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Validation for use with coyotes (*Canis latrans*) of a commercially available enzyme-linked immunosorbent assay for *Dirofilaria immitis*

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Abstract

Serological tests offer a potentially powerful tool for monitoring parasites in wildlife populations. However, such tests must be validated before using them with target wildlife populations. We evaluated in coyotes (*Canis latrans*) the performance of a commercially available serological test used to detect canine heartworm (*Dirofilaria immitis*) in domestic dogs. We obtained 265 coyote carcasses and serological specimens from 54 additional coyotes from several regions of California, USA. We necropsied coyotes to determine the adult heartworm infection status. Blood was collected at necropsy on filter paper strips and allowed to dry; it was later eluted in a buffer solution, and the supernatant was tested for heartworm. Receiver operating characteristic (ROC) analysis was used to assess discriminatory power of the test and indicated a 93% probability that a randomly selected infected coyote would exhibit a higher enzyme-linked immunosorbent assay (ELISA) value than a randomly selected uninfected coyote. We estimated specificity at 96% (95% CI: 92–98%) for 165 uninfected coyotes and sensitivity at 85% (77–91%) for 100 infected coyotes, results similar to published values for the commercial serological test used with dog serum or plasma. Test performance was similar for filter paper specimens and supernatant of frozen whole blood collected in EDTA tubes (i.e. hemolyzed plasma). We found no difference in test performance

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among geographic or demographic coyote groups. Our findings support application of the test to filter paper or standard serological specimens for detection of heartworm in coyote populations.
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1. Introduction

The accuracy of serological tests for detecting pathogens in wildlife populations is often unknown, limiting wildlife disease investigations (Gardner et al., 1996). For example, the apparent prevalence of infection in a population as measured by a serological test (i.e. seroprevalence) provides a biased estimate of the true prevalence (Gardner et al., 1996; Salman and Gardner, 2000). To estimate the true prevalence of a pathogen using a serological test, the sensitivity and specificity of the test must be known. Here we address the performance on coyotes (*Canis latrans*) of a serological test designed to detect canine heartworm (*Dirofilaria immitis*) in dogs.

Research has shown that commercially available enzyme-linked immuno-sorbent assays (ELISAs) are highly sensitive and specific for detection of heartworm antigen in domestic dogs (Courtney and Zeng, 1993, 2001). However, the sensitivity and specificity have not been determined for wild canids. It is possible that other parasites in wild canids that do not occur in dogs have antigens that cross-react with test antibodies or that wild canid blood chemistry disrupts the test (Gardner et al., 1996; Roemer et al., 2000). Conversely, although many wild canids have heartworms that are morphologically similar to the *D. immitis* in dogs (Sacks, 1998; Sacks and Blejwas, 2000), the equivalence of the heartworm antigens from the different hosts is unknown.

Serological tests have often been evaluated solely on their sensitivity and specificity under a restricted set of circumstances (Greiner et al., 2000). Our investigation included more general aspects of test performance. Microwell ELISAs, which produce a continuous optical density (OD) (i.e. ELISA value), require an arbitrary cutoff value to classify the test results as positive or negative and hence, to calculate sensitivity and specificity. Comparisons of diagnostic tests based solely on sensitivities and/or specificities maybe confounded by particular choices of cutoff value. We used receiver operating characteristic (ROC) curve analysis to provide a measure of test performance independent of cutoff value (Greiner et al., 2000). While ROC analysis allows an objective assessment of the diagnostic potential of a test, the eventual practical use of the test (and characterization of its sensitivity and specificity) still requires a defined cutoff value. Thus, another important consideration in evaluating the practical utility of a diagnostic test is robustness to errors in cutoff selection. Because selection of a particular cutoff value is arbitrary, a desirable attribute of a diagnostic test is for the optimal (depending on the purpose) sensitivity and specificity to be approached by a broad range of cutoff values.

