Performance of a commercially available in-clinic ELISA for the detection of antibodies against *Anaplasma phagocytophilum, Ehrlichia canis,* and *Borrelia burgdorferi* and *Dirofilaria immitis* antigen in dogs

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Objective—To evaluate the sensitivity and specificity of a commercially available in-clinic ELISA for detection of heartworm infection and tick-borne diseases in dogs.

Sample Population—846 serum, plasma, or blood samples obtained from dogs.

Procedures—Samples were evaluated via the in-clinic ELISA to detect antibodies against *Anaplasma phagocytophilum*, *Ehrlichia canis*, and *Borrelia burgdorferi* and *Dirofilaria immitis* (heartworm) antigen. True infection or immunologic status of samples was assessed by use of results of necropsy, an antigen assay for *D immitis*, and immunofluorescence assay or western blot analysis for antibodies against *B burgdorferi*, *E canis*, and *A phagocytophilum*.

Results—Sensitivity and specificity of the in-clinic ELISA for detection of heartworm antigen (99.2% and 100%, respectively), antibodies against *B burgdorferi* (98.8% and 100%, respectively), and antibodies against *E canis* (96.2% and 100%, respectively) were similar to results for a similar commercial ELISA. In samples obtained from dogs in the northeast and upper Midwest of the United States, sensitivity and specificity of the in-clinic ELISA for antibodies against *Anaplasma* spp were 99.1% and 100%, respectively, compared with results for an immunofluorescence assay. Samples from 2 dogs experimentally infected with the NY18 strain of *A phagocytophilum* were tested by use of the in-clinic ELISA, and antibodies against *A phagocytophilum* were detected by 8 days after inoculation. Antibodies against *Anaplasma platys* in experimentally infected dogs cross-reacted with the *A phagocytophilum* analyte. Coinfections were identified in several of the canine serum samples.

Conclusions and Clinical Relevance—The commercially available in-clinic ELISA could be used by veterinarians to screen dogs for heartworm infection and for exposure to tick-borne pathogens. (*Am J Vet Res* 2010;71:1443–1450)

Granulocytic anaplasmosis is an emerging tick-borne disease that is widely distributed in dogs in the upper Midwest, New England, parts of the Mid-Atlantic States, and northern California in the United States; southern Canada; and many parts of Europe. *Ixodes* ticks are endemic in all of these areas. Anaplasmosis in dogs is caused by the rickettsial agent *Anaplasma phagocytophilum* (formerly *Ehrlichia equi*). *Anaplasma phagocytophilum* is an obligate intracellular bacterium that targets granulocytes of mammalian hosts.[1,2] The principal vectors are *Ixodes scapularis* in the eastern United States, *Ixodes pacificus* in the western United States, *Ixodes ricinus* in Europe, and probably *Ixodes persulcatus* in parts of Asia.[1]

Microscopic diagnosis of *A phagocytophilum* infection relies on identification of morulae that can be seen in Wright-stained blood films. However, within days after an animal becomes infected, the organism becomes more difficult to detect in a blood film, which makes this technique unreliable.[3] An IFA is commonly used

Abbreviations

HRP	Horseradish peroxidase
IFA	Immunofluorescence assay

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for the diagnosis of anaplasmosis in dogs. An IFA will detect antibodies against both *A phagocytophilum* and *Anaplasma platys*. *Anaplasma platys* is the causative agents of thrombocytotrophic anaplasmosis (cyclic thrombocytopenia). There can be false-positive test results when whole-cell antigen-based ELISAs or IFAs are used.[4,5] Evidence of seroconversion detected with an ELISA or IFA and PCR amplification of *A phagocytophilum* or *A platys* DNA can also be used to confirm a diagnosis of anaplasmosis.

Borrelia burgdorferi, transmitted by *Ixodes* spp, is the causative agent of borreliosis (ie, Lyme disease) and can infect a wide range of mammals, including humans and dogs; it is the most common agent for vector-borne diseases in the United States.[6] Historically, serologic assays, such as whole-cell–based IFAs, ELISAs, and western blot assays, have been used to confirm a clinical diagnosis of borreliosis in dogs. Interpretation of such assays is complicated by the widespread use of commercial vaccines. These vaccines induce an antibody response that cross-reacts in whole-cell IFAs and ELISAs commonly used to detect antibodies against *B burgdorferi*.[6] An ELISA with a 25-amino acid synthetic peptide (C_6 peptide) derived from the VlsE protein of *B burgdorferi* has been developed to detect antibodies in dogs.[7,8] The C_6 peptide–based ELISA reportedly does not react with serum samples from animals vaccinated with the OspA or the whole-cell (bacterin) borreliosis vaccines.[7,8]

