Evaluation of peptide- and recombinant protein–based assays for detection of anti–Ehrlichia ewingii antibodies in experimentally infected dogs

Thomas P. O’Connor, PhD; Jill M. Saucier, BS; Daryn Daniluk, BS; Brett A. Stillman, PhD; Regis Krah, PhD; Yasuko Rikihisa, PhD; Qingming Xiong, PhD; Michael J. Yabsley, PhD; Dustin S. Adams, DVM; Pedro Paulo V. P. Diniz, DVM, PhD; Edward B. Breitschwerdt, DVM; Stephen D. Gaunt, DVM, PhD; Ramaswamy Chandrashekar, PhD

Objective—To evaluate microtiter-plate format ELISAs constructed by use of different diagnostic targets derived from the Ehrlichia ewingii p28 outer membrane protein for detection of E ewingii antibodies in experimentally and naturally infected dogs.

Sample Population—Serum samples from 87 kenneled dogs, 9 dogs experimentally infected with anti–E ewingii, and 180 potentially naturally exposed dogs from Missouri.

Procedures—The capacities of the synthetic peptide and truncated recombinant protein to function as detection reagents in ELISAs were compared by use of PCR assay, western blot analysis, and a full-length recombinant protein ELISA. Diagnostic targets included an E ewingii synthetic peptide (EESP) and 2 recombinant proteins: a full-length E ewingii outer membrane protein (EEp28) and a truncated E ewingii outer membrane protein (EETp28).

Results—A subset of Ehrlichia canis–positive samples cross-reacted in the EEp28 ELISA; none were reactive in the EESP and EETp28 ELISAs. The EESP- and EETp28-based ELISAs detected E ewingii seroconversion at approximately the same time after infection as the EEp28 ELISAs. In a field population, each of the ELISAs identified the same 35 samples as reactive and 27 samples as nonreactive. Anaplasma and E canis peptides used in a commercially available ELISA platform did not detect anti–E ewingii antibodies in experimentally infected dogs.

Conclusions and Clinical Relevance—The EESP and EETp28 ELISAs were suitable for specifically detecting anti–E ewingii antibodies in experimentally and naturally infected dogs. (Am J Vet Res 2010;71:1195–1200)

Canine granulocytic ehrlichiosis, caused by Ehrlichia ewingii, is characterized by fever, lethargy, anorexia, weight loss, vomiting, diarrhea, lameness, neutrophilic polyarthritis, severe but transient thrombocytopenia, and transient mild nonregenerative anemia with ineffective erythropoiesis.1–3 Ehrlichia ewingii infection has been reported in dogs in several states, including Missouri, Oklahoma, North Carolina, and Virginia.4–6 Amblyomma americanum, which is distributed throughout the southeastern and south central United States, is the only confirmed vector for the transmission of E ewingii.5,6 Serologic tests such as the IFA have been used diagnostically for detection of antibodies against several ehrlichial pathogens. However, because E ewingii has not been cultured in vitro, a sensitive and specific serologic test to detect E ewingii antibody in dogs is not presently available. Ehrlichia chaffeensis and Ehrlichia
canis whole cell organisms have been used as surrogate antigen targets in IFAs; however, these assays have variable specificity and lack standardization among IFA tests performed in different laboratories. Although microscopic diagnosis of *E. ewingii* infection can be made tentatively following identification of morulae in circulating neutrophils in Giemsa-stained blood smears, this method is technologically difficult, is not widely used in a clinical setting because it has poor sensitivity, and does not distinguish between *E. ewingii*, *Anaplasma phagocytophilum*, or other organisms that might form morulae in granulocytes. Polymerase chain reaction assays can be used to distinguish *E. ewingii*, *E. canis*, and *A. phagocytophilum* but have not been widely used in a clinical setting because of the lack of specialized instruments, expertise needed to perform and interpret results, and lack of assay performance data obtained from dogs in a clinical setting. All present options for detection of *E. ewingii*–specific antibody have deficiencies, and thus a sensitive and specific method is needed.

