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Diagnosis of Human Ehrlichiosis by PCR Assay of Acute-Phase Serum

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A PCR assay of 43 acute-phase serum samples was evaluated as a method for early detection of human granulocytic ehrlichiosis (HGE) and determination of etiology when serologic testing is inconclusive. Sequence-confirmed products of the HGE agent were amplified from three individuals residing or having exposure history in Minnesota or Wisconsin, and similarly confirmed products from *Ehrlichia chaffeensis* were amplified from three individuals from Florida or Maryland. Etiology, as determined by PCR and serology, was the same whenever there was a fourfold difference between the maximum titers of antibodies to both antigens, indicating that presumptive determination of etiology may be based on fourfold differences in titers. PCR testing determined that *E. chaffeensis* was the etiologic agent for one individual who had similar titers of antibodies to both agents. PCR assay of acute-phase serum in the absence of whole blood specimens may be a useful method for early detection of human ehrlichiosis and determination of etiology when serologic testing is inconclusive.

In the United States, human ehrlichiosis results from infection with either the human granulocytic ehrlichiosis (HGE) agent (3, 6) or *Ehrlichia chaffeensis* (9, 10). The Council of State and Territorial Epidemiologists established a surveillance case definition for ehrlichiosis which requires the presence of illness clinically compatible with human ehrlichiosis and relies on indirect immunofluorescence assays (IFA) and PCR assays for confirmation (5). Laboratory confirmation requires a fourfold change in IFA titer (seroconversion) of antibody to *Ehrlichia* sp. antigen, amplification of specific ehrlichial DNA sequences by PCR assay, or demonstration of intracytoplasmic microcolonies (morulae) together with a reciprocal titer of ≥64 (5). Seroconversion has been used to identify most cases of ehrlichiosis, but it may take 1 month or longer to obtain an adequate rise in titer (5), and it is often difficult to obtain convalescent-phase serum samples. Detection of morulae is not a sensitive technique, especially for *E. chaffeensis* (7). PCR assays offer an additional opportunity for early confirmation of ehrlichiosis.

PCR assays based on the 16S rRNA gene have been used to detect HGE agent and *E. chaffeensis* DNAs in acute-phase EDTA-anticoagulated whole blood (2, 6, 12, 15). Serum can also serve as a substrate for PCR testing. HGE agent DNA has been successfully amplified from acute-phase serum from HGE patients by using two rounds of amplification with the same primer set (8) or amplification with nested primer sets (12). In this study, we evaluated a 16S rRNA gene-targeted nested PCR assay of acute-phase serum as an alternative method for laboratory diagnosis of human ehrlichiosis.

MATERIALS AND METHODS

Samples. Serum samples, along with patient histories, were submitted to the Centers for Disease Control and Prevention (CDC) from 1987 to 1997 for serologic testing by IFA for suspected ehrlichial or rickettsial illness. Samples were collected from patients with probable or confirmed HGE, i.e., individuals who had clinically compatible illness and who had at least one titer of ≥64 of antibody to the HGE agent (5). We tested three groups of serum samples by PCR assay: (i) samples collected during the acute phase of illness from patients who subsequently seroconverted to either the HGE agent or to the HGE agent and to *E. chaffeensis* ($n = 20$); (ii) samples from suspected HGE cases, when only one sample had been tested by IFA or when seroconversion did not occur in paired serum samples ($n = 9$); and (iii) samples that were collected from individuals who were seropositive for both antigens but for whom there was a less-than-fourfold difference between the maximum titers of antibodies to one antigen and the other ($n = 14$).

We included three samples in which we surmised that ehrlichial microcolonies (morulae) had been seen in stained peripheral blood smears by the submitting physicians. Morulae were positively identified in one case, suspected in another, and referred to as neutrophilic inclusion bodies in the third. Samples originated from 14 states, including Arkansas ($n = 1$), California ($n = 3$), Connecticut ($n = 4$), Florida ($n = 4$), Georgia ($n = 1$), Maryland ($n = 2$), Minnesota ($n = 2$), Missouri ($n = 3$), Montana ($n = 1$), New York ($n = 11$), North Carolina ($n = 3$), Oklahoma ($n = 1$), Washington ($n = 1$), and Wisconsin ($n = 6$). The first sample submitted to CDC from each suspected case was tested. When an initial sample was positive, all subsequent serum samples from that patient were tested. Archived, frozen (−70°C), EDTA-anticoagulated whole blood samples from any of the individuals were tested when available.

