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Inter- and Intralaboratory Comparison of *Ehrlichia equi* and Human Granulocytic Ehrlichiosis (HGE) Agent Strains for Serodiagnosis of HGE by the Immunofluorescent-Antibody Test

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Human granulocytic ehrlichiosis (HGE) is usually diagnosed by immunofluorescent antibody (IFA) serology with *Ehrlichia equi*-infected neutrophils or HGE agent-infected cultured HL60 cells. The HGE agent and *E. equi* are antigenically diverse, and interpretation of serologic results is also often variable. Thus, we investigated the sensitivity and specificity of various HGE agent and *E. equi* antigens used for IFA diagnosis by three different laboratories. Serum samples from 28 patients with well-characterized HGE and 9 patients with suspected HGE who were investigated by PCR, blood smear examinations, and serology were used, along with 9 serum samples from patients with other rickettsial and ehrlichial infections. Each serum sample was tested with up to 10 different antigen preparations. Overall, qualitative IFA results agreed in 70% of the samples. Titers among antigens were similar ($r = 0.89$ to 0.96), but titers of individual samples varied by fourfold or more in 5 of 81 (6%) of the serum samples. Sensitivity ranged from 100% to 82%, and specificity varied from 100% to 67%, but these differences were not significant, even among those tested in the same laboratory or between two different laboratories. Antibodies were detected in 14 to 44% of acute-phase sera from confirmed HGE patients. Most false-positive reactions resulted with *Ehrlichia chaffeensis*; when these sera were excluded, the specificity of most antigens was 91 to 100%. These data indicate that IFA results often agree and that IFA is useful for diagnosis of HGE in convalescence. However, without further standardization, variability among serologic tests using *E. equi* and HGE agent isolates for diagnosis of HGE will occasionally provide discrepant results and confound diagnosis.

The agent of human granulocytic ehrlichiosis (HGE) has recently been recognized as an emerging, tick-borne infectious agent that causes disease throughout the United States and Europe (22). Infection with the HGE agent is mild to severe or even fatal (3). The clinical manifestations and laboratory findings of HGE are nonspecific and often lead to misdiagnosis. HGE may be confirmed by examination of a peripheral blood smear, culture, or PCR that detects HGE agent DNA in acute-phase blood (3, 6, 9). The indirect immunofluorescent antibody test (IFA) is the most frequently used diagnostic tool. However, diagnostic confirmation by IFA is often retrospective, since most HGE patients do not have specific antibodies in acute-phase sera (3, 5, 12, 19). Currently, a patient is diagnosed with HGE when the appropriate history and clinical manifestations are observed and a fourfold increase in antibody titer between acute- and convalescent-phase sera is detected.

A definitive diagnosis of HGE is achieved when serologic and PCR tests are positive and is further supported by blood smear analysis (1, 3). On occasion, these diagnostic tests are contradictory, confusing the diagnosis. A number of HGE patients, who have a negative PCR, are later found to seroconvert or, rarely, vice versa (1, 11). The antigens used for detection of HGE agent antibodies by IFA were initially *Ehrlichia equi*-infected neutrophils derived from experimentally infected horses (5). Since the recent cultivation of the HGE agent in HL60 cells (pending patent [5a]), different HGE agent and *E. equi* isolates can now be used as IFA antigens (2, 9). The discovery that isolates of the HGE agent and *E. equi* are antigenically diverse suggests that differences in the sensitivity and specificity of the antigens used for IFA may exist and may help explain some of the variability seen in diagnostic testing (2, 15, 18). Increasingly, sera for HGE diagnosis are submitted to reference laboratories that use various antigens and methods for which reproducibility has not been assessed. Thus, we investigated the sensitivity and specificity of and agreement among various HGE agent and *E. equi* antigens used by three different laboratories for the serodiagnosis of HGE by IFA.

