

# Comparison of an indirect immunofluorescence assay, western blot analysis, and a commercially available ELISA for detection of *Ehrlichia canis* antibodies in canine sera

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**Objective**—To examine the correlation between results for an indirect immunofluorescence assay (IFA) that uses *Ehrlichia canis* antigen as a substrate (ie, *E canis*-IFA), 2 western blot (WB) analyses, and a commercially available ELISA in the detection of *E canis* antibody in dog sera.

**Sample Population**—54 canine serum samples that were reactive on *E canis*-IFA and 16 canine serum samples that were *E canis*-IFA nonreactive.

**Procedure**—Serum samples were evaluated by use of 2 WB analyses and a commercially available ELISA. Correlation between results of the 3 testing modalities (ie, IFA, WB analyses, and the ELISA) was examined by use of nonreactive (*E canis*-IFA reciprocal titer, < 20), low-titer (reciprocal titer, 80 to 160), medium-titer (reciprocal titer, 320 to 2,560), and high-titer (reciprocal titer, 5,120 to > 20,480) serum samples.

**Results**—For all serum samples in the nonreactive (n = 16), medium-titer (17), and high-titer (18) groups, correlation of results among IFA, WB analyses, and the commercially available ELISA was excellent. A poor correlation was found between IFA results and those of WB analyses and the ELISA for serum samples in the low-titer group (19), with only 4 of the 19 serum samples having positive results on both WB analyses and the commercially available ELISA.

**Conclusions and Clinical Relevance**—The discrepancy between *E canis*-IFA, WB analyses, and the commercially available ELISA results for the low-titer serum samples may be related to a high IFA sensitivity or, more likely, a lack of specificity associated with cross-reactivity among *Ehrlichia* spp. (*Am J Vet Res* 2006;67:206–210)

Canine ehrlichiosis is caused by infection with  $\geq 1$  tick-borne *Ehrlichia* spp. In the southeastern United States, the following 3 species of *Ehrlichia* are most commonly implicated: *Ehrlichia canis*, *Ehrlichia chaffeensis*, and *Ehrlichia ewingii*.<sup>1-3</sup> Sera from *Ehrlichia*-infected dogs will typically cross-react in immunoassays.<sup>4</sup> An IFA that uses *E canis* antigen as a substrate (ie, the *E canis*-IFA) is commonly used to screen canine sera for prior exposure to any of the 3 *Ehrlichia* organ-

isms.<sup>5</sup> Thus, seroreactivity to *E canis* antigens on IFA can indicate exposure to 1 of the listed agents but not necessarily exposure to *E canis*.

Although not widely available, WB immunoassays that use native *E canis* antigens have been developed to confirm IFA results.<sup>6</sup> However, because infection with  $\geq 1$  *Ehrlichia* spp induces cross-reactive antibodies and because unique WB patterns have not been consistently reported, the WB analysis cannot be performed to confirm infection by a specific *Ehrlichia* spp.<sup>1</sup>

An ELISA<sup>a</sup> that detects heartworm (*Dirofilaria immitis*) antigen, antibody to *Borrelia burgdorferi* C<sub>6</sub> peptide, and *E canis* antibody is commercially available. The ELISA kit uses synthetic *E canis* peptides (P30, P30-1) derived from *E canis* immunodominant epitopes.<sup>7</sup>

The purpose of the study reported here was to compare results of the *E canis*-IFA, WB analyses, and commercially available ELISA for titers against *Ehrlichia* spp in serum samples from dogs for which a diagnosis of ehrlichiosis was suspected by the attending clinician. An approximately equal number of *E canis* nonreactive serum samples and serum samples with low, medium, and high titers against *Ehrlichia* spp were chosen from diagnostic accessions submitted to the NCSU-Vector Borne Diagnostic Laboratory for serologic testing. Serum samples were chosen on the basis of IFA titers in an effort to fully represent a range of results among the 4 groups. Serum samples were tested with WB analyses developed independently at NCSU and at a commercial laboratory<sup>b</sup> and with the commercially available ELISA.

