK; DiroCHEK® Heartworm Antigen Test Kit; Zoetis LLC, Parsippany, NJ) separately and this data was collected with personnel blinded to the *D. immitis* status of each dog. A total of 250 sera were divided into one of the five following groups based on necropsy results: 1) 50 dogs in which no *D. immitis* (male or female) or microfilaria were found (N), 2) 50 dogs with 1–5 female *D. immitis* (low, L), 3) 50 dogs with 6–20 female *D. immitis* (moderate, M), 4) 50 dogs with 21–40 female *D. immitis* (heavy, H), and 5) 50 dogs with more than 40 female *D. immitis* (very heavy, VH). Although in almost all cases, male *D. immitis* were found, collection into groups for testing only reflected female *D. immitis* status (Table 1). Both sera and test order were randomized and *D. immitis* status was blinded to personnel performing the assays. Prior to testing, each sample was thawed, brought to room temperature, and vortexed; then each sample was analyzed simultaneously by all tests. All assay results were un-blinded by other personnel.

2.3. Statistical analysis

Estimated Se and Sp were calculated using 2 × 2 contingency tables comparing results of each test to cardiopulmonary *D. immitis* status

Table 1
Mean number and standard deviation of *Dirofilaria immitis* worms detected in dogs at necropsy and sorted into subclasses reflecting low (L), medium (M), high (H) and very high (VH) numbers of worms for testing sera with commercial HW kits. Each group consisted of 50 dogs. Mean numbers of *D. immitis* represent the average number of *D. immitis* per dog in each group.

D. immitis Burden	% Dogs with Microfilariae (n)	Mean # Total *D. immitis* ± S.D.	Mean # Female *D. immitis* ± S.D.	Mean # Male *D. immitis* ± S.D.
1–5 (L)	58% (29)	7.62 ± 4.58	2.80 ± 1.30	4.20 ± 3.42
6–20 (M)	84% (42)	24.62 ± 9.71	12.36 ± 4.03	11.44 ± 6.27
21–40 (H)	90% (45)	53.86 ± 15.05	28.26 ± 5.13	23.62 ± 8.79
> 40 (VH)	92% (46)	104.92 (34.18)	58.84 (16.61)	46.90 (18.27)

(Altman and Bland, 1994a,b; Sackett, 1969). Ninety-five percent confidence intervals (CI) were calculated (Campbell and Gardner, 1988; Morris and Gardner, 1988) using a commercial statistical software program (Medcalc v 17.4). True positive and true negative status was based on cardiopulmonary *D. immitis* status. The estimated Se of a test was calculated as 100% x TP/(TP + FN), and the estimated Sp was calculated as 100% x TN/(FP + TN) where TP is True Positive, TN is True Negative, FP is False Positive, and FN is False Negative. Overall % positive agreement was calculated as 100 x (+ TN)/(TP + FP + FN + TN). Likelihood ratios (LR) were calculated as a means to estimate the post-test probabilities of a dog having *D. immitis* (Gardner and Greiner, 2006). The LR^+ was calculated as the Se/(1-Sp) and LR^- was calculated as (1-Se/Sp). The Kappa statistic was calculated as another method of reliability (McHugh, 2012).

3. Results

3.1. Study population

Three north central Florida counties, Duval, Gilchrist, and Alachua, were represented, and consisted of 133 male and 117 female dogs. A total of 250 sera were tested with 200 dogs harboring between 1 and 107 female *D. immitis* and between 1 and 213 total *D. immitis* (Table 1). Dogs with *D. immitis* (n = 200) were tested for microfilariae, of which, 81% (n = 162) were microfilariae-positive (Table 1). In the L group, 29 of 50 dogs had demonstrable microfilaria, 27/29 all agreed between tests and, of the remaining 21 dogs negative for microfilaria, one was discordant between tests. In the M and H groups, eight and five dogs, respectively did not have microfilaria, and all five patient-side tests as well as the DRK were positive. In the VH group of dogs, four dogs did not have microfilaria positive on all tests and all tested positive for all tests. The dogs were classified by weight in 4.5 kg increments and there was no significant difference between weight subclass of dog and total number of *D. immitis*, number of female *D. immitis*, or number of male *D. immitis* (Table 2).