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MATERIALS AND METHODS

Sample selection. Archived serum samples from 37 patients with suspected HGE were chosen for IFA testing by using different isolates of the HGE agent and *E. equi* as an antigen. Of these, 28 patients were proven (HGE confirmed) (1, 3) and 9 were never proven (non-HGE) to have HGE. Patients had presented with compatible exposure history along with typical clinical and laboratory findings that included fever, headache, malaise, myalgia, leukopenia, thrombocyto-

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TABLE 1. Antigen preparations used among the JHU, NYMC, and MN laboratories

Antigen	Laboratory testing site	Origin of isolate	Passage history
E. equi MRK in horse neutrophils	JHU	Calif.	In vivo
E. equi MRK in HL60 cells	JHU	Calif.	16 in vitro
HGE agent Webster strain	JHU	Wis.	8 in vitro
HGE agent Spooner strain	JHU	Wis.	7 in vitro
HGE agent NY-3 strain	NYMC	N.Y.	1 in vitro
HGE agent NY-6 strain	NYMC	N.Y.	1 in vitro
HGE agent NY-8 strain	JHU, NYMC	N.Y.	2, 1 in vitro
HGE agent HGE-2 strain	MN	Minn.	<20 in vitro

penia, anemia, and elevated serum hepatic transaminases (3). All of the patients were previously tested for HGE by blood smear examination and/or PCR. Twenty-five of the patients were confirmed to have HGE by a positive blood smear ($n = 16$) and/or a positive PCR ($n = 19$). Three patients were negative by these diagnostic methods; however, the illness was most consistent with HGE and occurred in a region in which HGE was highly endemic, and each patient had a therapeutic response to doxycycline. The nine patients with suspected HGE were negative by all three diagnostic tests, and the final clinical diagnosis was not HGE. Acute- and convalescent-phase paired serum samples from 35 patients and unpaired convalescent-phase serum samples from 2 patients were examined. Two patients were from N.Y., and the remaining patients were from the upper Midwest. To challenge the IFA systems, in addition to the serum samples from nine non-HGE patients, three acute- and convalescent-phase paired serum samples and one unpaired convalescent-phase serum sample from patients with PCR- and/or IFA-confirmed human monocytic ehrlichiosis (HME [or *E. chaffeensis* infection]) and two unpaired convalescent-phase serum samples from patients with serologically confirmed Rocky Mountain spotted fever (RMSF [*Rickettsia rickettsii* infection]) and scrub typhus (*Orientia tsutsugamushi* infection) were included in the testing. A total of 81 serum samples were tested. Sera were coded, and aliquots were submitted to each laboratory for blinded testing.

Interlaboratory comparisons. To compare the antigens used in different laboratories, the archived sera were tested at The Johns Hopkins University School of Medicine in Baltimore, Md. (JHU); the Westchester County Medical Center and New York Medical College, Valhalla, N.Y. (NYMC); and the University of Minnesota School of Medicine, Minneapolis, Minn. (MN). All 37 patients with confirmed or suspected HGE and all 6 patients with other rickettsial diseases were tested with all antigens from the JHU laboratory and with the NY-6 and NY-8 antigens from the NYMC laboratory. Thirty-one of the suspected HGE patients and 4 of the patients with other rickettsial diseases were tested with the NY-3 isolate from the NYMC laboratory. The HGE-2 isolate was employed by the MN laboratory to test serum samples from a total of 34 of the suspected HGE patients (only 21 patients were tested for both immunoglobulin M [IgM] and IgG) and from 5 of the patients with other rickettsial diseases.

IFA antigens. A total of 10 different antigen preparations made from eight different strains of either *E. equi* or the HGE agent were used among the three different laboratories (Table 1). Antigen preparations used by the JHU laboratory included two different preparations of *E. equi* MRK-infected horse neutrophils (courtesy of John Madigan, University of California, Davis), and the following strains cultivated in HL60 cells: *E. equi* MRK, Webster, and Spooner and the NY-8 strain of the HGE agent (2). The antigen preparations used by the NYMC laboratory included the NY-3, NY-6, and NY-8 strains of the HGE agent, all isolated from patients from Westchester County, N.Y., and cultivated in HL60 cells. The MN laboratory's antigen preparation was the HGE-2 strain of the HGE agent that was cultivated in HL60 cells and isolated from a patient in Minn. (9).

