

Evaluation of a polyvalent enzyme-linked immunosorbent assay incorporating a recombinant p44 antigen for diagnosis of granulocytic ehrlichiosis in dogs and horses

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Objective—To develop and evaluate a polyvalent ELISA incorporating a highly specific recombinant antigen (p44) for diagnosis of granulocytic ehrlichiosis in dogs and horses.

Animals—32 dogs and 43 horses.

Procedure—Results of the ELISA were compared with results of indirect fluorescent antibody (IFA) staining and western immunoblotting incorporating whole-cell antigen.

Results—For the canine and equine samples, percentages of samples with positive IFA staining, western immunoblotting, and ELISA results were similar. For 29 (91%) canine samples and 30 (70%) equine samples, results of IFA staining, western immunoblotting, and the ELISA were in complete agreement. Results of the ELISA for 3 canine serum samples known to contain antibodies to *Ehrlichia canis* and 12 equine serum samples known to contain antibodies to *E. risticii* were negative.

Conclusions and Clinical Relevance—Results of the present study suggest that a polyvalent ELISA incorporating a recombinant p44 antigen is suitable for detecting antibodies to *E. equi* in dogs and horses. (*Am J Vet Res* 2001;62:29–32)

Granulocytic ehrlichiosis, caused by infection with *Ehrlichia equi* or a closely related organism in the *Ehrlichia phagocytophila* genogroup, occurs in mammals living in areas where *Ixodes scapularis*, *I. pacificus*, and *I. ricinus* ticks abound.^{1,4} In the United States, this disease is most common in the northeastern^{5,7} and upper mid-western states¹ and in California.^{8,9} Leukopenia and thrombocytopenia are frequently reported clinical signs

of granulocytic ehrlichiosis in humans.¹ Fever and anorexia are common signs of illness in dogs and horses.³

A laboratory diagnosis of infection with organisms of the *E. phagocytophila* genogroup can be made by isolating the pathogen,^{10,12} detecting serum antibodies to bacterial antigens by use of indirect fluorescent antibody (IFA) staining methods or immunoblot procedures,^{6,9,13} observing inclusion bodies (ie, morulae) in stained neutrophils,¹⁴ and detecting bacterial DNA in blood samples from affected animals by use of a polymerase chain reaction assay or other methods.^{6,10,14} Although these methods are useful for research purposes, they are laborious and expensive and, therefore, of limited value in testing large numbers of samples. The purposes of the study reported here were to develop and evaluate a polyvalent ELISA incorporating a highly specific recombinant antigen for diagnosis of granulocytic ehrlichiosis in dogs and horses.

Materials and Methods

Serum samples—Serum samples obtained from blood samples collected from dogs and horses by veterinarians in New York state and Connecticut during 1985 and 1986 (dogs) or during 1985, 1995, and 1996 (horses) were used. All dogs and horses lived in tick-infested areas and had 1 or more signs of illness, such as lethargy, fever, lymphadenopathy, icterus, anorexia, or limb edema, at the time blood samples were collected. All serum samples had been stored at –60 C at the Connecticut Agricultural Experiment Station since the time of collection. Individual samples had been thawed and refrozen multiple times for various analyses prior to the present study; however, all samples had been promptly refrozen after being thawed to prevent losses in antibody titers.

Results of previous analyses of some of the samples used in the present study have been published.^{6,15} Ehrlichial DNA was detected by use of polymerase chain reaction methods in 19 of 38 equine samples.⁶ Twelve canine samples and 34 equine samples had antibodies to *E. equi*, as determined by use of IFA staining methods or western immunoblot procedures. The 34 equine samples also had reactivity to a 44-kd protein of the NCH-1 strain of the human granulocytic ehrlichiosis (HGE) agent. Antibody reactivity to this protein has been shown to be a sensitive and highly specific diagnostic marker for *E. phagocytophila* genogroup infection in human beings,^{13,16} horses,⁹ dogs,¹⁵ and white-footed mice (*Peromyscus leucopus*).¹⁷

Serologic testing—Thirty-two canine and 43 equine serum samples from as many subjects were tested for total antibodies to the NCH-1 strain of the HGE agent by use of IFA staining and western immunoblotting, as described.^{6,15} An additional 4 serum samples from 4 of these horses, collected from blood samples obtained 2 to 5 months after ini-

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tial sampling were available for analysis. Previous testing of human serum samples with various antigen strains (eg, horse-derived infected neutrophils or infected human promyelocytic leukemia cells) has not revealed any significant differences in prevalence of seropositive reactions.¹⁶ The NCH-1 strain was chosen for use in the present study because it is widely available, originates from the northeastern United States, and was the source of genetic material used to produce the recombinant 44-kd antigen used for developing the ELISA evaluated in the present study.