Robustness to cutoff value is especially important when testing serological specimens that vary in quality. This consideration is especially relevant to wildlife epidemiological applications where it is desirable to test large numbers of specimens from many regions, which may be collected and handled by individuals untrained in serological techniques. It

is useful to have a test that performs equally well on a broad range of types and conditions of serological specimens. For example, specimens of clotted whole blood, which can be extracted post-mortem, maybe easier to obtain in large numbers than serum collected from live animals. We were especially interested in the performance of the test on desiccated blood specimens collected post-mortem on filter paper strips. Collection of blood on filter paper strips is a simple procedure that requires little training and represents a potentially powerful method for collection of large numbers of blood specimens. The California Plague Surveillance Program (CPSP), which involves many wildlife trappers in blood collection, has resulted in the collection of numerous coyote specimens from throughout California. These specimens already have been validated for and used in seroepidemiological studies of *Bartonella* spp. (Chang et al., 1999) and *Ehrlichia* spp. (Pusterla et al., 2000). It is unknown how heartworm tests designed for use with standard serological specimens (e.g. plasma or serum) would perform when used with such filter paper specimens.

We evaluated a commercially available ELISA for heartworm in dogs for use on coyotes. Objectives were to characterize discriminatory power of the test as used on filter paper specimens, estimate sensitivity and specificity, and compare performance of the ELISA between filter paper specimens and standard serological specimens. We determined true infection status of a sample of coyotes by necropsy, against which we compared serological testing of filter paper specimens. We used a different sample of coyotes to assess the agreement between test classifications using filter paper specimens and supernatant of frozen whole blood collected in EDTA-coated tubes (i.e. hemolyzed plasma) from live or freshly killed coyotes.

2. Materials and methods

2.1. Field collections

We obtained 265 carcasses of coyotes that were trapped or shot by USDA/Wildlife Services and Santa Clara County Vector Control District specialists as part of livestock depredation control and public health programs in 15 counties of central and northern California. Wildlife specialists usually placed carcasses in freezers within the same day of death, although some carcasses appeared to have been subject to ambient temperatures for a few days. On a subset of 42 of these coyotes, specialists in the field obtained filter paper blood specimens immediately after coyotes were killed. The field-collected specimens were compared with those we acquired at necropsy to assess unwanted effects of our experimental procedure (see below). We also obtained filter paper specimens and whole blood from 54 additional live or freshly killed coyotes from the Santa Clara County Vector Control District that were used to compare ELISA results on filter paper specimens versus (hemolyzed) plasma specimens (see below).

2.2. Necropsy

Filter paper strips designed to absorb 40 μl of blood (Nobuto® strips, Advantec MFS, Pleasanton, CA) were submerged in blood from the heart or thoracic cavity, allowed to dry, and stored in manila envelopes. Clotted whole blood specimens were collected and frozen.

Coyotes were aged by enumeration of cementum annuli of a lower canine or premolar root (Linhart and Knowlton, 1967; Matsons Laboratory, Milltown, MT). Heartworm infection status and number of adult heartworms were determined for each coyote via inspection of the right ventricle through both branches of the pulmonary artery, the right atrium, and thoracic vena cavae. It is likely that a small number of coyotes (1%) determined in this way to be "uninfected" had ectopic patent infections, which would cause diagnostic potential (see below) and specificity to be slightly underestimated (Courtney and Cornell, 1990). Heartworms were sexed, measured, and frozen in physiological saline. Lung smears were performed to detect microfilariae (Weinmann and Garcia, 1980) in all coyotes for which $\geq$1 male and $\geq$1 female heartworm were found. Specimens were accessioned in the US National Parasite Collection (National Parasitology Institute, Beltsville, MD: accession number 87091).

2.3. Serological testing

Blood was eluted from filter paper strips using the CPSP protocol. This procedure involved soaking the strip overnight in 400 μl of borate buffer solution (1.5 M NaCl, 0.5 M H_3BO_3, 1.0 M NaOH, and distilled H_2O) at 4 °C; heat inactivating in a 56 °C water bath for 30 min, adding washed, sheep red blood cells, and centrifuging to separate supernatant. Because the strips were designed to absorb 40 μl of blood, the eluted solution represented a minimum 1:10 dilution (if all blood eluted from the strip into the 400 μl of buffer). Serological tests of these specimens, and of supernatant of whole blood collected at necropsy (NBS) and supernatant of whole blood collected from live coyotes or immediately post-mortem in the field (whole blood supernatant (WBS)) were conducted with the DiroChek microwell ELISA (Synbiotics Corporation, San Diego, CA, lot numbers 11186, 11190, and 11199). This commercially available ELISA was designed for use on serum or plasma, such that our use on NBS, WBS, and filter paper specimens represented off-label uses of the assay. All specimens were anonymously labeled so that testing was blind. Reactions were carried out in 96-microwell plates, including a positive and negative control supplied by the manufacturer. Reactions were stopped with 50 μl of 1% sodium dodecyl sulfate 5 min after addition of the chromogenic substrate buffer ("reagent 2"), and results were quantified with a microplate spectrophotometer (SPECTRAmax 340, Molecular Devices Corporation, Sunnyvale, CA). ELISA values were calculated as the difference between OD readings at 650 and 490 nm.