Ehrlichia canis is a tick-borne obligate intracellular bacterium that infects canine monocytes. An IFA based on whole-cell organisms can be used to detect antibodies against *E canis*. However, an IFA does not facilitate differentiation of the infecting *Ehrlichia* spp, particularly among organisms within the same genogroup, because of cross-reacting epitopes within the immunodominant antigens.[9] There are 3 species in the *Ehrlichia* genogroup known to infect dogs (*E canis*, *Ehrlichia ewingii*, and *Ehrlichia chaffeensis*).[5]

Heartworm infection in dogs is widely distributed in the United States and is transmitted by mosquitoes. Heartworm infection is caused by *Dirofilaria immitis*, a parasitic worm that lives as an adult in the right side of the heart and in the pulmonary arteries. Heartworm infection in dogs is diagnosed in blood samples via the detection of microfilariae or circulating antigens released by the adult female worms. Several antigen tests are available that use antibodies (monoclonal or polyclonal) to capture antigens.[10]

The primary purpose of the study reported here was to evaluate the performance of a commercially available in-clinic ELISA by testing sera from a variety of dogs for antibodies against *A phagocytophilum*, *E canis*, and *B burgdorferi* and for *D immitis* antigen. We also intended to determine the performance of each analyte in the in-clinic ELISA by testing serum obtained from dogs with natural infections and from dogs experimentally infected with *A platys* and *A phagocytophilum*.

Materials and Methods

Sample population—Samples (n = 846) of canine serum, plasma, or whole blood in a commercial company's sample collection[a] were obtained from a diagnostic laboratory,[b] a research center,[c] and veterinary clinics located throughout the United States. Samples were obtained from dogs with a single infection or coinfections. All samples were divided into aliquots and stored at –20°C until use. Performance of a commercially available in-clinic ELISA[d] was tested on matched whole blood, plasma, and serum samples.

Additional samples were obtained from 2 adult male Beagles inoculated IV with the NY18 strain of *A phagocytophilum*.[11,e] This infection trial was conducted at the University of Florida Department of Physiological Sciences. The study protocol was approved by the Institutional Animal Care and Use Committee, Department of Physiological Sciences, University of Florida. Samples were obtained from the dogs at various intervals for approximately 1 year after inoculation. In total, approximately 30 serum samples were obtained and tested for each dog.

Samples also were obtained from six 6-month-old female hound-type dogs with experimentally induced *A platys* infection.[12] This experimental-infection experiment was conducted at the Louisiana State University Department of Pathobiological Sciences. The study protocol was approved by the Institutional Animal Care and Use Committee, Department of Pathobiological Sciences, Louisiana State University. The dogs were inoculated IV with a Louisiana isolate of *A platys* (1-mL aliquots of infected canine platelets that had been stored at –70°C in 10% dimethyl sulfoxide). Blood samples collected from the dogs were processed, and sera were harvested and stored at –20°C until use. Approximately 4 samples from each of these 6 dogs were tested during a 5-week period.

Commercially available in-clinic ELISA—A commercially available in-clinic ELISA[d] was used for the simultaneous detection of antibodies against *A phagocytophilum*, *B burgdorferi*, and *E canis* and *D immitis* antigen in blood, plasma, or serum obtained from dogs. This ELISA had the same assay reagents for *E canis*, *B burgdorferi*, and *D immitis* as were included in another ELISA[f] from the same manufacturer.

The commercially available in-clinic ELISA involved the use of reversible chromatographic flow of sample and automatic, sequential flow of wash solution and enzyme substrate. It detected antibodies against *A phagocytophilum* by use of a synthetic peptide derived from the major outer surface protein[13,14] (immunodominant p44 protein) and antibodies against *B burgdorferi* by use of the C_6 peptide derived from the IR_6 region within the *Borrelia* membrane protein VlsE. The commercially available in-clinic ELISA detected antibodies generated against peptides from the p30 and p30-1 proteins of *E canis*,[15] and it included 2 polyclonal antibodies (one for capture and the other for detection) of *D immitis* antigen.[16]

Briefly, 3 drops of test sample were mixed with 4 drops of conjugate and applied to the flow matrix, as described in the manufacturer's instructions. The analyte-specific antigen (*D immitis*), if present in the sample, would bind to the heartworm antibody–HRP.[g] Similarly, peptide-specific antibody (*Anaplasma*, *E canis*, or *B burgdorferi*), if present in the sample, would bind to the peptide-HRP conjugate. Immune complexes that

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formed would bind to the heartworm antibody or the peptide-bovine serum albumin[h] conjugates on the flow matrix. The ELISA was then exposed to wash solution and substrate reagents. Color development in the area of the deposition of the immune complexes indicated a positive result for the various analytes.

