Reactivity of serum samples of dogs and horses tested by use of class-specific recombinant-based enzyme-linked immunosorbent assays for detection of granulocytic ehrlichiosis

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Objective—To test serum samples of dogs and horses by use of class-specific recombinant-based ELISA for establishing a diagnosis of granulocytic ehrlichiosis attributable to infection with organisms from the *Ehrlichia phagocytophila* genogroup.

Sample Population—Serum samples from 43 client-owned dogs and 131 horses (81 with signs of acute illness and 50 without signs of disease).

Procedure—Serum samples were analyzed, using ELISA with a recombinant 44-kd protein antigen for IgM and IgG antibodies to the human granulocytic ehrlichiosis (HGE) agent (NCH-1 strain). Western blot analyses, using infected human promyelocytic leukemia cells, were conducted on 38 serum samples of horses and 11 serum samples of dogs to verify reactivity to the 44-kd peptide.

Results—IgM or IgG antibodies to the HGE agent were detected in 5 to 28% of dog serum samples and 5 to 37% of horse serum samples. Thirty-five of 38 (92%) horse serum samples had corresponding results on both tests (2 positive results for 26 samples and 2 negative results for 9 samples), using an ELISA for IgG antibodies or immunoblotting for total immunoglobulins. All 11 serum samples of dogs had positive results for both methods.

Conclusion and Clinical Relevance—These ELISA with recombinant 44-kd antigen are suitable for detecting IgM or IgG antibodies to the HGE agent in serum samples of dogs and horses. Positive results for serum samples of horses from Connecticut, New York, Virginia, and Georgia indicate that the HGE agent is widely distributed in tick-infested areas of the eastern United States.

Closely related bacteria in the *Ehrlichia phagocytophila* genogroup cause granulocytic ehrlichiosis in humans, dogs, horses, and other domesticated mammals in North America and Europe.19 This disease is prevalent in areas where *Ixodes pacificus*, *I. ricinus*, or *I. scapularis* ticks are abundant. In humans, leukopenia and thrombocytopenia are commonly reported clinical features.2,10 Fever, lethargy, and anorexia are common components of illness in horses and dogs.5,9

Laboratory diagnosis of infections caused by *E phagocytophila* genogroup organisms, such as *E. equi*, *E. phagocytophila*, and the human granulocytic ehrlichiosis (HGE) agent, can be accomplished by isolating the pathogen, observing morulae in stained neutrophils, or detecting DNA of the agent in blood. However, serologic testing for antibodies is relied on most commonly for routine analyses. Use of a purified recombinant 44-kd protein antigen of the NCH-1 strain of the HGE agent in polyvalent ELISA is suitable for detecting serum antibodies in people with HGE.11 The automated and easily standardized ELISA are preferred over indirect fluorescent antibody (IFA) methods and are more practical than immunoblotting procedures when a large number of serum samples must be tested. Detection of IgM antibodies is particularly helpful in identifying recent infections. The purposes of the study reported here were to develop class-specific ELISA to measure concentrations of IgM and IgG antibodies to *E. phagocytophila* genogroup organisms in serum samples obtained from dogs and horses and to compare results of ELISA with those of other antibody tests and DNA analysis.

Materials and Methods

Serum samples—Serum samples from dogs and horses that had been tested in previous studies9,12-14 were reanalyzed by use of a polyvalent ELISA and screened by use of newly developed class-specific ELISA. Samples had been obtained during a passive surveillance program conducted by veterinarians from 1985 through 1996 in Connecticut and the lower Hudson River Valley region of New York. Twenty-seven serum samples of horses from Assateague Island, Va, and 23 serum samples of horses from Cumberland Island, Ga, that had been obtained from 1986 through 1989 were included in the study.

*Ixodes scapularis* ticks are abundant in each of these locations.

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All serum samples were stored at –60 C at the Connecticut Agricultural Experiment Station; to prevent decreases in antibody concentrations, they had been promptly returned to these conditions after being thawed for the previous analyses.

