

Comparison of *Anaplasma* and *Ehrlichia* species-specific peptide ELISAs with whole organism-based immunofluorescent assays for serologic diagnosis of anaplasmosis and ehrlichiosis in dogs

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Received February 7, 2020.

Accepted May 15, 2020.

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Canine anaplasmosis and ehrlichiosis are tick-borne diseases with a worldwide distribution and are caused by obligate intracellular bacteria of the genera *Anaplasma* and *Ehrlichia*, respectively.¹ Recognized *Anaplasma* species include *Anaplasma bovis*, *Anaplasma marginale* (and *A marginale* subsp *centrale*), *Anaplasma ovis*, *Anaplasma phagocytophilum*, and *Anaplasma platys*.² Of these, *A bovis*, *A marginale*, *A phagocytophilum*, and *A platys* have been documented to cause infections in dogs.³⁻⁸ *Anaplasma phagocytophilum* is transmitted by *Ixodes* spp ticks and causes granulocytic anaplasmosis in dogs, cats, horses, and humans.⁹⁻¹¹ *Anaplasma plat-*

ABBREVIATIONS

DAI Days after inoculation
IFA Immunofluorescent assay
TBP Tick-borne pathogen

OBJECTIVE

To compare the performance of 5 synthetic peptide-based ELISAs with that of 3 commercially available immunofluorescent assays (IFAs) for serologic diagnosis of anaplasmosis and ehrlichiosis in dogs.

SAMPLE

A convenience set of 109 serum samples obtained before and at various times after inoculation for 23 dogs that were experimentally infected with *Anaplasma phagocytophilum*, *Anaplasma platys*, *Ehrlichia canis*, *Ehrlichia chaffeensis*, or *Ehrlichia ewingii* and 1 uninfected control dog in previous studies.

PROCEDURES

All serum samples were assessed with 5 synthetic peptide-based ELISAs designed to detect antibodies against *A phagocytophilum*, *A platys*, *E canis*, *E chaffeensis*, and *E ewingii* and 3 whole organism-based IFAs designed to detect antibodies against *A phagocytophilum*, *E canis*, and *E chaffeensis*. The species-specific seroreactivity, cross-reactivity with the other tick-borne pathogens (TBPs), and diagnostic sensitivity and specificity were calculated for each assay and compared among assays.

RESULTS

All serum samples obtained from dogs experimentally infected with a TBP yielded positive results on a serologic assay specific for that pathogen. In general, sensitivity was comparable between ELISAs and IFAs and tended to increase with duration after inoculation. Compared with the IFAs, the corresponding ELISAs were highly specific and rarely cross-reacted with antibodies against other TBPs.

CONCLUSIONS AND CLINICAL RELEVANCE

Results suggested that peptide-based ELISAs had enhanced specificity relative to whole organism-based IFAs for detection of antibodies against *Anaplasma* and *Ehrlichia* spp, which should facilitate accurate diagnosis and may help detect dogs coinfecting with multiple TBPs. (*Am J Vet Res* 2021;82:71-80)

ys infects platelets in dogs, cats, humans, and ruminants and is likely transmitted by the brown dog tick, *Rhipicephalus sanguineus* sensu lato.^{7,12-14} In dogs, *A platys* infection can cause cyclic thrombocytopenia.⁷

Recognized *Ehrlichia* spp include *Ehrlichia canis*, *Ehrlichia chaffeensis*, *Ehrlichia ewingii*, *Ehrlichia muris*, and *Ehrlichia ruminantium*.² In North America, dogs are primarily exposed to *E canis* and *E ewingii*; however, *E chaffeensis*, *E muris*, and Panola Mountain *Ehrlichia* spp have been detected in a small number of dogs with clinical disease.¹⁵⁻¹⁹ For dogs infected with *Ehrlichia* spp, clinical signs range from none to severe acute or chronic illnesses. *Ehrlichia canis* is also transmitted by the brown dog tick and causes monocytic ehrlichiosis in dogs. Review of the veterinary literature suggests that approximately 80% to 100% of dogs experimentally infected with *E canis* recover following treatment with doxy-

cycline; however, dogs that are not treated or that do not respond to treatment may develop chronic ehrlichiosis, which is associated with severe clinical disease and a poor prognosis.^{20,21} *Ehrlichia ewingii* and *E chaffeensis* are transmitted by *Amblyomma americanum* (commonly known as the lone star tick, northeastern water tick, and turkey tick); infect granulocytes and monocytes, respectively; and cause clinical disease of varying severity in dogs.^{15,22,23} Because TBPs cause similar clinical signs in dogs, it can be difficult to distinguish among infections caused by different genera and species.