PCR analysis and serologic testing—Dogs experimentally infected with *A platys* were monitored via both PCR analysis and IFA. For PCR analysis, DNA was purified from 200 µL of EDTA-anticoagulated blood with a commercially available kit[i] used in accordance with the manufacturer's instructions. The PCR assay was performed by use of a thermocycler[j] and accompanying probe master mix.[k] The PCR primers for the *A platys* GroEL gene (GenBank accession No. AF478129) and *A phagocytophilum* GroEL gene (GenBank accession Nos. AY529489 and U96729) were designed. Forward and reverse primers for the *A platys* GroEL gene were 5′-GCTACCATCTCTGCTAACGGCGAT-3′ and 5′-CACTTCCTATTATTGTCTTTGTA-3′, respectively. Forward and reverse primers for the *A phagocytophilum* GroEL gene were 5′-GCAACATTGTCTGCTAATGGAGAC-3′ and 5′-CACTACCTATTATAGTAGTTGCG-3′, respectively. The PCR reaction was performed in a final volume of 20 µL that included 0.5µM of each primer and 2 µL of purified DNA. The cycling program was a single hot-start cycle (95°C for 15 minutes) followed by 45 cycles (denaturation at 95°C for 30 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 20 seconds). A melting curve analysis was performed on amplification products.

Antibodies against *A platys* in experimentally infected dogs were determined by use of IFA as described elsewhere.[15] Briefly, serum samples were serially diluted (2-fold dilutions; starting dilution, 1:40) with PBS solution (pH, 7.2). Results were reported as the highest dilution that yielded specific fluorescence. Test control samples included sera from seropositive and seronegative dogs (a dog with experimentally induced *A platys* infection and a noninfected control dog, respectively). Fifteen microliters of diluted serum was added to acetone-fixed drop suspension slides of *A phagocytophilum*[l] and incubated for 30 minutes at 37°C in a moist chamber. Slides were then rinsed twice with PBS solution and once with distilled water. An affinity-purified, fluorescein isothiocyanate–conjugated, rabbit anti-canine IgG (H + L) antibody[m] (dilution, 1:100) was added to each well; wells were incubated for 30 minutes and then washed. Slides were coated with 10% glycerol in PBS solution; coverslips were applied, and slides were examined by use of a fluorescent microscope.

Screening and confirmatory tests—Samples used to assess the sensitivity and specificity for the *D immitis* analyte in the commercially available in-clinic ELISA were evaluated during necropsy or tested by use of a heartworm antigen test.[n] The heartworm antigen test had sensitivity of 98% and specificity of 100% and was performed in accordance with the manufacturer's instructions. Samples were tested by use of an indirect IFA at a diagnostic laboratory[b] to detect antibodies against *A phagocytophilum*, *E canis*, and *B burgdorferi*.

Samples with discrepant results between the commercially available in-clinic ELISA and the IFA were subsequently evaluated via western blot analysis. Commercial western blot analysis kits[o] were used for *E canis*; western blot analyses were performed as described elsewhere.[16] In addition to native *E canis*–enriched membrane fractions, the western blots also contained synthetic peptide–based *E canis* antigens applied with airbrushing techniques. Strips were incubated with serum samples (dilution, 1:100) for 1 hour; strips were then washed and incubated with alkaline phosphatase–conjugated anti-canine IgG[p] (dilution, 1:3,000) for 1 hour. Strips were then washed and developed with a commercial substrate system.[h] Immunoblot results were categorized as positive on the basis of recognition of 27- to 30-kDa native proteins of *E canis* and one of the synthetic peptide antigens on the western blot. Negative results were defined as serum samples that failed to react during western blot analysis.

