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Serologic confirmation of *Ehrlichia equi* and *Borrelia burgdorferi* infections in horses from the northeastern United States

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Objective—To determine whether horses living in tick-infested areas of northeastern United States with clinical signs of borreliosis or granulocytic ehrlichiosis had detectable serum antibodies to both *Borrelia burgdorferi* and *Ehrlichia equi*.

Design—Prospective study.

Animals—Serum samples from 51 clinically normal horses, 14 horses with clinical signs of borreliosis, and 17 horses with clinical signs of granulocytic ehrlichiosis.

Procedure—Serum *B burgdorferi* or *E equi* antibodies were measured by use of an ELISA, immunoblot analysis, or indirect fluorescent antibody (IFA) staining.

Results—Of the 82 serum samples tested, 37 (45.1%) and 13 (15.9%) had detectable antibodies to *B burgdorferi* or *E equi*, respectively. Test results indicated that 12 horses had been exposed to both agents, 11 of these horses had granulocytic ehrlichiosis. The ELISA regularly detected antibodies to the following recombinant protein (p) antigens of *B burgdorferi*: p29, p37, p39, and p41-G. The use of immunoblot analysis confirmed ELISA results by indicating antibody reactivities to antigens of whole-cell *B burgdorferi* having molecular masses of predominantly 31, 34, 37, 39, 41, 58, and 93 kd.

Conclusions and Clinical Relevance—Horses living in areas where ticks (*Ixodes scapularis*) abound are sometimes exposed to multiple pathogens. Analyses for specific recombinant borrelial antibodies using an ELISA can help separate horses with borreliosis from those with granulocytic ehrlichiosis, even when antibodies to both etiologic agents are detected in serum samples. Analysis using immunoblots is sensitive, and along with ELISA or IFA procedures, is suitable for confirming a clinical diagnosis of each disease (*J Am Vet Med Assoc* 2000;217:1045–1050)

Mammals living in tick-infested areas may be exposed to *Borrelia burgdorferi* sensu lato and *Ehrlichia equi* (a member of the *Ehrlichia phagocytophila* genogroup), causative agents of borreliosis or equine granulocytic ehrlichiosis (EGE), respectively. *Ixodes scapularis* ticks transmit both organisms in northeastern and upper midwestern United States,[1] whereas *I pacificus* is the chief vector in California.[2] There are reports describing separate *B burgdorferi* or *E equi* infections in horses,[3-9] but little is known about possible concurrent infections in horses. Laboratory diagnosis of borreliosis in horses is difficult. Culture and polymerase chain reaction (PCR) methods to detect *B burgdorferi* have been low yielding, compared with procedures used to detect *E equi*. Results of serologic studies have been useful in demonstrating coexistence of antibodies to *B burgdorferi*, *E equi*, and *Babesia microti* in human beings[10] and white-footed mice (*Peromyscus leucopus*).[11] In dogs, exposure to *B burgdorferi* and *E equi* has also been documented.[12] The purposes of the study reported here were to determine whether horses living in tick-infested areas of Connecticut with clinical signs of borreliosis or EGE had been exposed to *B burgdorferi* and *E equi* and to further evaluate the use of recombinant antigens of *B burgdorferi* in an ELISA.

Materials and Methods

The 82 serum samples tested in our study included 65 samples collected in 1985[3] and 17 samples obtained in 1995 and 1996.[13] Twenty-nine of the 65 serum samples collected in 1985 had antibodies to *B burgdorferi* and 12 of the 17 samples collected in 1995 and 1996 had antibodies to *E equi*. All serum samples were stored at –60 C at the Connecticut Agricultural Experiment Station. The sources of test serum samples and positive and negative control serum samples have been reported.[3,13] Moreover, horses in our study were not vaccinated against borreliosis.

Sera from 1985 borreliosis surveillance—Sixty-five of the serum samples used in our study had been obtained originally in a passive surveillance program during April through July of 1985.[3] From that same program, an additional 15 serum samples were used that had originally served as negative control samples. Serum samples had been collected by veterinarians from 80 privately owned horses living in 19 towns in all counties of Connecticut except Hartford County. Of the 65 test serum samples, 51 were from clinically normal horses (ie, no signs of illness) and 14 were from clinically affected horses with borreliosis. Acute or convalescent-phase serum samples from 14 ill horses had been tested. Clinical

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signs of possible borreliosis included lethargy, low-grade fever (38.6 to 39.1 C [101.5 to 102.4 F]) and of single or multiple painful joints resulting in lameness, or stiffness and reluctance to move.