Current options for diagnosing anaplasmosis and ehrlichiosis include blood smear analysis to visualize morulae, PCR amplification of bacterial DNA, and serologic testing to detect antibodies against *Anaplasma* or *Ehrlichia* spp. Because all diagnostic modalities have limitations, it is recommended that blood smear examination, PCR assay, and serologic testing all be performed, particularly for dogs with clinical signs of anaplasmosis or ehrlichiosis.²⁴ Blood smear examination may provide a rapid diagnosis if morulae are detected, but the sensitivity of that method is low, with some reports^{23,25} indicating that morulae are detected in only 4% to 16% of dogs infected with TBPs. Diagnostic tests that use PCR technology are sensitive and can be broad (genus-specific) or specific (species-specific), but even highly sensitive diagnostic tests can yield false-negative results when the pathogen load is below the limit of detection.²⁴ Immunofluorescent assays for *Anaplasma* and *Ehrlichia* spp are reliant on the detection of fluorescence resulting from the binding of antibodies in a test sample to whole *Anaplasma* or *Ehrlichia* organisms grown in cell cultures and affixed to glass slides (ie, antibody reactivity). Immunofluorescent assays to detect antibody reactivity with numerous *Anaplasma* and *Ehrlichia* spp have been developed. Because *A platys* and *E ewingii* have not been successfully isolated in cell culture systems, IFAs for those organisms are not currently available. Paired quantification of antibody titers by conducting serial IFAs can help distinguish patients with subclinical, acute, or chronic infections. A 4-fold increase in antibody titer during the acute phase of anaplasmosis or ehrlichiosis is indicative of infection, whereas antibody titers tend to remain stable during subclinical and chronic ehrlichiosis.²⁰ Immunofluorescent assays are considered to have high diagnostic sensitivity but can have poor diagnostic specificity owing to cross-reaction of antibodies within or across the *Anaplasma* and *Ehrlichia* genera because those phylogenetically related organisms share common outer membrane proteins.²⁶⁻²⁸ Specifically, cross-reactivity has been reported between *A phagocytophilum* and *E canis*, *A phagocytophilum* and *E chaffeensis*, and *A phagocytophilum* and *A platys*, and among *E canis*, *E ewingii*, and *E chaffeensis*.^{26,28-30} Results of studies conducted to assess IFA cross-reactivity in dogs naturally infected with TBPs should be interpreted with caution because coinfection with more than 1 TBP

genera is not uncommon. To minimize cross-reactivity within and across *Anaplasma* and *Ehrlichia* genera, ELISAs that use synthetic peptides were designed to detect antibodies against species-specific immunodominant proteins of *A phagocytophilum*, *A platys*, *E canis*, *E chaffeensis*, and *E ewingii*.^{18,19}

The objective of the study reported here was to compare the performance of 5 synthetic peptide-based ELISAs designed to detect canine antibodies against *A phagocytophilum*, *A platys*, *E canis*, *E chaffeensis*, and *E ewingii* with the performance of 3 commercially available IFAs that use cell culture-grown whole *A phagocytophilum*, *E canis*, and *E chaffeensis* to detect antibodies against those bacteria. Serum samples obtained from dogs experimentally infected with *Anaplasma* or *Ehrlichia* spp were preferentially analyzed so that the diagnostic specificity could be evaluated as well as the earliest point after infection at which each serologic assay detected antibodies against each bacterium.

Materials and Methods

Serum samples

A convenience set of 109 frozen serum samples obtained from 24 dogs that were or were not (uninfected control) experimentally infected with *Anaplasma* and *Ehrlichia* spp in other studies^{10,31-33} (Table I) was analyzed in the study reported here. Serum samples were serially collected from each dog at predetermined times following experimental inoculation. Specifically, 24 serum samples from 6 *A phagocytophilum*-infected dogs, 30 serum samples from 6 *A platys*-infected dogs, 30 serum samples from 6 *E canis*-infected dogs, 8 serum samples from 2 *E chaffeensis*-infected dogs, 15 serum samples from 3 *E ewingii*-infected dogs, and 2 serum samples from an uninfected control dog were analyzed. Protocols regarding the care, feeding, housing, and handling for all dogs were reviewed and approved by the institutional animal care and use committee of the respective institutions where the studies³¹⁻³³ were performed or were conducted in accordance with the National Research Council's Guide for the Care and Use of Laboratory Animals and the Animal Welfare Act.¹⁰

ELISAs

Anaplasma and *Ehrlichia* species-specific peptide-based ELISAs designed to detect antibodies against *A phagocytophilum*, *A platys*, *E canis*, *E chaffeensis*, and *E ewingii* were performed on all 109 serum samples. A similar direct-format protocol was used for all ELISAs, except for the *E chaffeensis* ELISA for which results were derived from both indirect and direct plate assay formats. The species-specific peptides used in this study have been previously described^{18,19,28,32,34} and included *A phagocytophilum* p44-4, *A platys* p44-4, *E canis* p16, *E chaffeensis* variable-length PCR target protein,

Table 1—Description of the dogs from which serum samples were obtained that were analyzed in a study conducted to compare the performance of *Anaplasma* and *Ehrlichia* species-specific peptide ELISAs with that of whole organism-based IFAs for serologic diagnosis of anaplasmosis and ehrlichiosis in dogs.

TBP	No. and type of dogs	No. of serum samples collected/dog	No. of serum samples collected	Inoculum	Inoculation route	Pre-experimental screening for TBPs	Study location	Reference No.
<i>Anaplasma phagocytophilum</i>	6 specific pathogen-free hounds obtained from a Class A USDA vendor	4	24	HL-60 or autologous neutrophils infected with either a canine- or human-derived strain of the pathogen	IV	Not described	Johns Hopkins University School of Medicine	10
<i>Anaplasma platys</i>	Six 6-month-old female hound-type dogs	5	30	Infected canine platelet-rich plasma	IV	Not described	Louisiana State University	31
<i>Ehrlichia canis</i>	Six 6-month-old female hound-type dogs	5	30	DH82 infected cells	IV	Not described	Louisiana State University	31
<i>Ehrlichia chaffeensis</i>	2 specific pathogen-free dogs	4	8	DH82 infected cells	IV	Not described	The Ohio State University	33
<i>Ehrlichia ewingii</i>	3 specific pathogen-free Beagles	5	15	Infected canine blood or buffy coat	IV	Negative PCR assay results for <i>A phagocytophilum</i> , <i>E canis</i> , <i>E chaffeensis</i> , and <i>E ewingii</i> and negative IFA results for <i>A phagocytophilum</i> , <i>E canis</i> , and <i>E chaffeensis</i>	The Ohio State University	32
Uninfected control dog	One 6-month-old female hound-type dog	2	2	NA	NA	Negative PCR assay results for <i>A platys</i> and <i>Ehrlichia</i> spp and negative point-of-care peptide-based ELISA results for <i>A phagocytophilum</i> , <i>A platys</i> , <i>Borrelia burgdorferi</i> , <i>E canis</i> , <i>E chaffeensis</i> , and <i>Dirofilaria immitis</i>	Louisiana State University	31