To evaluate class-specific ELISA and conduct comparative studies, it was important to include serum samples of dogs and horses that had positive results when analyzed by other methods to document granulocytic ehrlichial antibodies or DNA of the etiologic agent. Data were available for 43 serum samples from dogs and 81 serum samples from horses that had been tested in other studies11–14 by use of IFA staining methods, immunoblotting procedures, or polyvalent ELISA. Most serum samples were reanalyzed by use of polyvalent ELISA. Results of western blot analyses (using whole-cell antigen) and polyvalent ELISA (using a recombinant 44-kd antigen) verified antibody reactivity to a 44-kd protein of the NCH-1 strain of the HGE agent.12,14 This antigen, common among North American strains of E. equi or the HGE agent, is a sensitive and specific marker for infections attributable to E. phagocytophila genogroup organisms.11,12,14 In addition, DNA of granulocytic ehrlichiae was detected, as detection of DNA was considered a positive result.11,13 In blood samples from 20–100% of clinically ill horses, using polymerase chain reaction (PCR) techniques. Three serum samples from dogs with antibodies to E. canis and 12 serum samples from horses with antibodies to E. risticii, all of which were used in a previous study,13 were screened by use of class-specific ELISA to assess specificity.

Class-specific ELISA—Serum samples obtained from dogs and horses were analyzed for IgM and IgG antibodies by use of an ELISA, which was modified from a polyvalent ELISA11–13 and contained the 44-kd recombinant antigen (also known as the HGE-44 or p44 antigen) of the NCH-1 strain of the HGE agent. In comparative tests of various isolates (ie, BDS, MRK, RCH, NCH-1, and Webster strains) with human serum samples,7 prevalence of seropositive reactions did not differ significantly when tested by use of IFA staining methods. The production and use of the 44-kd recombinant antigen, expressed and purified as a maltose-binding protein (MBP) fusion peptide, has been described for polyvalent ELISA.11,13 The MBP replaced the glutathione transferase fusion partner, commonly used in recombinant antigens of Borrelia burgdorferi, to improve solubility and yield after purification.

Positive- and negative-control samples that had been used in previous studies11–13 were included in class-specific ELISA to determine optimal antigen concentrations and to calculate net absorbance values that were used to define critical regions for positive results. An antigen concentration of 2.5 μg of protein per milliliter was selected for detecting antibodies in positive control samples for a dog and a horse. Twelve serum samples from dogs and 24 serum samples from horses, all of which had negative results, were diluted in PBS solution (pH, 7.2) to achieve dilutions of 1:1,600, 1:320, and ≥ 1:640 and were tested by use of the p44 antigen and conjugated antibodies to determine cut-off values for positive results. Secondary antibodies used to detect IgM antibodies consisted of affinity-purified horseradish peroxidase-labeled goat anti-dog or goat anti-horse immunoglobulins (μ-chain specific) diluted 1:2,000 or 1:2,500, respectively, in PBS solution. Conjugates used for detection of IgG antibodies were affinity-purified peroxidase-labeled goat anti-dog or goat anti-horse immunoglobulins (γ-chain specific) diluted 1:12,000 or 1:2,000, respectively, in PBS solution. For color production, 60 μl of commercially prepared 2, 2-azino-di-3-(ethylbenzthiazoline sulfonate) was added to each well. Absorbance value (ie, optical density [OD]) of each serum dilution was recorded at 414 nm, using a microplate spectrophotometer.

