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An Indirect Immunofluorescence Assay Using a Cell Culture-Derived Antigen for Detection of Antibodies to the Agent of Human Granulocytic Ehrlichiosis

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An indirect immunofluorescence assay for the detection of human antibodies to the agent of human granulocytic ehrlichiosis (HGE) was developed and standardized. Antigen was prepared from a human promyelocytic leukemia cell line (HL-60) infected with a tick-derived isolate of the HGE agent (USG3). Suitable antigen presentation and preservation of cellular morphology were obtained when infected cells were applied and cultured on the slide, excess medium was removed, and cells were fixed with acetone. Use of a buffer containing bovine serum albumin and goat serum reduced background fluorescence, and use of an immunoglobulin G (γ-specific) conjugate reduced nonspecific binding. The assay readily detected specific antibody from HGE patients and did not detect antibody from healthy individuals. No significant reactivity was noted in sera from patients with high titers of antibodies to other rickettsial species. We were able to identify antibodies reactive to USG3 antigen in samples from areas where HGE is endemic that had tested negative to other rickettsial agents. Animal sera reactive against *Ehrlichia equi* or *Ehrlichia phagocytophila* bound to the HGE antigen, indicating that the assay may be useful for veterinary use. Comparability between two different laboratories was assessed by using coded human sera exchanged between laboratories. Results from the two laboratories were similar, indicating that the assay can be easily integrated into use for routine testing for HGE. The assay was then compared to an assay using horse neutrophils infected with ehrlichiae. The two assays gave comparable results, indicating that the cell culture-derived antigen can be used for testing samples that have been previously tested with *E. equi* as an antigen. The new assay offers several advantages over other immunofluorescence methods that use animal-derived antigen and is suitable for use in testing for human antibodies to the HGE agent.

Cases of human granulocytic ehrlichiosis (HGE) were first reported in 1994 (2, 5) from Minnesota and Wisconsin. Since that time, at least 150 patients with this infection, primarily from the upper Midwest and northeastern United States, have been described. The pathogen has been identified in Connecticut, Florida, Maryland, Massachusetts, Minnesota, New York, and Wisconsin by PCR amplification and detection with primers specific to the 16S rRNA gene of this agent and subsequent identification by sequencing of amplified products (10). Human seroreactivity to *Ehrlichia phagocytophila* and *Ehrlichia equi* with antigens obtained from ovine or equine sources has been noted in several other states (10, 20) and in Europe (4, 26).

Genetic sequence analysis of the 16S rRNA gene indicates that the etiologic agent of HGE clusters with several veterinary pathogens in the *E. phagocytophila* genogroup. The HGE agent is similar, if not identical, to *E. equi* (5), and there is growing evidence that the granulocytic agents of equine ehrlichiosis (*E. equi*), tick-borne fever (*E. phagocytophila*), and HGE may be the same species. Further studies are needed to resolve the taxonomy of these closely related organisms; however, this close genetic similarity has allowed a number of antigens to be used to detect serum antibodies from humans and domesticated animals (2, 5, 9, 12).

The routine detection of antibodies to the HGE agent had been limited by the inability to isolate granulocytic ehrlichiae in cell culture for the production of large quantities of standardized antigen. In the past, antibodies to this group of ehrlichiae were detected with fixed neutrophils harvested from the blood of infected animals (7, 9, 13, 15, 17, 22). Initial serologic studies in the United States used horse neutrophils infected with *E. equi* (MRK strain) as a surrogate antigen for testing human sera (2, 20). In Europe, testing of human sera has been conducted with sheep-derived *E. phagocytophila* (26) and horse-derived *E. equi* (4), but the production of antigen in this manner is time-consuming and expensive and requires facilities to maintain large animals. Recently, organisms genetically indistinguishable (by 16S rRNA gene analysis) from the HGE agent were isolated from the blood of human patients (11) and from laboratory-reared dogs fed upon by field-collected, infected ticks (8). Cell culture-derived antigen was found to bind with antibody in serum from an HGE patient (11). This report details the development and evaluation of a serological assay using an in vitro-cultured isolate of the HGE agent.

MATERIALS AND METHODS

Origin of cells infected with the HGE agent. We used the USG3 isolate of the HGE agent, which was one of nine isolates made in 1994 by Aquila Biopharmaceuticals (Wochester, Mass.) (8). This isolate was recovered from the infected blood of a laboratory-reared beagle after the dog was fed upon by six adult *Ixodes*

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scapularis Say (Acari: Ixodidae) ticks that were field collected in Westchester County, N.Y., and Montgomery County, Pa. Venous blood from the infected dog was inoculated onto a suspension culture of the human promyelocytic leukemia cell line HL-60 (6). The cells were maintained at 37°C (5% CO_2 in air) in RPMI 1640 medium supplemented with 20% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 1 mM sodium pyruvate, and 0.1 mM nonessential amino acid mixture. Ehrlichial infection was indicated by the lysis of infected HL-60 cells within 35 days. When the cells began to lyse, they were passaged onto fresh cells.