NA = Not applicable.

and *E ewingii* p28. For the direct assay format, 96-well microtiter plates^a were coated with the respective synthetic peptides^{b,c} (concentration, 0.25 to 1.5 µg/mL). One-step incubations (neat sample + 0.5- to 2-µg/mL detection conjugate) were carried out for 60 minutes. Detection conjugates consisted of the respective synthetic peptides described above conjugated to the enzyme horseradish peroxidase. Plates were washed with PBS solution and then incubated with 3,3',5,5'-tetramethylbenzidine substrate solution. The reaction was stopped after 10 minutes by the addition of 0.1% SDS stop solution. The optical density of each well was determined with a plate reader set at a wavelength of 650 nm. Depending on each ELISA, reactive (positive) samples were defined as those with an absorbance value > 2 or 3 times that for the sample obtained from the uninfected control dog.

For the indirect assay format, each well of a 96-well microtiter plate was incubated with a 1:50 dilution of a test serum sample, washed with PBS solution, and then incubated with a 1:1,000 dilution of rabbit anti-dog antibody (IgG)-horseradish peroxidase for 30 minutes. Subsequent plate washing and reading (ie, antibody detection) were performed as described for the direct assay format. The optical density cut-off value for a positive serum sample was 0.106 for *A phagocytophilum*, 0.078 for *A platys*, 0.132 for *E canis*, 0.070 for *E chaffeensis*, and 0.110 for *E ewingii*.

IFAs

All serum samples underwent IFAs for detection of antibodies against *A phagocytophilum*, *E canis*, and *E chaffeensis*, except for 24 serum samples obtained from *A platys*-infected dogs, which did not undergo the *E chaffeensis* IFA. The *E canis* and *E chaffeensis* IFAs were conducted as described³⁵ at the Intracellular Pathogens Research Laboratory at North Carolina State University, Raleigh, NC. For serum samples obtained from *A phagocytophilum*-

infected dogs, an *A phagocytophilum* IFA was performed as described¹⁰ at the Johns Hopkins School of Medicine Molecular and Comparative Pathology Laboratory, Baltimore. For serum samples obtained from dogs that were not experimentally infected with *A phagocytophilum*, an *A phagocytophilum* IFA was performed with a commercially available test kit^d in accordance with the manufacturer's instructions at Idexx Reference Laboratories, Westbrook, Me.

All IFAs for *A phagocytophilum* used whole-organism antigens from an *A phagocytophilum* strain isolated from a human patient in New York. The IFA for *E canis* used whole-organism antigens from an *E canis* strain identified as K9 Jake. The IFA for *E chaffeensis* used whole-organism antigens from an *E chaffeensis* strain isolated from a human patient. To minimize IFA slide usage, serum samples obtained from *A phagocytophilum*-infected dogs that were analyzed at the Johns Hopkins School of Medicine Molecular and Comparative Pathology Laboratory were not diluted to achieve an end point antibody titer for samples collected at 0, 17, and 30 DAI. Instead, those samples were screened at 1:40, 1:80, and 1:160 dilutions, and samples that were seroreactive at the 1:160 dilution were considered positive. End point antibody titers were performed for positive samples collected at 60 DAI, which were titered to 1:2,560. For all other IFAs, 2-fold dilutions of each serum sample were screened at 1:16, 1:32, and 1:64 dilutions or 1:32 and 1:64 dilutions. Samples that were seroreactive at the 1:64 dilution were considered positive, and end point titers were determined to the 1:8,192 dilution.

PCR assay

Genomic DNA was isolated from serum samples obtained from *E ewingii*-infected dogs and evaluated with an *A platys* p44 real-time PCR hybridization probe assay as described³¹ to identify the *A platys* p44 polynucleotide (GenBank accession No. GP282016).

Data analysis

Serum samples were grouped by the TBPs with which the dogs were experimentally infected and were determined to be seropositive or seronegative on the basis of the antibody titer or absorbance value determined by the IFA or ELISA, respectively. For each group of experimentally infected dogs, the proportion of serum samples collected ≥ 10 DAI with positive results was calculated for each assay to assess the potential for cross-reactivity among tests.