Net absorbance value is the difference in OD between positive-antigen and negative-control (PBS solution) preparations for each serum dilution. A mean + 3 SD of net OD values for each set of negative-control serum samples was used to define critical regions. For dogs, net OD values of 0.12, 0.05, and 0.04 were considered positive for IgM antibodies in serum samples diluted to 1:160, 1:320, and ≥ 1:640, respectively, whereas a net OD value of 0.03 was considered positive for IgG antibodies in all dilutions of serum samples obtained from dogs and horses. Critical regions for IgM antibodies in horses were 0.06 and 0.03 for serum samples diluted to 1:160 and ≥ 1:320, respectively. There was weak background reactivity in PBS solution and MBP control wells in analyses of both sets of negative control serum samples; for horses, net OD values for serum samples diluted 1:160 were ≤ 0.04 in IgM antibody analyses. All polystyrene plates contained the same positive- and negative-control serum samples and wells to evaluate conjugated antibodies, PBS solution, MBP, and substrate. Titration endpoints were determined for all positive serum samples. In addition, 5 positive and 5 negative serum samples from dogs and horses were reanalyzed to assess reproducibility of test results.

Statistical analyses—Percentages of positive results were compared, using a z-test in a statistical software program.7 The Yates correction was applied to all calculations as a part of this program. Values of P < 0.05 were considered significant.

Results

Antibodies to the 44-kd recombinant antigen were detected in serum samples of dogs and horses from Connecticut and serum samples of horses from Virginia, Georgia, and New York. Detection of total immunoglobulins and class-specific IgG antibodies by use of ELISA was more frequent than identification of serum IgM antibodies in all categories (Table 1). In general, seropositivity rates for total immunoglobulins and IgG antibodies, as determined by results of ELISA, compared favorably with results of IFA staining methods. Sensitivities of the ELISA for polyvalent and class-specific IgG antibody results that had been recorded for serum samples obtained from horses in 1995 and 1996 paralleled those recorded when IFA staining methods were used; values ranged from 67 to 80% and did not differ significantly (z = 0.541; P = 0.589). Seropositivity rates for total antibodies in samples of horses from Connecticut and New York were at least 5-fold greater than those calculated for samples of horses from Georgia and Virginia. Percentages of positive serum samples of horses from Connecticut (75%; 15 of 20) and Georgia (9%; 2 of 23) differed significantly (z = 4.100; P < 0.001). When results of ELISA were compared for all groups of horses, sensitivity values were highest in tests for class-specific IgG antibodies.

Analyses for detection of DNA or antibodies to the HGE agent in horses from Connecticut and New York revealed that positive samples were obtained in all seasons (Table 2). In many instances, results of DNA analyses agreed with those of antibody detection determined by use of immunoblotting methods and ELISA for corresponding paired blood and serum samples. Of 38 paired samples, 20 (53%) and 31 (82%) contained DNA or antibodies, respectively, of the HGE agent. Results of PCR and antibody testing methods were positive for both testing methods in 19 paired samples and were negative for both testing methods in 7 other corresponding samples. Eleven additional paired blood and serum samples were negative by PCR methods but...
Table 1—Results of serologic testing for antibodies to whole-cell or recombinant p44 antigens of the NCH-1 strain of the human granulocytic ehrlichiosis (HGE) agent in serum samples obtained from dogs or horses in the eastern United States

<table>
<thead>
<tr>
<th>Animals</th>
<th>Serum samples</th>
<th>No. of samples tested</th>
<th>IFA No. (%) positive*</th>
<th>ELISA No. (%) positive* for antibodies</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Total Ig IgM IgG</td>
</tr>
<tr>
<td>Horses</td>
<td>1985 Connecticut</td>
<td>43</td>
<td>3 (7)</td>
<td>3 (7) 0 4 (9)</td>
</tr>
<tr>
<td></td>
<td>1986–1988 Georgia</td>
<td>23</td>
<td>1 (4)</td>
<td>2 (9) 0 4 (17)</td>
</tr>
<tr>
<td></td>
<td>1988–1989 Virginia</td>
<td>27</td>
<td>2 (7)</td>
<td>4 (15) 0 12 (44)</td>
</tr>
<tr>
<td></td>
<td>1995–1996 Connecticut</td>
<td>20</td>
<td>16 (60)</td>
<td>15 (75) 3 (15) 16 (60)</td>
</tr>
<tr>
<td></td>
<td>1995–1996 New York</td>
<td>18</td>
<td>14 (78)</td>
<td>12 (67) 4 (22) 13 (72)</td>
</tr>
</tbody>
</table>

*Positive results for total or class-specific antibodies by use of ELISA with recombinant p44 antigen or for total antibodies by indirect fluorescent antibody (IFA) staining methods with whole cells of the NCH-1 strain of the HGE agent.