A polyvalent ELISA to detect total immunoglobulins to the recombinant 44-kd protein of the HGE agent was developed, using modifications of described methods.¹⁹ Production of this antigen, expressed and purified as a maltose-binding protein fusion peptide, in analyses of human serum samples for antibodies to the HGE agent has been described.²⁰ Briefly, the p44 gene was cloned and expressed, and the fusion protein was modified by replacing the glutathione transferase fusion partner with maltose-binding protein to improve solubility and yield after purification. Polystyrene plates were used for the ELISA. Commercially available horseradish peroxidase-labeled goat anti-dog immunoglobulins and goat anti-horse immunoglobulins,* diluted to 1:10,000 and 1:5,500, respectively, in phosphate-buffered saline solution (PBSS, pH, 7.2), were used. Sixty microliters of commercially available 2,2'-azino-di-(3-ethylbenzthiazoline sulfonate) substrate* was added to each well for color production. Appropriate positive and negative control samples were included on each plate. Samples that yielded positive results were reanalyzed to determine titration endpoints and verify reproducibility of results. Thirteen canine and 9 equine samples with positive results and 4 canine and 4 equine samples with negative results were used for this analysis.

Twelve canine and 24 equine serum samples lacking antibodies to the HGE agent by western immunoblot procedures or IFA staining methods^{6,15} were used to determine cutoff values for the ELISA. Samples were diluted with PBSS (pH, 7.2) to concentrations of 1:160, 1:320, and $\geq 1:640$ and tested with antigen (2.5 μ g of protein/ml) by an ELISA. This antigen concentration was determined by testing a series of dilutions ranging from 1.0 to 3.0 μ g of protein/ml with serum samples from 2 dogs or 2 horses previously found to contain antibodies to the HGE agent by immunoblotting methods.^{6,15} The antigen concentration chosen for analyses allowed for maximum antigen-antibody complex formation and corresponding color production in plate wells. The cutoff for a positive result was calculated as mean net optical density (OD) + 3 SD. For the 12 canine samples, cutoffs for positive results were calculated as net OD of 0.07, 0.07, and 0.06 for samples diluted 1:160, 1:320, and $\geq 1:640$, respectively. For the 24 equine samples, cutoffs for positive results were calculated as net OD of 0.07, 0.07, and 0.05 for samples diluted 1:160, 1:320, and $\geq 1:640$, respectively.

Three canine serum samples determined by use of IFA staining methods to contain antibodies to *E canis* and 12 equine serum samples determined by use of these procedures to contain antibodies to *E risticii* were used to evaluate specificity of the ELISA. Sources of these samples have been reported.^{19,21}

Statistical analyses—The z -test with Yates correction was used to compare proportions of the 32 canine samples and the 43 equine samples with positive results for IFA staining, western immunoblotting, and the ELISA. Analyses were performed by use of a statistical software program.⁸

Results

Percentages of the canine samples seropositive for antibodies to *E equi* by use of IFA staining, western

immunoblotting, and the ELISA were similar ($P = 0.744$; Table 1). Similarly, percentages of the equine samples seropositive for antibodies to *E equi* by use of these 3 methods were similar ($P = 0.504$). However, percentage of seropositive equine samples was higher than the percentage of seropositive canine samples.

For 29 of the 32 (91%) canine samples and for 30 of the 43 (70%) equine samples, results of IFA staining, western immunoblotting, and the ELISA were in complete agreement (Table 2). Results for the remaining 3 canine samples and 13 equine samples were discordant. Percentages of concordant versus discordant results were significantly different for the canine ($z = 6.310$, $P < 0.001$) and the equine samples ($z = 3.494$, $P < 0.001$).