3.2. Patient-side testing

The Se of five rapid *D. immitis* detection kits developed for patient-side testing was compared using a single round of testing in order to replicate a clinical scenario (Table 3). The Se for dogs having between 1 and 108 female worms using a single round of testing was between 98% and 99.5% for the five tests (Table 3). All of the tests missed one dog with a *D. immitis* burden of 55 worms of which 29 were female. The sera of 50 dogs in which *D. immitis* were not detected upon necropsy were tested and three of these *D. immitis* negative dogs were positive for all five tests resulting in an overall Sp of 94%. All of the tests had a $LR^+ > 16$ and a LR^- between 0.01 and 0.03. Overall percent agreement for the five tests and necropsy findings ranged between 98% and 99.6% (Table 3).

For dogs in the L subclass of female *D. immitis* burden, the Se was at least 96% for all five tests (Table 4). For dogs in the M subclass of female *D. immitis* the Se was slightly higher for the five tests with the minimum Se of 98% (Table 3). For dogs in the H subclass of female *D. immitis*, all tests had a Se of 98%, and, at least 98% for the VH subclass (Table 3). One dog with 158 worms of both sexes tested negative on both the SNP and VSN tests. Agreement for weight subclasses of *D. immitis* burden was between 99% and 100% (Table 5).

Table 3
Overall sensitivity, specificity, and positive and negative likelihood ratios of test results from five commercial HW kits for patient-side testing of *Dirofilaria immitis* 200 positive and 50 negative necropsy verified dogs.

Test	Sensitivity (%) (95% C.I.)	Specificity (%) (95% C.I.)	LR^+ (95% C.I.)	LR^- (95% C.I.)
ANG	99.50 (97.25–99.99)	94.00 (83.45–98.75)	16.58 (5.54–49.68)	0.01(0.00–0.04)
SNAP	97.50 (94.26–99.18)	94.00 (83.45–98.75)	16.25 (5.42–48.69)	0.03 (0.01–0.06)
WIT	99.00 (96.43–99.88)	94.00 (83.45–98.75)	16.5 (5.51–49.43)	0.01(0.00–0.04)
VSC	98.50 (95.68–99.69)	94.00 (83.45–98.75)	16.42 (5.48–49.18)	0.02 (0.01–0.05)
SLO	98.00 (94.96–99.45)	94.00 (83.45–98.75)	16.33 (5.45–48.94)	0.02 (0.01–0.06)

ANG = Anigen Rapid One Step® (Bionote), SNAP = SNAP® 4Dx Plus Test Kit (IDEXX), WIT = WITNESS® Heartworm Canine Heartworm Antigen Test Kit (Zoetis), VSC = VetScan® Canine Heartworm Rapid Test (Abaxis), and SLO = Solo Step® CH Canine Heartworm Antigen Test (Heska).

Table 4
Numbers of positive test results from five commercial test kits for patient-side testing for *Dirofilaria immitis*. Two hundred dogs, identified as infected by necropsy, were sorted into groups of 50 based on numbers of female *D. immitis*.

D. immitis Burden	ANG # (%)	SNAP # (%)	WIT # (%)	VSC # (%)	SLO # (%)
1–5 (L)	50 (100)	48 (94)	49 (98)	49 (98)	48 (96)
6–20 (M)	50 (100)	49 (98)	50 (100)	50 (100)	49 (98)
21–40 (H)	49 (98)	49 (98)	49 (98)	49 (98)	49 (98)
> 40 (VH)	50 (100)	49 (98)	50 (100)	49 (98)	50 (100)

ANG = Anigen Rapid One Step® (Bionote), SNAP = SNAP® 4Dx Plus Test Kit (IDEXX), WIT = WITNESS® Heartworm Canine Heartworm Antigen Test Kit (Zoetis), VSC = VetScan® Canine Heartworm Rapid Test (Abaxis), and SLO = Solo Step® CH Canine Heartworm Antigen Test (Heska).