Serum samples were then classified into 3 infection stages on the basis of the duration between experimental inoculation and sample collection (early infection stage [7 to 10 DAI], mid infection stage [13 to 21 DAI], and late infection stage [≥ 28 DAI]). For each infection stage category and assay, the number of true-positive, true-negative, false-positive, and false-negative test results was tabulated, and the sensitivity and specificity and corresponding 95% CIs were calculated. A true-positive result was defined as a sample with a positive result for the pathogen with which the dog was experimentally inoculated. A true-negative result was defined as a sample with a negative result obtained from the uninfected control dog, a dog prior to experimental inoculation, or a dog following experimental inoculation with a pathogen other than that detected by the assay in question. A false-positive result was defined as a sample with a positive result for any pathogen with which the dog was not experimentally inoculated. A false-negative result was defined as a sample with a negative result for the pathogen with which the dog was experimentally inoculated. For TBPs (*A phagocytophilum*, *E canis*, and *E chaffeensis*) for which both an IFA and ELISA were evaluated, the extent of agreement between the 2 assays was assessed by means of the kappa (κ) statistic with linear weighting. All calculations were performed by use of a statistical computation website.^c

Results

Serologic results for all serum samples are available elsewhere (**Supplementary Table SI**, available at: avmajournals.avma.org/doi/suppl/10.2460/ajvr.82.1.71). For each assay, the number of samples with positive results was summarized on the basis of experimental infection group and the DAI the samples were collected (**Table 2**).

For the 6 *A phagocytophilum*-infected dogs, antibodies against the organism were consistently detected by both the *A phagocytophilum* IFA and ELISA in all serum samples collected after experimental inoculation but were not detected in any of the serum samples collected before experimental inoculation or obtained from the uninfected control dog (Table 2). Serum samples collected at various times after experimental inoculation with *A phagocytophilum* yielded false-positive results on the *A platys* ELISA, *E canis* IFA, and *E chaffeensis* IFA. None of the serum

samples collected from *A phagocytophilum*-infected dogs reacted to the *E canis* ELISA, *E chaffeensis* ELISA, or *E ewingii* ELISA.

For the 6 *A platys*-infected dogs, the *A platys* ELISA detected antibodies against the organism in all serum samples collected after experimental inoculation except 1 sample collected 90 or 91 DAI (Table 2). The *A platys* ELISA did not yield positive results for any serum sample collected before experimental inoculation with the organism or any serum sample obtained from the uninfected control dog. Serum samples collected at various times after experimental inoculation with *A platys* yielded false-positive results on the *A phagocytophilum* IFA, *E canis* IFA, and *E chaffeensis* IFA. None of the serum samples collected from *A platys*-infected dogs reacted to the *A phagocytophilum* ELISA, *E canis* ELISA, *E chaffeensis* ELISA, or *E ewingii* ELISA.

For the 6 *E canis*-infected dogs, antibodies against the organism were not consistently detected by the *E canis* IFA until 13 or 14 DAI and were not consistently detected by the *E canis* ELISA until 17 DAI (Table 2). Antibodies against *E canis* were not detected in any of the serum samples collected from the *E canis*-infected dogs before experimental inoculation or from the uninfected control dog. Serum samples collected from *E canis*-infected dogs at various times after experimental inoculation yielded false-positive results on the *A phagocytophilum* IFA and *E chaffeensis* IFA. None of the serum samples collected from *E canis*-infected dogs reacted to the *A phagocytophilum* ELISA, *A platys* ELISA, *E chaffeensis* ELISA, or *E ewingii* ELISA.

For the 2 *E chaffeensis*-infected dogs, antibodies against the organism were consistently detected by the *E chaffeensis* IFA beginning 14 DAI and by the *E chaffeensis* ELISA beginning 7 DAI (Table 2). Antibodies against *E chaffeensis* were not detected in any of the samples collected from *E chaffeensis*-infected dogs prior to experimental inoculation with the organism or from the uninfected control dog. Serum samples from both *E chaffeensis*-infected dogs yielded false-positive results on the *E canis* IFA but did not react to the *A phagocytophilum* IFA, *A phagocytophilum* ELISA, *A platys* ELISA, *E canis* ELISA, or *E ewingii* ELISA.

For the 3 *E ewingii*-infected dogs, antibodies against the organism were detected by the *E ewingii* ELISA in serum samples from 2 of the dogs beginning 28 DAI and from all 3 dogs beginning 35 or 42 DAI (Table 2). Antibodies against *E ewingii* were not detected in serum samples collected from *E ewingii*-infected dogs prior to experimental inoculation or from the uninfected control dog. Serum samples obtained from *E ewingii*-infected dogs at various times after experimental inoculation occasionally yielded false-positive results on the *A platys* ELISA, *E canis* IFA, and *E chaffeensis* IFA but did not react to the *A phagocytophilum* IFA, *A phagocytophilum* ELISA, *E canis* ELISA, or *E chaffeensis* ELISA.

Table 2—Number of canine serum samples described in Table 1 that yielded positive results for each of the 8 assays performed.

Infection group	No. of dogs	DAI	<i>A phagocytophilum</i>		<i>A platys</i>	<i>E canis</i>		<i>E chaffeensis</i>		<i>E ewingii</i>
			IFA	ELISA	ELISA	IFA	ELISA	IFA	ELISA	ELISA
<i>A phagocytophilum</i>	6	0	0	0	0	0	0	0	0	0
		17	6	6	1*	3*	0	0	0	0
		30	6	6	0	0	0	1*	0	0
		60	6	6	0	1*	0	1*	0	0
<i>A platys</i>	6	0	0	0	0	0	0	0†	0	0
		10	0	0	6	0	0	NP	0	0
		13 or 14	0	0	6	1*	0	NP	0	0
		17	0	0	6	1*	0	NP	0	0
<i>E canis</i>	6	90 or 91	4*	0	5	0	0	3†	0	0
		0	0	0	0	0	0	0	0	0
		10	0	0	0	1	2	0	0	0
		13 or 14	1*	0	0	6	4	1*	0	0
<i>E chaffeensis</i>	2	17	2*	0	0	6	6	2*	0	0
		90 or 91	1*	0	0	5	6	5*	0	0
		0	0	0	0	0	0	0	0	0
		7	0	0	0	0	0	0	2	0
<i>E ewingii</i>	3‡	14	0	0	0	2*	0	2	2	0
		63	0	0	0	2*	0	2	2	0
		0	0	0	0	0	0	0	0	0
		21	0	0	2*	0	0	0	0	0
Uninfected control dog	1	28	0	0	2*	0	0	0	0	2
		35 or 42	0	0	2*	1*	0	2*	0	3
		126 or 133	0	0	0	0	0	3*	0	3
		0	0	0	0	0	0	0	0	0
		91	0	0	0	0	0	0	0	