Characterization of the isolate. DNA from the USG3 cells was extracted by a standard phenol-chloroform method (1). A PCR assay was performed using the primers GE9 and GE10 (5). This primer set permits amplification of a 919-bp region of the 5′ end of the 16S rRNA gene of granulocytic ehrlichiae. Amplified products were isolated and purified from bands excised from the gel, and DNA was sequenced with a Prism Ready Reaction DyeDeoxy Cycle Sequencing kit (Applied Biosystems, Foster City, Calif.). Unincorporated, fluorescence-labeled deoxynucleoside triphosphates were removed with Centri-Sep columns (Princeton Separations, Inc., Adelphia, N.J.). Samples were loaded onto a 4.25% polyacrylamide gel for electrophoresis and detection on an ABI 377 automated sequencer. The resultant nucleotide sequence was aligned with sequence data deposited in GenBank.

Preparation of antigen slides. The USG3 culture was maintained at Aquila Biopharmaceuticals and was shipped at ambient temperature overnight to the Centers for Disease Control and Prevention (CDC) for processing. Actively growing (2 days from last passage), infected USG3 cells (passage 5) were enumerated in a hemacytometer and examined after gentle cytocentrifugation of the cells and Diff-Quik (Baxter Healthcare, McGaw, Ill.) staining of the preparation. The suspension (6.6×10^6 cells/ml) was pipetted onto cleaned 12-well (5-mm diameter), Teflon-templated slides (catalog no. 10-111; Cel-line Associates, Newfield, N.J.). The antigen slides were placed into a humidified chamber and incubated for 15 min at 37°C, allowing the cells to settle and adhere to the glass surface of the slide. The excess culture medium was carefully aspirated from the wells by using a pipette tip attached to a vacuum flask. The antigen wells were allowed to air dry for 20 min, and the antigen was fixed to the wells by immersion in acetone at room temperature for 10 min. In preliminary experiments, several common organic fixatives were tested (acetone, methanol, methanol-acetone, and acid-alcohol); acetone provided simple and effective fixation of the antigen to the slides and permeabilization of the cell membrane. Fixed slides were air dried, placed into plastic five-slide shipping containers, sealed with Parafilm (American National Can, Greenwich, Conn.), and stored at −70°C until used. A single lot of prepared antigen slides was used for all assays reported in this paper.

Indirect immunofluorescence assay (IFA). Frozen slides were placed over calcium sulfate dessicant (Drierite; W. A. Hammond Drierite Co., Xenia, Ohio) in a vacuum dessicator until they warmed to room temperature. The slides were then removed, rinsed once with phosphate-buffered saline (PBS; pH 7.4), and allowed to dry.

Twofold serial dilutions of test sera were made in PBS containing 1% bovine serum albumin, 1% goat serum, and 0.1% sodium azide (PBS-BSA) to help reduce nonspecific binding. The dilutions were applied, incubated in a humidified chamber at 37°C for 30 min, and washed with stirring in PBS three times (5 min for each wash). The wells were covered with an optimized dilution (1/150) of fluorescein isothiocyanate (FITC)-labeled, goat anti-human immunoglobulin G (IgG) (γ-specific) conjugate (Kirkegaard & Perry Laboratories, Gaithersburg, Md.) in PBS-BSA. The working solution of the conjugate was centrifuged at $10{,}000 \times g$ for 5 min to remove particulate complexes of FITC-labeled antibodies that can form upon freezing or long-term storage in the refrigerator. The slides were incubated and washed as before. During the next-to-last wash, four drops (per ca. 125-ml wash) of 1.65% eriochrome black T counterstain (Sigma Chemical Co., St. Louis, Mo.) in distilled water were added to reduce cellular autofluorescence and increase contrast with the specific fluorescence. Eriochrome black T stains host cells orange to red and does not reduce the intensity of specific fluorescence (25). A coverslip was mounted with an antifading mounting medium prepared as 0.3 M 1, 4-diazabicyclo[2.2.2]octane (DABCO) (Aldrich Chemical Co., Milwaukee, Wis.) in glycerol-PBS (9:1) buffer at pH 9.0 (3). The slides were examined with a UV epifluorescence microscope at ×200 and ×400 magnification for specific ehrlichial fluorescence. Host cells across the entire well were examined to avoid misinterpretation of patches of artifactual fluorescence associated with trapping of conjugate by clumped cells. Each separate assay included a complete dilution series of the positive and negative controls so that any variation in reactivity could be detected.

Endpoint titers were recorded as the reciprocal of the last serial dilution at which specific fluorescence of the individual ehrlichial organisms or microcolonies (morulae) was distinct. Positive fluorescence was defined as distinct apple-green fluorescence (not the dull green of autofluorescence) of ehrlichial inclusion bodies focally located in the cytoplasm of infected cells. The slides were initially screened at a magnification of ×200, and endpoint titers were evaluated at a magnification of ×400.

Origin of controls. The original positive control plasma was obtained from a Rhode Island resident who had physician-diagnosed HGE and who had previously been found to have a reciprocal IgG antibody titer of 1,024 in IFAs using either *E. equi*, *E. phagocytophila*, or HGE antigen. This patient had an illness clinically compatible with HGE and was positive in both serological and PCR assays, thus meeting the Council of State and Territorial Epidemiologists definition of a confirmed case of HGE. The negative control serum was obtained from a healthy laboratory worker with no history of ehrlichial exposure (IFA IgG titer <16). Numerous other sera from known or suspected HGE patients were also evaluated, as were sera from patients with other rickettsial diseases (see below).

Sera from clinically healthy blood donors residing in Seattle, Wash., were tested for reactivity to the HGE antigen. Samples ($n = 198$) were tested at initial dilutions of 1/16, 1/32, and 1/64, and any reactive sera were titrated to endpoint.