Antibody titers for the seropositive canine and equine samples ranged widely. Maximal titration endpoints determined by use of the ELISA greatly exceeded those determined by use of IFA staining (Table 3). For the 9 canine samples and the 27 equine samples seropositive for ehrlichiosis by all 3 assays, geometric mean antibody titers determined with the ELISA (mean titer for canine samples, 8,128; mean titer for equine samples, 1,372) were greater than mean titers determined by use of IFA staining meth-

Table 1—Comparison of results of indirect fluorescent antibody staining methods (IFA), western immunoblotting (WB), and an enzyme-linked immunosorbent assay (ELISA) incorporating a recombinant p44 antigen of the NCH-1 strain of the human granulocytic ehrlichiosis agent for diagnosis of granulocytic ehrlichiosis, using serum samples from dogs and horses

Assay	Dogs (n = 32)	Horses (n = 43)
	No. seropositive (%)	No. seropositive (%)
IFA	10 (31)	34 (79)
WB	12 (38)	34 (79)
ELISA	11 (34)	31 (72)

Table 2—Concordance of results of IFA, WB, and an ELISA incorporating a recombinant p44 antigen for diagnosis of granulocytic ehrlichiosis, using serum samples from 32 dogs and 43 horses

Assay result			No. of dogs (%)	No. of horses (%)
IFA	WB	ELISA		
+	+	+	9 (28)	27 (63)
+	+	—	1 (3)	1 (2)
—	+	+	2 (6)	4 (9)
+	—	—	0 (0)	6 (14)
—	+	—	0 (0)	2 (5)
—	—	—	20 (63)	3 (7)

All dogs and horses were febrile and suspected to have tick-borne infections. + = Positive result. — = Negative result.

Table 3—Antibody titers for dogs and horses seropositive for granulocytic ehrlichiosis by means of IFA incorporating whole-cell antigen or an ELISA incorporating a recombinant p44 antigen of the NCH-1 strain of the human granulocytic ehrlichiosis agent

Reciprocal antibody titer	No. of dogs		No. of horses	
	IFA	ELISA	IFA	ELISA
80 to 160	6	0	20	4
320 to 640	3	2	13	13
1,280 to 2,560	1	1	1	7
5,120 to 10,240	0	4	0	4
20,480 to 40,960	0	4	0	3

ods (mean titer for canine samples, 187; mean titer for equine samples, 184).

Results of the ELISA for the 3 canine serum samples known to contain antibodies to *E. canis* and for the 12 equine serum samples known to contain antibodies to *E. risticii* were negative.

Reproducibility of results of the ELISA for the 13 seropositive and 4 seronegative canine serum samples tested twice on separate days was high. For the seropositive samples, antibody titers were the same ($n = 6$), differed by a factor of 2 (6), or differed by a factor of 4 (1). For the sample for which results differed by a factor of 4, net OD was 0.19 on the first day of testing and 0.08 on the second at a dilution of 1:160. Results for the 4 seronegative samples were negative on the second day of testing.

Similarly, reproducibility of results of the ELISA for the 9 seropositive and 4 seronegative equine samples tested twice on separate days was high. For the seropositive samples, antibody titers were the same ($n = 1$), differed by a factor of 2 (7), or differed by a factor of 4 (1). For the sample for which results differed by a factor of 4, net OD was 1.03 on the first day of testing and 0.95 on the second at a dilution of 1:160. Results for the 4 seronegative samples were negative on the second day of testing.

For the 4 horses found to be carrying antibodies to *E. equi* during 1995 or 1996 that were treated with antibiotics, ELISA antibody titers ranged from 1:640 to 1:40,960 at the time of initial testing. For all 4 horses, titers 2 to 5 months later were lower by a factor of 4.

Discussion

Results of the present study suggest that a polyvalent ELISA incorporating a recombinant p44 antigen is suitable for detecting antibodies to *E. equi* in dogs and horses. Results of this assay were sensitive, specific, and reproducible. Use of other recombinant antigens in ELISA has improved laboratory diagnosis of borreliosis in humans²² and horses,¹⁹ but sensitivities and specificities of these assays are usually lower.