*Assay results suggestive of serologic cross-reactivity between the 2 given TBPs. †Only 3 of the 6 serum samples obtained from *A platys*-infected dogs on this day underwent this assay. ‡Two of the 3 dogs were subsequently determined to have been inoculated with blood from a blood donor dog that was coinfecting with *E ewingii* and *A platys* (ie, those 2 dogs were inadvertently inoculated with *A platys* in addition to *E ewingii*). NP = Not performed.

Table 3—Proportion of canine serum samples obtained ≥ 10 DAI that yielded positive test results for each assay.

Infection group	<i>A phagocytophilum</i>		<i>A platys</i>	<i>E canis</i>		<i>E chaffeensis</i>		<i>E ewingii</i>
	IFA	ELISA	ELISA	IFA	ELISA	IFA	ELISA	ELISA
<i>A phagocytophilum</i>	18/18	18/18	1/18*	4/18*	0/18	2/18*	0/18	0/18
<i>A platys</i>	4/24*	0/24	23/24	2/24*	0/24	3/3*†	0/24	0/24
<i>E canis</i>	4/24*	0/24	0/24	18/24	18/24	8/24*	0/24	0/24
<i>E chaffeensis</i>	0/6	0/6	0/6	4/6*	0/6	4/6	6/6	0/6
<i>E ewingii</i> ‡	0/12	0/12	6/12*	1/12*	0/13	5/12*	0/12	8/12

*Assay results suggestive of serologic cross-reactivity between the 2 TBPs. †Only 3 serum samples that were collected from 3 dogs ≥ 10 DAI were tested by the *E chaffeensis* IFA. ‡Two of the 3 dogs (ie, 8 serum samples) represented in this infection group were subsequently determined to have been inoculated with blood from a donor dog that was infected with *A platys*.

Serum samples collected from 2 of the 3 *E ewingii*-infected dogs after experimental inoculation yielded positive results on the *A platys* ELISA. Further investigation revealed that the *E ewingii*-infected blood used to inoculate those 2 dogs was obtained from the same blood donor dog. A PCR assay to detect *A platys* p44 DNA was performed on serum samples collected 21 and 28 DAI from the 2 *E ewingii*-infected dogs that tested positive for antibodies against *A platys* and a serum sample collected from the donor dog that provided the blood used for the *E ewingii* inoculum for those 2 dogs. All samples tested positive for *A platys* DNA. Consequently, it was determined that the blood donor dog was infected with *A platys*, and *A platys* was transferred to the 2 *E ewingii*-

infected dogs during experimental inoculation with *E ewingii*. The blood used for the *E ewingii* inoculation of the third dog was obtained from a different blood donor dog, and none of the serum samples obtained from that dog yielded positive results on the *A platys* ELISA.

Among the 231 individual whole organism-based IFAs performed on serum samples collected ≥ 10 DAI, 37 (16%) yielded false-positive results (the serum sample yielded positive results [ie, cross-reacted with the antigen used in the IFA] for an organism other than the TBP with which the dog was experimentally inoculated). Only 7 of 420 (1.7%) peptide-based ELISAs performed on serum samples collected ≥ 10 DAI yielded positive results for an organism other than the TBP

Table 4—Summary of assay results, sensitivity, and specificity for the canine serum samples described in Table 1 stratified on the basis of infection stage.

Infection stage	Assay variable	<i>A phagocytophilum</i>		<i>A platys</i>	<i>E canis</i>		<i>E chaffeensis</i>		<i>E ewingii</i>
		IFA*	ELISA*	ELISA†	IFA	ELISA	IFA‡	ELISA	ELISA§
Early	True positive (No.)	—	—	6	1	2	0	2	—
	True negative (No.)	38	38	32	32	32	27	36	38
	False positive (No.)	0	0	0	0	0	0	0	0
	False negative (No.)	—	—	0	5	4	2	0	—
	Sensitivity (95% CI; %)	—	—	100 (52–87)	17 (7–32)	33 (6–76)	0 (0–80)	100 (20–100)	—
	Specificity (95% CI; %)	100 (89–100)	100 (89–100)	100 (84–100)	100 (87–100)	100 (87–100)	100 (85–100)	100 (88–100)	100 (89–100)
Mid	True positive (No.)	6	6	12	12	10	2	2	0
	True negative (No.)	26	29	20	24	32	18	31	32
	False positive (No.)	3	0	1	8	0	3	2	0
	False negative (No.)	0	0	0	0	2	0	0	3
	Sensitivity (95% CI; %)	100 (52–100)	100 (52–100)	100 (70–100)	100 (70–100)	83 (51–97)	100 (20–100)	100 (20–100)	0 (0–69)
	Specificity (95% CI; %)	90 (72–97)	100 (85–100)	95 (74–100)	75 (56–89)	100 (87–100)	86 (63–96)	94 (78–99)	100 (87–100)
Late	True positive (No.)	12	12	5	5	6	2	2	8
	True negative (No.)	19	24	26	18	21	16	34	27
	False positive (No.)	5	0	0	3	0	15	0	0
	False negative (No.)	0	0	1	1	0	0	0	1
	Sensitivity (95% CI; %)	100 (70–100)	100 (70–100)	83 (37–99)	83 (37–99)	100 (52–100)	100 (20–100)	100 (20–100)	89 (51–99)
	Specificity (95% CI; %)	79 (57–92)	100 (83–100)	100 (84–100)	86 (63–96)	100 (81–100)	52 (33–69)	100 (87–100)	100 (85–100)