Homologous and heterologous reactivities among ehrlichial species. To assess the reactivity of sera from animals and humans infected with *E. canis*, *E. chaffeensis*, *E. equi*, *E. phagocytophila*, *E. risticii*, or *E. sennetsu* to our HGE antigen, as well as our HGE-reactive sera to other ehrlichial antigens, a number of comparisons were performed. Serum specimens from patients with human ehrlichiosis due to the HGE agent or *E. chaffeensis* were obtained from sera submitted for laboratory confirmation. We obtained or prepared antisera to a number of other ehrlichial pathogens of animals (horse anti-*E. equi*, canine anti-*E. canis*, bovine anti-*E. phagocytophila*, and murine anti-*E. risticii*) and of humans (canine anti-*E. chaffeensis*, canine anti-HGE agent, and murine anti-*E. sennetsu*). Twofold serial dilutions of the sera were prepared from an initial dilution of 1/16 and tested against antigen slides of each *Ehrlichia* species prepared as described for the HGE antigen. The species-specific FITC-labeled conjugates (Kirkegaard & Perry Laboratories) were used at optimal dilutions as determined by checkerboard titration for each of the animal species.

Serological cross-reactivity with other rickettsial agents. Sera from patients with confirmed Rocky Mountain spotted fever ($n = 5$), murine typhus ($n = 5$), scrub typhus ($n = 5$), rickettsialpox ($n = 5$), and Q fever ($n = 5$) were obtained from the CDC serum bank. These were tested for reactivity to the HGE antigen at dilutions of 1/64 and 1/128. For this portion of the study, IgG antibody titers of <64 were considered nonreactive with the HGE antigen.

Interlaboratory and antigen comparisons. To assess the use of this antigen in an additional setting, 47 human serum samples from both the CDC laboratory (CDC set; $n = 26$) and the Connecticut Agricultural Experiment Station (CAES) laboratory (CAES set; $n = 21$) were coded and exchanged. The CDC set was derived from patients in 11 states, while the CAES set represented only patients from Connecticut. Sets from each laboratory were selected to contain sera from patients that had met either the Council of State and Territorial Epidemiologists (CDC set) or Connecticut Emerging Infections Program (CAES set) case definition for human ehrlichiosis, nonreactive sera, and a number of problematic sera (sera with borderline titers, reactivity to other antigens, or high background). Each laboratory tested the sera in separate assays using the HGE antigen and a horse-derived antigen. Each of the laboratories performed the assay with its routine methodology. No attempt was made to standardize reagents or protocols between laboratories; however, the endpoint titers were obtained in each laboratory with IgG (γ-specific) conjugate.

Comparisons were made for both the concordance of outcome, classified as positive or negative by using the individual laboratory's established threshold of ≥64 (CDC) or ≥80 (CAES) to define the positive values, and differences between endpoint titers obtained between antigens within laboratories and between laboratories using different antigens. For the purposes of this study, the corresponding twofold serial dilutions made from each of these initial values were assumed to be equivalent in the determination of level of discrepancy in the titers. A twofold variation in titer was considered normal and expected for an immunofluorescence assay. Negative outcomes in tests at the two laboratories were considered equivalent titers for analysis.

Commercially available *E. equi* slides (Spirochete and Rickettsia Laboratory, University of California School of Veterinary Medicine, Davis, Calif.) were used to compare the HGE antigen to horse-derived antigen for the detection of human antibodies to the HGE agent. These slides were prepared (19) with the BDS strain of a granulocytic ehrlichia isolated from a woman in Wisconsin by inoculation of her blood into a healthy horse (18). In the CAES laboratory, this strain had given results equivalent to those obtained with an isolate (MRK strain) from a naturally infected horse from northern California (23).

Assays were set up in similar fashion, using optimal concentrations of conjugate determined by checkerboard titration with the positive and negative controls. These dilutions were 1/150 and 1/125 for HGE antigen slides and *E. equi* slides, respectively. Prior to use in assays at the CDC, equine immunoglobulins were eluted from the horse-derived antigen slides with glycine buffer (21).

Application of the assay for routine diagnostic samples. Fifty samples were randomly chosen from sera submitted to CDC for serologic diagnosis of suspected rickettsial diseases from each of two states with areas where HGE was known to be endemic, Wisconsin (1991 to 1994) and Connecticut (1993 to 1995). The samples had previously tested negative for antibodies to other rickettsial agents (*Rickettsia rickettsii*, *Rickettsia typhi*, *Coxiella burnetii*, and *E. chaffeensis*) routinely used in the CDC rickettsial screening panel. These samples were tested in the assay described above, except that the sera were screened with an FITC-labeled, goat anti-human IgG (heavy plus light chains) conjugate which reacts with IgG and other immunoglobulins with similar light chains. Sera reactive at either screening dilution were titered to endpoint with a heavy chain (γ)-specific conjugate (Kirkegaard and Perry Laboratories).

Statistical methods. Tallies of concordant and discordant results obtained between laboratories and between the two antigens were assessed by computing kappa statistics. The kappa statistic measures the percent agreement between

two measurements over that expected by chance alone, with values of less than 0.4 considered poor, values of 0.4 to 0.75 considered good, and values of greater than 0.75 considered to be excellent correspondence (16).

