Antibodies to granulocytic ehrlichiae in cattle from Connecticut

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Enzyme-linked immunosorbent assays (ELISA) with a purified recombinant 44-kDa protein and indirect fluorescent antibody (IFA) staining methods incorporating whole-cell antigens of the human granulocytic ehrlichiosis (HGE) agent were used to detect antibodies to Ehrlichia phagocytophila genogroup organisms in cattle sera. The cattle lived in tick-infested areas of Connecticut, USA and were healthy at the times blood samples were collected in 1990, 1999 and 2000. Of the 339 serum samples analysed, 40 (12%) and 15 (4%) were positive by ELISA and IFA, respectively. Western immunoblots of a subset of sera verified antibody reactivity of six serum samples, positive by ELISA with titres of 640–2560, to a protein with a molecular mass of c. 44 kDa. Although seroprevalence rates were low, cattle were exposed to the HGE agent at different sites and should be monitored for anaemia, leukopenia or thrombocytopenia, especially if there is evidence of unexplained decreased milk production. Different serological testing methods should be used to detect immunoglobulins.

Introduction

Hard-bodied ticks, Ixodes pacificus, I. scapularis and I. ricinus, transmit bacteria of the Ehrlichia phagocytophila genogroup to mammals in the USA and Europe. E. phagocytophila was first recognised as the causative agent of tick-borne fever in goats, cattle and sheep in Europe [1–5]. Subsequently, E. equi and the agent of human granulocytic ehrlichiosis (HGE) were found infecting horses, dogs, white-tailed deer (Odocoileus virginianus) and man in the USA [6–16]. Based on analyses of 16S rRNA gene sequences, these pathogens are nearly identical [14–16] with 99.8% homology and are probably strains of the same species.

Fever, leukopenia and thrombocytopenia are common signs of illness in human patients [6] and cattle [17]. In European studies of infected cows, decreased milk production was noted [17]; E. phagocytophila was detected in leucocytes from milk samples obtained during the acute phase of disease [18]. Although there are numerous reports on HGE in the USA [6, 7, 14–16, 19], little is known about patterns of immune responses, antibody production and seroprevalence of granulocytic ehrlichial infections in cattle. Preliminary studies revealed that cattle living in tick-infested areas of Connecticut, USA, had antibodies to the HGE agent. The purposes of the study reported here were to determine seroprevalence rates for healthy cattle living at different sites in Connecticut and to evaluate different serological testing methods for detecting antibodies.

Materials and methods

Sources of serum samples

During 1999 and 2000, 196 serum samples were obtained by veterinarians from 196 clinically normal dairy or beef cattle living in 19 towns (6 counties), primarily in eastern and southern Connecticut where I. scapularis ticks abound and where HGE or equine granulocytic ehrlichiosis (EGE) have been reported [10–12, 20]. Another 143 serum samples, obtained in 1990 from 143 dairy cattle in Durham and Lyme, Connecticut, were selected from archived collections for analyses. Although the majority of animals were Holstein, other breeds (Brown Swiss, Ayrshire, Guern-
sey and Jersey) were included in the study. All serum samples were obtained from cattle that had grazed in pastures and were kept at −60°C (−76°F) at the Connecticut Agricultural Experiment Station until required for analyses. Serum samples were collected from 15 clinically normal Holstein cattle during the autumn of 1996 in Vermont, USA, a state where *I. scapularis* is rare and where EGE and HGE are not known to occur, and were used as negative controls in serological testing.

**Indirect fluorescent antibody (IFA) staining methods**

Materials and procedures used previously [21] were applied in analyses of serum samples to measure total immunoglobulins (Ig) to the NCH-1 strain of the HGE agent. This strain, originally isolated from a human patient in Nantucket, MA, USA, and grown in human promyelocytic leukaemia cell (HL-60, American Type Culture Collection CCL-240) cultures, has been used extensively in serological testing [11, 19, 21, 22]. Serum samples were diluted to 1 in 80 in phosphate-buffered saline (PBS; pH 7.2) and placed in 12-μl volumes over each antigen preparation on glass microscope slides. After incubation for 30 min and washing with PBS, a 1 in 5 dilution of fluorescein isothiocyanate-conjugated goat anti-bovine IgG (heavy and light chain specific) antibodies (Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA) was used at a protein concentration of 2.5 μg/ml. The second antibody was commercially prepared (Kirkegaard & Perry Laboratories) and consisted of a 1 in 5000 dilution of affinity-purified, horseradish peroxidase-labelled goat anti-bovine antibodies (heavy and light chain specific) (Kirkegaard & Perry Laboratories) to calculate net absorbance values. For colour production, 60 μl of commercially prepared 2, 2′-azino-di (3-ethyl-benzthiazoline sulphonate) substrate (Kirkegaard & Perry Laboratories) were introduced into each plate well. A microplate spectrophotometer measured absorbance values (optical densities; OD) of each preparation at 414 nm [23]. A net absorbance value is the difference in OD readings between preparations with or without antigen (i.e., PBS control) for each serum dilution. Critical regions for positive results were determined by statistical analyses (mean ± 3 SD) of net OD values for each set of negative control sera. Net OD values of 0.31, 0.25 and 0.21 were used for serum dilutions of 1 in 160, 1 in 320 and ≥1 in 640, respectively. The same positive and negative serum controls used in IFA analyses were included in each plate to check antigen, conjugate, MBP and PBS. Positive test sera were reanalysed to determine titration endpoints and to assess reproducibility of results.