2.4. Data analyses

2.4.1. Accuracy of the ELISA used on filter paper specimens

We used ROC curves to quantify the diagnostic potential (i.e. discriminatory power) of the test (Greiner et al., 2000). The ROC curve is a plot (sometimes smoothed) of sensitivity on the quantity $(1 - \text{Sp})$ of a test as calculated for several sequential cutoff values. The discriminatory power of a test corresponds to the area under the ROC curve (AUC), ranging from 50% for a test with no discriminatory power to 100% for a perfect test. The main advantage of using AUC to indicate test performance is that it is independent of cutoff value used. The AUC is directly interpreted as the probability that a randomly selected infected individual will have a higher ELISA value than a randomly selected uninfected individual

(Hanley and McNeil, 1982). Because ELISA value distributions for our uninfected and infected coyotes were non-normal, we used non-parametric AUCs and associated standard errors (Greiner et al., 2000).

We estimated the sensitivity (the proportion of infected individuals classified correctly) and specificity (the proportion of uninfected individuals classified correctly) of the testing procedure. Sensitivity and specificity, which depend on the choice of cutoff value, relate to the AUC as follows. A test with high discriminatory power (a high AUC) is one that minimizes the tradeoff between sensitivity and specificity i.e. enabling increases in sensitivity (specificity) with little loss in specificity (sensitivity); a perfect test allows both parameters to be 100% at the same cutoff. To select a cutoff, we used the Youden index, J ($J = \mathrm{Se} + \mathrm{Sp} - 1$) (Greiner et al., 2000). We chose as the cutoff value one that maximized J while erring on the side of reduced sensitivity (i.e. within the range of OD values corresponding to similarly high J values). Maximizing J results in a cutoff value that minimizes the number of false classifications (positive and negative combined), when true prevalence is at 50%. Sensitivity was calculated as the proportion of infected (necropsy-positive) animals that tested positive and specificity was calculated as the proportion of uninfected (necropsy-negative) animals that tested negative. The 95% CI were calculated for these parameters according to Zar (1984, p. 378).

To assess the population-level accuracy of the test, we compared the heartworm seroprevalence (as determined with the filter paper specimens) to the true prevalence (as determined at necropsy) among seven geographically separated populations (Mendocino County, Sonoma County, Napa County, El Dorado County, Santa Clara/San Mateo Counties, Yuba/Sutter Counties, Butte/Glenn/Tehama Counties). We also calculated an unbiased prevalence estimate from the seroprevalence, based on the sensitivity and specificity determined as described above. This estimate was calculated as follows (Gardner et al., 1996):

$$\text{unbiased prevalence} = \frac{\text{seroprevalence} + \mathrm{Sp} - 1}{\mathrm{Se} - 1 + \mathrm{Sp} - 1}.$$

2.4.2. Comparison of types of serological specimens

Possible unwanted effects of our specimen collection procedures (waiting until necropsy as opposed to collecting blood immediately post-mortem in the field), such as degradation and excess clotting of blood, were assessed using a subset of 42 coyotes for which we had filter paper blood specimens taken by trappers in the field as part of the CPSP (i.e. blood taken immediately after death). To assess effects on discriminatory power of tests, we compared ROC curves generated from field and necropsy specimens. The AUCs were compared using a non-parametric paired test based on the Mann–Whitney U-statistic (Greiner et al., 2000). To assess systematic effects on the ELISA value of tests, we performed a Wilcoxon paired-sample test (Zar, 1984, p. 154) between field and necropsy specimens; this was done separately for uninfected and infected coyotes because absence or presence of antigen was likely to affect differences. The magnitude of a significant difference provided the basis for an adjustment of the cutoff value for use with field-collected specimens.