RESULTS

Characterization of isolate. A product of the expected size (919 bp) was amplified when the GE9-GE10 primer set was used in the PCR assay. This primer set is specific for the *E. phagocytophila* genogroup but does not differentiate among species in this group. The nucleotide sequence obtained by automated sequencing was aligned with all GenBank entries, as well as sequences from two HGE patients determined in our laboratory. The USG3 isolate had 100% identity with the corresponding region of the original sequence of the HGE agent (GenBank accession number U02521) and to the HGE sequences from two other patients. This isolate also had bases identical to those in the HGE agent in two signature positions (84 and 886) found in the amplified region (5). Thus, using the recognized criterion of the 16S rRNA gene sequence (27), we identified our isolate of tick origin as the HGE agent.

Preparation of antigen slides. Antigen slides produced with a cell suspension containing 6.6×10^6 cells/ml (75% infected) of growth medium, according to the described methods, resulted in predominantly intact host cells that adhered to the wells. Cells retained normal cellular morphology, which allowed the observation of individual or clustered ehrlichial organisms localized within the cytoplasm (Fig. 1a).

IFA. The original positive control sample had an IgG titer of 1,024 to the USG3 antigen. In repeated testing throughout the study, the positive control consistently gave this endpoint titer and only rarely varied by an expected twofold dilution. The negative control sample showed no specific binding at dilutions of 1/16 or greater (Fig. 2a) in all assays. In the positive control sample, the morulae and individual ehrlichiae fluoresced brightly, and no host cellular reactivity was noted. The infection level per cell ranged from a few ehrlichiae to large clusters filling the cytoplasm (Fig. 2b). Occasionally, the ehrlichiae could be seen as cell-free fluorescing particles outside of lysed host cells, but only cell-associated ehrlichiae were used to determine the endpoint titers.

Only 1 of 198 sera from clinically healthy residents in an area where HGE is not endemic was found to have a detectable antibody titer of 64. None of the remaining sera reacted at our initial serum dilution of 1/16. The seroreactive sample bound to ehrlichiae in only a fraction of the infected host cells.

Homologous and heterologous reactivities among ehrlichial species. Reactivities among species tested by using various specific antisera and ehrlichial antigens are shown in Table 1. Significant serologic reactivities to heterologous antigens were usually seen in genetically related species (e.g., *E. canis* and *E. chaffeensis*), but only minor cross-reactivities were noted for heterologous sera to ehrlichiae pathogenic to humans. Antisera to *E. risticii* and *E. sennetsu* were reactive with the heterologous antigen but rarely with other ehrlichial species. The positive control sample for the HGE antigen did not cross-react with *E. chaffeensis* antigen and vice versa in these samples. In most of the antisera tested, similar endpoint titers were observed for the *E. equi*, *E. phagocytophila*, and HGE antigens.

Significant reactivities to heterologous antigens were seen in serum from a dog that had been hyperimmunized with *E. canis*. In contrast, an experimentally *E. chaffeensis*-infected dog showed minimal reactivity to *E. canis*, and a dog experimentally infected with the HGE agent exhibited reactivity to only the granulocytic ehrlichia group.

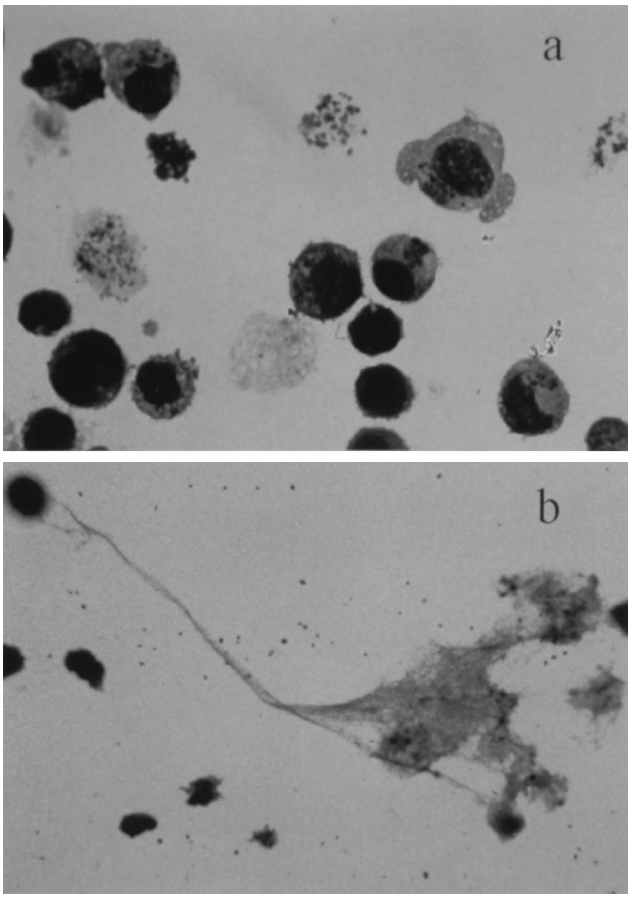

FIG. 1. Appearance of HGE agent-infected cells in the antigen slides after Diff-Quik staining. (a) Host cells allowed to adhere to the slides, showing conservation of cellular detail; (b) host cells dotted with a capillary tube, showing excessive shearing of the cells.