Table 5
Percent agreement with 95% C.I. and Kappa statistics comparing each of five commercial Heartworm tests with a commercial microwell ELISA format.

Tests	# Matched	% Agreement	95% C.I.	Kappa	SE Kappa
DRK vs ANG	248	99.2	97.14, 99.90	0.97	0.06
DRK vs SNAP	246	98.4	95.95, 99.56	0.95	0.063
DRK vs WIT	247	98.8	96.53, 99.75	0.96	0.063
DRK vs VSC	246	98.4	95.95, 99.56	0.95	0.063
DRK vs SLO	247	98.8	96.53, 99.75	0.96	0.06

DRK = microplate ELISA (DiroCHEK®; Zoetis), ANG = Anigen Rapid One Step® (Bionote), SNAP = SNAP® 4Dx Plus Test Kit (IDEXX), WIT = WITNESS® Heartworm Canine Heartworm Antigen Test Kit (Zoetis), VSC = VetScan® Canine Heartworm Rapid Test (Abaxis), and SLO = Solo Step® CH Canine Heartworm Antigen Test (Heska).

Table 2
Mean number of total *D. immitis* worms ± standard deviation per dog detected in the heart and lungs at necropsy presented by weight class of dog.

Weight Class (kg)	# of dogs	Mean Number Female *D. immitis* (± S.D.)	Mean Number Total *D. immitis* (± S.D.)
< 9.1	9	5.9 (6.8)	10.9 (11.6)
> 9.1–13.6	21	16.95 (17.0)	34.7 (34.0)
> 13.6–18.1	31	18.7 (18.8)	35.7 (36.0)
> 18.1–22.7	53	18.7 (23.7)	33.9 (44.3)
> 22.7–27.2	49	24.2 (24.0)	45.1 (43.5)
> 27.2–31.8	19	21.3 (21.0)	42.9 (41.25)
> 31.8	18	9.9 (12.2)	17.9 (24.2)

3.3. Comparison of DiroCHEK® to patient-side testing

The Se and Sp for the DRK test compared to cardiopulmonary necropsy results on this cohort of dogs was 99% (95% CI = 96.43–99.88%) and 96% (95% CI = 86.29–99.51%), respectively, with a LR^+ of 24.75 (95% CI = 6.37 to 96.24) and LR^- of 0.01 (95% CI = 0.00 to 0.04). Agreement between all tests and DRK and within all of the tests irrespective of test format was between 97 and 100%, and the Fliess's Kappa was > 0.95 (Table 5).

4. Discussion

The goal of this study was to evaluate the diagnostic Se and Sp of currently available commercial tests. In this study, the reference sera was derived from a cohort of dogs obtained from multiple Florida counties. Dogs were evaluated for *D. immitis* burden solely by necropsy examination of the heart and pulmonary vasculature. Thus, this reference sera was defined by various levels of cardiopulmonary worm burden, not aberrant worm migration. These samples do not reflect status of clinical *D. immitis* disease because none of the dogs was evaluated clinically before euthanasia. Of these dogs, 81% were positive for microfilaria, which was slightly higher but consistent with previous studies that identified 60–75% of dogs as blood microfilariae positive (Courtney and Zeng, 2001b). The percentage of dogs positive for microfilariae also increased as worm burden increased. No correlation was found between dog weight class *D. immitis* burden.

Using these defined sera, all of the rapid antigen detection tests were highly sensitive for the presence of female *D. immitis* in the heart and lungs, with the current results between 97.5 and 99.0%. Notably in this study, the Se was high even for dogs with low worm burdens, between 94.0% and 100%. Previous studies have indicated lower Se when compared with necropsy results, especially for dogs with a low worm burden (Courtney and Zeng, 2001a; Martini et al., 1996; McCall et al., 2004; Atkins, 2003). Courtney and Zeng (2001a) tested dogs with a low worm burden, defined as having 1–10 *D. immitis*, with Se between 52 and 67%, respectively. Atkins (2003) classified dogs with a low worm burden as having 1–4 *D. immitis* and the Se of the SNP, VSN, and SLO tests was 84%, 78%, and 79%, respectively. The study by Lee et al. (2011) examined the SNAP® Heartworm RT test and reported a Se a 96% in 1–4 *D. immitis*-positive dogs. Many of the tests undergo modest changes that do not require revalidation for licensing, which could affect overall readability and test performance. In a recent study, examining canine plasma samples submitted for diagnostic *D. immitis* testing and presumed negative samples from purpose bread beagles, the WIT, and SNP were 96.4% and 98.9% specific when the DRK was used as the reference standard (Starkey et al., 2017). Additionally, several of the early studies had results from more than one technician from within a practice or between practices, which also could have affected study results. The high Se in our study may also reflect higher amounts of detectable antigen within samples derived from heart bleeds, however the earlier study by Courtney and Zeng (2001a) used serum derived from heart blood.

