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Outcome of Diagnostic Tests Using Samples from Patients with Culture-Proven Human Monocytic Ehrlichiosis: Implications for Surveillance

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We describe the concordance among results from various laboratory tests using samples derived from nine culture-proven cases of human monocytic ehrlichiosis (HME) caused by *Ehrlichia chaffeensis*. A class-specific indirect immunofluorescence assay for immunoglobulin M (IgM) and IgG, using *E. chaffeensis* antigen, identified 44 and 33% of the isolation-confirmed HME patients on the basis of samples obtained at initial clinical presentation, respectively; detection of morulae in blood smears was similarly insensitive (22% positive). PCR amplifications of ehrlichial DNA targeting the 16S rRNA gene, the variable-length PCR target gene, and the *groESL* operon were positive for whole blood specimens obtained from all patients at initial presentation. As most case definitions of HME require a serologic response with compatible illness for a categorization of even probable disease, PCR would have been required to confirm the diagnosis of HME in all nine of these patients without the submission of a convalescent-phase serum sample. These data suggest that many, if not most, cases of HME in patients who present early in the course of the disease may be missed and underscore the limitations of serologically based surveillance systems.

Since the first isolation of *Ehrlichia chaffeensis* in cell culture in 1990 (5), only three additional isolates had been recovered by 1997 (14). The most common impediments to obtaining isolates of *E. chaffeensis* have been ascribed to the fastidious nature of the organism or to problems in obtaining whole blood samples from acutely ill persons prior to the initiation of antibiotic therapy. Disease caused by this tick-borne pathogen is not commonly reported; however, more than 300 cases of serologically confirmed human monocytic ehrlichiosis (HME) have been identified through testing of paired acute- and convalescent-phase specimens submitted to the Centers for Disease Control and Prevention (CDC) since 1986 (2a).

Increasingly, cases of HME and human granulocytic ehrlichiosis (HGE) are being identified through mandated reporting programs initiated by state health departments. The case definitions adopted by the Council of State and Territorial Epidemiologists for the surveillance of the human ehrlichioses include categories of "probable" disease (compatible illness with a single serum sample that tests positive above a certain minimum endpoint antibody titer) and "confirmed" disease (compatible illness with a fourfold-or-greater change in antibody titer between paired acute- and convalescent-phase serum specimens) (2). Other laboratory criteria that provide confirmation of HME or HGE are the presence of membrane-bound, intracytoplasmic bacterial aggregates (morulae) in blood, cerebrospinal fluid, or bone marrow, concomitant with a single positive serum sample, and the detection of specific ehrlichial DNA sequences after DNA extraction of patient tissue and PCR amplification by using specific primers for *E. chaffeensis* or the HGE agent. The latter two confirmatory tests are not universally available at state health laboratories and require special expertise in their routine application and interpretation.

Because the diagnosis of HME depends primarily on laboratory methods designed to detect the patients' immune response to the pathogen or the presence of ehrlichial DNA, it is essential to establish the concordance among various test results when using samples derived from culture-proven cases. As with any cultivable pathogen, culture confirmation of *E. chaffeensis* infection must be regarded the "gold standard" for defining the parameters of other diagnostic tests. In this report, we summarize the laboratory findings from nine patients with culture-confirmed HME. The findings from this case series underscore the limitations of serologically based surveillance systems and suggest that a secondary level of testing is required to identify the majority of HME cases when only a single acute-phase blood specimen is available for testing.

MATERIALS AND METHODS

Patient samples. Whole blood samples were submitted to CDC by physicians who treat patients with suspected HME. Six isolates of *E. chaffeensis* were obtained from samples submitted to CDC during 1997 and 1998. Serum or plasma specimens were available from each HME patient for acute-phase antibody titer determinations, and a second serum sample was obtained from five of six patients. Isolates of *E. chaffeensis* were also obtained from three patients who presented with suspected HME at Vanderbilt University, Nashville, Tenn. Coded samples of whole blood specimens yielding these isolates were frozen at −80°C until being forwarded to CDC along with three pairs of EDTA-anticoagulated blood samples from non-HME patients. Paired acute- and convalescent-phase sera were available from these three patients. Cell culture growth characteristics and limited genetic sequence comparisons of two of the *E. chaffeensis* isolates have been reported previously (14), as have detailed comparisons of variable-length PCR target (VLPT) gene sequences for six of the isolates (18).