Serologic cross-reactivity with other rickettsial agents. None of the sera from patients with Rocky Mountain spotted fever, murine typhus, scrub typhus, rickettsialpox, or Q fever were reactive in the HGE assay at the two dilutions tested (1/64 and 1/128). Significant homologous reactivity was shown in the respective IFAs: Rocky Mountain spotted fever (geometric mean titer [GMT] = 891; range, 512 to 2,048), murine typhus (GMT = 891; range, 512 to 4,096), rickettsialpox (GMT = 776; range, 256 to 2,048), and Q fever (GMT = 388; range, 256 to 512).

Interlaboratory and antigen comparisons. The overall agreements between the two laboratories using the two different antigens were good to excellent (as defined by the kappa statistic) for both sets of sera (CDC and CAES) (Table 2). Agreement between the two laboratories was satisfactory, regardless of the antigen used. Two of the samples tested with culture-derived antigen and four of those tested with horse-derived antigen gave positive reactions in the CAES laboratory but were negative in the CDC laboratory. Results for all other samples were concordant.

There was also good to excellent agreement between horse-derived antigen and the culture-derived antigen within each laboratory. The culture-derived antigen identified a greater number of positive specimens (23 of 47 sera) in each of the laboratories than did the horse-derived antigen (20 of 47). Only in the CAES laboratory were some samples that reacted

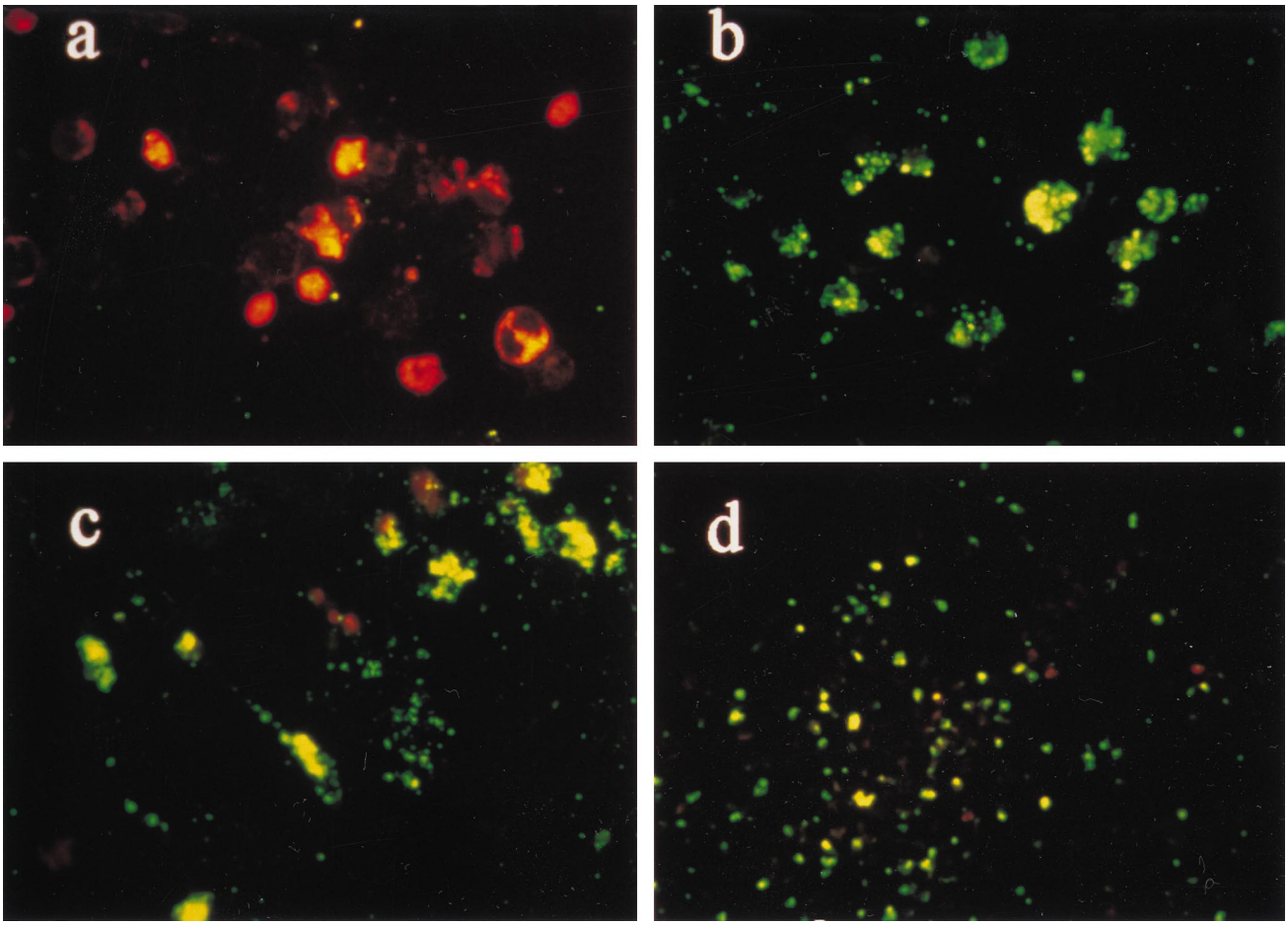

FIG. 2. Appearance of HGE agent-infected cells in the IFA after reaction with negative control serum on culture-derived antigen (a) and after reaction with positive control serum on culture-derived antigen (b), capillary-dotted antigen (c), and infected horse neutrophils (horse-derived antigen) (d).

with the horse-derived antigen but not with the cultured antigen found.