## Materials and Methods

**Serum sample selection**—Serum samples that were reactive on *E canis*-IFA were selected from dogs for which a diagnosis of canine ehrlichiosis was suspected by the attending clinician (n = 53). Serum samples were collected over a 12-year period (1988 to 2000) and stored at  $-70^{\circ}\text{C}$ . Serum samples underwent an undetermined number of freeze-thaw cycles during the period prior to testing. The commercially available ELISA and the WB analyses were performed by use of the same sample aliquot at a single site. Personnel at the NCSU-Vector Borne Diagnostic Laboratory performed all of the testing reported in our study. Serum samples were obtained from canine blood samples that were usually free of gross evidence of hemolysis or lipemia. Serum samples were

IFA	Immunofluorescence assay
WB	Western blot
NCSU	North Carolina State University

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chosen on the basis of the recorded titer on the *E canis*-IFA to include an approximately equal number of low-, medium- and high-titer serum samples. In our study, 19 low-titer serum samples (reciprocal titer, 80 to 160), 17 medium-titer serum samples (reciprocal titer, 320 to 2,560), and 18 high-titer serum samples (reciprocal titer, 5,120 to > 20,480) were used. Nonreactive control sera consisted of 8 clinical accessions that had IFA reciprocal titers of < 20 and 8 serum samples collected from specific pathogen-free dogs that were raised in a tick-free environment. A single serum sample that was reactive on *E canis*-IFA was from a dog experimentally infected with *E canis* (14 days after infection).

**Serologic testing**—An indirect IFA was performed at NCSU by use of *E canis* (Florida strain) organisms grown in DH82 cells. Starting with an initial serum dilution of 1:20, serial 2-fold dilutions of sera were applied to IFA slides, which were washed and reacted with fluorescein isothiocyanate anti-canine IgG.<sup>c</sup> Endpoint titers were determined as the last dilution at which brightly stained organisms could be detected with a fluorescence microscope with exciter and barrier filters. Seroreactivity against *E canis* antigens was defined as a titer of  $\geq 80$  (cutoff titer).

**Western immunoblotting**—Western blot analyses were performed by use of assays developed independently at NCSU (ie, NCSU WB analysis) and at a commercial laboratory (ie, commercial WB analysis).<sup>b</sup> For NCSU WB analysis, *E canis* (Florida strain) grown in DH82 cells, a continuous canine monocytoic cell line, was purified by sucrose gradient centrifugation and the protein concentration determined. Dilutions of antigen were made in final sample buffer (0.062M Tris-HCl, 5% 2-mercaptoethanol, 10% glycerol, 2% sodium dodecyl sulfate, 0.01% bromphenol blue) at a concentration of 7.5 mg/mL. Antigen was loaded by use of a volume of 20  $\mu$ L/well and electrophoresed on 12.5% SDS-PAGE mini gels. Proteins were electrotransferred to 0.45- $\mu$ m nitrocellulose membrane. After blocking with 5% milk in PBS solution or a solution of PBS with 0.05% Tween 20, 0.5% nonfat dry milk, and 1% normal goat serum, proteins were reacted with canine serum samples at a 1:50 dilution and with peroxidase-conjugated goat anti-canine IgG at a 1:400 dilution in 1% milk in PBS solution. Bands were detected with the color reagent 4-chloro-1-naphthol. Serum samples from a dog experimentally infected with *E canis* (Florida strain), with a reciprocal titer of 10,240, were used as a positive control. Prestained color molecular weight standards run with each gel were used to calculate the molecular weight of protein bands.

Immunoblot patterns of the NCSU WB analysis were categorized as positive, nonreactive, or indeterminate. Sera in the positive category had immunoblot patterns of antigenic proteins that were indicative of *E canis* exposure with detection of the 27- to 30-kd multigene family of proteins as well as detection of 0 to 4 other proteins that are also detected routinely by IFA in serum samples from dogs experimentally infected with *E canis*. Sera from dogs that reacted with  $\geq 1$  *E canis* antigens but did not recognize the 27- to 30-kd, genus-specific, multigene family of proteins on NCSU WB analysis were classified as indeterminate. Nonreactive sera were defined as not reacting with any *E canis* antigens on NCSU WB analysis.