Sera from 1995-1996 EGE surveillance—An additional 17 serum samples were used in our study that had been obtained originally from 17 acutely ill horses that had EGE in Connecticut and New York State during 1995 and 1996.[8,13] Serum samples were obtained 20 to 82 days after clinical signs of EGE were observed by veterinarians participating in a passive surveillance program. Horses with acute EGE had high rectal temperatures (≥ 38.9 C [≥ 102 F]) and clinical signs that included depression, icterus, anorexia, or limb edema. Confirmation of *E equi* infection was made on the basis of granulocytic ehrlichial DNA detection by use of polymerase chain reaction (PCR). Ten whole blood samples obtained from horses within 2 days after onset of illness were analyzed by PCR,[7,8,13] a time when serum samples usually lack antibodies to *E equi*.[8] Serum samples from the remaining 7 horses had negative results by PCR analysis but were confirmed as having antibodies to *E equi*.[8,13] All horses were successfully treated with tetracycline.[8]

Serologic analyses—A polyvalent ELISA was used to determine total antibody titers to whole cell *B burgdorferi* (strain 2591) and the following recombinant antigens: protein (p) 22, p41-G, p37, p39, outer surface protein (Osp)B (34 kd), OspC (23 kd), OspE (19 kd), and OspF (29 kd). All recombinant antigens, produced at Yale University or the University of Connecticut,[14-17] were cloned and expressed as fusion proteins in *Escherichia coli*. Recombinant 39 antigen was made from DNA of *B burgdorferi* (strain 2591) using primers (upstream primer 5'-TAGTGGTAAAGGTACTCTT-3' and downstream primer 5'-TTAAATAAATTCTTTAA-GAAAC-3') on the basis of published sequence[18] (GenBank accession number L24194). The product was cloned into pGEX-2T and expressed as a fusion protein similar to the other recombinant antigens with glutathione S-transferase in *E coli*. Recombinant OspA (31 kd) antigen was unavailable. The materials and procedures used to analyze equine serum samples and the cut-off values for positive results for assays with the p41-G and the Osp antigens have been reported.[14,15]

Recombinant antigens of p22, p37, and p39 were evaluated with control serum samples to determine critical regions for positive results. Antigen concentrations of 2 to 5 µg of protein/ml were most suitable for obtaining optimal serologic reactivity with serum samples with positive results. Fifteen equine serum samples with negative results, analyzed in earlier studies,[3,15] were diluted to 1:160, 1:320, and ≥ 1:640 and used to determine cut-off values in separate ELISA with these antigens. Statistical analyses (Mean values ± 3 SD) of net absorbance values for the respective data sets were used to calculate critical regions. Net optical density (OD) values of 0.10, 0.05, and 0.04 were considered positive results for the serum dilutions of 1:160, 1:320, and ≥ 1:640 when p22 antigen was used, whereas OD values of 0.06, 0.05, and 0.04 indicated antibody presence when ELISA contained the p37 antigen. Higher net OD values were computed for ELISA with the p39 antigen (0.35, 0.25, and 0.13). Polystyrene plates contained the same positive and negative control serum samples and tests for phosphate-buffered saline solution (PBSS) and commercially produced peroxidase-labeled antibodies.[a]

Immunoblot analysis, used earlier,[14] verified the presence of antibodies to whole cell *B burgdorferi* in positive control serum samples and determined total antibody measurements in 13 serum samples obtained in 1985. The blocking reagent was PBSS containing 5% nonfat dry milk. Modifications of procedures included the use of commercially prepared alkaline phosphatase-labeled goat antisera[a] (diluted to 1:2,000 in PBSS containing 5% bovine serum albumin) against horse immunoglobulin. Blots were developed as before[10,13] and quenched in distilled water. Analyses contained positive and negative control serum samples and molecular mass standards.[b]