Most of the samples showed little or no difference in endpoint titer, regardless of where they were tested (Fig. 3) or which antigen was used (Fig. 4). At CDC, over 96% of the titers obtained either were identical or fell within the expected ± twofold dilution when results for the cultured antigen were compared with those for the horse-derived antigen. The CAES laboratory noted wider variation in endpoint titers, as only 66% of the results fell within the expected range.

Characteristics of samples giving discordant results. To further investigate causes of discordant results obtained from the two laboratories, we examined the 10 discordant sera in greater detail. Seven of the 10 samples were also reactive to *E.*

TABLE 1. IFA reactivities with homologous and heterologous antigens

Antiserum	Species[a]	Titer with indicated antigen[b]						
		E. canis	*E. chaffeensis*	*E. equi*	HGE agent	*E. phagocytophila*	*E. sennetsu*	*E. risticii*
E. canis	Dog (E)	16,384	16,384	1,024	2,048	2,048	ND	512
E. chaffeensis	Human (N)	<64	1,024	<64	<64	<64	<64	<64
	Dog (E)	64	1,024	<64	<64	<64	<64	<64
	Mouse (E)	<64	1,024	<64	<64	<64	<64	<64
E. equi	Horse (E)	<64	<64	512	1,024	1,024	<64	<64
	Mouse (E)	<64	<64	1,024	256	512	<64	<64
HGE agent	Human (N)	<64	<64	512	1,024	1,024	<64	<64
	Dog (E)	<64	<64	512	512	256	<64	<64
E. phagocytophila	Cow (E)	<64	<64	ND	<64	128	<64	<64
E. sennetsu	Mouse (E)	<64	<64	<64	<64	<64	2,048	256
E. risticii	Mouse (E)	<64	<64	<64	<64	<64	256	8,096

[a] E, experimental infection; N, natural infection.
[b] Homologous (or presumed to be homologous) reactions are underlined. ND, not done.

TABLE 2. Comparison of test results of sera provided by and tested in two laboratories (CDC and CAES) with culture-derived and horse-derived antigen

Comparison[a]	Test sera	No. of results				% Agreement	Kappa statistic[c]
		Concordant		Discordant			
		Positive/positive[b]	Negative/negative	Positive/negative	Negative/positive		
Between laboratories							
Culture-derived antigen	Both sets	23	22	0	2	95.7	0.91**
	CDC set	11	13	0	2	92.3	0.85**
	CAES set	12	9	0	0	100	1.0**
Horse-derived antigen	Both sets	20	23	0	4	91.5	0.83**
	CDC set	8	18	0	0	100	1.0**
	CAES set	12	5	0	4	81	0.59*
Between antigens							
CDC laboratory	Both sets	20	24	3	0	93.6	0.87**
	CDC set	8	15	3	0	88.5	0.76**
	CAES set	12	9	0	0	100	1.0**
CAES laboratory	Both sets	20	18	5	4	80.9	0.62*
	CDC set	8	13	5	0	80.8	0.62*
	CAES set	12	5	0	4	81	0.59*

[a] For each of four comparisons (two labs by two antigens) we have indicated the numbers and source of concordant and discordant results and computed the kappa statistic. For reporting concordant and discordant results, the reference laboratory was the CDC (i.e., laboratory results are CDC/CAES) and the culture-derived antigen was the reference antigen (culture derived/horse derived).
[b] Titers ≥64 (CDC) or ≥80 (CAES).
[c] **, excellent agreement; *, good agreement (16).

chaffeensis antigen. Dually reactive samples have also been seen in other specimens submitted for routine diagnostic testing, and almost all of these samples (most with high *E. chaffeensis*-reactive titers) showed a pattern of subset-type fluorescence against the culture-derived HGE antigen. Intensely fluorescing ehrlichiae were noted in a subset of approximately one-half of the number of infected host cells observed when a positive HGE serum was examined against the homologous antigen. The apparent size of the individual fluorescing ehrlichiae was distinctly smaller in the *E. chaffeensis*-reactive samples.

Application of the assay for routine diagnostic samples. Of the 100 samples tested that previously had been negative when tested for antibodies to other rickettsial agents, 12 (12%) were found to contain antibodies reactive with the HGE agent. Four (8%) of 50 samples from Connecticut (from 1993 and 1995) and 8 (16%) of 50 samples from Wisconsin (from 1992 and 1993) were reactive at endpoint titers ranging from 256 to 1,024 (GMT = 512) in Connecticut and from 1,024 to 4,096 (GMT = 1,579) in Wisconsin.