Western blot analysis for *B burgdorferi* was performed by use of commercial western blot kits[q]; western blot analyses were performed in accordance with the manufacturer's instructions. A checkerboard titration was performed by use of serum samples with positive (n = 10) and negative (10) results and various dilutions of goat anti-canine IgG conjugate.[p] A serum dilution of 1:50 and conjugate dilution of 1:750 were found to be optimal and were used to test clinical samples. Results obtained for clinical samples were compared with results obtained with the positive control sample supplied with the kit.

Escherichia coli–derived *A phagocytophilum*–specific rP44 protein[13] was used for western blot analysis. Briefly, rP44 protein was separated by use of 4% to 12% gradient PAGE and transferred to a nitrocellulose membrane. Membranes were blocked by incubation in Tris-buffered saline solution (150mM NaCl and 50mM Tris-HCl [pH, 7.4]) containing 0.05% Tween 20 and 5% milk at 22°C for 1 hour. Nitrocellulose membranes were incubated with canine sera (dilution, 1:100) and then with alkaline phosphatase–conjugated affinity-purified anti-dog IgG (dilution, 1:3,000). Membranes then were washed and developed with a commercial substrate system.[h]

Statistical analysis—Results obtained with the commercially available in-clinic ELISA and various confirmatory tests were analyzed by use of statistical software.[r] Sensitivity and specificity were calculated relative to necropsy results or results of the heartworm antigen test for the heartworm analyte and relative to IFA results for the other analytes. Discrepant samples were tested by use of additional confirmatory tests, but those results were not used to calculate sensitivity and specificity. Concordant and discordant results between tests were assessed by computing κ statistics. The κ statistic measures the degree of agreement between variables above the agreement that would be expected by chance alone. Values of $\kappa < 0.40$ are considered poor agreement, values of κ of 0.50 to 0.75 are considered good agreement, and values of $\kappa > 0.75$ are considered excellent agreement.[17]

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Table 1—Sensitivity and specificity of a commercially available in-clinic ELISA[d] for the detection of various diseases in serum or plasma samples obtained from dogs.

Analyte	Test results*					Sensitivity (%)†	Specificity (%)†	κ value
	+/+	–/+	+/–	–/–	Total			
Dirofilaria immitis‡	118	1	0	236	355	99.2 (94.8–100)	100 (98–100)	0.99
Anaplasma phagocytophilum§	217	2	0	236	455	99.1 (96.5–100)	100 (98–100)	0.99
Ehrlichia canis§	100	4	0	236	340	96.2 (90.1–98.8)	100 (98–100)	0.97
Borrelia burgdorferi§	166	2	0	236	404	98.8 (95.4–99.9)	100 (98–100)	0.99

*Represents results (positive [+] and negative [–]) for the commercially available in-clinic ELISA/reference test. †Value reported is mean (95% confidence limit). ‡Reference tests were necropsy and a heartworm antigen test.[n] §Reference test was an IFA.

Results

***A phagocytophilum*—**A population of 219 serum samples obtained from dogs in the northeast and upper Midwest that had positive results for antibodies against *A phagocytophilum* (as determined via IFA) was tested with the commercially available in-clinic ELISA. The in-clinic ELISA yielded positive results for 217 of 219 samples. The 2 samples that had negative results also had negative results for the rP44 western blot analysis. An additional 236 samples with negative results for antibodies against *A phagocytophilum* (as determined via IFA) were tested by use of the in-clinic ELISA, and all had negative results. Sensitivity and specificity of the commercially available in-clinic ELISA for the detection of antibodies against *A phagocytophilum* were 99.1% and 100%, respectively, relative to results for the IFA (**Table 1**).

Samples obtained at intervals over the course of a year from 2 dogs experimentally infected with *A phagocytophilum* were used to monitor seroconversion by use of the in-clinic ELISA. Antibodies against *A phagocytophilum* were detected by use of the in-clinic ELISA and IFA as early as day 8 after inoculation, and the dogs remained seropositive by both methods throughout the duration of the study (1 year; data not shown).