Serum samples were classified into 3 infection stages on the basis of the duration between experimental inoculation and sample collection (early [7–10 DAI], mid [13–21 DAI], and late [\geq 28 DAI]). A true-positive result was defined as a sample with a positive result for the pathogen with which the dog was experimentally inoculated. A true-negative result was defined as a sample with a negative result obtained from the uninfected control dog, a dog prior to experimental inoculation, or a dog following experimental inoculation with a pathogen other than that detected by the assay in question. A false-positive result was defined as a sample with a positive result for any pathogen with which the dog was not experimentally inoculated. A false-negative result was defined as a sample with a negative result for the pathogen with which the dog was experimentally inoculated. *No serum samples were collected from *A phagocytophilum*-infected dogs during the early infection stage; thus, there were no samples available to serve as true-positive references for calculation of assay sensitivity. †Six serum samples were excluded from the sensitivity and specificity calculations for the *A platys* ELISA because subsequent testing revealed that 2 of the 3 *E ewingii*-infected dogs were inoculated with blood that was coinfecting with *A platys*; thus, those samples could not be considered as true negatives. ‡Twenty-four serum samples collected from *A platys*-infected dogs were not evaluated by the *E chaffeensis* IFA. §No serum samples were collected from *E ewingii*-infected dogs during the early infection stage; thus, there were no samples available to serve as true-positive references for calculation of assay sensitivity.

— = Not applicable.

with which the dog was experimentally inoculated, and the *A platys* ELISA yielded all 7 of those positive results. However, 6 of the 7 serum samples that yielded apparently erroneous results on the *A platys* ELISA were obtained from the 2 aforementioned dogs that were inadvertently inoculated with *A platys* in addition to *E ewingii* (Table 3). Thus, the positive results for those 6 samples were likely true-positive results.

Assay results and sensitivity and specificity were summarized on the basis of infection stage (early [7 to 10 DAI], mid [13 to 21 DAI], and late [\geq 28 DAI]; Table 4). In general, the sensitivity of all serologic assays improved and the specificity for the IFAs decreased as the stage of infection advanced from the early to late stages.

The κ statistic for the extent of agreement between the IFA and ELISA was 1 (95% CI, 1 to 1) for *A phagocytophilum*, 0.72 (95% CI, 0.47 to 0.98) for *E canis*, and 0.5 (95% CI, 0 to 1) for *E chaffeensis*.

Discussion

In the study reported here, the performance of peptide-based ELISAs for detection of antibodies against *Anaplasma* and *Ehrlichia* pathogens was compared with that of species-specific whole organism-based IFAs for a convenience set of canine serum samples collected at various times before and after experimental inoculation with an *Anaplasma* or *Ehrlichia* sp. Results indicated that the ELISAs were highly specific relative to the corresponding IFAs and

rarely cross-reacted with antibodies against other *Anaplasma* or *Ehrlichia* spp. In fact, only the *A platys* ELISA yielded what were initially considered false-positive results. However, 6 of those 7 results were recorded for serum samples that were subsequently determined to have been obtained from dogs that were inadvertently inoculated with *A platys* in addition to the intended *E ewingii* inoculum, because the dog that donated the blood for the experimental inoculum was infected with *A platys*. Therefore, it is likely that the positive *A platys* ELISA results for those 6 samples were true-positive results owing to occult *A platys* infection. One serum sample from an *A phagocytophilum*-infected dog yielded positive results on the *A platys* ELISA. Homology is limited between the DNA sequences that encode *A platys*-specific p44 and *A phagocytophilum*-specific p44. Therefore, that test result may have been a false-positive result, or the dog from which the sample was obtained may have been previously exposed to *A platys*.

When the performance of the *A phagocytophilum*, *E canis*, and *E chaffeensis* ELISAs was compared with the corresponding whole organism-based IFAs, the sensitivity for the ELISA was generally greater than that for the IFA during the early infection stage (7 to 10 DAI) but became comparable between the 2 assay modalities during the mid (13 to 21 DAI) and late (\geq 28 DAI) infection stages. The specificity for both assay modalities was high (ie, 100%) during the early infection stage likely owing to the lack of

detectable antibody titers during the first few DAI. The specificity for the IFAs typically decreased as the stage of infection progressed, potentially because of time-dependent changes in the recognition of immunoreactive proteins. The extent of agreement between the IFA and ELISA was perfect for *A phagocytophilum* (κ , 1) and very good for *E canis* (κ , 0.72) but was only moderate for *E chaffeensis* (κ , 0.50).