Cultivation of *Ehrlichia* strains. All HGE agent isolates and one *E. equi* isolate were cultivated in the HL60 cell line (CCL240: American Type Culture Collection). Infected HL60 cells were propagated in RPMI 1640 (Gibco, Grand Island, N.Y.) with 3% fetal bovine serum (Gibco) at 37°C with 5% CO_2. Infected cell cultures were maintained at approximately 2×10^5 cells/ml. When cells were from 70 to 100% infected, cell cultures were split at a 1:3 ratio of infected cells to uninfected cells. The infectivity of the cells was determined by microscopic examination of cytospin preparations stained with LeukoStat solutions (Fisher, Pittsburgh, Pa.). Uninfected HL60 cells were grown in RPMI 1640 with 10% fetal bovine serum at 37°C with 5% CO_2. The HGE-2 isolate from the MN laboratory was cultivated under slightly modified conditions described by Ravyn et al. that included the addition of 30 mM HEPES, 20 mM sodium bicarbonate, and 10% fetal calf serum to the RPMI 1640 culture medium (19).

Antigen preparation. HL60 cells that were 90 to 100% infected with the isolates from the JHU and NYMC laboratories were centrifuged at low speed (1,500 rpm) for 10 min and resuspended in 25 ml of 0.1 M phosphate-buffered saline (PBS) with 2% fetal bovine serum–0.05% sodium azide solution. The optimal cell concentration was empirically determined by microscopic inspection of LeukoStat-stained preparations. Ten microliters of the cell suspension was added to each well of 12-well Teflon-coated slides that were then air dried, fixed in acetone for 10 min, and stored at −80°C.

The HGE-2 antigen preparation from the MN laboratory was prepared as described by Ravyn et al. (19). Briefly, HL60 cells that were greater than 95% infected with the HGE-2 isolate were centrifuged at low speed and resuspended in 10 mM PBS. The resuspended infected cells were diluted to a concentration of 10^7 cells/ml, and 5 μl of cells was applied to each well of 18-well coated slides. The slides were air dried, fixed in a 1:1 solution of methanol and acetone for 10 min, and stored at −70°C.

E. equi-infected horse neutrophil antigens were prepared as previously described (5). Briefly, infected leukocytes were prepared from the buffy coat of infected blood after sedimentation at $1 \times g$ at 4°C overnight. Erythrocytes were removed by osmotic lysis, and the *E. equi*-infected leukocytes were harvested by centrifugation. The infected cell pellet was reconstituted to approximately 10^5 leukocytes/ml in PBS with 2% fetal bovine serum and 0.05% sodium azide solution. The suspension was applied to 12-well Teflon-coated slides that were air dried, fixed in acetone for 10 min, and stored at −80°C.

IFA method. Serum samples from all patients were tested for antibodies reactive with each of the different antigens by using the indirect IFA test (5). All sera were screened at a 1:80 dilution in PBS (pH 7.4) with 0.5% nonfat dry milk (PBSM) and were incubated with each of the different antigens in a humidified chamber for 1 h. After being washed three times with PBS, fluorescein isothiocyanate (FITC)-labeled goat anti-human IgG, IgA, and IgM (heavy plus light chains) diluted 1:50 in PBSM were added in the JHU and NYMC laboratories, and the slides were incubated for 1 h, optimized as previously described (4). The MN laboratory used FITC-labeled goat anti-human IgG (heavy plus light chains) diluted 1:240 or an FITC-labeled goat anti-IgM (Mu chain-specific) antibody diluted 1:40 separately as the secondary antibody. (All fluorescent antibodies were obtained from Kirkegaard and Perry Laboratories, Gaithersburg, Md.) After three more washes with PBS, the slides were incubated for 5 min with 0.005% Evans blue in PBS and then rinsed with distilled water and air dried. An antiquenching mounting solution was added to each well, and the slides were examined by fluorescent microscopy. Positive fluorescent staining was determined by the presence of fluorescent morulae within the cytoplasm of the HL60 cells and the distribution of fluorescent morula-containing cells on the slides. All sera that contained antibodies at a 1:80 dilution were titrated to at least 2,560. For the MN laboratory, any sample that had a titer of ≥80 by either IgM or IgG testing was considered positive, but since the other laboratories did not specifically assay IgM and IgG, the results of isotype titrations were not used in interlaboratory comparisons. For generation of the receiver operator characteristic (ROC) curve and sensitivity-specificity analyses for the HGE-2 isolate, only samples that had both IgM and IgG results were used, and the higher of the IgM and IgG titers was used as the overall titer for that serum.