Table 2—Detection of HGE DNA and antibodies to the p44 recombinant antigen of the HGE agent in corresponding blood and serum samples obtained from ill horses in Connecticut and New York during 1995 and 1996

<table>
<thead>
<tr>
<th>Sample collection period</th>
<th>No. of paired blood and sera tested</th>
<th>No. positive* for DNA</th>
<th>Antibodies</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Total Ig IgM IgG</td>
</tr>
<tr>
<td>1995</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nov</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Dec</td>
<td>9</td>
<td>6</td>
<td>7</td>
</tr>
<tr>
<td>1996</td>
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<tr>
<td>Jan</td>
<td>9</td>
<td>6</td>
<td>8</td>
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<td>Jun</td>
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<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Jul</td>
<td>5</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>Total</td>
<td>38</td>
<td>20</td>
<td>27</td>
</tr>
</tbody>
</table>

*DNA of the HGE agent was detected in blood samples by use of polymerase chain reaction techniques. Antibodies were detected by use of immunoblotting.

Results of class-specific ELISA and polyvalent western blot analysis were compared for the 38 serum samples obtained from horses in Connecticut and New York during 1995 and 1996 and for 11 serum samples obtained from dogs in Connecticut. The ELISA contained the 44-kd recombinant antigen, whereas the blots had lysates of human promyelocytic leukemia cells (HL-60 cells) infected with the NCH-1 strain of the HGE agent. Thirty-nine (92%) of the serum samples had positive (n = 26) or negative (9) results for both assays for IgG antibodies. The other 3 serum samples were weakly positive by use of immunoblotting methods to p44 but were negative by use of an ELISA. In analyses for class-specific IgM antibodies in horses, 26 of 38 (68%) serum samples had positive results when tested by both methods, whereas 7 (18%) serum samples had negative results for each assay. Three additional serum samples had positive results when tested by use of immunoblotting methods but had negative results when tested by use of an ELISA. Conversely, 2 other serum samples had positive results when tested by use of an ELISA at a dilution of 1:160 and negative results when tested by use of western blot analysis at a dilution of 1:100. Three negative-control serum samples of horses were nonreactive in ELISA and immunoblotting procedures. All 11 serum samples from dogs had positive results when tested by immunoblotting methods and polyvalent ELISA. An additional 10 negative-control serum samples obtained from dogs did not have reactivity when tested by use of an ELISA or blots.

A wide range of antibody titers and geometric mean values were recorded for the polyvalent and class-specific ELISA when serum samples of dogs and horses were tested. Ranges of reciprocal antibody titers were identical in analyses of serum samples of dogs from New York for total immunoglobulins and class-specific IgG antibodies (Table 3). Maximal titers were nearly 4-fold higher than those calculated for IgM antibodies. In each instance, geometric mean values for categories of dogs exceeded those calculated for horses. In analyses of serum samples of horses, geometric mean value for results of a polyvalent ELISA was nearly 2-fold greater than the mean calculated for class-specific IgG antibodies. Although a similar maximal IgM titration endpoint of 1:10,240 was recorded for a serum sample of a horse from Connecticut, compared with values for 2 serum samples with the same IgG antibody titer, a geometric mean of 640 for the former was the lowest recorded for all categories. Maximal antibody titers for serum samples of horses from Assateague Island and Cumberland Island ranged from 1:160 to 1:1,280 for the class-specific ELISA. Tests on the reproducibility of results for 3 positive serum samples of dogs (including 3 positive-control samples) and 5 positive serum samples of horses that had antibody titers ranging from 1:320 to 1:10,240 for the class-specific ELISA did not reveal differences in antibody titers (n = 6 trials) or revealed 2-fold (8), or 4-fold (6) changes in titers. Four-fold changes in titers were recorded in tests for IgG antibodies. Results for an additional 10 negative serum samples from dogs and horses (5 dogs, 5 horses) remained negative in the second trial for both class-specific ELISA.