IFA. Titers of antibody to the HGE agent were determined by a previously described IFA that used the USG3 isolate of the HGE agent cultivated in HL-60 cells (13). Titers of antibody to *E. chaffeensis* had been determined prior to HGE testing as described previously (10) and were obtained by examination of CDC records. Samples submitted to CDC prior to 1991 had been tested by using *E. canis* as a surrogate antigen for *E. chaffeensis*, and results for these samples were combined with those for *E. chaffeensis*.

Extraction of DNA. Serum was measured (volume in microliters), transferred into a 1.5-ml conical microtube, and centrifuged at 10,000 × *g* for 20 min at 4°C to pellet any particulate matter. We determined that this method was sufficient to concentrate cell-free *Ehrlichia*. Supernatant serum was then removed and retained for serologic analysis. DNA was extracted from the pellet and the remaining serum (approximately 20 μl) by using a QIAamp tissue kit (QIAGEN, Chatsworth, Calif.). Manufacturer recommendations, modified as follows, were followed. Briefly, 180 μl of lysis buffer (buffer ATL) and 20 μl of proteinase K (10 mg/ml) were added to the remaining pellet suspension. The suspension was vortexed and incubated at 55°C for 3 h; 200 μl of buffer AL was added, and the mixture was incubated at 70°C for 10 min. Absolute ethanol (210 μl) was added, and the entire mixture was placed into a QIAamp column and centrifuged at 6,000 × *g* for 1 min. The column was washed twice with 500 μl of buffer AW by centrifugation (6,000 × *g* for 1 min), followed by an additional centrifugation at 10,000 × *g* for 2 min to completely remove the wash buffer. DNA was eluted from the column with 200 μl of buffer AE preheated to 70°C. The loaded column was incubated at 70°C for 5 min and at room temperature for 1 min before centrifugation at 6,000 × *g* for 1 min. To increase the DNA yield, the 200 μl of

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TABLE 1. Geographic locations, times from onset of illness to sample collection, titers, and PCR results for individuals from whose serum ehrlichial DNA was amplified

No.	State	Sample no.	Days PO[a]	Titer[b]		PCR result	Sequencing result
				E. chaffeensis	HGE		
1	Wis.	1	8	<64	<64	Positive	HGE agent
		2	25	<64	512	Negative	
2	Minn.[c]	1	21	<64	512	Positive	HGE agent[d]
		2	31	<64	4,096	Negative	
3	Minn.	1	14	<64	65,536	Positive	HGE agent[d]
		2	30	<64	1,024	Negative	
4	Md.	1	Unavailable	<64	256	Positive	*E. chaffeensis*[e]
		2	Unavailable + 9	256	512	Negative	
		3	Unavailable + 26	512	512	Negative	
5	Fla.	1	9	1,024	<64	Positive	*E. chaffeensis*
		2	27	4,096	128	Negative	
6	Md.	1	Unavailable	256	64	Positive	*E. chaffeensis*
		2	Unavailable + 4	4,096	1,024	Negative	

[a] Days postonset (PO) of symptoms until collection of serum sample.
[b] *E. chaffeensis* was tested by using a polyvalent conjugate specific for immunoglobulin G (heavy and light chains); HGE was tested by using a monovalent conjugate specific for immunoglobulin G (heavy chain) (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Md.).
[c] This individual resided in Florida but was exposed and presented in Minnesota.
[d] Suspected intracytoplasmic inclusion bodies (morulae) were noted on a stained blood smear.
[e] Acute-phase EDTA-anticoagulated whole blood was also positive by PCR for *E. chaffeensis*; blood collected 9 days later was negative by PCR.

eluted DNA solution was transferred back to the same column, and the elution step was repeated. Whole blood (200 μl) was extracted similarly. Final DNA extracts were stored at 4°C until tested.

PCR assays. PCR assays were prepared by using commercial amplification reagent kits (GeneAmp; Roche Molecular Systems, Branchburg, N.J.). Five microliters of template DNA was added to 45 μl of a master mixture in 0.5-ml reaction tubes prepared as previously described (14). A nested reaction was conducted on all samples for a total of 80 cycles by using eubacterial 16S rRNA primers EC9 and EC12 (2) in the first round, followed by primers GE9f and GE10r for the HGE agent (6). A second PCR substituted primers HE1 and HE3 for *E. chaffeensis* (1) in the second round. In the first round, the reaction mixture cycled for 3 preliminary cycles (94°C, 1 min; 48°C, 2 min; 68°C, 1.5 min), followed by 37 amplification cycles (88°C, 1 min; 55°C, 2 min; 6°C, 1.5 min). An additional extension period of 5 min at 68°C was added to the final cycle. During the second round of amplification, the annealing temperature of the reaction was raised to 55°C and the extension temperature was raised to 72°C. A negative control (gamma-irradiated water), an HGE-positive control (USG3 isolate grown in HL-60 cells), and an *E. chaffeensis*-positive control (Arkansas strain grown in DH-82 cells) were included in every test. PCR products were separated by agarose gel electrophoresis, stained with ethidium bromide, visualized by UV illumination, and photographed.