Statistical analysis. The consensus geometric mean titer (GMT) and standard deviation were calculated for each sample. Individual titers with each antigen preparation were compared by linear regression analysis with the consensus GMT for that sample. Additionally, the GMT for antigens from each geographic region (N.Y., upper Midwest, Calif.) was calculated, and a paired Student's t test was performed to determine whether a statistical difference between titers of regional antigens and overall GMT existed. ROC curves were derived for each antigen by using dilutions of <80 through 2,560 as cutoff points. Curves were evaluated for statistical differences by calculating the area under each curve (GraphROC for Windows 2.0) by using two-tailed unpaired nonparametric tests, as previously described (10). P values of <0.05 were considered significant.

Using a cutoff titer of 80, the consensus qualitative results for all antigens from one region were compared for agreement to the overall consensus results of all antigens. Qualitative results were also used to determine consensus sensitivity and specificity for antigens from each region. In addition, for each convalescent-phase sample, a GMT was established for that geographic region, and these results were used to construct ROC curves. By paired analyses, the areas under the curves were calculated to detect significant differences in serologic reactions attributable to the geographic origin of the antigens.

Acute-phase sera were stratified by the interval of time that elapsed after onset of fever until collection of the serum sample to assess the sensitivity of antibody detection in early active disease. Results were compared by Student's t test to determine significant differences between this interval in antibody-positive and -negative acute-phase sera.

TABLE 2. Overall qualitative agreement among serologic results[a]

No. of antigens	Agreement	
	No. of samples	% of samples
All	46	70
9	10	15
8	3	5
7	2	3
6	4	6
5	1	2

[a] Results represent overall qualitative agreement for 10 different antigen preparations (eight strains) tested by using 66 serum samples with confirmed or suspected HGE and other rickettsial infections.

RESULTS

Qualitative agreement. Of 81 serum samples selected for testing, all three laboratories tested 66. Overall, 46 of the 66 (70%) serum samples were either all positive or all negative for HGE agent antibodies with the 10 different antigen preparations. Discrepancies (Table 2) with 1, 2, or ≥3 antigen preparations were seen in 10 (15%), 3 (5%), and 7 (13%) serum samples, respectively. Discrepant results occurred in serum samples from 12 confirmed HGE patients and in 8 serum samples from non-HGE patients, including 3 serum samples from patients with *E. chaffeensis* infection. The consensus GMT of the sera with discrepant results was 115, and the consensus GMT for the sera for which all antigens agreed was 105. Both discrepant and nondiscrepant sera had titers ranging from 80 to ≥2,560. By Student's *t* test, there was not a significant difference between the titers of the discrepant sera versus the nondiscrepant sera ($P = 0.79$).

The consensus qualitative results for all N.Y. antigens, upper Midwest antigens, and Calif. antigens were compared to the total consensus qualitative results for all antigens. The N.Y., upper Midwest, and Calif. antigens agreed with 91, 98, and 99% of the total consensus results, respectively.

Quantitative agreement. The GMT and standard deviation were calculated for each sample tested with the various antigens used by both the JHU and NYMC laboratories. In the logarithmic transformation used for comparison of titer results, a standard deviation of 0.3 is equivalent to a twofold difference in antibody titer. Since a fourfold change in antibody titer is routinely considered to be significant when comparing diagnostic serologic results, titers among different antigens were considered to be similar when the standard deviation of the GMT was less than 0.6. Thus, a significant difference was defined as the equivalent of a fourfold or greater variation (standard deviation, ≥0.6). Seventy-six of the 81 (94%) serum samples tested had GMT standard deviations of less than 0.6, reflecting similar titers among antigens; 74% had identical titers.

The GMT was calculated for all antigens by geographical region. The GMT for the Calif. antigens was 130, that for the N.Y. antigens was 140, and that for the upper Midwest antigens was 117, differences that were not statistically significant (paired Student's *t* test, Calif. versus N.Y. antigens, $P = 0.38$; Calif. versus upper Midwest antigens, $P = 0.09$; and N.Y. versus upper Midwest antigens, $P = 0.06$).