Additional tests were conducted to assess specificity and for verification of homologous antibody reac-
tivity of positive-control serum samples to the 44-kd antigen. All 12 serum samples of horses that had homologous antibodies to *E risticii* (IFA titers, 1:80 to 1:1,280) and 3 serum samples of dogs that had homologous antibodies to *E canis* (IFA titers, 1:80 to 1:320) had negative results when tested by use of polyvalent or class-specific ELISA with the 44-kd recombinant antigen. In other tests of positive-control serum samples (1 dog, 1 horse) that contained antibodies to whole-cell antigen of the HGE agent (IFA titer, ≥ 5,120, respectively), results for both class-specific ELISA verified antibody reactivity to the 44-kd antigen (titers, 1:640 to 1:20,480). Control samples for 3 serum samples of dogs with negative results, 12 serum samples of horses with negative results, conjugates, PBS solution, and MBP were nonreactive in the polyvalent and both class-specific ELISA.

### Discussion

We developed class-specific ELISA to measure concentrations of IgM and IgG antibodies to *E phagocytophila* genogroup organisms and compared results for these ELISA with those of other assays. Class-specific and polyvalent ELISA with recombinant 44-kd antigen are useful for detecting IgM, IgG, or total immunoglobulins to the HGE agent, similar to IFA staining methods and immunoblotting procedures with whole-cell antigens. Sensitivities and specificities of ELISA for total immunoglobulins or class-specific IgG antibodies were comparable. Detection of IgM antibody in dogs and horses, however, was infrequent. In a previous study,7 antibody detection in these serum samples that had been collected during early days of illness from naturally infected horses was of limited value. Horses inoculated with *E equi*, however, produce IgM and IgG antibodies within 10 days after infection.17 In another study,16 antibodies were detected in horses 2 to 3 weeks after inoculation with *E equi*-infected whole blood. Immune responses of dogs and horses to *E phagocytophila* genogroup organisms vary, and with repeated exposure to infected ticks, it appears that class-specific IgG antibodies are more likely to be detected. It may be difficult to define recent *ehrlichia* infections on the basis of results of antibody testing alone. Reliance on other assays, such as PCR methods, detection of morulae in stained neutrophils, or culturing of the infectious agent, may be required to more efficiently identify recent infections.

Results of class-specific ELISA with p44 antigen paralleled findings obtained by immunoblotting methods with whole-cell antigen for serum samples obtained from dogs and horses. A similar pattern of concordance in assay results was reported in analyses of human serum samples.11 The discrepant finding for 3 serum samples of horses that had positive results for immunoblotting methods but negative results for ELISA was probably attributable to differences in initial serum dilutions tested (1:100 for blots and 1:160 for ELISA). In tests of blood and serum samples of horses, however, DNA analyses verified antibody results in some instances but were less helpful when blood samples were obtained several days after onset of illness.14 When antibody titers begin to increase, prevalence of DNA-positive samples decreases, probably because horses mount immune responses that are sufficient to suppress direct detection of the pathogen in blood by means of PCR techniques.

Investigators in other laboratories have reported that there is a family of closely related proteins with molecular masses of 42 to 47 kd and that there is a high degree of nucleotide similarity in conserved parts of the gene sequence.5,10,12 Moreover, proteins of the p44 family are differentially expressed.1,12 Therefore, similar to the situation in humans, it is possible that some dogs and horses may develop antibodies to different homologues. Nonetheless, the high degree of concordance among our results for immunoblotting, which contained proteins from infected whole cells, and for our ELISA for total immunoglobulins or class-specific IgG antibodies indicated that our p44 recombinant antigen was a suitable reagent that can be used to verify past or current infections caused by *E phagocytophila* genogroup organisms.

