Reactivity of Human Sera to Different Strains of Granulocytic Ehrlichiae in Immunodiagnostic Assays

Louis A. Magnarelli, Jacob W. IJdo, J. Stephen Dumler, Robert Heimer, and Erol Fikrig

Sera from 35 patients diagnosed with human granulocytic ehrlichiosis in Connecticut were tested by indirect IFA staining methods with 5 strains of *Ehrlichia equi* or the human granulocytic ehrlichiosis agent to assess the suitability of different strains in laboratory analyses. Antigens included horse-derived infected neutrophils (MRK and BDS strains) and human isolates cultured in human promelocytic leukemia cells (NCH-1, RCH, and Webster). Of 35 sera, 23 (65.7%) reacted to all 5 strains. Seropositivity was highest (97.1%) in assays that contained the MRK strain from California and lowest (71.4%) in tests with the NCH-1 strain from Nantucket, Massachusetts. In parallel testing of 32 sera with the NCH-1 strain by indirect IFA and Western blot analyses, results were concordant for 30 samples (93.8%). All strains of ehrlichiae can be used in IFA analyses for antibody detection, but assay sensitivity varied with the strain used.

Human and equine infections caused by a granulocytic bacterium, *Ehrlichia equi* or a closely related agent, occur in the northeastern and upper midwestern United States and California [1–5]. A closely related organism, *Ehrlichia phagocytophila*, causes tickborne fever in cattle and sheep in Europe [4] and is probably also a human pathogen. Having many common antigens and a high degree of genetic relatedness, as determined by 16S rRNA gene analyses, these ehrlichiae are recognized as members of the *E. phagocytophila* genogroup. The likely tick vectors in or near forested areas of the Northern Hemisphere are *Ixodes pacificus*, *Ixodes ricinus*, and *Ixodes scapularis* [4, 6].

Diagnosis of human granulocytic ehrlichiosis (HGE) is based on evidence of leukopenia or thrombocytopenia with a mild to moderate increase in serum hepatic transaminase activity, the presence of ehrlichial inclusion bodies (i.e., morulae) in granulocytes, the detection of ehrlichial DNA in blood, a positive culture of the pathogen, or a positive serologic test for antibodies [3, 4, 7–9]. Routine, cost-effective laboratory diagnosis of HGE relies most heavily on serum antibody detection. IFA staining methods are frequently used for initial screening [4, 9, 10], while Western blot analyses are appropriate for confirming IFA results [4, 9].

Advances made in growing the HGE agent in human promyelocytic leukemia cell (HL-60) cultures [7, 8] and in recovering isolates from horses [2, 4, 11] have yielded a variety of strains, some of which are antigenically different [11]. Preliminary studies of dog sera revealed differences in the reactivity of 2 sera to *E. equi* (MRK strain) and an HGE agent (NCH-1 strain) in IFA staining methods and Western blot analyses [12]. The present study was conducted to determine if serum antibody test results for humans also vary with the use of different North American strains of *E. equi* or the HGE agent and if polymerase chain reaction (PCR) test results correlate with antibody presence.

Materials and Methods

Human sera were obtained during all seasons in 1995 and 1996 as a part of a statewide surveillance program in Connecticut on emerging infectious diseases. Physicians obtained sera 4–381 days after onset of illness from 35 patients who lived in or entered tick-infested areas and had developed acute, febrile illnesses with leukopenia or thrombocytopenia. All patients had a high probability of having HGE. The majority of sera were collected 2–10 weeks after onset. Sera were submitted to the Connecticut Department of Health, and, when leukocyte or blood platelet counts were abnormally low, specimens were shipped to the Connecticut Agricultural Experiment Station for antibody analyses. The sera chosen for this study contained ehrlichiosis antibodies, as detected by IFA staining methods with different strains of antigen and (or) Western blot analyses and were analyzed for total immunoglobulins [10, 11].
Results

Previous studies [12, 13] showed close agreement in results of both assays. Acute-phase, whole blood samples, available from 16 patients, were analyzed by PCR methods [8] to detect the *E. equi* DNA.

Five strains of *E. equi* or the HGE agent were selected for comparative studies. *E. equi* MRK was isolated from a horse in California [11] and had been maintained in horses by subsequent inoculations. The BDS strain, a Minnesota isolate, was recovered from a horse inoculated with infected human blood. The NCH-1, RCH, and Webster strains were isolated from the blood of HGE patients in Massachusetts, Connecticut, and Wisconsin [11, 13], respectively, and grown in HL-60 cell cultures. These strains had been passed in cultures 120, 19, and 6 times, respectively. Two cultures of the NCH-1 strain, maintained for 2 months (30 passages) or 12 months (120 passages), were available for comparative analyses. Antigens consisted of infected horse neutrophils (~40% infected cells per microscope field) or HL-60 cells (≥60% infected cells per field) fixed to glass microscope slides by cold acetone treatment. Sera also were tested by Western blot analyses [13] with the NCH-1 strain of the HGE agent. All analyses contained positive and negative serum controls [10, 13]. Since slides contained separate strains of antigen, tests on the reproducibility of antibody titers were conducted to assess assay variability.