The peptide-based ELISAs evaluated in the present study consistently distinguished antibodies against each *Ehrlichia* species without evidence of serologic cross-reactivity. Of particular importance, the peptide-based ELISA reliably detected antibodies against *E ewingii*; that organism has not yet been successfully cultured, and a whole organism-based IFA and ELISA are not currently available for *E ewingii*. The use of whole organism-based IFAs designed to detect antibodies against *E canis* or *E chaffeensis* for the detection of antibodies against *E ewingii* lacks diagnostic sensitivity and can lead to false-negative test results. Results of the present study indicated that there was poor cross-reactivity between anti-*E ewingii* antibodies and the antigens in the whole organism-based IFA for *E canis*. Results of other studies^{18,19} that involved the use of *Ehrlichia* species-specific peptide assays suggest that *E ewingii* is the most seroprevalent *Ehrlichia* species infecting dogs in the United States. Because *E ewingii* causes disease in dogs, an accurate serologic assay is clinically important.²³ Reliance on *E ewingii* cross-reactivity with *E canis* serologic assays for the identification of anti-*E ewingii* antibodies in dogs could lead to misdiagnoses. Another study³⁶ describes the use of an in-clinic peptide-based ELISA^f with both *E ewingii*- and *E canis*-specific peptides. For example, dogs with a positive test result on the in-clinic *Ehrlichia* peptide-based ELISA but a negative test result on the *E canis* whole organism-based IFA have likely been exposed to *E ewingii*.²³

In the present study, the specificities for the whole organism-based IFAs for *A phagocytophilum*, *E canis*, and *E chaffeensis* were substantially lower than the specificities for the corresponding peptide-based ELISAs for those TBPs, which suggested that the respective IFAs were more likely to cross-react with antibodies against other *Anaplasma* and *Ehrlichia* spp than were the ELISAs. The veterinary literature contains conflicting information regarding the extent of cross-reactivity between *Anaplasma* and *Ehrlichia* spp in dogs. Sainz et al¹ suggest that there is little serologic cross-reactivity between *Anaplasma* and *Ehrlichia* spp, whereas investigators of other studies^{26,29} report that there is cross-reactivity between those 2 TBPs. In the present study, results of the whole organism-based IFAs provided evidence that there was inconsistent cross-reactivity both across and within the *Anaplasma* and *Ehrlichia* genera at various DAI. For example, 2 *A phagocytophilum*-infected dogs had high antibody titers (1:4,096 and 1:512) on the *E canis* IFA at 17 DAI but yielded negative results on that assay at 30 DAI. One of those dogs also yielded positive results on the *E chaffeensis*

IFA at 30 and 60 DAI. Two *A platys*-infected dogs had moderate antibody titers (1:128) against *E canis* at approximately 14 DAI; however, serum samples obtained from those 2 dogs > 14 DAI consistently yielded negative results on the *E canis* IFA. Both of those *A platys*-infected dogs yielded positive results on the *E chaffeensis* IFA at 90 DAI. Two *E canis*-infected dogs yielded weakly positive results on the *A phagocytophilum* IFA at approximately 14 DAI. Interestingly, those 2 dogs had fairly low antibody titers (1:128 and 1:256) against *E canis* at approximately 14 DAI, whereas the *E canis*-infected dogs that had extremely high anti-*E canis* antibody titers (1:1,024 and 1:8,192) at 14 DAI did not yield positive results on the *A phagocytophilum* IFA. If *E canis* antibodies were truly cross-reactive with the *A phagocytophilum* IFA, we might expect high antibody titers to correlate with cross-reactivity. The inconsistencies in seroreactivity across the *Anaplasma* and *Ehrlichia* genera observed on the basis of the results of the whole organism-based IFAs evaluated in the present study are difficult to explain. Eight of 24 (33%) serum samples collected from *E canis*-infected dogs \geq 10 DAI tested positive on the *E chaffeensis* IFA, which suggested that anti-*E canis* antibodies frequently cross-react with the *E chaffeensis* antigen in the IFA. Serologic cross-reactivity between *E canis* and *E chaffeensis* has been widely documented and is likely owing to the large number of similar protein epitopes within the outer membranes of the 2 organisms.³⁷ Serologic cross-reactivity between species within the same genera during the late stage of infection (eg, the serum samples obtained at 90 and 91 DAI from 2 *A platys*-infected dogs that yielded positive test results on the *A phagocytophilum* IFA) may have represented time-dependent changes in the recognition of immunoreactive proteins. Although not always reliable, serologic cross-reactivity can be useful for detection of broad genus-level pathogen exposure but is not optimal for distinguishing pathogen exposure at the species level.¹⁷ The serologic cross-reactivity observed within and across genera in the present study might have represented true cross-reactivity, or dogs that were assumed to be pathogen-free might in fact have been exposed to other TBPs prior to enrollment in the studies^{10,31-33} during which the serum samples evaluated in this study were collected. For example, serum samples from all 3 *E ewingii*-infected dogs yielded positive results on the *E chaffeensis* IFA. Both *E ewingii* and *E chaffeensis* are transmitted by *A americanum*; thus, it is possible that the *E ewingii*-infected dogs were concurrently infected with *E chaffeensis* when they were administered the *E ewingii*-infected inoculum. It is also possible that some of the apparent serologic cross-reactions observed in the present study were actually false-positive test results owing to nonspecific IFA fluorescence or variability in interpretation of immunofluorescence by IFA technicians.