For commercial WB analysis, an *E canis* isolate<sup>d</sup> was used that was cultured in canine O30.9 cells. An *E canis*-enriched membrane fraction was prepared by differential centrifugation and solubilized in 1% SDS. Standard methods were used for SDS-PAGE and electrophoretic transfer of these culture-derived *E canis* antigens to nitrocellulose. Synthetic-peptide-based *E canis* antigens were synthesized, conjugated to bovine serum albumin, and applied to the

nitrocellulose by airbrushing. Immunoblots were blocked with nonspecific proteins; then, strips were cut from blot sheets, incubated with serum-plasma samples at a 1:50 dilution for 2 hours, washed, incubated with peroxidase-conjugated anti-canine IgG<sup>e</sup> at a 1:3,000 dilution for 1 hour, washed, and peroxidase signal-developed for 15 minutes with a commercial substrate system.<sup>f</sup>

Immunoblot patterns of the commercial WB analysis were categorized as positive, nonreactive, or indeterminate. Sera in the positive category recognized the 27- to 30-kd proteins of the culture-derived *E canis* antigen and 1 or both of the synthetic peptide antigens. Sera that reacted with only the culture-derived 27- to 30-kd antigens or 1 of the synthetic peptide antigens on the commercial WB analysis were classified as indeterminate. Nonreactive sera were defined as not reacting with the culture-derived or synthetic *E canis* antigens.

**Commercially available ELISA**—Detection of *E canis* antibody was performed by use of the commercially available ELISA.<sup>a</sup> The ELISA provides for simultaneous detection of canine heartworm antigen, *E canis* antibodies, and antibodies to *B burgdorferi* C<sub>6</sub> peptide in canine serum, plasma, or whole blood. The ELISA format uses the proprietary assay device of the manufacturer, which provides reversible chromatographic flow of sample and automatic sequential flow of wash and enzyme substrate. Synthetic peptides that duplicate immunodominant regions of *E canis* surface proteins were made by use of an automated peptide synthesizer.<sup>8,8</sup> The synthetic peptides were conjugated to bovine serum albumin and to horseradish peroxidase by use of standard methods.<sup>9</sup> The bovine serum albumin-peptide conjugate was deposited onto a porous polyethylene flow matrix. The horseradish peroxidase-peptide conjugate was added to a conjugate diluent containing nonspecific proteins and detergent. Two drops of test sample were mixed with 5 drops of conjugate and applied to the flow matrix. Ehrlichia canis antibody (if present) in the sample binds to the synthetic peptide-horseradish peroxidase conjugate and to the synthetic peptide-bovine serum albumin conjugate. The deposited synthetic peptide-bovine serum albumin conjugate was then exposed to wash and substrate reagents in the course of the assay. Results of the commercially available ELISA were considered to be positive if blue color developed in the area of deposition of the synthetic peptide-bovine serum albumin conjugate.

## Results

The WB analyses and commercially available ELISA serum sample results were arranged on the basis of IFA-titer groups (ie, nonreactive, low, medium, or high; Table 1). Of the 16 serum samples in the nonreactive group (ie, titer on *E canis*-IFA of  $\leq 20$ ), all were nonreactive on both WB analyses and the commercially available ELISA. Likewise, of the 17 serum samples in the medium-titer group (ie, *E canis*-IFA reciprocal titer of 320 to 2,560) and the 18 serum samples in the high-titer group (ie, *E canis*-IFA reciprocal titer of 5,120 to > 20,480), all had positive results on both WB analyses and the commercially available ELISA. Results for both WB analyses were positive or nonreactive for the 51 serum samples in these 3 groups; no indeterminate results were found on NCSU or commercial WB analyses.

A variety of results were found on WB analyses and the commercially available ELISA for the 19 serum samples in the low-titer group (*E canis*-IFA reciprocal titer of 80 to 160; Table 2). Of the 19 serum samples in

Table 1—Results of WB analyses and the commercially available ELISA<sup>a</sup> for serum samples with various indirect IFA titers for detection of *Ehrlichia canis*.

<i>E canis</i> -IFA reciprocal titers	No. of serum samples	No. of positive results				
		NCSU WB	COM WB	NCSU WB and COM WB	ELISA	NCSU WB, COM WB, and ELISA
Nonreactive (< 20)	16	0	0	0	0	0
Low (1:80 to 1:160)	19	10	11	7	6	4
Medium (320 to 2,560)	17	17	17	17	17	17
High (5,120 to > 20,480)	18	18	18	18	18	18

<sup>a</sup> *E canis*-IFA = An IFA that uses *E canis* antigen as a substrate. NCSU WB = WB analysis was performed by use of assays developed independently at NCSU. COM WB = WB analysis developed at a commercial laboratory.