Correlation of antibody titers among antigens and testing sites. The degree of correlation of the GMT of individual antigens (except NY-3 and HGE-2) with the overall consensus GMT was calculated by linear regression. The NY-3 and HGE-2 antigens were excluded from these analyses, since these antigens were not tested with all 81 serum samples and since the HGE-2 antigen tests were performed with IgG and IgM conjugates only. The *R* values for the JHU antigens were similar, ranging from 0.89 to 0.96, and the *R* values for the NYMC antigens were 0.91 for both the NY-6 and NY-8 strains. Slight differences in *R* values were observed for the three *E. equi* antigens tested at JHU and for the NY-8 antigen tested by the JHU and NYMC laboratories.

Sensitivity and specificity. Evaluation of ROC curves showed similar features for each antigen evaluated; in general, a cutoff titer of 80 resulted in the highest concurrent sensitivity and specificity for each antigen. No statistically significant differences were observed for any of the ROC curves generated. The overall results of sensitivity and specificity analyses with and without *E. chaffeensis* sera are shown in Table 3. For specificity analysis, the serologic results from the nine non-HGE patients, four HME patients, one RMSF patient, and one scrub typhus patient were used.

In the JHU laboratory, sensitivity among antigens ranged from 82 to 100% and specificity ranged from 67 to 100%. All HGE agent antigen preparations used in the JHU laboratory had similar high degrees of sensitivity and specificity. Sensitivity and specificity also varied between the two *E. equi*-infected horse neutrophil preparations and among the *E. equi*-infected horse neutrophil preparations and *E. equi* antigen cultivated in HL60 cells. In the NYMC laboratory, sensitivity ranged from 89 to 95% and specificity ranged from 73 to 85%, and the HGE-2 antigen used by the MN laboratory yielded a 100% sensitivity and a 79% specificity (Table 3).

To estimate the potential diagnostic differences attributable

TABLE 3. Number of single convalescent-phase sera reactive with and sensitivities and specificities of the various antigen preparations[a]

Antigen (testing laboratory)	No. of HGE samples reactive/no. tested	% Sensitivity (95% CI)[b]	No. of HME samples reactive/no. tested[c]	% Specificity (95% CI)	% Specificity without HME
E. equi neutrophils 1 (JHU)	28/28	100 (83–100)	1/4	93 (71–100)	100
E. equi neutrophils 2 (JHU)	23/28	82 (62–90)	0/4	100 (82–100)	100
E. equi HL60 (JHU)	28/28	100 (75–97)	3/4	67 (42–86)	82
Webster strain (JHU)	27/28	96 (79–99)	1/4	93 (79–100)	100
Spooner strain (JHU)	26/28	93 (75–97)	1/4	93 (71–100)	100
NY-8 strain (JHU)	26/28	93 (75–97)	1/4	93 (71–100)	100
NY-3 strain (NY)	21/22	95 (74–98)	1/2	85 (67–100)	91
NY-6 strain (NY)	26/28	93 (71–95)	3/4	73 (49–90)	91
NY-8 strain (NY)	25/28	89 (71–95)	3/4	73 (55–94)	91
HGE-2 strain (MN)	9/9	100 (60–90)	2/3	79 (61–97)	91

[a] None of the antigens reacted with sera from the RMSF or scrub typhus patients.
[b] CI, confidence interval.
[c] *E. chaffeensis* infection.

TABLE 4. Sensitivity of various HGE agent and *E. equi* antigens in detection of antibodies in acute-phase sera from HGE patients

Antigen(s)	No. of samples		% Sensitivity
	Positive	Tested	
E. equi HL60	9	26	35
E. equi neutrophils	4	26	15
HGE agent strains (JHU)	4	26	15
NY-3 strain	3	21	14
NY-6 and NY-8 strains	5	24	21
HGE-2 strain	4	9	44

to regional variation in antigens, the consensus sensitivity and specificity of all N.Y. antigens, upper Midwest antigens, and Calif. antigens were individually calculated with convalescent-phase sera only. The N.Y. antigens had a sensitivity of 92% and specificity of 73%, the upper Midwest antigens had 93% sensitivity and 93% specificity, and the Calif. antigens yielded 100% sensitivity and 93% specificity. However, the areas under the ROC curves significantly differed only when Calif. antigens were compared with those from N.Y. ($P = 0.0473$).