The Sp of all tests was also high (94%) with same three dogs testing positive on all of the patient-side tests. The DRK results were in agreement for two of these three dogs. These dogs were consistently positive on all three tests which may demonstrate either false positive results or an original misclassification by necropsy. In addition, these dogs could have undergone recent treatment with a macrocyclic lactone leaving retained circulating antigen (Martini et al., 1996; Rohrbach and Patton, 2013).

One dog with a high worm burden tested false negative on all five tests and the DRK assay. In a previous study, animals with false negative findings have been postulated as having high amounts of antibody against *D. immitis* which may mask antigen reactivity (Drake et al., 2015). In the Drake et al. (2015) study, animals had been treated with the slow kill regimen and heat treatment of sera demonstrated an increase in reactivity. In another recent study, unmasking was also demonstrated by heat treatment (Venco et al., 2017). The original work by Weil (1987) demonstrated that antigen was retrieved by trichloroacetic acid (TCA), heat, and DNase treatments. We did not pursue heat treatment due to sample amount limitation; in this situation addition of TCA or DNase treatment may offer alternatives when samples amount is limited. Nonetheless, these false negative animals are a conundrum for veterinarians testing dogs, which reside in areas of high *D. immitis* prevalence. Careful evaluation of dogs residing in areas with high *D. immitis* prevalence with doubtful history of prophylaxis should be carefully examined with multiple test formats.

The DRK assay is considered a second test to be performed in pursuit of confirmatory diagnosis. In this study, DRK had a slightly increased Sp than the patient-side formats. The Se was higher but if there was disagreement between tests by more than one test (two dogs only), agreement between DRK and certain tests still did not rectify status of the dog based on testing alone. One dog in the group with a low worm burden tested positive on VSN and ANG, and DRK agreed with this result. However, another dog with a medium worm burden tested positive on ANG and WIT, but DRK agreed with the negative results of the SNP and SLO tests. Nonetheless, the Kappa statistic, developed as a measurement of reliability between either raters or methods (as in quality assurance testing), was high irrespective of combination of tests. The very high Kappa values indicate that any combination of testing is likely to result in the same degree of agreement. The ANG and WIT tests agree most highly with DRK, and agreement between ANG and WIT was highest for two consecutively performed patient-side tests.

The positive and negative predictive value of a test is also dependent upon prevalence (Gardner and Greiner, 2006; Sackett, 1969; Vezzani et al., 2008). Since these predictive indicators are different depending on geographic location or more importantly previous prophylaxis in areas of high prevalence, we chose LR^+ and LR^- ratios, which are calculated on pre- and post-test results, as a means to determine what a positive or a negative test means in terms of reliably predicting infection. The LR^+, which is the likelihood of dogs having *D. immitis* based on a positive result, would be 16 times more likely of having *D. immitis* infection than not having *D. immitis*. Likewise, a dog with negative test results would be 0.02 times more likely of having *D. immitis* infection than not.

All of the tests we evaluated (except for the DRK) are marketed as patient-side screening tests, and for a screening test, Se should be theoretically higher than Sp (Gardner and Greiner, 2006; Sackett, 1969). Thus, a screening test-positive result should require additional confirmatory and clinical testing as recommended by the AHS (2014). The testing should include a plate antigen ELISA and test for microfilariae via direct smear and/or Knott's test. Regarding clinical status and confirmation of *D. immitis* infection, radiography and echocardiography (AHS, 2014) provides both the most objective and definitive diagnosis, respectively, of adult *D. immitis* infection (AHS, 2014).