*Ehrlichia ewingii* peptides and recombinant proteins are ideally suited for use in place of culture-derived organisms to develop sensitive and specific assays for *E. ewingii* antibody. The *E. ewingii* p28 gene was initially identified by sequencing a partial *E. ewingii* gene, which had sequence homology with the OMP genes from *E. canis* p30, *E. chaffeensis* p28, and *Ehrlichia ruminantium* MAP1. Subsequently, in a systematic study, the entire OMP locus for *E. ewingii* was sequenced and, consistent with OMPs of other *Ehrlichia* spp, was found to contain multiple OMP paralogs (n = 19). Synthetic peptides were made to reproduce predicted antigenic regions of extracellular loops for each of the 19 paralogs to identify species-specific and paralog-specific antigens. By use of screening serum samples obtained from 3 dogs experimentally infected with *E. ewingii* and 3 dogs experimentally infected with *E. canis*, several peptides were identified as candidate antigens for use in assays to specifically identify and distinguish *E. ewingii* infection from infection with *E. chaffeensis* or *E. canis*.

The purpose of the study reported here was to evaluate microtiter-plate–format ELISAs constructed by use of various diagnostic targets derived from the *E. ewingii* p28 OMP for detection of *E. ewingii* antibodies in experimentally and naturally infected dogs.

### Materials and Methods

**Canine field samples**—Two sets of field samples were obtained from dogs housed in a Missouri kennel. The dogs originated from Missouri and neighboring states (Oklahoma, Texas, Kansas, and Iowa), were primarily outdoor dogs, and were supplied without knowledge of previous tick exposure. The primary vector for *E. ewingii* (*A. americanum*) is endemic in Missouri, and *E. ewingii* is the primary etiologic agent of canine ehrlichiosis throughout Missouri. An initial set of 87 samples, obtained between October 2004 and July 2005, was used in experiments to determine and compare the sensitivity and specificity of the *E. ewingii* assays. The second set of 180 samples was obtained between November 2005 and July 2006 and was used to assess the prevalence of *E. ewingii* infection in dogs from this region. A subset of 105 samples from the second group was tested by use of PCR assay for *E. ewingii* DNA.

The *E. canis* seropositive samples were from 27 dogs from the *E. canis*–endemic northeastern region of Arizona. In a previous study, use of PCR assay detected *E. canis* DNA in blood from 35% (n = 233) of dogs sampled from this area; none of these were PCR-assay positive for *E. chaffeensis* or *E. ewingii* DNA. *Rhipicephalus sanguineus*, the natural vector for *E. canis*, was the only species identified among 442 ticks found attached to dogs from this area.

**E. canis** experimental infection samples—Six female hound-type dogs (6 months old) were inoculated IV with a single Louisiana isolate of *E. canis*–infected canine histiocytic cell inoculum that had been stored at −80°C and thawed immediately before use. The study was conducted at Louisiana State University under compliance of Institutional Animal Care and Use Committee–approved protocol No. 06-054. Serum samples were obtained from all dogs prior to infection and at 3- to 4-day intervals up to 35 days after infection. Serum samples were frozen at −20°C until use.

**E. ewingii** experimental infection samples—Two experimental infection studies were conducted to generate samples. In the first study, specific-pathogen–free Beagles were used that initially had negative results via PCR assay for *E. chaffeensis*, *E. canis*, *E. ewingii*, and *A. phagocytophilum* and via IFA by use of *E. chaffeensis*, *E. canis*, and *A. phagocytophilum* as antigens. Three dogs were inoculated IV 2 or 3 times with 10 to 15 mL of blood or 5 mL of buffy coat from *E. ewingii* PCR-assay–positive (*E. chaffeensis*–, *E. canis*–, and *A. phagocytophilum*–PCR-assay–negative) dogs. The dogs were administered cyclophosphamide (10 mg/kg) by IV injection either 1 or 2 times. Dogs became *E. ewingii*–PCR-assay positive between 14 and 28 days after the final inoculation. Clinical signs associated with *E. ewingii* infection were not evident in these dogs. Severe thrombocytopenia after the second and third injections with *E. ewingii*–positive blood in 2 dogs may have been caused by cyclophosphamide treatment.