Interlaboratory comparison using a single HGE agent strain. The NY-8 strain of the HGE agent was tested with a total of 79 archived serum samples by both the JHU and NYMC laboratories, where similar antigen preparation methods and assay methods were used. Qualitative results revealed agreement between these antigens in 69 of the 79 (87%) serum samples tested, which was better than overall agreement among all antigens. The sensitivity and specificity with the NY-8 strain differed between the JHU and NYMC laboratories, although this difference was not significant when ROC curves were compared (Table 3). The apparent lower specificity for the NYMC was due to cross-reactions in serum samples from three *E. chaffeensis* patients (Table 3) and a false-positive result for one of the non-HGE patients. The lower sensitivity for the NYMC was due to false-negative results for three confirmed HGE patients (consensus titers of 67, 226, and 293).

Cross-reactivity of non-HGE patient sera. Most false-positive results for all antigens resulted from cross-reactions of antibodies to *E. chaffeensis* (Table 3) with HGE agent or *E. equi* titers ranging from 80 to 1,280. When sera from HME patients were excluded from analyses, all HGE agent antigens and both *E. equi*-infected horse neutrophil antigens tested at JHU had a 100% specificity, and *E. equi*-infected HL60 cell antigen specificity increased from 67% to 82%. All NYMC HGE agent antigens and the MN HGE-2 antigen specificity increased to 91% with the exclusion of the *E. chaffeensis* patient sera. None of the antigen preparations reacted with the sera from the RMSF or scrub typhus patients.

Sensitivity and specificity in acute-phase samples. The interval of fever varied for each patient and ranged from 2 to 21 days (mean 5 days) of fever before acute-phase serum was obtained. The mean interval of fever for the HGE patients who had antibodies detected in their acute-phase sera (by a consensus of serologic tests) was 7 days, while the mean interval of fever for HGE patients without detectable antibodies was 4 days ($P = 0.06$). In polyvalent IFA tests, the antigen preparation made from *E. equi* cultivated in HL60 cells detected HGE agent antibodies in 9 of the 26 (35%) acute-phase samples from confirmed HGE patients (Table 4), but also detected four false positives. The three HGE agent isolates and the two *E. equi* horse neutrophil preparations tested at JHU detected antibodies in the same (15%) acute-phase samples from confirmed HGE patients (Table 4). Of the 24 acute-phase samples from confirmed HGE patients tested with the NY-6 and NY-8 isolates, 5 (21%) contained HGE agent antibodies, and 3 of 21 (14%) acute-phase samples tested with the NY-3 isolate had HGE agent antibodies detected (Table 4).

IgM and IgG IFA for convalescent- and acute-phase sera. A limited number of sera were tested for IgG and/or IgM separately, and with only the HGE-2 isolate as an antigen. In 40 convalescent-phase serum samples tested by IgG IFA, the sensitivity was 88.5% and specificity was 93% (100% if all three *E. chaffeensis* serum samples tested by IgG IFA were excluded), whereas only 24 convalescent-phase serum samples were tested for IgM alone, yielding a sensitivity of 30% and a specificity of 79%. Among the 23 convalescent-phase serum samples that were tested for both IgG and IgM antibodies, the overall sensitivity and specificity using either an IgG or IgM titer of $\geq$80 were 100 and 79%, respectively.

Among 21 acute-phase serum samples for which IgM and IgG tests were both performed, the sensitivity of IgM IFA was 33% and the specificity was 83%, whereas the sensitivity of IgG IFA in the same cohort was 44% and the specificity was 100%. When a cutoff titer of 160 was used to determine the presence of IgM antibodies, the specificity increased to 100% and the sensitivity remained unchanged. Among nine acute-phase and nine convalescent-phase serum samples from the 12 patients with HGE for whom both IgM and IgG tests were performed, IgG and IgM antibodies were detected at medians of 39 days (range, 5 to 145 days) and 18 days (range, 7 to 41 days), respectively.