To assess whether the dilution involved in the processing of filter paper specimens reduced sensitivity, we compared test results between blood on filter paper strips and undiluted blood, both collected at necropsy from a subset of 80 coyotes. Comparisons were made

with respect to ROC curves and ELISA values using methods similar to the comparison described previously for field- and necropsy-collected filter paper strip specimens.

We wished to determine the accuracy of the test applied to a more standard form of serological specimen. Unfortunately, we had no such specimens from the necropsied coyotes. So, filter paper and WBS specimens from 54 coyotes that were not necropsied were compared to assess agreement of infection classification from the two specimen types. High agreement would indicate that sensitivity and specificity were similar for the two specimen types. We used Fisher's exact tests to define significant differences in putative sensitivities and specificities between specimen types. Because the coyotes were not necropsied, their true infection status was unknown requiring that putative sensitivities and specificities be calculated under different assumptions. First, we assumed the filter paper specimens reflected the sensitivity and specificity estimated from the necropsied coyotes, the expected case. Next, to encompass more extreme scenarios we assumed all filter paper test results were correct and then assumed all WBS test results were correct. The two latter assumptions were conservative because they maximized the probability of finding a significant difference between specimen types under a broad range of possibilities.

3. Results

3.1. Validation using necropsy specimens

The AUC was estimated at 93% (±S.E. = 1.8%; Fig. 1). Based on the Youden index and erring on the side of reduced sensitivity (Fig. 2), we selected a cutoff of 0.07 OD, which produced estimates of Sp = 96% (95% CI: 92–98%) and Se = 85% (77–91%). The sensitivity of the test increased with female heartworm burden (Fig. 3). Sensitivity was

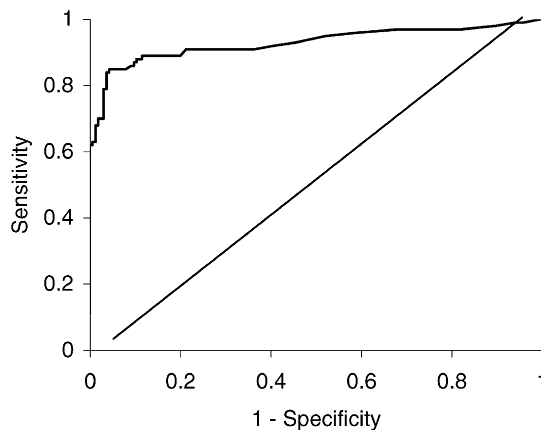

Fig. 1. ROC curve for the DiroChek ELISA used with filter paper specimens of coyote blood supernatant illustrating range of possible sensitivity–specificity combinations ($n = 165$ uninfected coyotes, $n = 100$ infected coyotes). Diagonal line represents hypothetical curve corresponding to a test with no discriminatory power.

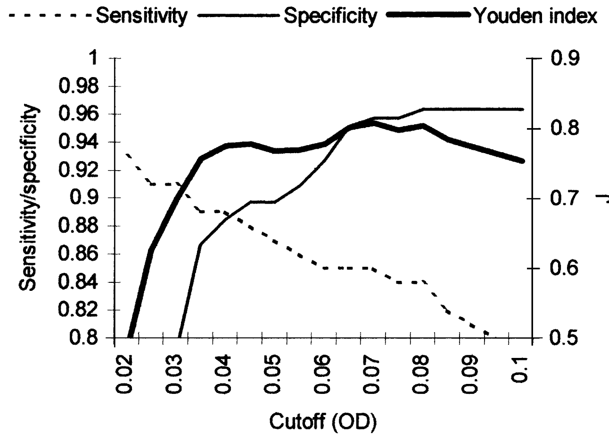

Fig. 2. Sensitivity, specificity, and Youden index (J) as functions of cutoff value, illustrating wide range of cutoff values with similar accuracy. The Youden index maximizes accuracy (efficiency) when prevalence is 0.50.

100% (92–100%) in 44 infected coyotes that were microfilaremic and 71% (55–84%) in 41 infected coyotes that were amicrofilaremic.