In the second experimental infection study, 6 young (< 1-year-old) mixed-breed dogs and Beagles were infected with *E. ewingii* by various routes (IV with *E. ewingii*–infected blood or with whole blood cell fractions or by exposure to experimentally infected ticks). Dogs were fully vaccinated and dewormed and were kept in a climate-controlled, tick-free facility for the duration of the study. Prior to inoculation, the dogs had negative results of PCR assays for *E. chaffeensis*, *E. canis*, *E. ewingii*, *A. phagocytophilum*, and *Borrelia* spp, for prior exposure by screening serum samples for anti–*E. ewingii* antibodies by use of the EESP ELISA, and for antibodies reactive to *E. chaffeensis*, *A. phagocytophilum*, and *Borrelia* spp by use of IFA tests. Blood from each of the dogs became *E. ewingii*–PCR-assay positive between 4 and 28 days after infection. Sequential blood samples from 4 dogs were tested by use of the EESP ELISA and had reactive antibodies between 28 and 46 days after infection. Three dogs had intermittent fever (maximum temperature, 40.2°C), thrombocytopenia, and leucopen-
nia and were hyperphosphatemic or had high serum alkaline phosphatase activity. The only abnormality found in the dog infected by exposure to ticks was a slight thrombocytopenia 35 days after infection.

The first study was conducted at The Ohio State University (Institutional Animal Care and Use Committee No. 99A0120), and the second study was conducted at the University of Georgia (Institutional Animal Care and Use Committee No. A2006 6-111). Serum samples from both studies were frozen at −20°C until use.

Expression and purification of recombinant E ewingii proteins—The EEp28 was made by use of a synthetic gene encoding the full-length p28 of E ewingii OMP-1-16 (GenBank accession No. EF116932.1).\(^{13}\) The EETp28 was made by expressing a synthetic gene product encoding the amino acid sequence of the EESP and a second region (14 amino acids) of the OMP-1-16 paralog containing an E ewingii–specific region joined by use of the linker sequence KS5KS5. The EEp28 and the EETp28 genes were synthesized with codons optimized for expression in E coli and were subcloned into the pET28a′ vector.\(^{10,17}\) The protein was expressed as an inclusion body in E coli strain BL21(DE3) and induced by use of isopropyl-\(\beta\)-thiogalactopyranoside (1 mM). Cells were collected via centrifugation and frozen at −70°C.

The cell pellet was subjected to successive rounds of resuspension, sonication, and centrifugation by use of buffers containing increasing concentrations of urea (2M urea and 6M urea, respectively). The solubilized recombinant material was purified via column chromatography by use of standard methods.\(^1\) Fractions were analyzed and pooled by use of SDS-PAGE.

Cell pellets containing the EEp28 were lysed and treated as described. The EETp28 derivative was soluble in urea-free lysis buffer and was purified by use of column chromatography as described, with minor modifications.

EESP—The EESP was identified by aligning the sequence of an immunodominant region of the E canis p28 protein with the partial sequence of the E ewingii p28 protein (GenBank accession No. AF287961) and the full-length OMP-1-16 (GenBank accession No. EF116932.1).\(^{12,13,17}\) The synthetic 21-mer peptide, which contained an N-terminal cysteine (C) used for conjugation chemistry, was obtained from a commercial vendor.\(^5\)

EEp28, EETp28, and EESP ELISAs—Microtiter plates\(^4\) were coated with 0.5 \(\mu\)g/mL (100 \(\mu\)L/well) of the EEp28, EETp28, or EESP in 0.05M sodium carbonate buffer (pH, 9.6), and washed and blocked by use of a Tris-buffered solution (pH, 7.6) containing Tween 20 and sucrose. Diluted samples were incubated in wells for 30 minutes, wells were washed, and horseradish peroxidase–labeled anti-canine antibody\(^4\) was added to each well and incubated (30 minutes). Plates were washed, 3,3′,5,5′ teteramethylbenzidine substrate solution was added, and optical density was determined at 650 nm; reactive samples were denoted by any absorbance value > 2 times the negative control value.