To determine total serum antibody titers to *E equi*, indirect fluorescent antibody (IFA) staining procedures were used with antigens of either the BDS or NCH-1 strains of granulocytic ehrlichiae. Obtained in Minnesota and Massachusetts, respectively, both strains are human isolates that had been propagated in horses (BDS strain) or grown in human promyelocytic leukemia cell cultures (NCH-1 strain). Results of comparative analyses with these strains[13] revealed little difference in the proportion of serum samples with positive results. Serum reactivities at dilutions of ≥ 1:80 were considered positive. Details on the sources of antigens, serum samples, fluorescein-labeled antibodies, and on the conservative grading of fluorescence have been described.[11] Positive and negative control serum samples were included in tests. Immunoblot analysis[13] confirmed past or current *E equi* infections by indicating reactivity to a 44 kd protein of the NCH-1 strain.

Analyses were conducted to further assess specificity of ELISA containing recombinant antigens of *B burgdorferi*. Twelve serum samples with antibodies to *Erhlichia risticii*, as determined by IFA staining methods,[15] were screened against all recombinant antigens. Earlier work[10,12,15,19] determined that horse, human, or dog antibodies to *B burgdorferi* or *E equi* typically did not cross-react by IFA staining methods with heterologous antigens at serum dilutions of > 1:80. Likewise, little or no cross-reactivity was observed in reciprocal antibody testing of *E equi* antigen and antisera with similar reagents of *E canis*, *E chaffeensis*, or *E risticii*.[10,19]

Results

Review of clinical records for horses that were seropositive for *B burgdorferi* in 1985 revealed no evidence of EGE. However, during that time, EGE was not a recognized disease in northeastern United States. Further, horses examined in 1995 and 1996 with acute EGE were not suspected of having current or past borreliosis. In each horse, exposure to *B burgdorferi* and *E equi* was unknown.

Sera from 1985 borreliosis surveillance—The 65 equine serum samples were reanalyzed for antibody titers to *B burgdorferi* and *E equi*. Of the 65 samples, 29 (44.6%) had antibodies to whole cell *B burgdorferi* detected by use of an ELISA. Of these, 1 serum sample from a clinically normal horse had antibodies to *E equi* at a titer of 1:80, as determined by IFA staining meth-

Table 1—Results of serologic testing for antibodies to whole-cell sonicated or recombinant antigens of *Borrelia burgdorferi*, using polyvalent ELISA and immunoblot analysis in serum samples from 14 horses examined and suspected of having borreliosis in 1985

	ELISA		Immunoblot
Antigen	No. of positive results*	Range of reciprocal titers	No. of positive results*
Whole cell	9	640–10,240	13
p22	3	640–2,560	0
p37	8	160–5,120	5
p39	11	160–5,120	4
p41-G	9	640–2,560	14
OspB	2	320–640	4
OspC	3	160–320	2
OspE	3	320–640	2
OspF	7	640–5,120	2

*Results for total antibody measurements. Horses with antibody titers determined by ELISA to be ≥ 1:160 were considered seropositive. Blots contained whole-cell antigen.