DISCUSSION

The antigenic diversity of different strains of *E. equi* and the HGE agent used as antigen for IFA may, in part, explain some of the variability seen in the serodiagnosis of HGE. The molecular basis of antigenic variation in ehrlichiae is under investigation (2, 24). Antigens that have been identified and cloned from the HGE agent indicate that a complex array of proteins may contribute to IFA reactivity (12, 14, 21, 23). A major outer membrane protein antigen that is approximately 44 kDa in molecular size and is encoded by a gene that is part of a multigene family has been identified in protein immunoblots and cloned (2, 5, 13, 16, 23). Whether more than one of the outer membrane protein-encoding genes is transcriptionally active at any one time point and whether expression occurs during the course of HGE are not known. It has recently been shown that a similar multigene complex that encodes the major immunodominant protein antigens of *E. chaffeensis* and the related *Anaplasma marginale* exists and that only one or a few of the genes are transcriptionally active during in vitro propagation or in vivo infection (8, 20). Regardless, further investigation will be required to assess the overall contribution of these factors to the variability observed in diagnostic serologic tests for HGE. Thus, we assessed the sensitivity, specificity, and reproducibility of IFA by using a variety of different antigens in three different laboratories that perform serodiagnostic testing for HGE.

Several previous reports have investigated serologic tests for the diagnosis of HGE (3, 12, 15, 17, 19). The authors of these reports suggested that IFA is an effective serodiagnostic method, but could not comprehensively evaluate the method because of a relative lack of samples from confirmed clinical cases. This report is the first attempt to assess the sensitivity and specificity of the IFA assay by using a large number of patients proven to have HGE based largely upon clinical manifestations and nonserologic laboratory confirmation. Bias toward seropositive samples may have been introduced into this

study in an attempt to maximize the numbers of infected patients analyzed; however, this bias is not likely to significantly affect the overall results of comparative laboratory tests or the ROC curves that determine test utility.

The 10 different antigen preparations tested by the three different laboratories frequently agreed, suggesting that the use of different antigens will usually yield similar results. However, 15% of the sera had discrepant results in which two or more antigen preparations differed. It was anticipated that discrepant results might occur most often for sera with low titers, a finding not confirmed here. These data indicate that other biological or technical factors must be in part responsible for some of the lack of reproducibility and that there is a reasonable chance that the use of different IFA antigens will lead to discrepant serologic results when testing for HGE.

Since the majority of patients in this study were from the upper Midwest, and only 2 were from N.Y., it is possible that the N.Y. isolates are less reactive with HGE agent antibodies from upper Midwest patients. When compared to the consensus qualitative results for all antigens, results obtained with the N.Y. antigens agreed 91% of the time, while the upper Midwest and Calif. antigens agreed 98 and 99% of the time, respectively. Additionally, the largest variation in titers among antigens from each region occurred between the N.Y. and upper Midwest antigens ($P = 0.06$), and ROC analysis of Calif. and N.Y. antigens indicated a significant difference. These results are consistent with the concept that greater antigenic heterogeneity exists among isolates from different geographical regions than among isolates from a single region (2, 24). We have recently identified seroconversions in two PCR-confirmed patients from Calif. by using the HGE agent Webster strain (7), and this strain was also successfully used to document seroconversion in a patient with HGE acquired in Slovenia (18), suggesting that these antigens may be appropriate substrates for IFA serologic testing globally.

Antigenic diversity between strains explains only part of the variability observed with IFA. The results with the NY-8 strain that was tested by both the JHU and the NYMC laboratories agreed in 87% of tests. Thus, at least part of the discrepancy is most likely due to differences in antigen preparation, fluorescence interpretation, and methodological technique, since the ehrlichial isolates used were the same. It is unlikely, however, that discrepancies in IFA serodiagnosis result from technical variation alone. Within the JHU laboratory, where six antigens prepared by identical protocols were studied, 77% of the results agreed among all antigens, while 21 and only 2% of results were discrepant with one and more than one antigen preparation, respectively. Since these results are comparable to the qualitative results calculated for all three laboratories, it is likely that both antigenic diversity and technical factors play a role in discrepant qualitative results.

Quantitative assessments suggest that results obtained with assays using the individual antigens are good predictors of the consensus titer. However, quantitative results differed between the *E. equi* antigens and the NY-8 antigen used at both the JHU and NYMC laboratories, and titers calculated for individual serum samples differed among the various antigen preparations by fourfold or more in 6% of the sera tested. These findings suggest that although different antigens often produce similar titers, antigenic variability in combination with technical differences can result in significant variations in antibody titer.