Isolation of *E. chaffeensis*. Aliquots of EDTA-anticoagulated whole blood were diluted with 2 volumes of Hanks' balanced salt solution and layered onto Histpaque 1083 (Sigma Diagnostics, St. Louis, Mo.). Following centrifugation at 800 × *g* for 20 min, the pelleted cells were resuspended and placed in 25-cm^2 flasks containing a semiconfluent layer of DH82 cells, as described in detail previously (14). Inocula were removed after 24 h, and fresh culture medium was

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TABLE 1. Results of diagnostic testing by thin smear, class-specific IFA serology, and amplification of three different gene targets by PCR[a] for nine isolation-confirmed HME cases caused by *E. chaffeensis*

Patient no./isolate	Presence of morulae[b]	IFA titer[c]				PCR target gene result		
		First serum sample		Second serum sample		16S rRNA	VLPT (no. of repeats)	*groESL* operon
		IgM (days postonset)	IgG	IgM (days postonset)	IgG			
1/Jax	−	16 (7)	256	32 (13)	1,024	+	+ (4)	+
2/St. Vincent	+	<16 (4)	<64	<16 (8)	<64	+	+ (3)	+
3/Osceola	+	16 (3)	<64	64 (9)	1,024	+	+ (4)	+
4/Wakulla	−	<16 (5)	512	256 (41)	2,048	+	+ (6)	+
5/Liberty	−	64 (3)	64	NT[f]	NT	+	+ (4)	+
6/West Paces	−	<16 (3)	<64	64 (51)	512	+[d]	+ (3)	NT
7/V5[e]	−	<16 (3)	<64	512 (43)	8,192	+	+ (4)	+
8/V6[e]	−	16 (3)	<64	512 (50)	2,048	+	+ (4)	+
9/V7[e]	−	<16 (4)	<64	128 (53)	256	+	+ (5)	+
No. positive/total (%)	2/9 (22.2)	4/9 (44.4)	3/9 (33.3)	7/8 (87.5)	7/8 (87.5)	9/9 (100)	9/9 (100)	8/8 (100)

[a] 16S rDNA was amplified by nested PCR, as required for patients 5 and 6.
[b] Intracytoplasmic aggregates of bacteria observed in peripheral blood smears.
[c] IFA titers for IgM of ≥16 and for IgG of ≥64 are considered evidence of current or past infection with *E. chaffeensis*.
[d] Detected by heminested assay.
[e] Isolates designated V5 to V7 were obtained at Vanderbilt University, although additional testing of whole blood and sera was conducted at CDC.
[f] NT, not tested.

added to the flasks, after which cultures were monitored for evidence of ehrlichial infection by using cytocentrifuged preparations stained with Diff-Quik.

IFA testing. Serum or plasma samples were tested in class-specific assays using the Arkansas isolate of *E. chaffeensis* as the antigen. The test details were as previously described (13) for the HGE agent and *E. chaffeensis* testing. To minimize variation, all samples were tested together by using the same lots of antigen slides, conjugates, and other reagents.

The assay for immunoglobulin M (IgM) determinations followed the general indirect immunofluorescence assay (IFA) protocol, but pretreatment of each sample was included to remove interference from rheumatoid factors and specific IgG. Prior to assay using fluorescein isothiocyanate-labeled conjugate to human IgM (μ chain specific) at a 1/100 dilution (Kirkegaard & Perry Laboratories, Gaithersburg, Md.), 50-μl serum samples were depleted of IgG by using a recombinant protein G affinity method (Quik-Sep IgM; Isolab, Inc., Akron, Ohio). For the reciprocal endpoint titers of each assay, see below and Table 1. Geometric mean titers (GMT) were calculated for reactive samples with IgM titers of ≥16 and IgG titers of ≥64.

Extraction of DNA from blood samples and cultured cells. DNA was extracted from 200 μl of patient whole blood by using the QIAmp Blood Kit (Qiagen Inc., Santa Clarita, Calif.) in accordance with the manufacturer's recommendations.

PCR analyses of whole blood. PCR assays targeting three different genes were used to detect and distinguish ehrlichial DNA. As these samples were initially processed for diagnostic evaluation in separate laboratories, each of these assays was performed independently and different DNA extractions were used for the 16S rRNA gene (rDNA) assays than for the VLPT and *groESL* operon assays. Therefore, the analytic sensitivities of the various assays could not be compared and were not assessed (17).