PCR products of the expected sizes (919 bp for the HGE agent, 389 bp for *E. chaffeensis*) were sequenced by using an ABI Prism 377 sequencer (ABI, Foster City, Calif.). Products from *E. chaffeensis* were further evaluated by amplification and sequencing of a repetitive-motif sequence (variable-length PCR target [VLPT]) that has been shown to vary in repeat numbers in different isolates of *E. chaffeensis* (15). The PCR primers and parameters used for amplification of the VLPT have been described previously (15).

Strict precautions were taken to minimize the risk of amplicon contamination and generation of false-positive results. All positive results were confirmed by reamplification from the original extract.

RESULTS

Fifty serum samples from 43 patients were tested by using a nested PCR assay. Products from either the HGE agent or *E. chaffeensis* were amplified from serum of six (14%) individuals residing in Florida, Maryland, Minnesota, and Wisconsin (Table 1). One of the patients who was PCR positive for HGE resided in Florida but had traveled to rural northern Minnesota 3 weeks prior to the onset of symptoms, where he was diagnosed. No ehrlichial DNA was detected in serum from the remaining 37 patients, including 12 confirmed and 9 probable cases of HGE, 3 confirmed cases of *E. chaffeensis*, and 13 individuals who had less-than-fourfold differences between maximum titers of antibodies to the agents and for whom etiology was not ascribed.

Of the three individuals who were positive for the HGE agent by PCR, one was simultaneously seronegative and two were simultaneously seropositive for HGE (Table 1). Suspected morulae were observed in blood smears of the latter two individuals. Samples that were seronegative or seropositive for *E. chaffeensis* were also simultaneously positive by PCR for *E. chaffeensis* in three individuals (Table 1). Two of the latter individuals also seroconverted to the HGE agent, but the titers of antibodies to *E. chaffeensis* were at least fourfold greater. One individual (no. 4) who was positive for *E. chaffeensis* by PCR had detectable antibodies to the HGE agent but not to *E. chaffeensis* in the acute-phase sample; subsequent samples demonstrated a rise in the titers of antibodies to both agents (Table 1). No ehrlichial DNA was detected in second ($n = 6$) or third ($n = 1$) serum samples which were collected from the six PCR-positive individuals.

Seven blood samples were available from 6 of the 43 individuals. *E. chaffeensis* DNA was amplified from the first of two blood samples from an individual (no. 4) whose serum was positive by PCR. No ehrlichial DNA was detected in the remaining six blood specimens.

PCR-positive serum samples were collected on days 8, 9, 14, and 21 following onset of illness (Table 1). Samples collected from the same individuals on days 25, 27, 30, and 31, respectively, after illness onset were negative by PCR. Dates of serum collection for 14 PCR-negative individuals ranged from 3 to 32 days (median, 5.5 days) following the onset of symptoms.

Nine individuals received doxycycline therapy during the course of their illness. Only one PCR-positive patient (no. 3) received doxycycline therapy, which was given the day after the serum sample was drawn. Three PCR-negative patients had received doxycycline therapy from 1 to 3 days before the collection of serum.

TABLE 2. Serologic categorization of 43 individuals whose serum samples were selected for testing by PCR

Serum sampling and inferred agent	Serologic evidence of infection	No. of samples tested	No. (%) positive
Multiple			
HGE agent	Seroconversion[a] to HGE agent; antibodies to HGE agent only present	12	3 (25)
	Seroconversion to HGE agent; maximum titer at least fourfold greater than that of *E. chaffeensis*	3	
	Antibodies to HGE agent only; titer of antibody to HGE agent ≥64 in both samples but no seroconversion	3	
E. chaffeensis	Seroconversion to *E. chaffeensis*; maximum titer at least fourfold greater than that of antibody to HGE agent	5	2 (40)
Undetermined	Maximum titer of antibody to one agent less than fourfold greater than maximum titer of antibody to other agent	9	
Single			
HGE agent	Antibodies to HGE agent only present	6	
Undetermined	Less-than-fourfold difference in titers	5	

[a] Defined as a fourfold or greater change in titers of immunoglobulin G (gamma chain) for HGE and immunoglobulin G (heavy and light chains) for *E. chaffeensis* in successive samples with at least one titer of ≥64.

The six PCR-positive samples were concentrated from serum volumes of 630 to 1,400 μl (mean, 988 μl). The 37 PCR-negative samples had volumes of 40 to 1,700 μl (mean, 655 μl), but the difference was not significant ($P > 0.05$, Wilcoxon W).