Analyses were conducted to determine the specificity of IFA and ELISA methods. In addition to the serum samples from Vermont cattle, the panel included four serum samples from cattle vaccinated against *Leptospira interrogans* serovars (Canicola, Grippotyphosa, Icterohaemorrhagiae and Pomona), one adult Jersey cow serum sample with *Brucella* antibodies and three adult Holstein cow serum samples from Connecticut with antibodies to *Borrelia burgdorferi*. These sera were selected from archived collections at the University of Connecticut and were known to contain antibodies homologous to the corresponding antigens by agglutination or immunoblotting methods.

**Immunoblotting**

Western blot analysis was used to identify banding patterns and to check results for 19 serum samples, which were positive or negative by IFA or ELISA. It was particularly important to confirm serological reactivity of positive sera to the p44 antigen of the HGE agent, a specific marker for HGE and EGE infections. Generally, immunoblotting procedures used before [19, 21] were employed in analyses of serum samples from cattle. Lysates of HL-60 cells infected with the NCH-1 strain of the HGE agent were included as antigen. The second antibody was commercially prepared (Kirkegaard & Perry Laboratories) and consisted of a 1 in 1000 dilution of alkaline phosphatase-labelled F(ab′)2 anti-bovine antibodies in PBS. The blocking reagent was PBS containing non-fat dried milk 5%. Incubation, washing procedures and methods of detecting specific bound antibodies were the same as used before. Analyses contained a positive deer serum sample [11] to verify antigen and conjugate reactivity and five negative control serum samples from Vermont cattle.
**DNA analysis**

Materials and methods used to detect the DNA of the HGE agent in whole blood samples from human patients [19], white-tailed deer [11] and horses [12, 13] were applied in analysis of nine EDTA-treated whole blood samples from nine cattle. Blood samples were collected from these animals in tick-infested areas of five towns during December 1999 and February 2000. All serum samples from these animals contained antibodies to the HGE agent, as determined by IFA analysis (titres 80–640), ELISA (titres 160–2560), or immunoblotting. Buffy coat samples from whole blood specimens were selected for PCR analyses. The primers used were those described previously [11, 26] and are based on the sequence of the hge-44 gene that encodes the immunodominant 44-kDa protein of the NCH-1 strain of the HGE agent. Positive (i.e., infected HL-60 cells) and negative controls were included in all PCR analyses to verify reagent reactivity and monitor for false positive results.

**Statistical analyses**

The z-test with Yates's correction (Sigma Stat, SPSS, Chicago, IL, USA) was used to compare proportions of positive results for IFA staining and ELISA methods.

**Results**

Analyses of 339 cattle serum samples from widely separated sites in Connecticut detected antibodies to the HGE agent during different seasons (Table 1). Overall percentages of positive serum samples determined by IFA (4%) and ELISA (12%) methods were statistically significant ($z = 3.698, p < 0.001$). By ELISA, seroprevalence was highest (22–29%) during December 1990 and 1999.

Western immunoblotting was performed to determine banding patterns for positive cattle serum samples and to compare results with those obtained by IFA and ELISA methods. Six serum samples, positive by ELISA and with antibody titres of 640–2560, were included along with 13 serum samples negative by ELISA. Few bands were observed for the seropositive samples (Fig. 1), but there was distinct reactivity to the 44-kDa protein of the HGE agent in five serum samples (lanes 2, 4, 10, 11 and 18) and a very weak reaction to this protein for the remaining serum sample (lane 13). There was additional antibody reactivity to proteins with molecular masses of c. 110 kDa for two serum samples, a pattern similar to that observed for the positive deer serum sample used as a positive control.