Western blot analyses have shown that the 44-kd peptide is a sensitive and specific marker for *E. phagocytophila* genogroup infection in humans,^{13,16,23,24} dogs,¹⁵ and horses.⁶ Because procedures for ELISA can be automated and standardized, these types of assays are the most practical method for initial analyses of large numbers of serum samples. It is advised, however, that a second method, such as western immunoblotting with whole-cell antigen, be used as an adjunct, particularly when results of the ELISA do not fit the clinical findings or other laboratory results (ie, blood cell counts) are inconclusive.

In the present study, geometric mean antibody titers obtained with the ELISA were greater than titers obtained by use of IFA staining, which incorporated whole-cell antigen. The greater sensitivity of the ELISA was likely attributable to the use of a highly purified recombinant antigen, which allowed for maximal amounts of antibody-antigen complexes to form in plate wells without interference or blocking caused by irrelevant components of the whole-cell antigen.

Use of the ELISA described in the present study

revealed a 4-fold decrease in antibody titers during a period of 2 to 5 months in 4 horses in which granulocytic ehrlichiosis was diagnosed and treated with antibiotics. Although a 4-fold decrease in titer was within the normal variation of results for this ELISA, findings for these 4 horses do indicate that antibodies can persist for several weeks. Similar results were reported for experimentally infected ponies.^{25,26} It is unlikely, however, that high concentrations of antibodies persist for several months in naturally exposed horses that receive antibiotics.²⁷ On the other hand, repeated exposure of naturally infected horses to infected ticks may cause temporary increases in antibody titers without signs of illness. Because the number of times animals are infected is usually unknown and it is unclear whether test results indicate active infection, antibody detection assays should be limited in dogs and horses to verifying exposure to the organism.

Because there is limited information on IgM and IgG antibodies in dogs and horses with granulocytic ehrlichiosis, class-specific ELISA should be developed to determine concentrations of specific types of antibodies in infected animals. Such differentiation of immunoglobulins, and particularly identification of IgM antibody, may be helpful in identifying recent infection and could possibly play a role in selection of other laboratory procedures to verify granulocytic ehrlichiosis.

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References

1. Walker DH, Dumler JS. Emergence of the ehrlichioses as human health problems. *Emerg Infect Dis* 1996;2:18-29.
2. Johansson KE, Pettersson B, Uhlen M, et al. Identification of the causative agent of granulocytic ehrlichiosis in Swedish dogs and horses by direct solid phase sequencing of PCR products from the 16S rRNA gene. *Res Vet Sci* 1995;58:109-112.
3. Engvall EO, Pettersson M, Persson M, et al. A 16S rRNA-based PCR assay for detection and identification of granulocytic *Ehrlichia* species in dogs, horses, and cattle. *J Clin Microbiol* 1996;34:2170-2174.
4. Richter PJ, Kimsey RB, Madigan JE, et al. *Ixodes pacificus* (Acari: Ixodidae) as a vector of *Ehrlichia equi* (Rickettsiales: Ehrlichieae). *J Med Entomol* 1996;33:1-5.
5. Centers for Disease Control and Prevention. Statewide surveillance for ehrlichiosis—Connecticut and New York. 1994-1997. *MMWR Morb Mortal Wkly Rep* 1998;47:476-480.
6. Magnarelli LA, Van Andel AE, IJdo JW, et al. Serologic testing of horses for granulocytic ehrlichiosis, using indirect fluorescent antibody staining and immunoblot analysis. *Am J Vet Res* 1999;60:631-635.
7. Madigan JE, Barlough JE, Dumler JS, et al. Equine granulocytic ehrlichiosis in Connecticut caused by an agent resembling the human granulocytotropic *Ehrlichia*. *J Clin Microbiol* 1996;34:434-435.
8. Madigan JE, Hietala S, Chalmers S, et al. Seroepidemiologic survey of antibodies to *Ehrlichia equi* in horses of northern California. *J Am Vet Med Assoc* 1990;196:1962-1964.
9. Madigan JE. Equine ehrlichiosis. *Vet Clin North Am Equine Pract* 1993;9:423-428.
10. Heimer R, Van Andel A, Wormser GP, et al. Propagation of granulocytic *Ehrlichia* spp. from human and equine sources in HL-60 cells induced to differentiate into functional granulocytes. *J Clin Microbiol* 1997;35:923-927.
11. Munderloh UG, Madigan JE, Dumler JS, et al. Isolation of equine granulocytic agent, *Ehrlichia equi*, in tick cell culture. *J Clin Microbiol* 1996;34:664-670.