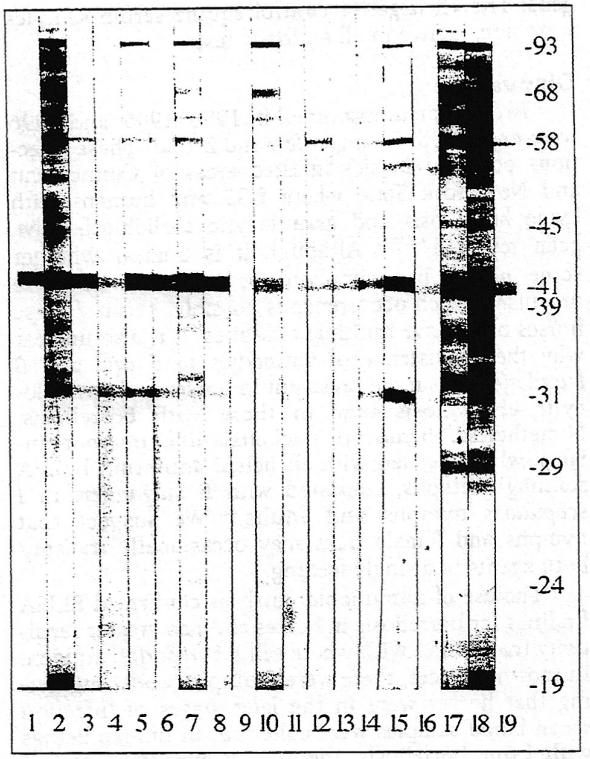

Figure 1—Representative results of immunoblot analysis of equine serum samples to determine total antibody to whole-cell *Borrelia burgdorferi*. Molecular masses for key bands are indicated in kilodaltons. Lane 1, negative control sample; lane 2, positive control sample; lanes 3 to 8, 10, 11, 14, 15, and 17 to 19 reveal reactivity of equine serum samples; lane 9, 12, 13, and 16 indicate no specific antibody reactivities.

ods. Test results of the additional negative control serum samples were nonreactive in both tests.

Analyses by use of an ELISA (Table 1) of 14 serum samples from clinically affected horses with borreliosis revealed low to high antibody titers of 1:160 to 1:10,240 to whole cell or recombinant antigens of *B burgdorferi*. Reactivities of antibodies to p37, p39, p41-G, and OspF antigens were most common. Less common reactivity was observed when serum samples were screened with p22, OspB, OspC, and OspE antigens.

Results of immunoblot analysis (Fig 1) with whole cell *B burgdorferi* confirmed ELISA results for 13 of 17 horses and had distinct reactivity to 2 or more key immunodominant proteins, including OspA, OspB, p37, p39, flagellin (41 kd), or 93 kd antigens. Distinct bands for the latter peptide were evident in blots of serum samples from 8 horses suspected of having borreliosis on the basis of clinical data. A single band for the 41 kd protein or weak bands for other antigens was not considered to be evidence of *B burgdorferi* infections, whereas distinct bands for OspA, OspB, OspE, OspF, p37, and the 93 kd protein were viewed as important markers. In general, when 3 or more distinct bands were visible, ELISA antibody titers were high and ranged from 1:640 to 1:40,960 (Table 2).

Sera from 1995–1996 EGE surveillance—Serum samples from 17 horses with acute EGE were reanalyzed for antibody titers to *B burgdorferi* and *E equi*. None of these horses had a record of prior immunization for borreliosis. Granulocytic ehrlichial DNA was detected by PCR in 10 of the 17 samples. Of these 17 serum samples, 11 had antibodies to whole cell *B burgdorferi* and 12 contained antibodies to either or both strains of *E equi*.

Table 2—Polyvalent immunoblot analysis and ELISA results for equine serum samples that were obtained originally in a passive surveillance program for borreliosis during April through July of 1985

	Immunoblot analysis*	ELISA*
Sample No.	Protein mass (kd)	Reciprocal titers
1	37 (weak), 41	N
2	19, 29, 31, 34, 35, 41, 45, 49, 58, 65, 68, 93	10,240
3	31, 41, 58	N
4	31, 41, 58, 60	N
5	31, 41, 58, 68, 93	2,560
6	31, 41, 58	1,280
7	19, 31, 34, 35, 37, 41, 58, 65, 68, 93	1,280
8	31, 37, 41, 58	N
9	41(weak)	N
10	19, 35, 41, 58, 65, 68, 93	1,280
11	31, 41, 93	640
12	41, 58	640
13	41	N
14	31, 41, 58	640
15	31, 41, 55, 58, 93	1,280
16	31, 41, 55	N
17	29, 31, 34, 41, 45, 58, 65, 68, 93	5,120
18	19, 29, 31, 34, 35, 37, 41, 45, 49, 55, 58, 65, 68, 93	40,960
19	41, 58	1,280

*Whole-cell antigen from *Borrelia burgdorferi* was used. Serum sample No. 1 was considered a negative control. Serum sample No. 2 was considered a positive control. Serum samples 9, 12, and 19 were obtained from horses that were clinically normal (ie, no signs of borreliosis). The remaining 14 serum samples were from horses clinically suspected of having borreliosis. N = Reciprocal antibody titer < 160, indicating that serum sample was from a seronegative horse.