**Table 1. Results of analyses for total antibodies to whole-cell or recombinant p44 antigens of the HGE agent in cattle serum samples in Connecticut**

<table>
<thead>
<tr>
<th>Sampling counties</th>
<th>period</th>
<th>Total serum samples tested</th>
<th>IFA</th>
<th>ELISA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fairfield</td>
<td>Dec. 1999</td>
<td>12</td>
<td>2 (17)</td>
<td>1 (8)</td>
</tr>
<tr>
<td></td>
<td>Feb. 2000</td>
<td>12</td>
<td>2 (17)</td>
<td>1 (8)</td>
</tr>
<tr>
<td>Litchfield</td>
<td>Aug. 1999</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Aug. 1990</td>
<td>97</td>
<td>1 (1)</td>
<td>5 (5)</td>
</tr>
<tr>
<td></td>
<td>July 1999</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Sep. 1999</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Mar. 2000</td>
<td>12</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>New Haven</td>
<td>Dec. 1999</td>
<td>12</td>
<td>2 (17)</td>
<td>3 (25)</td>
</tr>
<tr>
<td>New London</td>
<td>Dec. 1990</td>
<td>46</td>
<td>4 (9)</td>
<td>10 (22)</td>
</tr>
<tr>
<td></td>
<td>Sep. 1999</td>
<td>61</td>
<td>1 (2)</td>
<td>6 (10)</td>
</tr>
<tr>
<td></td>
<td>Dec. 1999</td>
<td>24</td>
<td>2 (8)</td>
<td>7 (29)</td>
</tr>
<tr>
<td></td>
<td>Mar. 2000</td>
<td>12</td>
<td>0</td>
<td>2 (17)</td>
</tr>
<tr>
<td>Windham</td>
<td>July 1999</td>
<td>5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Sep. 1999</td>
<td>6</td>
<td>0</td>
<td>1 (17)</td>
</tr>
<tr>
<td></td>
<td>Dec. 1999</td>
<td>36</td>
<td>1 (3)</td>
<td>4 (11)</td>
</tr>
<tr>
<td><strong>Totals</strong></td>
<td></td>
<td><strong>339</strong></td>
<td><strong>15 (4)</strong></td>
<td><strong>40 (12)</strong></td>
</tr>
</tbody>
</table>

*Whole-cell antigens (infected HL-60 cells) were used in IFA analysis and a recombinant p44 antigen was incorporated in an ELISA.
control (lane 22). All five serum samples from Vermont cattle were negative.

Results of IFA staining, Western immunoblotting and ELISA were in complete agreement for 10 (53%) of 19 cattle serum samples (Table 3). Results for the remaining nine serum samples were discordant. With immunoblotting as a standard, results for 13 (68%) of 19 serum samples were in agreement with those of IFA analysis, whereas blot findings for 15 (79%) of 19 serum samples supported the ELISA results. Differences in these percentages were statistically insignificant ($z = 0.401$, $p = 0.689$).

Specificity tests were conducted to determine if four serum samples from cattle vaccinated against and having antibodies to \textit{L. interrogans} serovars, one serum sample containing antibodies to \textit{Brucella} species, or three serum samples from cattle with immunoglobulins to \textit{B. burgdorferi} cross-react with whole-cell or recombinant p44 antigens. Results of IFA analyses and ELISA were negative for all specimens, as were 15 negative control serum samples from Vermont cattle.

Buffy coat cells of nine EDTA-treated whole blood samples from nine cattle that had serum antibodies to the HGE agent by one or more assay methods were tested for the DNA of the HGE agent. Results of PCR analyses were negative.

**Discussion**

Cattle living in widely separated tick-infested areas of Connecticut, where granulocytic ehrlichiosis is reported in horses and man, contained antibodies to the HGE agent. The cattle grazed in open pastures, where \textit{I. scapularis} ticks are not usually abundant, but some pastures border forests where these ticks and deer are abundant. In Switzerland [5] seroprevalence of ehrlichial infections increased from 16% to 63% when cattle grazed in pastures during summer months. In California [27], 0.18% of 2725 healthy cattle had antibodies to \textit{E. equi}. Furthermore, cattle in the present study normally had low concentrations of antibodies to the HGE agent during late summer, autumn and winter and, like horses [13, 21], were probably bitten earlier by nymphal or female ticks during periods extending over several weeks or months.

Results of DNA analyses ofuffy coat preparations of whole blood specimens, obtained from cattle that had antibodies to the HGE agent, were negative. In a study of cattle inoculated with \textit{E. equi} or the HGE agent [27], ehrlichiae were not observed in leucocytes; PCR analyses were negative. In studies of horses [12, 13],

**Table 2.** Frequency distributions of antibody titres and geometric mean titres for cow serum samples with antibodies to the HGE agent in Connecticut

<table>
<thead>
<tr>
<th>Assay method</th>
<th>Total positive</th>
<th>80</th>
<th>160</th>
<th>320</th>
<th>640</th>
<th>1280</th>
<th>2560</th>
<th>Geometric mean titre</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFA</td>
<td>15</td>
<td>9 (60)</td>
<td>4 (27)</td>
<td>0</td>
<td>2 (13)</td>
<td>0</td>
<td>0</td>
<td>127</td>
</tr>
<tr>
<td>ELISA</td>
<td>40</td>
<td>0</td>
<td>16 (40)</td>
<td>9 (23)</td>
<td>8 (20)</td>
<td>4 (10)</td>
<td>3 (8)</td>
<td>374</td>
</tr>
</tbody>
</table>

*Starting serum dilutions for IFA and ELISA were 1 in 80 and 1 in 160, respectively.