Of the 15 false-negative coyotes, six had single-sex infections (male-only) and eight involved immature female heartworms (length $\bar{x} = 120$ mm, range $= 81$–206 mm). Eight false-negative coyotes were female and seven were males, which was not significantly different from the sex ratio of true positives (43F:57M, Fisher's exact test $P = 0.17$). Neither false-negative nor false-positive coyotes were geographically clustered, although sample sizes were insufficient for statistical assessment (Tables 1 and 2). Based on a subset of coyotes for which age was determined, three false-negative coyotes were <1 year old and five were ≥ 1 year old compared to 11 and 44, respectively, of the true-positive coyotes, which was not a significant difference (Fisher's exact test $P = 0.11$).

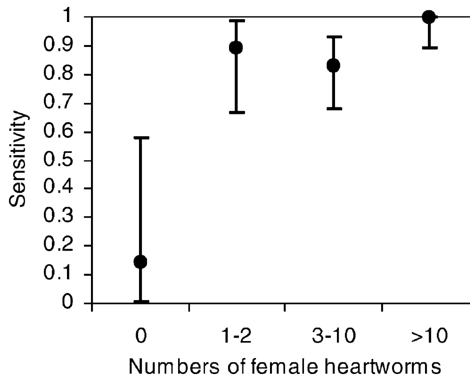

Fig. 3. Sensitivity estimates (±95% CI) for the DiroChek ELISA as a function of female heartworm burden as applied to filter paper specimens of coyote blood supernatant ($n = 7$, 19, 41, and 33 coyotes with 0 (male heartworms only), 1–2, 3–10, and >10 female heartworms, respectively).

Table 1
Distribution of false-negative coyotes among central and northern California counties

County	No. infected	Expected no. false-negatives	Observed no. false-negatives
Mendocino	39	5.9	6
Sonoma	17	2.6	4
El Dorado	11	1.7	3
Napa	10	1.5	1
Santa Clara	10	1.5	0
Other[a]	13	2.0	1

[a] Butte, Glenn, Sutter, Yolo, and Yuba.

3.2. *Population-level accuracy and bias of the test*

Both the seroprevalence and unbiased prevalence estimates were highly correlated with the true prevalence (both $r = 0.97$; Fig. 4). As expected, the unbiased estimate of prevalence was, on average, closer to the true prevalence, although differences were slight.

3.3. *Effect of waiting until necropsy to collect blood on filter paper strips*

Based on the subset of 42 coyotes with field-collected blood specimens, the AUC for necropsy-collected specimens was not significantly different from field-collected specimens ($z = 0.29$; $P = 0.77$; Fig. 5). For uninfected coyotes, ELISA values were higher for necropsy-collected specimens than field-collected specimens (median difference = 0.01 OD; Wilcoxon $T = 70$; $P < 0.001$). For infected coyotes, ELISA values did not differ significantly between field-collected and necropsy-collected specimens (Wilcoxon $T = 27$; $P = 0.35$). Because ELISA values associated with uninfected coyotes tended to be 0.01 OD less for field-collected than necropsy-collected specimens, we used the cutoff value, 0.06 OD (i.e. 0.07–0.01 OD), for field-collected specimens.

3.4. *Effect of dilution associated with filter paper strips*

Based on the subset of 80 coyotes for which we tested both filter paper specimens and NBS, the AUCs were not significantly different ($z = 0.23$; $P = 0.82$; Fig. 6). ELISA values were higher for NBS than filter paper specimens for uninfected coyotes

Table 2
Distribution of false-positive coyotes among central and northern California counties

County	No. uninfected	Expected no. false-positives	Observed no. false-positives
Yuba	39	1.7	3
Napa	22	0.9	0
Santa Clara	21	0.9	0
Mendocino	15	0.6	2
Other[a]	68	2.9	2

[a] Alpine, Butte, El Dorado, Glenn, Placer, Solano, Sonoma, Sutter, Tehama, Yolo, and Yuba.