Table 2—Number of the commercially available ELISA<sup>a</sup> positive and nonreactive results and results of WB analyses for serum samples with low *E canis*-IFA titers.

No. of low-titer serum samples*	WB analyses		Commercially available ELISA	
	NCSU	COM	Pos	Neg
7	Pos	Pos	4	3
1	Pos	Ind	0	1
2	Pos	Neg	0	2
4	Ind	Pos	2	2
1	Ind	Ind	0	1
4	Ind	Neg	0	4
0	Neg	Neg	0	0

\* *E canis*-IFA reciprocal titer of 80 to 160. Pos = Positive result. Neg = Negative (ie, nonreactive) result. Ind = Indeterminate result. See Table 1 for remainder of key.

the low-titer group, 10 had positive results by use of the NCSU WB analysis, 11 had positive results by use of the commercial WB analysis, and 6 had positive results by use of the commercially available ELISA. Seven of the 19 serum samples had positive results on both WB analyses, and 4 of the 19 serum samples had positive results on both WB analyses and the commercially available ELISA. Ten of the 19 serum samples had indeterminate results in 1 or both WB analyses. Nine indeterminate results were found by use of the NCSU WB analysis and 2 by use of the commercial WB analysis. The serum sample from the single experimentally infected dog was in the low-titer group (ie, titer on *E canis*-IFA of 160) and had positive results on both WB analyses but was nonreactive on the commercially available ELISA.

## Discussion

Sera from dogs infected with different *Ehrlichia* spp can have several possible reaction patterns when evaluated by use of native *E canis* antigens on WB analyses, which will vary depending upon the *Ehrlichia* spp.<sup>1,6</sup> Sera from *E canis*-, *E ewingii*-, and *E chaffeensis*-infected dogs all react with similar higher molecular weight proteins ranging from 34- to 110-kd but have several possible reactions to the 27- to 30-kd proteins. On the basis of our criteria, sera must react with the 27- to 30-kd proteins to confirm prior exposure to *E canis* by WB analysis.<sup>10</sup> Sera from *E canis*-infected dogs react, sera

from *E ewingii*-infected dogs do not react, and sera from *E chaffeensis*-infected dogs have variable reaction to the 27- to 30-kd proteins.<sup>6,11</sup> The lack of consistent reaction of *E chaffeensis*-positive serum samples to the 27- to 30-kd proteins on WB analysis for *E canis* has been observed for canine and human serum samples and may be related to antigenic differences among *E chaffeensis* strains.<sup>6,11,12</sup> Sera that react to  $\geq 1$  *E canis* proteins in the range of 34- to 110-kd but do not react to the 27- to 30-kd proteins were classified as indeterminate on NCSU WB analysis and nonreactive on commercial WB analysis. Therefore, WB analyses results by use of *E canis* antigens would be reported as positive for serum samples from *E canis*-infected dogs; as indeterminate (by NCSU WB analysis) or nonreactive (by the commercial WB analysis) for serum samples from *E ewingii*-infected dogs; and as positive, indeterminate (NCSU and the commercial WB analyses), or nonreactive (the commercial WB analysis) for *E chaffeensis*-infected dogs. The large proportion of serum samples (10/19) with indeterminate WB analysis results in the low-titer group, compared with serum samples in the nonreactive, medium-, and high-titer groups where no indeterminate results were found for 51 serum samples, provides evidence that a high proportion of these sera was from dogs infected with an *Ehrlichia* spp other than *E canis*.

The commercially available ELISA includes synthetic peptide reagents that duplicate amino acid

sequences found in specific *E canis* proteins and is designed to react with sera from dogs infected with *E canis*. Homologous proteins expressed in strains of *E chaffeensis* contain the same amino acid sequence<sup>8,11</sup> and induce antibodies in sera that are cross-reactive in the commercially available ELISA. However, not all sera from *E chaffeensis*-infected dogs recognize this epitope, suggesting that it is not expressed in all strains of *E chaffeensis* or that it is differentially expressed during the infection cycle.