The Wilcoxon signed rank test [14], a nonparametric statistical procedure, was used to determine whether the use of a particular strain in IFA staining methods resulted in significantly improved assay performance, compared with results for a local Connecticut strain (RCH). Results were based on the statistic T, the absolute value of the smaller of the two sums of ranks, where n was defined as the number of nonzero difference values for antibody titers. Analyses were performed on results of paired sera at the level of significance of *P* < .01.

### Table 1. Reactivity of 35 human sera from Connecticut to different strains of *Ehrlichia equi* or the HGE agent by polyvalent indirect IFA staining methods.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Site</th>
<th>Host</th>
<th>No. (%) positive</th>
<th>Geometric mean</th>
<th>Antibody titers, range</th>
</tr>
</thead>
<tbody>
<tr>
<td>BDS</td>
<td>Minnesota</td>
<td>Human/horse</td>
<td>29 (82.9)</td>
<td>188</td>
<td>80–1280</td>
</tr>
<tr>
<td>MRK</td>
<td>California</td>
<td>Horse</td>
<td>34 (97.1)</td>
<td>191</td>
<td>80–5120</td>
</tr>
<tr>
<td>NCH-1</td>
<td>Massachusetts</td>
<td>Human</td>
<td>25 (71.4)</td>
<td>247</td>
<td>80–1280</td>
</tr>
<tr>
<td>RCH</td>
<td>Connecticut</td>
<td>Human</td>
<td>29 (82.9)</td>
<td>273</td>
<td>80–2560</td>
</tr>
<tr>
<td>Webster</td>
<td>Wisconsin</td>
<td>Human</td>
<td>28 (80)</td>
<td>215</td>
<td>80–2560</td>
</tr>
</tbody>
</table>

* Antibody titers of ≥1:80 were considered positive by IFA staining methods.
with uninfected horse neutrophils by IFA. There were sufficient amounts of sera available for 32 samples for parallel tests with homologous NCH-1 antigen. Of 32 sera, 25 (78.1%) were positive by IFA and Western blot analyses, and 5 (15.6%) were negative in both tests. Discordant results were recorded for the remaining 2 sera (6.3%), both of which were positive in immunoblots and nonreactive by IFA staining methods. Thus, when the NCH-1 antigen was used in both assays, immunoblotting had greater assay sensitivity (84.4% positive) than IFA (78.1%).

Acute-phase, whole blood samples from 16 subjects whose convalescent-phase sera contained antibodies were screened by PCR analyses. Five samples (31.3%) contained the DNA of *E. equi*. Of these 5 sera, 4 were positive by IFA staining methods to all 5 strains and by Western blot analysis; the other serum was negative to all strains except the MRK antigen. Confirmed infections were documented in May, July, November, and December. The remaining 11 sera, matched with blood samples that had no detectable DNA, contained antibodies to at least 3 strains, including the RCH antigen.

**Discussion**

Antigens from all 5 strains can be used for detecting serum antibodies by IFA staining methods. Results for antibody titers within tests with a given antigen were highly reproducible. There were no significant differences in assay performance among tests with different antigens when the RCH strain was used as a standard. However, the seropositivity rate for the assay with the NCH-1 antigen was lower than rates calculated for the other strains and was significantly different from the strain (MRK) with the highest rate. Western blot analysis with the NCH-1 strain had higher assay sensitivity than did IFA with this antigen.

Differences in the reactivities of sera from HGE patients to various isolates of the HGE agent have been reported [11]; 2 sera from a Wisconsin patient reacted weakly or not at all with a New York State isolate (NY-8). Immunoblots with the NY-8 antigen revealed fewer and weaker bands than the Wisconsin isolates. Similar observations were made in the analyses of dog sera in Connecticut [12]. Clearly, antigenic differences exist among HGE isolates, and such diversity, in some instances, might affect the sensitivity of antibody tests. Different antigens of the HGE agent, such as the MRK and BDS strains, have been used successfully in laboratory diagnosis [9–11], but further work is needed to identify the strains (local or not) that will yield the most accurate results.

When infected horse neutrophils were used in IFA, seropositivity rates were higher than those obtained for strains cultured in HL-60 cells. Although the latter are easier to examine by fluorescence microscopy [15], we suspect that the use of higher densities of infected HL-60 cells, when combined with low concentrations of serum antibodies, might occasionally result in a loss of assay sensitivity because of formation of lower amounts of detectable antibody-antigen complex. Under these conditions, there can be insufficient staining with the fluorescein-labeled antibodies. Moreover, ehrlichiae in a horse may be antigenically different from those maintained in HL-60 cells. Therefore, it is important to determine if structural or molecular variations exist among strains from different antigen sources and to determine which strains are more desirable for immuno-diagnostic testing.

Immunoblotting was helpful in interpreting and confirming IFA findings. The infrequent discrepant findings were attributed to variable reactivity of low-titered sera and possible decreases in antibody concentrations in some samples because of repeated thawing and freezing. Studies have shown that IFA staining methods are acceptable for confirming HGE infections [9–13, 15]. However, whenever possible, Western blot analyses should be utilized to further assess the performance of IFA staining methods. Further, we detected ehrlichial DNA in human blood, but the overall rate of positivity was lower than antibody test results. Most sera were obtained ≥2 weeks after onset of illness. We suspect that the PCR methods are probably more suitable for testing acute blood samples rather than convalescent specimens and suggest that the latter be analyzed for antibodies.

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**References**