Samples obtained from 6 dogs on days 7, 14, 21, and 35 after inoculation with *A platys* were tested for evidence of *A phagocytophilum* infection by use of the commercially available in-clinic ELISA, IFA, and PCR analysis. Prior to inoculation, all dogs had negative results for antibodies against *Anaplasma* spp when screened by use of IFA and the in-clinic ELISA. Three of 6 dogs experimentally infected with *A platys* were seroreactive for *A phagocytophilum* on day 7 (IFA result), and all 6 dogs had positive results for the IFA by day 14 (**Table 2**). Results for the in-clinic ELISA revealed that 4 of 6 dogs had cross-reactive antibodies against *A phagocytophilum* by day 7 after inoculation of *A platys*, and all 6 dogs were seropositive by day 14 after inoculation. The other 3 analytes on the in-clinic ELISA did not react with samples obtained at all time points. A PCR analysis for the *A platys* GroEL gene was performed on samples obtained 7 and 35 days after inoculation, and all 6 dogs had positive results at day 7 after inoculation. However, only 5 of 6 dogs had positive results at day 35 after inoculation. All 6 dogs experimentally infected with *A platys* had negative results for *A phagocytophilum* DNA when tested with the PCR assay.

Table 2—Results for serum samples obtained from 6 dogs experimentally inoculated with *Anaplasma platys*.

Time after inoculation (d)*	Commercially available in-clinic ELISA	*A phagocytophilum* IFA	*A platys* GroEL PCR assay
0	0/6	0/6	0/6
7	4/6	3/6	6/6
14	6/6	6/6	NT
21	6/6	6/6	NT
35	6/6	NT	5/6

Values reported represent the number of dogs with positive test results/number of dogs tested.

*Day of inoculation was designated as day 0.

NT = Not tested.

***D immitis*—**Each of 236 samples that had negative results for heartworm antigen (results of the heartworm antigen test) also had negative results when tested by use of the commercially available in-clinic ELISA. Of the 119 necropsy-confirmed samples that had positive results for heartworm, 118 had positive results for *D immitis* antigen via the in-clinic ELISA. Sensitivity and specificity of the in-clinic ELISA for detection of heartworm antigen were 99.2% and 100%, respectively, for this selected population of samples.

***E canis*—**One hundred of 104 samples that had positive results for *E canis* on the basis of results of an IFA had positive results when tested by use of the commercially available in-clinic ELISA. When the 4 IFA-positive, in-clinic ELISA–negative samples were retested via western blot analysis, they were confirmed as having negative results on the basis of a lack of reactivity to the 27- to 30-kDa native proteins of *E canis* and one of the synthetic peptide antigens. Each of the 236 samples that had negative results for *E canis* (IFA or western blot analysis [or both]) also had negative results when tested via the in-clinic ELISA. Thus, for the samples evaluated in this study, the sensitivity and specificity of the commercially available in-clinic ELISA were 96.2% and 100%, respectively, relative to results for the IFA.

***B burgdorferi*—**Results for detection of *B burgdorferi* in serum samples obtained from field populations of dogs were summarized (Table 1). Of 168 samples with positive results when tested by use of the IFA, the commercially available in-clinic ELISA detected positive results for antibody against *B burgdorferi* in 166. When the 2 IFA-positive, in-clinic ELISA–negative samples were retested via western blot analysis, they were confirmed as having positive results. An addition-

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Table 3—Results for 846 samples obtained from dogs and tested with a commercially available in-clinic ELISA.

Organism	No. with positive results
A phagocytophilum–A platys	160
A phagocytophilum–A platys + *E canis*	9
A phagocytophilum–A platys + *B burgdorferi*	48
E canis	84
E canis + *B burgdorferi*	6
E canis + *D immitis*	1
B burgdorferi	108
B burgdorferi + *D immitis*	4
D immitis	113

al 236 samples with negative results for *B burgdorferi* by use of the IFA also had negative results when tested by use of the in-clinic ELISA. For the samples evaluated in this study, the sensitivity and specificity of the in-clinic ELISA for the detection of antibodies against *B burgdorferi* were 98.8% and 100%, respectively, relative to results for the confirmatory tests.

Evidence of coinfection—The use of the multiple-analyte commercially available in-clinic ELISA allowed identification of coexposures in dogs. Of the samples tested in this study, 48 were seroreactive for both *A phagocytophilum* and *B burgdorferi* (**Table 3**). Similarly, 6 samples were seroreactive for *E canis* and *B burgdorferi*, whereas 9 samples were seroreactive for both *Anaplasma* and *E canis* analytes.