In addition to the commercially available ELISA used in our study, several other ELISAs have been developed for detection of *E canis* antibody. These include an ELISA<sup>13,b</sup> that uses cultured whole organism as the detection antigen and an ELISA that uses the recombinant major antigenic protein 2 of *E canis* as the detection antigen.<sup>14</sup> In a study comparing the performance of these 3 ELISAs with that of the *E canis*-IFA, the sensitivity of the 3 ELISAs ranged from 71% to 86%, which was improved to a range of 91% to 100% when only serum samples with IFA titers of > 1:320 were considered.<sup>15</sup>

In our study, the correlation of IFA, WB analyses, and the commercially available ELISA results for the 16 non-reactive, 17 medium-titer, and 18 high-titer serum samples was excellent. Each of the serum samples in the non-reactive group was nonreactive on all assays, and each of the medium- and high-titer serum samples had positive results on all assays. No indeterminate WB analyses results were found among these 3 groups. Results of IFA, WB analysis, and the commercially available ELISA correlated poorly for serum samples in the low-titer group. Only 4 of 19 low-titer serum samples had positive results in each of the WB analyses and the commercially available ELISA. These discordant results may reflect enhanced IFA sensitivity, poor IFA specificity, or exposure of dogs with low IFA titers to *Ehrlichia* spp other than *E canis*. Sera from dogs reactive to *E canis* antigen as measured by IFA have been found to be infected with *Ehrlichia* from several related spp, including *E canis*, *E chaffeensis*, and *E ewingii*.<sup>6,10,16</sup> The overall less intense reaction observed in sera from dogs infected with an *Ehrlichia* spp other than *E canis* to the heterologous *E canis* antigen has been observed by use of IFA and WB analyses.<sup>2,6</sup> This would result in a measurable but low IFA titer that is reduced in comparison to that of *E canis*-infected dogs and could explain the clustering of unconfirmed IFA results in the low-titer group.

An advantage of the commercially available ELISA is that it supplies a rapid result (approx 8 minutes) and can be used in a clinical setting. An advantage of the IFA is that results are reported in a quantitative fashion. A high IFA titer ( $\geq 320$ ) would be indicative of an *E canis* infection. A low titer on the *E canis*-IFA ( $\leq 160$ ) may result from a weak response to an *E canis* infection but may also result from an *Ehrlichia* infection that is not caused by *E canis*. The lack of specificity on IFA for *E canis* antibody detection, presumably reflecting potential cross-reactivity to other *Ehrlichia* spp, resulted in poor correlation for low-titer serum samples in our study.

In our study, serum samples with IFA reciprocal titers of  $\geq 320$  had uniformly positive results on both

WB analyses and the commercially available ELISA. The correlation between specific IFA titers and results of the WB analyses and the commercially available ELISA is accurate for the IFA as performed in the NCSU-Vector Borne Diagnostic Laboratory. The *E canis*-IFA has not been standardized among veterinary diagnostic laboratories. There can be variation in IFA procedure, variation in the quality and quantity of *E canis* antigen used to prepare slides for IFA, and variation in the technical capacity to discern positive results and nonreactive results by fluorescence microscopy that yield substantial differences in reported IFA titer values among laboratories.<sup>1,17</sup> A study using sequential serum samples from dogs experimentally infected with *E canis*, *E chaffeensis*, and *E ewingii* would be required to further define the relative sensitivity and specificity of the *E canis*-IFA in comparison to WB analyses and the commercially available ELISA results.

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- a. SNAP 3Dx, IDEXX Laboratories Inc, Westbrook, Me.
  - b. IDEXX Laboratories Inc, Westbrook, Me.
  - c. Cappel, ICN Pharmaceuticals Inc, Costa Mesa, Calif.
  - d. "Jake" isolate, North Carolina State University, Raleigh, NC.
  - e. Jackson Immunodiagnosics, West Grove, Pa.
  - f. BioRad, Richmond, Calif.
  - g. Perkin Elmer, Applied Biosystems Division, Foster City, Calif.
  - h. Immucomb, Biogal, Kibbutz Gal'ed, Israel.
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