PCR assay—At The Ohio State University, PCR assay testing was carried out for the dogs from the first study that were experimentally infected with E ewingii. Total DNA was extracted from the blood specimens by use of standard methods.\(^1\) Specimens were screened for E ewingii, E chaffeensis, E canis, and A phagocytophilum infection by use of nested PCR assay to detect 16S rRNA gene, as described.\(^2,16-21\)

The PCR assay testing for E ewingii DNA in blood samples was amplified by use of 16S rRNA oligonucleotide primers that were designed to specifically amplify a 395-bp fragment of E ewingii DNA.\(^2\) The E canis PCR assay was performed as described.\(^2\)

E canis p16 and E chaffeensis p120 peptide ELISAs—Samples were tested by separate microtiter-plate–format assays by use of a synthetic peptide from the E canis p16 protein (also referred to as gp19)\(^2,12,24\) and a synthetic peptide from the tandem repeat sequence of the E chaffeensis p120 protein.\(^2,25-27\) Peptides, which contained an N-terminal cysteine (C) for conjugation, were synthesized\(^2\) and coated directly on microtiter plates\(^2\) at a concentration of 0.5 to 1 \(\mu\)g/mL in 0.05M sodium carbonate (pH, 9.6); plates were washed, blocked with 1% bovine serum albumin, and dried. Peptides were conjugated to horseradish peroxidase by use of standard methods.\(^28\) Test sample (30 \(\mu\)L) and 50 \(\mu\)L of peptide: horseradish peroxidase conjugate (1 \(\mu\)g/mL) in a conjugate diluent containing nonspecific protein and detergent were added to coated wells and incubated for 60 minutes. Microtiter wells were aspirated, and the aspirate was washed 6 times and incubated with a substrate solution containing 3,3′,5,5′ teteramethylbenzidine; optical density values were determined at 650 nm.

Commercially available ELISA—An ELISA was used for the simultaneous detection of canine heartworm antigen and antibodies against E canis, Borrelia burgdorferi, and A phagocytophilum in canine serum, plasma, or blood.\(^2,21\) The samples were tested per the manufacturer’s instructions.

IFA and western blot analysis—Indirect IFA was performed as described by use of E chaffeensis Arkansas–infected DH82 cells and E canis Oklahoma–infected DH82 cells.\(^20\) Western blot analysis was performed by use of purified E chaffeensis organisms as described.\(^27\)

Results

Detection of antibody in dogs experimentally infected with E ewingii—Temporal samples from 3 dogs experimentally infected with E ewingii were tested by use of PCR assay, western blot analysis, and microtiter-plate–format ELISAs made by use of the EEp28, EETp28, and EESP constructs. Sera from
dogs experimentally infected with *E. ewingii* reacted with higher–molecular-weight *E. chaffeensis* antigens in the western blot assay by 63 days after infection but reacted poorly with the 28-kDa antigen that typically reacts strongly with *E. chaffeensis* antisera. Temporal serum samples were reactive in microtiter-plate assays made by use of each of the 3 *E. ewingii* constructs between 28 and 35 days after infection and remained seroreactive for the 200-day duration of the study (Figure 1).