For TBPs, serologic cross-reactivity within and across genera can lead to misdiagnoses and prevent detection of dogs coinfecting with multiple pathogens.^{1,26,29} Polymerase chain reaction methods are

recommended for speciation of TBPs, even though a low pathogen load may lead to false-negative PCR assay results and prevent TBP speciation.²⁴ In TBP-infected patients for which PCR methods fail to identify the infecting species, use of species-specific serologic testing modalities might facilitate accurate diagnosis of the infecting species. However, coinfection with *Anaplasma* and *Ehrlichia* spp can complicate interpretation of serologic test results and clinical signs in ill dogs,^{1,26,31} and treatment duration and response can vary depending on the infecting pathogen or pathogens. Common tick species that parasitize dogs can cotransmit *Anaplasma* and *Ehrlichia* spp.³⁸ *Rhipicephalus sanguineus* transmits *A platys* and *E canis*, *A americanum* transmits *E chaffeensis* and *E ewingii*, and *Ixodes scapularis* transmits *A phagocytophilum* and *E muris*. It is likely that those tick species naturally establish transmission dynamics that support coinfection because dogs living in *R sanguineus*-, *A americanum*-, or *I scapularis*-endemic regions can be coinfecting or coexposed to *Anaplasma* and *Ehrlichia* spp.^{7,39} Results of the present study indicated that, in dogs experimentally infected with 1 of 5 TBPs, species-specific peptide-based ELISAs were more effective than whole organism-based IFAs for the differentiation of antibodies against the infecting pathogen.

The present study had several limitations. The serum samples evaluated represented a convenience set of samples from dogs that were experimentally infected with a TBP by use of various inoculation protocols in previous studies.^{10,31-33} Also, a small number of serum samples were evaluated from dogs experimentally infected with each TBP, and the number of DAI at which the serum samples were collected varied among the original experimental studies.^{10,31-33} Intravenous injection of blood containing a TBP to induce experimental infection does not necessarily mimic the progression of disease and antibody kinetics in naturally infected animals; however, that method of inoculation is well established for inducing tick-borne disease in experimental studies of disease and diagnostic test performance.^{31,40} Not all dogs were screened for TBPs prior to experimental inoculation. Results of a recent study⁴¹ suggest that dogs enrolled in experimental studies of TBPs should be tested for the presence of a TBP, preferably multiple times, before experimental inoculation. Among the dogs from which serum samples assessed in the present study were obtained, preinoculation testing for TBPs was not described for dogs experimentally infected with *A phagocytophilum*,¹⁰ *A platys*,³¹ *E canis*,³¹ and *E chaffeensis*.³³ Although the dogs experimentally infected with *A phagocytophilum*¹⁰ and *E chaffeensis*³³ were described as specific pathogen-free dogs (and there was a reasonable expectation that those dogs were indeed free of TBPs), kennels rearing specific pathogen-free dogs do not routinely administer acaricides to prevent TBP infections.^{10,33} The *E ewingii*-infected dogs were

screened for TBPs prior to experimental inoculation and were negative for *A phagocytophilum*, *E canis*, *E chaffeensis*, and *E ewingii* on the basis of PCR assay results and seronegative for antibodies against *A phagocytophilum*, *E canis*, and *E chaffeensis* on the basis IFA results.³² However, it was subsequently determined that 2 of the 3 *E ewingii*-infected dogs were inoculated with blood from a blood donor dog that was coinfecting with *E ewingii* and *A platys*, which likely accounted for those dogs yielding positive results on the *A platys* peptide-based ELISA. Furthermore, the *E ewingii*-infected dogs were housed in kennels in Missouri and may have been exposed to ticks carrying *A platys* or other TBPs prior to experimental inoculation. Thus, dog origin, history of acaricide administration, inoculum source, and rigorousness of TBP screening prior to inoculation are important considerations for experimental TBP infection studies.

Results of the present study indicated that, for dogs experimentally infected with a TBP, the specificity of TBP species-specific peptide-based ELISAs was superior to that of whole organism-based IFAs. Findings also indicated that the species-specific ELISAs for *A platys* and *E ewingii* (ie, TBPs for which whole organism-based IFAs are not currently available) could be useful for identification of dogs that are seropositive for antibodies against those organisms. Peptide-based ELISAs are useful for the detection of antibodies against *Anaplasma* and *Ehrlichia* spp, which should facilitate accurate diagnosis and may help detect dogs coinfecting with multiple TBPs.

Acknowledgments

Supported by Idexx Laboratories Inc, Westbrook, Me. Drs. Stillman, Beall, and Chandrashekar and Ms. Foster are employees of Idexx Laboratories Inc. Dr. Qurollo is codirector of the Vector Borne Disease Diagnostic Laboratory within the Department of Clinical Sciences, College of Veterinary Medicine, North Carolina State University, a position that receives salary support from Idexx Laboratories Inc. Dr. Breitschwerdt is codirector of the Vector Borne Disease Diagnostic Laboratory and the Intracellular Pathogens Research Laboratory within the Department of Clinical Sciences, College of Veterinary Medicine, North Carolina State University; chief scientific officer at Galaxy Diagnostics, Research Triangle, NC; and a paid consultant for Idexx Laboratories Inc.

Presented, in part, as a research abstract at the American College of Veterinary Internal Medicine Forum in Anaheim, Calif, June 2010.

The authors thank Kathleen Newcomb, Blythewood Consulting LLC, Nathalie, Va, for editorial support during manuscript preparation and James B. Robertson, College of Veterinary Medicine, North Carolina State University, for assistance with data analysis.

Footnotes

- a. VWR, Radnor, Penn.
- b. AnaSpec, Fremont, Calif.
- c. New England Peptide, Gardner, Mass.
- d. Fuller Laboratories, Fullerton, Calif.
- e. VassarStats, Vassar College, Poughkeepsie, NY. Available at: vassarstats.net. Accessed Aug 26, 2020.
- f. SNAP 4Dx Plus Test, Idexx Laboratories Inc, Westbrook, Me.

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