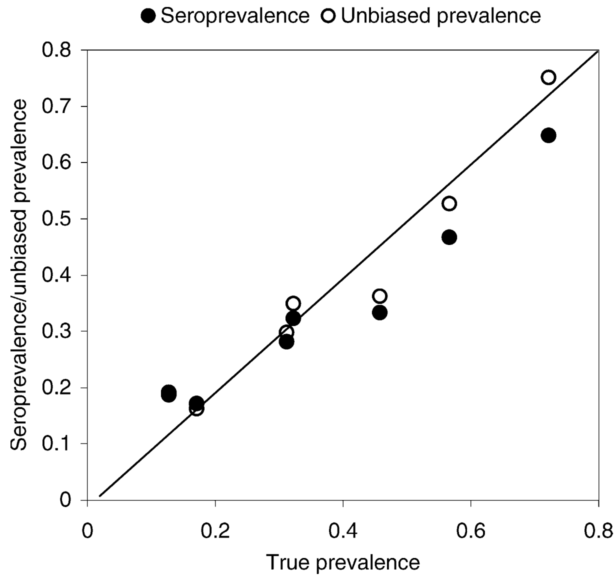

Fig. 4. Relationship between observed seroprevalence, unbiased prevalence, and true prevalence as determined by necropsy, among seven coyote populations (total $n = 252$, population $n = 24$–53). Adjusted seroprevalence $= 1.23 \times$ seroprevalence $\times$ 0.05, based on the formula given by Gardner et al. (1996), assuming $Se = 0.85$ and $Sp = 0.96$. The diagonal line indicates equality between estimates and true prevalence.

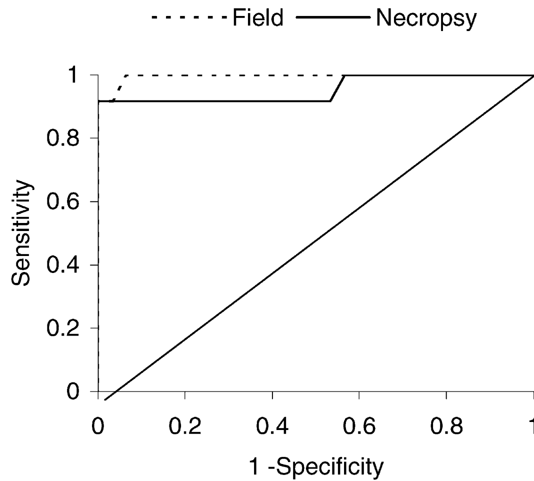

Fig. 5. ROC curves for the DiroChek ELISA used with filter paper specimens of coyote blood supernatant eight collected in field versus at necropsy ($n = 30$ uninfected coyotes, $n = 12$ infected coyotes). Diagonal line represents hypothetical curve corresponding to a test with no discriminatory power.

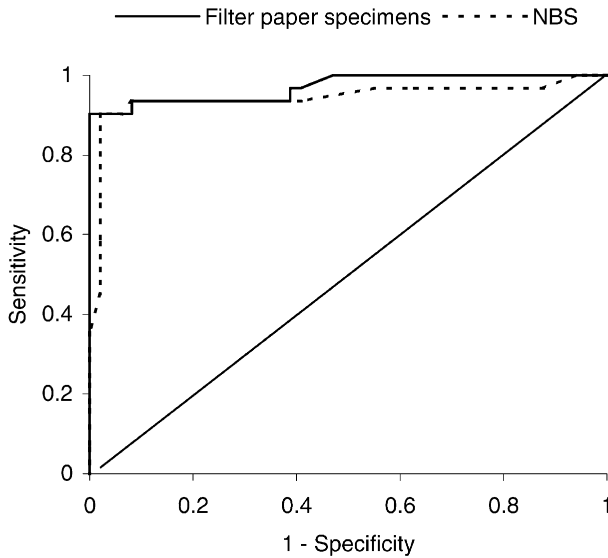

Fig. 6. ROC curves for the DiroChek ELISA used with filter paper specimens versus when used with necropsy blood supernatant (NBS; $n = 49$ uninfected coyotes, $n = 31$ infected coyotes). Diagonal line represents hypothetical curve corresponding to a test with no discriminatory power.