Results of specificity testing by class-specific ELISA with the p44 recombinant antigen did not reveal cross-reactivity with antibodies to *E canis* or *E risticii* in serum samples obtained from dogs and horses, respectively. This agrees with findings recorded for results of a polyvalent ELISA.15 High specificity also was evident when serum samples from people who had syphilis, Lyme borreliosis, human monocytic *ehrlichiosis*, and rheumatoid arthritis were tested.15,20 The recombinant p44 antigen appears to be a highly specific marker for *E equi* or HGE infection in horses.

The DNA of *E equi* or class-specific antibodies to the HGE agent was detected in samples obtained from horses during all seasons. The DNA of granulocytic *ehrlichiae* can be detected in blood samples within 2 days after onset of illness,3 and serum antibodies can persist for several weeks or months but usually do not remain for more than 7 months in naturally infected animals. Titers gradually decrease to baseline values with time.1 Detection of *ehrlichial* DNA and IgM antibodies in horses during the fall (November and December) also correlates with detection of granulocytic *ehrlichial* DNA of the 44-kd gene of the HGE agent in white-tailed deer (*Odocoileus virginianus*) during that time of year.22 Female *I scapularis* ticks are abundant during that season in the northeastern United States and probably transmit the HGE agent to medium- and large-size mammals. Detection of *ehrlichial* DNA and class-specific IgM antibody in blood or serum samples obtained from horses during

<table>
<thead>
<tr>
<th>ELISA</th>
<th>Range</th>
<th>Mean</th>
<th>Range</th>
<th>Mean</th>
</tr>
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<tbody>
<tr>
<td>Polyvalent</td>
<td>640–40,960</td>
<td>5,120</td>
<td>160–40,960</td>
<td>1,309</td>
</tr>
<tr>
<td>Class-specific IgM</td>
<td>840–5,120</td>
<td>1,810</td>
<td>220–10,240</td>
<td>640</td>
</tr>
<tr>
<td>Class-specific IgG</td>
<td>640–40,960</td>
<td>5,120</td>
<td>160–10,240</td>
<td>723</td>
</tr>
</tbody>
</table>

*Serum samples were obtained from dogs in New York during 1985 and 1986 and from horses in Connecticut during 1985, 1995, and 1996 and New York during 1995 and 1996. Geometric means are computed only for samples that had positive results.

See Table 1 for total No. of positive serum samples in respective groups.
June and July coincides with peak feedings of nymphal *I. scapularis* and indicates possible transmission of the HGE agent during the summer as well.

Analyses of serum samples of horses from Assateague Island, Va, and Cumberland Island, Ga, revealed antibodies to the HGE agent. Although most cases of granulocytic ehrlichiosis in humans are reported by people who live in northern states, there is serologic evidence of the HGE agent in deer and mice in the mid-Atlantic and southeastern United States. Antibodies to the HGE agent have been detected in cotton mice (*Peromyscus gossypinus*) in Sapelo Island, Ga, and Amelia Island, Fla. Therefore, the HGE agent appears to be widely distributed throughout the range of *I. scapularis* in the eastern United States. More attention should be given to identifying granulocytic ehrlichial infections in dogs, horses, and humans in the southeastern United States.

Class-specific ELISA with the 44-kd recombinant antigen are alternative methods that can be used to verify past or current infections caused by *E* phagocytophila *genogroup organisms in dogs and horses*. We suggest that western blot analysis continue to be used as a reference test to further evaluate the performance of ELISA containing the p44 antigen. Automated ELISA are more easily standardized and can include computer analysis of data. These methods are more practical for screening a large number of serum samples. However, more precise information is needed to determine periods of IgM and IgG antibody production and persistence of antibodies relative to times when the etiologic agent can be cultured from naturally or experimentally infected dogs and horses.

**References**