Although not statistically significant, the sensitivity and specificity of IFA also varied among the different laboratories. These differences were evident even within the same laboratory, particularly when *E. equi* MRK was used as an antigen. A high degree of variability was detected among the two different *E. equi*-infected horse neutrophil preparations and the *E. equi* cultivated in HL60 cells. Whether these differences are due to the biological variation induced by in vivo propagation or are due to technical variation within the laboratory is not known. Our results suggest that antigens produced by in vitro cultivation under standardized conditions will reduce the variability observed when infected equine neutrophils are used.

Moreover, differences in sensitivity and specificity among the in vitro-propagated antigens could be due to changes that occur during in vitro propagation. *E. equi* MRK was passaged at least two times more than any other antigen. Since an *E. equi* MRK strain antigen with a low number of passages was not tested and compared, what effect, if any, passage history has on the variability of sensitivity and specificity cannot be determined. The NY-8 isolates from the JHU and NYMC laboratories both had a low number of in vitro passages and still demonstrated differences in sensitivity, specificity, titers, and qualitative agreement, perhaps due to interlaboratory variability.

The sensitivities of the antigens were comparable regardless of the geographical origin of the isolate, but the specificity obtained when N.Y. antigens were used was comparatively low, mainly due to cross-reactions with *E. chaffeensis*. In fact, the majority of false-positive reactions observed for each antigen could be attributed to *E. chaffeensis* antibodies. The titers obtained from these sera were not all low, as would be expected. Several of these sera had high HGE agent titers ($\geq$320) that could be misinterpreted as evidence of HGE unless concurrent serologic tests for *E. chaffeensis* are performed. Although *E. equi* or HGE agent titers in *E. chaffeensis* sera ranged from 80 to 1280, these were always at least a twofold dilution lower than that obtained with homologous *E. chaffeensis* antigen (data not shown). As demonstrated in previous studies, we have shown that other rickettsial infections do not cause false-positive reactions (5, 19). These results indicate that when IFA is used for the serodiagnosis of HGE, it may be advantageous to also test for antibodies against *E. chaffeensis* or to use confirmatory immunoblots (2, 5, 19) in order to rule out possible cross-reactivity.

IFA is most commonly used to detect antibodies in convalescent-phase sera, since only about 25 to 40% of HGE patients have detectable antibodies in their acute-phase samples (reference 3 and unpublished data). *E. equi* antigen cultivated in HL60 cells was the most sensitive antigen for detecting antibodies in acute-phase samples from HGE patients; however, this antigen had the lowest specificity. The three HGE agent isolates tested in the JHU laboratory detected antibodies in the same four acute-phase samples from HGE patients, and these antigens all had a high specificity. In fact, 46% of patients with HGE had antibodies detected in acute-phase serum, and these antibodies were more frequently detected when patients had clinical manifestations for longer intervals, although the differences were not statistically significant ($P = 0.06$).

Evaluation of paired acute- and convalescent-phase sera should still be considered the optimal method for serodiagnosis of HGE, since up to 15% of people residing in areas in which HGE is endemic have preexisting HGE agent antibody titers in the absence of active infection (4). Given this high rate, tests on single or even paired samples may not always be adequate to detect a significant rise in antibody titer. Separate IgG and IgM tests for HGE agent antibodies, while not different in sensitivity during the acute and early convalescent phases, may offer reliable methods to distinguish recent infections, since IgM antibodies were not detected after 41 days postonset in a small cohort of individuals tested by this

method. Care must be exercised in the use of IgM tests, because the specificity was lower than that of most tests that also detected IgG antibodies. Moreover, since IgM tests were not conducted under conditions that would exclude rheumatoid factors or after IgG removal, further confirmatory studies must be conducted.

This study is limited by the retrospective review of a relatively small number of patients with HGE and other diagnoses that potentially introduces bias into the sensitivity and specificity results. Thus, prospective epidemiological studies still need to be performed to better evaluate the IFA assay or other serologic tools for the diagnosis of HGE. However, these data indicate that differences in technical antigen preparation, assay performance, and antigenic variability among different *E. equi* and HGE agent isolates are associated with qualitative discrepancies and variation in antibody titer when used for IFA. While this variation was generally small and each antigen was comparable for use in the diagnostic serology of HGE, discrepant results that occasionally confound diagnosis will occur. Therefore, it would be desirable for all laboratories that perform serodiagnosis of HGE to adopt the use of one of several standard antigen strains and standardized methods that yield the most optimal sensitivity and specificity.

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