12. Goodman JL, Nelson C, Vitale B, et al. Direct cultivation of the causative agent of human granulocytic ehrlichiosis. *N Engl J Med* 1996;334:209-215.
13. Ijdo JW, Zhang Y, Hodzic E, et al. The early humoral response in human granulocytic ehrlichiosis. *J Infect Dis* 1997; 176:687-692.
14. Madigan JE, Richter PJ, Kimsey RB, et al. Transmission and passage in horses of the agent of human granulocytic ehrlichiosis. *J Infect Dis* 1995;172:1141-1144.
15. Magnarelli LA, Ijdo JW, Anderson JF, et al. Antibodies to *Ehrlichia equi* in dogs from northeastern United States. *J Am Vet Med Assoc* 1997;211:1134-1137.
16. Wong SJ, Brady GS, Dumler JS. Serological responses to *Ehrlichia equi*, *Ehrlichia chaffeensis*, and *Borrelia burgdorferi* in patients from New York State. *J Clin Microbiol* 1997;35:2198-2205.
17. Magnarelli LA, Stafford KC III, Ijdo JW, et al. Antibodies to granulocytic ehrlichiae in white-footed and cotton mice in eastern United States. *J Wildl Dis* 1999;35:259-265.
18. Magnarelli LA, Ijdo JW, Dumler JS, et al. Reactivity of human sera to different strains of granulocytic ehrlichiae in immuno diagnostic assays. *J Infect Dis* 1998;178:1835-1838.
19. Magnarelli LA, Flavell RA, Padula SJ, et al. Serologic diagnosis of canine and equine borreliosis: use of recombinant antigens in enzyme-linked immunosorbent assays. *J Clin Microbiol* 1997;35: 169-173.
20. Ijdo JW, Wu C, Magnarelli LA, et al. Serodiagnosis of human granulocytic ehrlichiosis by a recombinant HGE-44-based enzyme-linked immunosorbent assay. *J Clin Microbiol* 1999;37: 3540-3544.
21. Magnarelli LA, Stafford KC III, Mather TN, et al. Hemocytic rickettsia-like organisms in ticks: serologic reactivity with antisera to ehrlichiae and detection of DNA of agent of human granulocytic ehrlichiosis by PCR. *J Clin Microbiol* 1995;33:2710-2714.
22. Magnarelli LA, Fikrig E, Padula SJ, et al. Use of recombinant antigens of *Borrelia burgdorferi* in serologic tests for diagnosis of Lyme borreliosis. *J Clin Microbiol* 1996;34:237-240.
23. Zhi N, Ohashi N, Rikihisa Y, et al. Cloning and expression of the 44-kilodalton major outer membrane protein gene of the human granulocytic ehrlichiosis agent and application of the recombinant protein to serodiagnosis. *J Clin Microbiol* 1998;36:1666-1673.
24. Ijdo JW, Sun W, Zhang Y, et al. Cloning of the gene encoding the 44-kilodalton antigen of the agent of human granulocytic ehrlichiosis and characterization of the humoral response. *Infect Immun* 1998;66:3264-3269.
25. Nyindo MB, Ristic M, Lewis GE, et al. Immune response of ponies to experimental infection with *Ehrlichia equi*. *Am J Vet Res* 1978;39:15-18.
26. Corstvet RE, Graunt SD, Karms PA, et al. Detection of humoral antigen and antibody by enzyme-linked immunosorbent assay in horses with experimentally induced *Ehrlichia equi* infection. *J Vet Diagn Invest* 1993;5:37-39.
27. Van Andel AE, Magnarelli LA, Heimer R, et al. Development and duration of antibody response against *Ehrlichia equi* in horses. *J Am Vet Med Assoc* 1998;212:1910-1914.