Archives of the sera of dogs used in our study could be improved by cardiopulmonary histopathological evaluation, cardiac morphometries, and examination of dogs for ectopic *D. immitis*. This would allow the development of clinical indices correlating *D. immitis* burden, disease state, and test status. In addition, pre-euthanasia cephalic vein samples may better reflect the antigen load of clinical dog samples, however, there is little experimental data which indicates this is necessary.

5. Conclusions

In this study, all of the commercial patient-side rapid screening tests were highly accurate, sensitive, and specific. All of the tests had more than 90% agreement with necropsy results, which is quite high, reaching confirmatory test reliability. Given the complexities of *D. immitis* infection, these tests can be used to provide patient-side information. This allows practitioners to make informed decisions based on test results, *D. immitis* prevention history, geographic location of the

dog, and the need for additional diagnostic work-up before instituting or continuing prophylaxis. When history, activity, and health of the dog do not coincide with *D. immitis* screening findings, confirmatory tests and complete clinical evaluation are always recommended.

Conflict of interest statement

This research was funded by Zoetis LLC under the ethical guidelines and research contracts developed by the University of Florida Office of Grants and Contracts and the research was conducted at the University of Florida. Student assistance was conducted as part of the Florida Veterinary Student Scholars Program, University of Florida College of Veterinary Medicine. This manuscript underwent legal review by Zoetis for proprietary reasons before first submission; no scientific content was revised. Additional mentorship was provided by the Florida Veterinary Scholars Program.

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References

AHS, 2014. Guides for the diagnosis, prevention, and management of heartworm (Dirofilaria immitis). Am. Heartworm Soc. https://www.heartwormsociety.org/images/pdf/2014-AHS-Canine-Guidelines.pdf.

Altman, D., Bland, J., 1994a. Diagnostic tests 2 - predictive values. Br. Med. J. 309, 102.

Altman, D., Bland, J., 1994b. Diagnostic tests 1 - sensitivity and specificity. Br. Med. J. 308, 1552.

Aroch, I., Rojas, A., Slon, P., Lavy, E., Segev, Gl, Baneth, G., 2015. Serological cross-reactivity of three commecial in-house immunoassays for detection of *Dirofilaria immitis* antigens with *Sprocerca lupi* in dogs with benigh esophageal spricercosis. Vet. Parasitol. 211, 303–305.

Atkins, C.E., 2003. Comparison of results of three commercial heartworm antigen test kits in dogs with low heartworm burdens. J. Am. Vet. Med. Assoc. 222, 1221–1223.

Brunner, C.J., Hendrix, C.M., Blagburn, B.L., Hanrahan, L.A., 1988. Comparison of serologic tests for detection of antigen in canine heartworm infections. J. Am. Vet. Med. Assoc. 192, 1423–1427.

Campbell, M., Gardner, M., 1988. Statistics in medicine - calculating confidence-intervals for some non-parametric analyses. Br. Med. J. 296, 1454–1456.

Courtney, C.H., Cornell, J.A., 1990. Evaluation of heartworm immunodiagnostic tests. J. Am. Vet. Med. Assoc. 197, 724–729.

Courtney, C., Zeng, Q., 2001a. Comparison of heartworm antigen test kit performance in dogs having low heartworm burdens. Vet. Parasitol. 96, 317–322.

Courtney, C., Zeng, Q., 2001b. Relationship between microfilarae count and sensitivity of the direct smear for diagnosis of canine dirofilariosis. Vet. Parasitol. 94, 199–204.

Drake, J., Gruntmeir, J., Merritt, H., Allen, L., Little, S.E., 2015. False negative antigen tests in dogs infected with heartworm and placed on macrocyclic lactone preventives. Parasit. Vectors 8, 68.

Gardner, I.A., Greiner, M., 2006. Receiver-operating characteristic curves and likelihood ratios: improvements over traditional methods for the evaluation and application of veterinary clinical pathology tests. Vet. Clin. Pathol. 35, 8–17.