Cross-reaction of *E. canis*–positive samples with EESP and recombinant proteins—In an effort to determine whether *E. canis* antibody–positive samples would cross-react with the 3 *E. ewingii* constructs, sera from 6 dogs experimentally infected with *E. canis* and 27 seroreactive field dogs naturally infected with *E. canis* were tested by use of ELISAs made with the EEp28, EETp28, and EESP antigens. Sera from the 6 dogs experimentally infected with *E. canis* were reactive in the p16 ELISA for *E. canis* antibody between 13 and 17 days after infection. All were nonreactive at all time points in the EESP and EETP ELISAs, whereas sera from 4 of the 6 dogs were reactive at 1 or more time points when tested by use of the full-length EEp28 ELISA. The ELISA results obtained with samples from *E. canis*–seroreactive field dogs were tabulated. Each of the samples was reactive to the *E. canis* p16 antigen, all were nonreactive in the EESP and EETp28 ELISAs, and 11 of 27 (40.7%) samples were reactive in the EEp28 ELISA.

Comparison of results for the EESP, EETp28, and the EEp28 ELISAs—In an effort to examine a population of field dogs that could be used to directly compare the relative reactivity of the 3 *E. ewingii* p28 constructs by use of ELISA, samples from the initial group of 87 Missouri kennel dogs were tested for the presence of antibodies against *E. canis* and the related *Ehrlichia* organism, *E. chaffeensis*. Twenty-five samples were reactive in the *E. canis* or *E. chaffeensis* ELISAs and were excluded from the population. Sera from 62 dogs with no evidence of *E. canis* or *E. chaffeensis* infection were tested by use of the EESP, EETp28, and EEp28 ELISAs and were compared by plotting of optical density readings for the ELISAs (Figure 2). The signals produced in the 3 assays were similar, and slopes-of-best-fit trend lines and correlation coefficients ($R^2$ values) were 1.07 and 0.93, respectively, for the EESP-EEp28 comparison and 0.73 and 0.92, respectively, for the EETp28-EEp28 comparison. All 62 samples had identical results (reactive or nonreactive) in each of the 3 assays.

Results of *E. ewingii* PCR assay and comparison with EESP ELISA—A second set of 180 samples obtained from the Missouri kennel was tested by use of the EESP ELISA, and a subset of these was tested by use of the *E. ewingii* PCR assay. Eighty-two of 180 (45.6%) dogs had antibodies reactive against the *E. ewingii* p28 peptide. Twenty-one of 81 (25.9%) ELISA-reactive samples that were available for PCR testing were *E. ewingii*–PCR-assay positive. All 24 EESP ELISA negative samples that were tested by use of the *E. ewingii* PCR assay yielded negative results (Table 1).

Use of the commercial ELISA with dogs experimentally infected with *E. ewingii*—Samples from each of the dogs from the second *E. ewingii* experimental infection study reacted in the EESP ELISA and were *E. ewingii*–PCR-assay positive. None of the samples reacted

<table>
<thead>
<tr>
<th><em>E. ewingii</em> PCR</th>
<th>Pos</th>
<th>Neg</th>
</tr>
</thead>
<tbody>
<tr>
<td>EESP ELISA</td>
<td>21</td>
<td>60</td>
</tr>
<tr>
<td>Neg</td>
<td>0</td>
<td>24</td>
</tr>
</tbody>
</table>

Table 1—Comparison of positive (pos) and negative (neg) results for an EESP ELISA and an *Ehrlichia ewingii* PCR assay in 105 dogs.
with the E canis analyte on the commercial ELISA, indicating a lack of cross-reactivity between E ewingii antibodies and the E canis peptides used in the test. In contrast to the E canis peptide test, sera from dogs experimentally infected with E canis were reactive for E canis antibody by use of the commercial ELISA (Table 2).