DISCUSSION

In this study, we describe the development and application of an IFA using cell culture-derived ehrlichiae for the detection of antibodies present in patients with HGE. Sequencing of ehrlichial DNA recovered from humans, dogs, and horses in the United States and Sweden showed little variation in the 16S rRNA gene and demonstrated that these sequences are identical to those of the HGE agent (12, 14, 18). The nucleo-

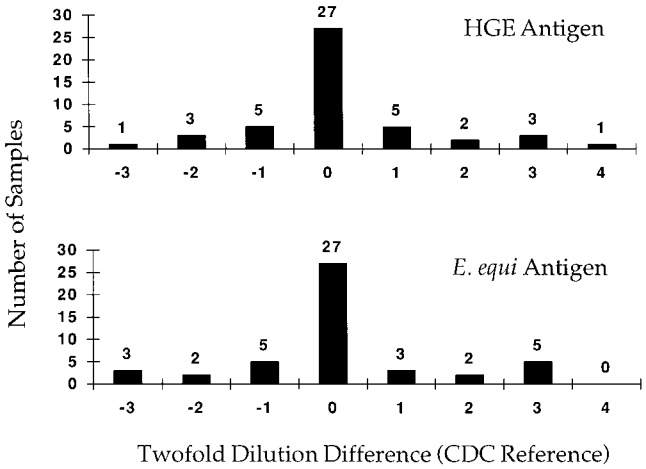

FIG. 3. Differences in antibody titer by antigen source in split samples analyzed at CDC and at CAES. Differences are expressed as the number of twofold dilutions from the value obtained at the CDC laboratory. Negative outcomes in tests at the two laboratories were considered equivalent titers.

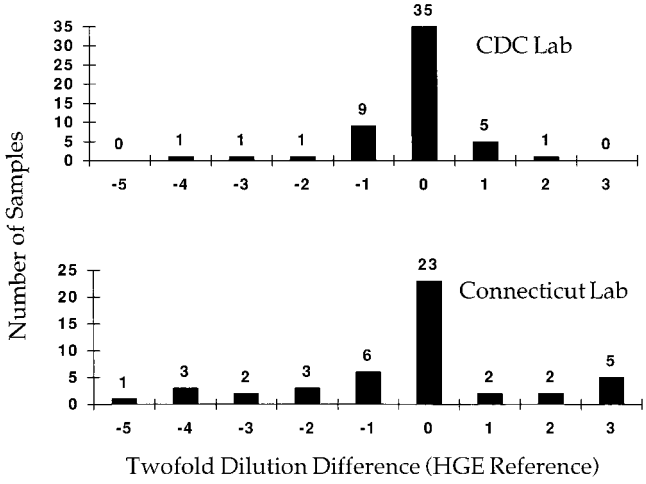

FIG. 4. Differences in antibody titer by laboratory in split samples analyzed using the two antigens. Differences are expressed as the number of twofold dilutions from the value obtained with the HGE antigen. Negative outcomes in tests with the two antigens were considered equivalent titers.

tide sequence of a variable region of the 16S rRNA gene of the USG3 isolate was shown to be identical to that of the HGE agent. On the basis of this criterion and the USG3 isolate's similarity to the HGE sequences obtained from two other human patients, the organism was considered to represent an isolate of the HGE agent and appropriate for use in a serologic assay to identify human disease. Furthermore, the similarity in antibody titers of HGE cases in tests using the culture-derived antigen and *E. equi* antigen from horses (9) (Table 2) provides additional evidence that the two strains are antigenically similar.

The USG3 antigen as used in our IFA was reactive with the sera from patients with disease clinically diagnosed as HGE but did not detect antibodies from patients with other rickettsial illnesses. This finding suggests that our assay should be useful in differentiating illness due to traditional rickettsial agents from illness due to ehrlichial agents. The assay rarely detected reactive antibody in healthy people. In the one reactive sample from a blood donor, the titer was low and the sample showed a distinct pattern of subset fluorescence not seen in sera from well-defined HGE patients.

In most cases, our control sera from humans or animals infected with other *Ehrlichia* species did not react with our culture-derived antigen. The exception was a hyperimmunized dog serum that was reactive against all ehrlichial antigens tested. The high homologous antibody titer (16,384) of this serum may account for the increased level of heterologous reactivity in this assay. A high degree of cross-reaction was noted in antisera to various granulocytic ehrlichiae (*E. equi*, *E. phagocytophila*, and HGE agent) when assayed against heterologous members of that group. These close antigenic relationships correlate with the currently recognized genetic relationships (10).

Prior to the isolation of the HGE agent in cell culture, serologic assays for diagnosing HGE infection used antigen derived from infected-horse neutrophils. Although an effective assay, this method of antigen production presents several drawbacks. The inconvenience and expense of maintaining and infecting live animals and the variation among lots (e.g., numbers of infected cells) limit the widespread use of the horse-derived antigen. Moreover, we have previously had difficulties in interpreting results when using commercially available preparations of the horse-derived antigen.

Problems encountered with the horse-derived antigen included the nonuniform distribution of infected host cells in some lots and background fluorescence, probably due to prebound immunoglobulin and entrapment of the conjugate by clumped neutrophils. In the CDC laboratory, we have been able to reduce background fluorescence due to prebound immunoglobulin by pretreating the slides with a low-pH glycine buffer to remove the existing antibody. The presence of this antibody might have contributed to some of our discordant results.

The cell culture-derived antigen resolved several of the problems noted above. The desired level of infection could be more readily adjusted in culture-derived antigen, reducing variation among lots. In subsequent routine testing at CDC, we have used at least seven antigen lots (passages 5 and 8 to 13) and have found little variation among the slides. Applying infected-cell suspensions to the slide wells allowed even settling of largely intact, morphologically normal cells that flattened and spread on the glass surface, providing easily located ehrlichial organisms within the host cell cytoplasm (Fig. 1a). This method was especially suitable for preparing antigen slides for the monocytic group of ehrlichiae (*E. chaffeensis*, *E. canis*, *E. risticii*, and *E. sennetsu*) because adherence to glass was more evident in the monocytic-macrophage cell lines. In parallel experiments, application of the cells in this manner was preferable to dotting the antigen onto slides with a capillary tube. Capillary dotting appeared to exert a mechanical shearing effect on the heavily infected cells that lysed many host cells and released large numbers of cell-free ehrlichiae (Fig. 1b and 2c). In addition, the dried dot contained excess medium components that increased background fluorescence.