Note: geometric means are for positive results only.

**Table 3.** Concordance or discordance of results of IFA, Western immunoblotting (WB) and an ELISA incorporating whole-cell or recombinant p44 antigen for detection of antibodies to the HGE agent in serum samples from 19 cows

<table>
<thead>
<tr>
<th>Assay results*</th>
<th>IFA</th>
<th>WB</th>
<th>ELISA</th>
<th>Number of cattle</th>
</tr>
</thead>
<tbody>
<tr>
<td>+++</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>5</td>
</tr>
<tr>
<td>++</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>3</td>
</tr>
<tr>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>1</td>
</tr>
<tr>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>5</td>
</tr>
</tbody>
</table>

*Results were positive for antibodies at titres $> 80$ (IFA), 100 (WB) or $> 160$ (ELISA).
DNA analyses were less helpful when blood specimens were obtained several days after onset of illness. As antibody titres rose with time, prevalence of DNA-positive specimens in horses declined. In the present study, cattle were healthy and precise times of ehrlichial infections were unknown. Blood samples may have been obtained beyond periods of acute infection when immune responses of the animals were sufficient to suppress direct PCR detection of the pathogen in blood. Alternatively, cattle in the present study may not be susceptible to infection by *E. equi* or the HGE agent [27].

These findings indicate that a polyvalent ELISA incorporating a recombinant p44 antigen is suitable for detecting antibodies to the HGE agent. Although results were not totally concordant with those obtained by IFA or immunoblotting methods incorporating whole-cell antigens, ELISA findings were sensitive, highly specific and reproducible. This agrees with earlier work [24, 25]. Discrepant findings in seroprevalence rates for the three methods were probably due to differences in initial serum samples tested (dilutions of 1 in 80 for IFA, 1 in 100 for blots and 1 in 160 for ELISA) and other technical factors (i.e., concentrations of different antigen preparations and conjugated antibodies). Similar differences in assay sensitivities have been reported in analyses of horse and dog serum samples [25]. If possible, different methods of antibody detection should be used to determine seroprevalence rates for cattle, other domesticated animals and human subjects. However, the automated and easily standardised ELISA procedures are more practical for initial testing of large numbers of serum samples.

Application of Western blot procedures revealed few bands when cattle serum samples were tested with lysed whole-cell antigen of the HGE agent. However, the recognition of the p44 antigen, a sensitive and specific marker for *E. phagocytophila* genogroup infections in man [24], dogs [25] and horses [25], was important because it verified results of ELISA. Blot results compared more favourably with ELISA findings than IFA results, although differences were statistically insignificant. Moreover, the presence of few bands for cattle serum samples closely parallels immunoblot results for deer serum samples [11]. Bovid and cervid antibody responses to HGE antigens, unlike humoral responses of horses [21], dogs [28] and man [19], appear to be more limited. Nonetheless, Western blot analyses can be used as an alternative method to determine if cattle were exposed to the HGE agent.

Seroprevalence rates for granulocytic ehrlichial infections in cattle were lower than those calculated for dogs, horses and man [20, 25], regardless of the antibody detection method used. This may be due, in part, to sampling bias when obtaining blood samples from healthy cattle. There were no overt signs of disease in these animals or indications of unexplained decreased milk production. Also, tick exposure rates for cattle were probably lower than those of dogs, horses or human subjects who live in or more frequently enter tick-infested, forested areas of Connecticut. Asymptomatic seroconversion is reported for cows and sheep [27]. In a low number of cattle inoculated with *E. equi* or the HGE agent in California [27], no abnormal clinical or haematological findings were observed. However, similar studies in Switzerland [17] revealed that cows inoculated with *E. phagocytophila* became ill and had fever, respiratory problems, leukopenia, thrombocytopenia and decreased milk production. Although *E. equi* and the HGE agent appear to be of low pathogenicity for some breeds of cattle [27], there is a need to monitor cattle in heavily tick-infested areas of north-eastern and upper mid-western USA and in Europe, particularly if there is evidence of decreased milk production in herds. If cattle are diagnosed with fever, lethargy, thrombocytopenia, anaemia or leukopenia, blood samples should be screened by PCR analyses and introduced into HL-60 or *I. scapularis* cell cultures [29, 30] to determine if *E. phagocytophila* genogroup organisms are causing infection.

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