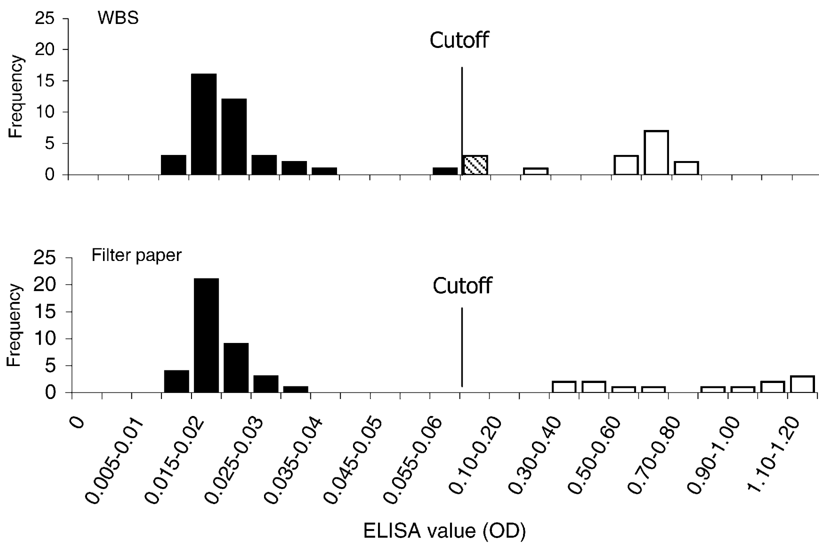

Fig. 7. Distributions of ELISA values for filter paper specimens and whole blood supernatant (WBS) of 54 coyotes, including those classified by both tests as negative (black bars), those classified by both tests as positive (white bars), and those classified as negative based on filter paper specimens but positive based on WBS (hatched bars). Note: scale differs on right and left sides of cutoff.

Table 3
Comparison of filter paper specimens and WBS with respect to test performance under expected and extreme (filter paper perfect, WBS perfect) assumptions regarding the realized sensitivity and specificity of tests

	Filter paper	WBS	Fisher's exact, P
Expected[a]			
Sensitivity	0.88	1.00	0.24
Specificity	0.99	0.96	0.48
Filter paper perfect[b]			
Sensitivity	1.00	1.00	–
Specificity	1.00	0.93	0.12
WBS perfect[c]			
Sensitivity	0.81	1.00	0.11
Specificity	1.00	1.00	–

[a] Expected sensitivity and specificity of filter paper specimens were based on estimation of these parameters from necropsy-confirmed cases. The parameter estimates for WBS were those consistent with both observed WBS test results and the estimated number of infected and uninfected coyotes as calculated from apparent prevalence, sensitivity, and specificity of filter paper tests.

[b] Assumes all classifications based on filter paper specimens were correct.

[c] Assumes all classifications based on WBS were correct.

(median difference $= 0.022$; Wilcoxon $T = 4.5$; $P < 0.001$) and infected coyotes (median difference $= 0.081$; Wilcoxon $T = 5$; $P < 0.001$).

3.5. Comparison of WBS versus filter paper specimens

Of 54 coyotes, 51 (94%) were classified the same way by tests on both filter paper specimens and WBS ($n = 38$ negative, $n = 13$ positive) and three coyotes were classified as positive by WBS tests and negative by filter paper tests (Fig. 7). We found a small but significant difference in ELISA values for seronegative coyotes with the three discrepant cases included (median difference $= 0.003$ OD; Wilcoxon $T = 246.5$; $P = 0.02$) but no significant difference when these cases were excluded (Wilcoxon $T = 247$; $P = 0.08$). We found no significant difference in ELISA values for seropositive coyotes whether or not the three borderline cases were included (Wilcoxon $T = 50$, 26, respectively; $P > 0.20$), although the variance in positive ELISA values was greater for filter paper specimens (S.D. $= 0.42$ OD) than for WBS (S.D. $= 0.24$ OD). Regardless of the true infection status of coyotes, there was no significant difference between specimen types in sensitivity or specificity (Table 3).

4. Discussion

The performance of the DiroCheck ELISA with filter paper specimens was excellent and better than we expected given the $\geq$10-fold dilution involved in the processing of specimens. Indeed, we found no difference in discriminatory power of the test applied to filter paper specimens versus the undiluted (i.e. NBS) specimens. Our estimates of sensitivity

and specificity with filter paper specimens also were similar to estimates for undiluted dog serum or plasma (Courtney and Zeng, 1993). In fact, for low worm burdens (≤10 females) our estimate of sensitivity was higher (Se = 0.77, 95% CI: 66–87%) than that for dogs (Courtney and Zeng, 2001, Se = 0.71, 95% CI: 62–79), although not significantly ($P > 0.05$). That our sensitivity for low worm burdens was not comparatively lower was especially surprising in light of previous findings indicating that sensitivity for low worm burdens was reduced by >50% by an eight-fold dilution (Courtney et al., 1989). Reagents used in the DiroChek ELISA have improved over time, which likely explains the generally high sensitivity observed in this study.