Sample type—A population of 103 matched canine whole blood, plasma, and serum samples was tested by use of the commercially available in-clinic ELISA. Regardless of the sample type (whole blood, plasma, or serum), 10 sample sets yielded positive results for heartworm antigen, 28 sample sets were seroreactive for *A phagocytophilum*, 35 sample sets were seroreactive for *E canis*, and 3 sample sets were seroreactive for *B burgdorferi*. Samples that had positive results for heartworm, *E canis*, and *B burgdorferi* when tested by use of the commercially available in-clinic ELISA also had positive results when tested by use of another ELISA[e] from the same manufacturer. All samples with positive results for *A phagocytophilum* by use of the commercially available in-clinic ELISA also had positive results when tested by use of the IFA. Thirty-seven matched sample sets had negative results for all analytes. Thus, all samples tested with the commercially available in-clinic ELISA had the same results regardless of the sample type. On the basis of this population of samples, there was no impact of sample type on the results of the commercially available in-clinic ELISA.

Discussion

In the study reported here, we evaluated the performance of a commercially available in-clinic ELISA for the detection of antibodies against *A phagocytophilum*, *A platys*, *E canis*, and *B burgdorferi* and *D immitis* antigen. The in-clinic ELISA used the synthetic peptide C_6 derived from the IR_6 region within the *Borrelia* membrane protein VlsE, peptides derived from the immunodominant p44 protein of *A phagocytophilum*, the p30 and p30-1 protein of *E canis*, and polyclonal antibodies against *D immitis*.

Conventional assays (such as IFAs) used to diagnose anaplasmosis have some limitations related to the nature of antigens used in these tests, the existence of cross-reactive antigens shared between the target diagnostic antigen and other bacteria, and the lack of standardization between IFAs performed in different laboratories.[5,16] Despite these limitations, there was very good agreement between results for the IFA and the commercially available in-clinic ELISA used in this study. In other studies,[14,18,19] investigators have reported that sera from dogs infected with *E canis* cross-react with *A phagocytophilum* antigens on the IFA. However, it is not clear whether this cross-reactivity was attributable, in part, to antibodies against *A platys* because dogs (particularly those in the southern half of the United States) are sometimes exposed to both *E canis* and *A platys*.

A screening test that is recommended for use in determining exposure among a general population of dogs should have a high degree of specificity. The major advantage of the ELISAs used in this study is the capability of generating accurate serologic results in a relatively short time within a hospital setting without the need for expensive equipment or highly trained laboratory technicians. In addition, ELISAs based on synthetic peptide antigens can eliminate specificity issues associated with conventional native antigen-based tests such as IFAs. The defined structure and highly purified nature of the synthetic reagents used in immunoassays minimize problems related to antigen variability, contamination of antigen preparations with unrelated proteins, lot-to-lot variability of antigen preparations that by necessity are grown in cell cultures, and concerns associated with large-scale growth and purification of antigens in cell cultures.[20,21] In addition, synthetic peptides can provide uniform and clearly defined antigen targets for developing species-specific antibody assays, which reduces interassay and intra-assay variation. Because of the highly specific nature of peptide reagents, several peptide-based assays can also be combined in a single assay without interference among tests.[10,22] Additionally, results in a recent study[s] indicated that samples from dogs infected with *E ewingii* will not cross-react with the tick-borne analytes in the commercially available in-clinic ELISA. However, the *A phagocytophilum* analyte in the commercially available in-clinic ELISA can be used to detect cross-reactive antibodies against a closely related species (ie, *A platys*) in experimentally infected dogs, and the *E canis* analyte will cross-react with anti–*E chaffeensis* antibodies.[16]

The *A phagocytophilum* analyte for the commercially available in-clinic ELISA used a peptide derived from the immunodominant P44 protein of the organism.[13] To our knowledge, the sequence from an analogous gene or protein for *A platys* has not been published. Analysis of data from the study reported here indicated that dogs experimentally infected with *A platys* can generate cross-reactive antibodies against the *A phagocytophilum* peptide used in the commercially available in-clinic ELISA. Although the commercially available in-clinic ELISA was found to be 99.1% specific for the detection of *A phagocytophilum* (relative to results for the IFA) in this study, it is important to mention that antibodies

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against *A platys* could have had similar cross-reactivity between the *A phagocytophilum* IFA and the commercially available in-clinic ELISA; thus, both tests would be seroreactive despite exposure to different *Anaplasma* spp. Although additional studies are needed to determine the diagnostic sensitivity of the *A phagocytophilum* analyte for detection of antibodies against *A platys*, veterinarians in regions endemic for *A platys*, which may overlap with regions endemic for *A phagocytophilum*, should be aware of cross-reactivity among these 2 *Anaplasma* organisms when using the commercially available in-clinic ELISA.