Goodwin, J.K., 1998. The serologic diagnosis of heartworm infection in dogs and cats. Clin. Tech. Small Anim. Pract. 13, 83–87.

Jackson, R., Otto, G., Bauman, P., Peacock, F., Hinrichs, W., Maltby, J., 1966. Distribution of heartworms in right side of heart and adjacent vessels of dog. J. Am. Vet. Med. Assoc. 149, 515–519.

Jackson, R., Otto, G., Vonlichtenberg, F., 1962. Occurrence of adult heartworms in venae cavae of dogs. J. Am. Vet. Med. Assoc. 141, 117–121.

Knight, D.H., 1977. Heartworm heart disease. Adv. Vet. Sci. Comp. Med. 21, 107–149.

Knight, D.H., 1987. Heartworm infection. Vet. Clin. North Am. Small Anim. Pract. 17, 1463–1518.

Lee, A.C., Bowman, D.D., Lucio-Forster, A., Beall, M.J., Liotta, J.L., Dillon, R., 2011. Evaluation of a new in-clinic method for the detection of canine heartworm antigen. Vet. Parasitol. 177, 387–391.

Martini, M., Capelli, G., Poglayen, G., Bertotti, F., Turilli, C., 1996. The validity of some haematological and ELISA methods for the diagnosis of canine heartworm disease. Vet. Res. Comm. 20, 331–339.

McCall, J.W., Guerrero, J., Genchi, C., Kramer, L., Bazzocchi, C., Simon, F., Martarino, M., Am Heartworm, S., 2004. Recent advances in heartworm disease. Vet. Parasitol. 125, 105–130.

McHugh, M.L., 2012. Interrater reliability: the kappa statistic. Biochem. Med. (Zagreb) 22, 276–282.

Morris, J., Gardner, M., 1988. Calculating confidence-intervals for relative risks (odds ratios) and standardized ratios and rates. Br. Med. J. 296, 1313–1316.

Rohrbach, B.W., Odoi, A., Patton, S., 2011. Risk factors associated with failure of heartworm prophylaxis among members of a national hunting dog club. J. Am. Vet. Med. Assoc. 238, 1150–1158.

Rohrbach, B.W., Patton, S., 2013. Effects of diagnostic test accuracy and treatment efficacy on the occurrence of suspected failure of heartworm prophylaxis in dogs. J. Vet. Intern. Med. 27, 791–797.

Sackett, D.L., 1969. Clinical epidemiology. Am. J. Epidemiol. 89, 125–127.

Schnyder, M., Deplazes, P., 2012. Cross-reactions of sera from dogs infected with Angiostrongylus vasorum in commercially available *Dirofilaria immitis* test kits. Parasit. Vectors 5, 258.

Venco, L., Manzocchi, S., Genchi, M., Kramer, L.H., 2017. Heat treatment and false-postitive heartworm antigen testing in ex vivo parasites and dogs naturally infected by *Dirofilaria repens* and Angiostrongylus vasorum. Parasit. Vectors 10, 129–134.

Starkey, L.A., Bowles, J.V., Payton, M.E., Byron, B.L., 2017. Comparative evaluation of available point-of-care heartworm antigen tests using well-characterized canine plasma. Parasit. Vectors 10, 145–149.

Vezzani, D., Fontanarrosa, M.F., Eiras, D.F., 2008. Are antigen test kits efficient for detecting heartworm-infected dogs at the southern distribution limit of the parasite in South America? Preliminary results. Res. Vet. Sci. 85, 113–115.

Weil, G.J., Malane, M.S., Powers, K.G., 1984. Detection of circulating parasite antigens in canine dirofilariasis by counterimmunoelectrophoresis. Am. J. Trop. Med. Hyg. 33, 425–430.

Weil, G.J., Malane, M.S., Powers, K.G., Blair, L.S., 1985. Monoclonal antibodies to parasite antigens found in the serum of Dirofilaria immitis-infected dogs. J. Immunol. 134, 1185–1191.

Weil, G.J., 1987. Dirofilaria immitis: identification and partial characterization of parasite antigens in the serum of infected dogs. Exp. Parasitol. 64, 244–251.