This study was designed not to standardize serologic assays between laboratories but rather to examine the utility of the culture-derived antigen as a replacement for other substrates with limited availability. In addition, some sera that were difficult to interpret because of low titer, high background fluorescence, or reactivity to other antigens were selected. The selection of such problematic sera increases the likelihood of obtaining discordant results. Our data provide evidence that this antigen was equivalent to the standard horse-derived antigen and was readily adapted for use in two laboratories. Our findings, as measured by the kappa statistic, indicate good to excellent agreement between the two antigens and between the two laboratories in identifying positive and negative samples. However, a few discrepant results were obtained.

The wider variation in endpoint titers at CAES may have been due to the HGE antigen being used by this laboratory in an IFA for the first time during this trial. Experience with any antigen and assay would reduce variability. Examination of discordant results certainly supports this conclusion, as the majority were obtained from sera with either a low titer or reactivity to an additional ehrlichial species. Frequently, the discrepant values were due to false-positive reactions seen in the culture-derived antigen when testing samples strongly reactive to *E. chaffeensis*. The level of serologic cross-reactivity between patients infected with these two species was originally thought to be low (10), although occasional sera reactive to both antigens have been recorded (20). In our experience with diagnostic samples received from many areas of the country, some sera have antibody titers to both agents. Typically, this dual reactivity is not a problem, as titers to one organism (presumably the agent responsible for disease) are at least fourfold higher than titers to the other.

Most dually reactive samples with high *E. chaffeensis*-reactive titers showed a distinctive pattern of subset fluorescence against the culture-derived HGE antigen. Intensely fluorescing ehrlichiae were noted in a subset of approximately one-half of the number of infected host cells observed when a positive HGE serum was examined against the homologous antigen. The apparent size of the individual fluorescing ehrlichiae was also smaller in the *E. chaffeensis*-reactive samples, suggesting reactivity to fewer epitopes. These patterns of reactivity to the culture-derived antigen were not seen in all *E. chaffeensis*-reactive samples but when present were a relatively accurate predictor that the sample would be reactive with *E. chaffeensis* antigen. Whether this finding represented cross-reactive epitopes or antibody induced by separate exposures to the two organisms was impossible to ascertain. This dual reactivity was not seen in any of our experimentally infected animals or in the *E. chaffeensis*-reactive human control sera. Further study of this dual reactivity is in progress.

Several problems must be anticipated by laboratories using this or any other culture-derived antigen in serologic assays. Some sera submitted for HGE testing may exhibit varying levels of background fluorescence, autofluorescence, or specific fluorescence to cellular components that interfere with the reader's ability to observe specific ehrlichial fluorescence. Background fluorescence can result from antibody binding to either the host cell, other constituents of the antigen prepara-

tion, or components of the serum. Nonspecific cellular fluorescence can be caused by a high level of heterophile antibodies sometimes seen in samples from patients with acute infections. Frequently, these nonspecific reactions, and others caused by autofluorescence or high lipid content of the serum, disappeared at the analytical threshold values used in each laboratory. A titer of 80 has been used as the cutoff in HGE cases to determine a positive response to horse-derived antigens in other studies (9, 21). Our study was not designed to establish diagnostic threshold values but provides useful information on the low level of nonspecific binding and cross-reactivity at these dilutions. Only 1 of 198 sera from healthy residents in an area where HGE is not endemic was found to have detectable antibody at our cutoff dilution. Correlation of laboratory data with clinical information will further refine diagnostic threshold values for serological confirmation of human cases (24).

Specific fluorescence to cellular organelles and to nuclear antigens could be detected in some samples but was distinguishable from specific ehrlichial fluorescence. Although nuclear staining was easily identified, organelle-specific binding could mimic ehrlichiae or obscure the proper evaluation of infected cells. In addition, fluorescing bacterial or fungal contaminants in a sample could mimic the appearance of intracellular ehrlichiae when attached to the surface of the host cells and might have led to erroneous or confusing results. Contaminating bacteria tended to be larger and more rod-like, ovoid, or elliptical than ehrlichiae. Centrifugation of serum samples through 0.22-μm-pore-size filters (Spin-X; Costar Corp., Cambridge, Mass.) can be used when necessary to remove contamination.

The culture-derived antigen was useful in detecting antibodies to the HGE agent in patient sera from areas where HGE is endemic. Approximately 10% of archival patient samples that were submitted for rickettsial testing from areas where HGE is endemic and were negative to the other agents had significant levels of antibody to the HGE agent. This finding suggests that a substantial number of HGE cases have previously gone unconfirmed.

We have shown that a cell culture-derived HGE antigen is suitable in an IFA for the detection of HGE-specific antibodies in human, domesticated animal, and rodent sera. Use of the culture-derived antigen offers several advantages over the use of horse neutrophils infected with granulocytic ehrlichiae, and the availability of culture-derived antigen will provide a readily accessible and more affordable means of serologic testing. The routine application of this assay will facilitate studies examining the public health significance, geographic distribution, and potential animal reservoirs of the HGE agent or other closely related organisms.

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