### Discussion

The EESP, EETp28, and EEp28 were used to detect antibodies in naturally and experimentally infected dogs. In an effort to determine species specificity, the E ewingii paralogs were compared with sera from E canis seroreactive dogs. By use of sera from dogs experimentally infected with E canis it was found that, in contrast to the EEp28, which cross-reacted with sera from 3 of 5 dogs, both the EESP and the EETp28 were nonreactive to sera from all 5 dogs at all time points. Cross-reaction to the EEp28, but not the EESP or EETp28, was found by use of ELISA for 11 of 27 known E canis–positive field samples. All E canis–seroreactive samples tested were nonreactive in the EESP and EETp28 ELISAs, and a subset was reactive in the EEp28 ELISA. To eliminate this differential reaction in experiments designed to directly compare the relative reactivity of field dogs with the E ewingii constructs, we excluded any dogs that were E canis or E chaffeensis seroreactive. It was necessary to exclude E chaffeensis–reactive dogs because of the homology of E chaffeensis OMPs with E canis and E ewingii.13,30–32

In ELISAs constructed by use of the EESP, EETp28, and EEp28 antigen targets, there was little difference in relative reactivity when temporal samples were tested from dogs experimentally infected with E ewingii. The relative assay signal produced in the EESP- and EETp28-based ELISAs was similar to the EEp28 ELISA when tested with 62 sera from field dogs with no evidence of exposure to E canis and E chaffeensis. Each of the 3 ELISAs identified the same 35 reactive and 27 nonreactive samples and produced signals of similar intensity for individual samples. Importantly, there were no samples that were reactive in the EEp28 ELISA that were not also reactive in both the EESP and EETp28 ELISAs, suggesting that these 2 constructs could be used in place of the full-length protein, which would substantially increase test specificity.

The second group of kennel dogs was used to estimate the prevalence of E ewingii seroreactivity by use of ELISA and to compare these results with results of PCR assay in a smaller set of dogs within this group. Eighty-two of 180 (45.6%) samples were reactive in the EESP ELISA, supporting previous results that suggested that E ewingii is the primary agent of canine ehrlichiosis in this region.2 The percentage of EESP ELISA-reactive samples that were positive when tested by use of the E ewingii PCR assay was 25.9% (21/81).

Although results of several experiments indicate that antibody is produced against multiple paralogs during Ehrlichia infection, it remains unclear whether detection of antibody to a single E ewingii paralog is sufficient for use as a sensitive assay for serodiagnosis of infection resulting from diverse strains of E ewingii.13,30–32 By use of 19 paralog-specific synthetic peptides, all 19 E ewingii paralogs were reactive with sera from 3 dogs experimentally infected with E ewingii.13 Similarly, sera from E chaffeensis–infected dogs were reactive with all 22 E chaffeensis paralogs, suggesting that the p28 paralogs are expressed concurrently during infection.13 The data presented in the present report indicated that the ELISA used for detection of antibody against EESP provides a sensitive assay for detection of antibody against the OMP 1–16 paralog. Additional studies are needed with dogs from widely separated locations to determine whether the sensitivity of the assay can be improved by incorporation of synthetic peptides from additional OMPs.

### References


6. Epoch Biologs Inc, Missouri City, Tex.

7. EMD, Novagen Brand, Madison, Wis.


9. Anaspec, San Jose, Calif.


12. QIAamp Blood Kit, Qiagen, Valencia, Calif.

13. Vector-Borne Disease Diagnostic Laboratory, College of Veterinary Medicine, North Carolina State University, Raleigh, NC.

14. SNAP 4Dx, IDEXX Laboratories Inc, Westbrook, Me.

---

<table>
<thead>
<tr>
<th>Sample acquisition</th>
<th>E. canis (commercial ELISA)</th>
<th>EESP (PCR assay)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before infection</td>
<td>0/18</td>
<td>0/18</td>
</tr>
<tr>
<td>After infection</td>
<td>0/18</td>
<td>0/18</td>
</tr>
<tr>
<td>After infection</td>
<td>0/12</td>
<td>0/12</td>
</tr>
</tbody>
</table>

Table 2—Assay results (number positive/number tested) for samples from 18 dogs with Ehrlichia canis or E ewingii experimental infection tested by use of the commercial ELISA for E canis antibody, an EESP ELISA, and an E ewingii PCR assay.