All PCR products were confirmed by nucleotide sequencing of 16S rRNA gene fragments as belonging to the HGE agent or *E. chaffeensis*. All samples that were positive for the *E. chaffeensis* 16S rRNA gene by PCR were confirmed by using VLPT primers. Products from two individuals contained four repeats, and a similar product was amplified from the blood of one of these individuals (Table 1). The third patient's VLPT product contained five repeats (15).

Etiology, as indicated by the highest serologic antibody titer, was the same as that indicated by PCR whenever there was a fourfold difference between the maximum titers of antibodies to the HGE agent and *E. chaffeensis* in patients with antibodies to both antigens (Table 2). By using this criterion, etiology was ascribed for nine of the individuals tested in this study. Overall, there were 24 confirmed or probable cases of HGE from nine states: California ($n = 1$), Connecticut ($n = 2$), Florida ($n = 3$), Georgia ($n = 1$), Minnesota ($n = 2$), Montana ($n = 1$), New York ($n = 9$), Washington ($n = 1$), and Wisconsin ($n = 4$). There were no significant travel histories for HGE cases from Florida, Georgia, or Montana. There were five cases of *E. chaffeensis* from Florida ($n = 1$), Maryland ($n = 1$), Missouri ($n = 2$), and North Carolina ($n = 1$). Etiology was not determined for 14 individuals from nine states.

DISCUSSION

During the early stages of human ehrlichiosis, serum may possibly include residual *Ehrlichia*-infected leukocytes, free ehrlichiae, or free ehrlichial DNA, all of which may result in a positive PCR assay. Although a specific and rapid laboratory diagnosis may occasionally be obtained by PCR testing of acute-phase serum, this method lacked satisfactory sensitivity in the present study, as only 6 (14%) of the 43 individuals tested were positive by PCR.

Because all of the individuals in our study were ill and had at least one sample that was seropositive for HGE, each represented at least a probable case of HGE by the Council of State and Territorial Epidemiologists definition (5). However, 22 (51%) of the 43 individuals were seropositive for *E. chaffeensis*, including five confirmed cases of *E. chaffeensis* (Table 2). This study and others (13, 16) suggest that a fourfold or greater difference in maximum titers may be used to infer the identity of the infecting ehrlichial agent for individuals who are seropositive for both antigens. Although the HGE agent and *E. chaffeensis* IFA used different conjugates, PCR results were in agreement when serologic results indicated a fourfold or greater difference in the maximum titers of antibodies to either agent.

Although only a limited number of samples were evaluated, the geographic distribution of PCR-positive individuals was consistent with the known geographic distributions of the two agents (3, 4, 10, 15, 17). Etiology was confirmed by PCR for one individual from Maryland whose serologic results were equivocal (Table 1). Had this individual's first sample only been tested by serology, a diagnosis of HGE would have been suggested. Although simultaneous or prior infection with the HGE agent cannot be ruled out, the initial response to the HGE agent underscores the need to use both antigens when testing for human ehrlichiosis in the United States.

Suspected morulae were detected in blood smears of two of the patients who were positive for the HGE agent by PCR and were also noted in a smear from an individual who was negative by PCR but who strongly seroconverted (titers, <64 to 4,096) to the HGE agent. The acute-phase sample from the latter patient had been collected 5 days after antibiotic therapy had been given. While this may be the cause of the negative results for this sample, as well as for the three PCR-negative samples from individuals who had received doxycycline treatment prior to the collection of serum, only 150 μl of this patient's serum was available for testing. The sensitivity of PCR assays has been previously demonstrated to be influenced by the volume of the clinical sample processed (11); however, although the mean volume of the positive samples was greater than the mean volume of the negative samples, the difference was not significant.

Ehrlichial DNA was detectable in the serum of patients from days 8 to 21 following the onset of illness, similar to findings reported by Dumler and Bakken (8), who recovered DNA of the HGE agent from patient serum from days 2 to 30 after the onset of illness. They recovered DNA of the HGE agent from serum that had titers of antibody to *E. equi* of 160 and 320 and from seronegative serum samples of two patients. We amplified the DNAs of both agents from seronegative and seropositive samples, although not simultaneously. To our knowledge, this is the first report of amplification of *E. chaffeensis* DNA from serum.

Our data suggest that PCR testing of acute-phase serum is not a highly sensitive method for the early diagnosis of human ehrlichiosis. The observed concordance of the PCR and serologic results supports the use of a fourfold difference between the maximum titers of antibodies to the antigens to infer etiology for patients who react to both antigens. Although the sensitivity is low, PCR of acute-phase serum in the absence of whole blood may be used to confirm a diagnosis or determine etiology when serologic testing is inconclusive.

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