The ROC analyses supported the high discriminatory power of the DiroChek test used on coyote filter paper specimens. In general, tests with AUC >90% are considered "highly accurate" (Swets, 1988). Therefore, our estimate of the area under the ROC curve (93%) for filter paper specimens qualifies as highly accurate. In addition to use with filter paper specimens, paired comparisons indicated that the DiroChek ELISA could be applied with similar accuracy to other specimen types. This was supported by our comparison of field-collected filter paper specimens and necropsy filter paper specimens, between necropsy filter paper specimens and NBS (undiluted, coagulated blood taken at necropsy), and between the classifications of tests on in-field filter paper specimens versus WBS (hemolyzed plasma). Although all specimen types used in this study were hemolyzed (representing off-label uses of the test), it is likely that the test would perform at least as well on coyote serum or plasma because hemolysis can reduce specificity of heartworm ELISAs by increasing the probability of non-specific binding with test antibodies (Tonelli, 1989).

Although we found no significant difference in the diagnostic potential associated with the various specimen types, realization of that potential would require appropriate cutoffs to be selected. The DiroChek ELISA has exhibited nearly perfect specificity when used on dog serum or plasma (Courtney and Zeng, 1993). Therefore, we erred on the side of reduced sensitivity in determining the cutoff for the experimental filter paper specimens. Because we found systematic differences in ELISA values between field-collected and necropsy-collected filter paper specimens, we determined a different cutoff for the field-collected specimens to maintain expected sensitivity and specificity. Although shifting the cutoff value in this way introduced more potential for error, the magnitude of such error was probably small. For example, the application of the field specimen cutoff to the experimental data (i.e. reducing the cutoff by 0.01 OD) resulted in no change in the sensitivity estimate, indicating a wide margin for error as can be seen in Fig. 2.

We found no evidence of heterogeneity in test performance among demographic and geographic groups of coyotes, indicating that the test is generally applicable to California coyotes. We recognize several concerns regarding our assessment. First, the sensitivity of the test could be biased by prevalence if populations with low prevalence have a proportionally higher prevalence of male-only infections (Courtney and Cornell, 1990). If sensitivity is low for male-only infections, as we found and as has been previously reported for dogs (Courtney and Zeng, 2001), the false-negative rate will be higher in the low-prevalence population. However, the low frequency of observed male-only infections (7% of infected coyotes) in this study suggests this bias is small. More importantly, the relationship between seroprevalence and true prevalence in seven populations here was close to that predicted without such a bias (Fig. 4). A second concern is the possibility that some populations of

coyotes not assessed here may have parasites with antigens that cross-react with antibodies used in the DiroChek ELISA. If so, the specificity of the test would be reduced.

Although we found no evidence of geographic or demographic biases in sensitivity, seasonal biases exist. Heartworm transmission in California (and throughout much of the world) is seasonal, which serves to synchronize the timing of new infections (Knight and Lok, 1995). In much of California, the peak transmission period occurs in June and July. The pre-patent period for heartworm is approximately 6 months although immature (L5) heartworms arrive in the heart or associated vasculature as early as 3 months post-infection (Orihel, 1961). In this study, as has been reported for dogs (Courtney and Zeng, 1995; McTier et al., 1995), sensitivity was strongly affected by the maturity of an infection and whether it was patent. Therefore, sensitivity is likely to increase progressively during September–February as new infections mature and more of them become patent. Thus, it is important when comparing heartworm prevalence estimates that samples be unbiased by time of year.

In conclusion, the DiroChek ELISA is highly sensitive and specific to the detection of adult heartworm infection in coyotes when used with filter paper blood specimens or hemolyzed plasma. Post-mortem specimens should be obtained as soon after death as possible, given the observed increase in ELISA values of filter paper specimens from field collection relative to necropsy collection. We advise against using the test with necropsy blood supernatant without experimental determination of a cutoff for that type of specimen.

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