Nine of 10 serum samples that contained ehrlichial DNA also had detectable antibodies to the NCH-1 strain of *E equi* and to whole cell or recombinant antigens of *B burgdorferi* (Table 3). Similar reactions occurred when the BDS strain of *E equi* was used as antigen. In analyses for *B burgdorferi* antibody titers, 7 serum samples had positive results to OspB antigen and 6 had positive results to whole cell and p37 antigens. There was infrequent reactivity to p22, p41-G, and OspC antigens by use of ELISA and no reactions to p39, OspE, and OspF antigens.

Results for 7 serum samples that did not contain granulocytic ehrlichial DNA revealed that 3 samples had antibodies to *E equi* and 5 had antibodies to whole cell and OspB antigens of *B burgdorferi*. Two serum samples had antibodies to both pathogens. Antibodies to p22, p37, p41-G, as well as p39 and OspF antigens were also detected.

Results of IFA staining methods with the NCH-1 strain of *E equi* were compared with those of ELISA containing whole cell, p37, or OspB antigens of *B burgdorferi* for the 17 serum samples from horses with EGE. When whole cell *B burgdorferi* was used in an ELISA, results for 8 serum samples (7 positive and 1 negative) agreed with those obtained by IFA staining methods for *E equi* antibodies. Mixed results were recorded for the remaining 9 serum samples. Further comparisons of findings for both assays revealed agreement (7 serum samples) or disagreement (10) when recombinant p37 antigen was used in an ELISA. Finally, in ELISA containing OspB antigen, mixed results (10 serum samples) once again exceeded the number of positive reactions (7) recorded in both tests.

Serologic testing of 12 equine serum samples containing antibodies to *E risticii* revealed that there was no reactivity in ELISA containing whole cell or recombinant antigens of *B burgdorferi* or by IFA staining methods with *E equi* antigen. Similarly, a positive control serum sample, having a homologous antibody titer of 1:2,560 to the MRK strain of *E equi* following inoculation, did not react with any of the *B burgdorferi* antigens. The 15 negative control equine serum samples were nonreactive in all antibody tests.

Discussion

Twelve horses examined in 1985, 1995, and 1996 were exposed to *B burgdorferi* and *E equi*. These infections occurred in tick-infested areas of Connecticut and New York State where EGE and humans with Lyme borreliosis and granulocytic ehrlichiosis have been reported.[6-8,10,13] Although it is unclear whether some horses had simultaneous infections of these organisms, such occurrence is possible, because these horses often have multiple tick bites. It is also unclear why the coexistence of antibodies to *E equi* and *B burgdorferi* was more prevalent in horses with granulocytic ehrlichiosis than in those with borreliosis. Nonetheless, hemocytic rickettsia-like microorganisms, which reacted with ehrlichial antiserum by IFA staining methods, coexisted with *B burgdorferi* in *I scapularis* nymphs and adults.[20] We suspect that nymphs and female ticks may occasionally transmit both agents in a single feeding.

The use of immunoblot analysis confirmed ELISA findings for borreliosis in horses and had greater sensitivity than ELISA with whole cell *B burgdorferi* antigen. In most instances, there were multiple bands, suggesting that horses were in the later stages of infection when blood samples were collected. In human beings with Lyme borreliosis, there is an expansion of IgG immune response to multiple antigens of the spirochete when disease progresses.[21] Antibody reactivity in equine serum samples to several immunodominant proteins of this spirochete is similar to that reported for human[22-24] and dog[25] serum samples. Immunoblot analysis reactions to proteins having molecular masses of about 31, 34 (OspB), 41, 58, and 93 kd occurred often, but reactivities to the 41 and 58 kd peptides are probably not specific. In a pony inoculated twice with *B burgdorferi*,[4] there was confirmation of IgG antibody production to OspA and OspB, highly specific antigens for *Borrelia* spp, but there was no evidence of antibody

Table 3—Results of serologic tests for antibodies to recombinant or whole-cell antigens of *Borrelia burgdorferi* and whole cell *Ehrlichia equi*, using an ELISA or indirect fluorescent antibody (IFA) staining methods, in serum samples from 17 horses examined in 1995 and 1996 with equine granulocytic ehrlichiosis