Infection attributable to *A phagocytophilum* can be diagnosed serologically (ie, confirmation of seroconversion) at most commercial laboratories with indirect IFAs. An IFA uses whole organisms grown in cell culture as the source of antigens.[4] Investigators have used this test and found that dogs may seroconvert as soon as 2 to 5 days after morulae appear in a blood sample.[23] In the present study, inoculated dogs had positive test results for both the IFA and the commercially available in-clinic ELISA as early as 8 days after inoculation; however, morulae were not observed in neutrophils during the early stages of this experimentally induced infection. These dogs did not develop clinical signs, and they remained persistently infected (as determined on the basis of results of PCR tests) for nearly 1 year.[e] Although the results of the present study and another study[24] support the potential for chronic infection in dogs after inoculation of *A phagocytophilum*, persistent infection has not been verified following natural infection in dogs. As described for testing via IFAs and illustrated by use of the commercially available in-clinic ELISA in experimentally infected dogs, the commercially available in-clinic ELISA can also be used to confirm seroconversion.

In addition to the detection of antibodies against *Anaplasma*, we also evaluated the performance of the commercially available in-clinic ELISA for detection of antibodies against *E canis* and *B burgdorferi* in dogs. The sensitivity and specificity for *E canis* and *B burgdorferi* were similar to that reported for another ELISA from the same manufacturer.[14] Addition of the *A phagocytophilum* analyte to the existing 3-analyte platform for that other ELISA did not affect the performance of those analytes in the commercially available in-clinic ELISA. A large number of samples with positive results for *D immitis* (as confirmed during necropsy or on the basis of results of the heartworm antigen test) were evaluated in the present study. The commercially available in-clinic ELISA had a sensitivity of 99.2% and specificity of 100%; none of 354 canine samples with negative results for the heartworm antigen test had a positive result when tested by use of the commercially available in-clinic ELISA. All heartworm antigen test kits and diagnostic kits, including the commercially available in-clinic ELISA, detect heartworm antigens released into the circulation primarily by adult female worms.[22] The sensitivity and specificity of the heartworm analyte in the multiple-analyte commercially available in-clinic ELISA were extremely similar to values reported for stand-alone heartworm antigen detection tests.[10,22]

The commercially available in-clinic ELISA can be used to detect antibodies against *A phagocytophilum*, *E canis*, and *B burgdorferi* and *D immitis* antigen in a single blood or serum sample. When combined with the medical history, physical examination findings, and results of appropriate diagnostic tests, the commercially available in-clinic ELISA will help practitioners rapidly identify serologic evidence supportive of a single infection or coinfection.[15,25–27] In the present study, we identified 68 of 846 canine samples that contained antibodies against > 1 species of tick-borne pathogen. More than 48 of 217 (22%) samples that were seroreactive for *A phagocytophilum* were also seroreactive for *B burgdorferi*. Evidence supporting coexposure of dogs to both *B burgdorferi* and *A phagocytophilum* has been reported,[26] particularly for dogs in areas endemic for both organisms. The prevalence of coinfection in dogs is caused by several factors, including sequential or cotransmission of organisms, the expanding range of the tick vectors, increased travel by pet owners accompanied by their dogs, and a tendency for families to move into tick-laden environments.[25,26]

Clinical signs of anaplasmosis in dogs may vary from mild to severe, and include fever, anorexia, weight loss, polyarthritis, and possibly meningitis. Most dogs have mild thrombocytopenia; anemia is uncommon, and changes in leukocyte counts may be variable among a population of sick dogs.[3,4,23,28,29] Elevated alkaline phosphatase activity has been reported[23] in dogs with anaplasmosis. Although neurologic disease is infrequent in dogs with anaplasmosis, signs may include seizures and neck pain. Tetracycline-type drugs are used to treat anaplasmosis in dogs. Limiting tick exposure and the routine use of acaracides is an important part of preventing these infections.[3]

Clinical signs in dogs with borreliosis include fever, lameness, anorexia, myocarditis, inflammatory joint disease, lymphadenopathy, glomerulonephritis, and (in rare circumstances) neurologic disease.[6] Ehrlichiosis in dogs may be either acute or chronic, with clinical signs that include fever, lethargy, myalgia, anorexia, and hemorrhage.[5] Abnormalities in laboratory tests may include anemia, thrombocytopenia, hyperglobulinemia, and proteinuria. Ehrlichiosis is transmitted through the bite of *Rhipicephalus sanguineus*, the brown dog tick.[5] Clinical signs of heartworm disease may include a dry chronic cough, shortness of breath, weakness, nervousness, and potentially tiring with exercise. The heart and lungs can be severely damaged in dogs with heavy worm burdens, which can lead to heart failure.[22]