	10 serum samples that contained ehrlichial DNA*		7 serum samples that did not contain ehrlichial DNA*	
Antigen	No. of positive results†	Range of reciprocal titers	No. of positive results†	Range of reciprocal titers
Ehrlichia equi‡	8	320	0	NA
Ehrlichia equi§	9	80–1,280	3	80–160
Borrelia burgdorferi	—	—	—	—
2591 whole cell	6	640–2,560	5	640–40,960
p22	2	640–1,280	1	1,280
p37	6	320–640	4	160–320
p39	0	NA	2	640–5,120
p41-G	1	640	3	640–5,120
OspB	7	320–1,280	5	160–1,280
OspC	2	640	0	NA
OspE	0	NA	0	NA
OspF	0	NA	3	1,280–2,560

*As determined by polymerase chain reaction (PCR) analysis. †Antibody titers to *E equi* or *B burgdorferi* were determined by IFA staining or ELISA methods, respectively. ‡Strain BDS. §Strain NCH-1. NA = Not applicable.

production to OspF, p37, p39, or p93 antigens. In our studies, one or more of these proteins were recognized immunologically by some horses suspected of having borreliosis and were, therefore, considered to be appropriate indicators of past or current *B burgdorferi* infections. As in the laboratory diagnosis of EGE,[13] immunoblot analysis is suitable for confirming clinical diagnosis of borreliosis in horses and can be used as an adjunct method with other antibody tests. However, immune responses vary among horses and differences in patterns of reactivities in immunoblot analysis should be considered when interpreting results.

Although the horses in our study were not vaccinated for borreliosis, use of vaccines to whole cell *B burgdorferi* may complicate interpretation of serologic test results. By contrast, if vaccines directed to OspA of *B burgdorferi* become widely used, they should only stimulate antibody production to OspA antigen. Therefore, if vaccines directed to whole cell *B burgdorferi* have not been used, then serum reactivity to the other specific immunodominant antigens, such as OspB, OspF, p37, and p93, can be viewed as evidence of natural exposure to *B burgdorferi*.

Use of recombinant antigens of *B burgdorferi* in an ELISA is acceptable for initial screening of equine serum samples for antibodies to this bacterium. Inclusion of whole cell *B burgdorferi* in ELISA or IFA staining methods can result in false-positive reactions primarily because of nonspecific reactivity of antibody to flagellin, the 60 kd protein, or to other common antigens such as heat shock proteins (60 to 70 kd range). The use of highly specific recombinant antigens, such as OspB, OspC, p41-G, OspE, or OspF, in class-specific or polyvalent ELISA has aided clinical diagnosis of borreliosis in people[10,16] and borreliosis in dogs and horses.[15] Important differences are observed, however, in antibody reactivity to certain antigens. For example, OspC is a reliable marker for early and late human Lyme borreliosis but appears to be a poor indicator of borreliosis in horses. When in horses, *B burgdorferi* may not express OspC as commonly as it apparently does during early and late stages of human Lyme borreliosis. We conclude that the use of p22 and OspE antigen in an ELISA is likewise of limited value in detecting antibodies to *B burgdorferi* in equine serum samples.

Consistent with earlier reports,[10,12,15,19] there was no cross-reactivity between *E equi* or *E risticii* antibodies and *B burgdorferi* whole cell or recombinant antigens. Moreover, in comparisons of IFA and ELISA results for *E equi* and *B burgdorferi* infections, respectively, findings often differed, regardless of the *B burgdorferi* antigens used. Although patterns of antibody reactivity in our study indicated no cross-reactivity, potential exists for infrequent false-positive reactions if ELISA with whole cell *B burgdorferi* antigen is used, because there are some shared antigens among these unrelated bacteria (ie, heat shock proteins).[26] The use of an ELISA with highly specific recombinant antigens of *B burgdorferi* or immunoblot analyses with whole cell *B burgdorferi* or *E equi* antigens (including human isolates) provide results that are easier to interpret. These antibody assays can be used to help separate borreliosis in horses from EGE, even when there are antibodies to both causative agents in a given serum sample. Results of our study indicate that if horses are suspected of having either tick-associated illness, additional laboratory analyses for antibodies are warranted to determine whether there has been exposure to either or both etiologic agents.

[a]Kirkegaard & Perry Laboratories, Gaithersburg, Md.
[b]Bio-Rad Laboratories, Hercules, Calif.

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