On the basis of the high degree of specificity found in the study reported here, a positive result for the commercially available in-clinic ELISA for a tick-borne organism indicates that the dog has been exposed to that pathogen but does not indicate whether the dog has an active infection. Therefore, a complete medical history, physical examination, and appropriate hematologic, biochemical, and confirmatory diagnostic tests are needed to define whether the disease process is caused by one of these pathogens or whether the illness is unrelated to positive results obtained by use of the commercially available in-clinic ELISA. However, the ability to accurately and rapidly determine exposure to a

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vector-borne pathogen may improve understanding of the clinical signs associated with each type of infection as well as the atypical and complex clinical signs that can be seen in association with coinfections. In addition, the capacity to identify exposure to multiple tick-borne pathogens will aid veterinarians in defining the prevalence of exposure to a larger spectrum of tick-borne pathogens that are transmitted by *Ixodes* spp and *R sanguineous* ticks. In a recent study,[27] investigators clarified that there can be borreliosis and anaplasmosis coinfection in dogs in defined endemic areas, whereas *E canis* and *A platys* generally are in different geographic locations. Coinfection with *B burgdorferi* and *A phagocytophilum* appears to increase the severity of illness and the likelihood that these highly adapted vector-borne organisms will induce clinical signs of illness in dogs.[25,30,t] Veterinarians are increasingly finding that dogs with multiple simultaneous vector-borne infections are more likely to become sick and can have more pronounced damage to internal organs.[25,30,31] The commercially available in-clinic ELISA can be used to detect evidence of infection with *D immitis* or exposure to *A phagocytophilum*, *A platys*, *B burgdorferi*, and *E canis* via testing of a single blood or serum sample.

a. IDEXX Laboratories, Westbrook, Me.
b. IDEXX Reference Laboratories, IDEXX Laboratories, Westbrook, Me.
c. Sinclair Animal Research, Columbia, Mo.
d. SNAP 4Dx test kit, IDEXX Laboratories, Westbrook, Me.
e. Alleman RA, Chandrashekar R, Beall MB, et al. Experimental inoculation of dogs with a human isolate (NY18) of *A phagocytophilum* and demonstration of persistent infection following doxycycline therapy (abstr). *J Vet Intern Med* 2006;3:763.
f. SNAP 3Dx test kit, IDEXX Laboratories, Westbrook, Me.
g. Roche Diagnostics Corp, Indianapolis, Ind.
h. Sigma Chemicals, St Louis, Mo.
i. QIAamp DNA mini kit, Qiagen, Valencia, Calif.
j. Roche LightCycler 2.0, Roche Diagnostics Corp, Indianapolis, Ind.
k. SYBRGreen, Roche Diagnostics Corp, Indianapolis, Ind.
l. ProtaTek International Inc, Saint Paul, Minn.
m. FITC-conjugated anti-canine IgG, Jackson ImmunoResearch Laboratories Inc, Westgrove, Pa.
n. PetChek heartworm PF antigen test, IDEXX Laboratories, Westbrook, Me.
o. *Ehrlichia canis* western blot kit, IDEXX Laboratories, Westbrook, Me.
p. Alkaline phosphatase–conjugated anti-canine IgG, Jackson ImmunoResearch Laboratories Inc, Westgrove, Pa.
q. MarBlot *Borrelia burgdorferi* IgG western blot kits, Mardx Diagnostics Inc, Carlsbad, Calif.
r. Analyse-it standard edition, Analyse-it Software Ltd, Leeds, West Yorkshire, England.
s. Daniluk D, Slauenwhite J, O'Connor T, et al. Preliminary evaluation of a peptide-based assay for detection of *Ehrlichia ewingii* antibodies in experimentally and naturally-infected dogs (abstr). *J Vet Intern Med* 2007;21:625.
t. Gaunt SD, Chandrashekar R, Beall M, et al. Potentiation of thrombocytopenia and anemia in dogs experimentally co-infected with *Anaplasma platys* and *Ehrlichia canis* (abstr). *J Vet Intern Med* 2007;21:576.

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