(i) 16S rDNA (primary and nested). The 16S rDNA was amplified by using direct (single-step) and heminested protocols. The direct assay used primers HE1 and HE3 and has already been described in detail (1). The heminested assay consisted of a 50-μl primary reaction mixture containing 5 μl of the purified DNA as the template and primers HE1 (1) and HE20 (5′ GAATTCCGCTAT CCTCTTTCGAC). All reagents were from the GeneAmp PCR Kit with AmpliTaq DNA Polymerase (Perkin-Elmer, Foster City, Calif.), and reactions were performed in a Perkin-Elmer 9600 thermal cycler. Each primary PCR amplification mixture contained 200 μM each deoxynucleoside triphosphate (dATP, dCTP, dGTP, and dTTP), 1.25 U of *Taq* polymerase, and 0.5 μM each primer. Cycling conditions involved an initial 2-min denaturation at 95°C and 40 cycles each consisting of a 30-s denaturation at 94°C, 30 s of annealing at 55°C, and a 1-min extension at 72°C. These 40 cycles were followed by a 5-min extension at 72°C. Reaction products were subsequently maintained at 4°C until analyzed by agarose gel electrophoresis or used as templates for nested reactions.

Heminested PCRs used 1 μl of the primary PCR product as the template. Each nested amplification contained 200 μM each deoxynucleoside triphosphate (dATP, dCTP, dGTP, and dTTP), 1.25 U of *Taq* polymerase, and primers HE1 and HE3 at 0.2 μM each in a total reaction volume of 50 μl. Nested cycling conditions were identical to those of the primary amplification, except that only 30 cycles were used.

(ii) VLPT. The VLPT is a repetitive-motif sequence that varies in the number of repeats among different isolates of *E. chaffeensis* (14). The number of repeats was determined for each whole blood sample and the corresponding *E. chaffeensis* isolate as an independent marker of the uniqueness of each isolate. The PCR method and primers used for amplification of the VLPT from blood samples originally submitted to CDC have been described previously in detail (18). Patient whole blood samples from which cell culture isolations were made at Vanderbilt University were tested at CDC to help assess interlaboratory consistency. The standard VLPT primers were used (14), but the originally described methods were changed by using Ready-To-Go PCR Beads in 0.2-ml tubes (Amersham Pharmacia Biotech, Piscataway, N.J.) and a different thermocycler, the Perkin-Elmer GenAmp 9600. For each 25-μl reaction mixture, individual beads were dissolved in 23 μl of water containing primers FB5A and FB3A (each at a concentration of 1 μM) to which 2 μl of the appropriate DNA extraction was added. Thermocycler parameters were 94°C for 2 min, followed by 40 cycles consisting of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min and then extention at 72°C for 7 min. PCR products were separated by agarose gel electrophoresis, stained with ethidium bromide, and visualized by UV illumination.

(iii) *groESL* operon. Primers HS1 and HS6 (primary reaction) and primers HS43 and HS45 (nested reaction) were used to amplify a 528-bp segment of the *groESL* heat shock operon from patient blood samples corresponding to the Osceola, Liberty, West Paces, and Wakulla isolates of *E. chaffeensis* by methods described in detail elsewhere (18). The same protocol was used for blood samples corresponding to the Jax and St. Vincent isolates, except that primer HS79 was substituted for primer HS43 to amplify a larger segment (1,302 bp) for nucleotide sequencing (14). As with the VLPT PCR, the blood samples from Vanderbilt University were tested by using Ready-To-Go PCR Beads and a Perkin-Elmer GenAmp 9600. Cycling parameters for primary and nested reactions were identical to those listed for the VLPT, except that the annealing temperature for the *groESL* PCR primary reaction (primers HS1 and HS6) was changed to 50°C.

Confirmation of PCR products. Purified PCR preparations from the VLPT reactions of samples originally obtained at CDC were sequenced by using the Prism Ready DyeDeoxy Cycle Sequencing kit (Applied Biosystems) as previously described (18, 19). Both strands were sequenced by using an Applied Biosystems 370A or 377 automated sequencer.

RESULTS

Whole blood specimens from nine clinically ill patients with a diagnosis of suspected HME were collected a median of 3 (range, 3 to 7) days after the initial onset of illness (Table 1). Morulae were noted in the stained peripheral blood smears from two of the nine patients. Four of the nine patients had a class-specific IgM antibody titer to *E. chaffeensis* at the time of initial clinical evaluation that would have been considered of diagnostic relevance with the IFA (GMT, 22; range, 16 to 64); three endpoint titers were at the minimum value tested, i.e., 16. Three of the nine patients had detectable titers of IgG anti-

body to *E. chaffeensis* at the time of their initial clinical evaluation that would have been considered of diagnostic relevance with the IFA (GMT, 204; range, 64 to 512); one endpoint titer was equal to the minimum value considered significant in this study, i.e., 64.

Of the eight patients from whom a second serum sample was collected at a median of 42 (range, 8 to 53) days postonset, four additional patients developed IgM titers of ≥16, bringing the number of patients with detectable IgM to seven of eight (GMT, 141; range, 32 to 512). The three patients with initial IgM titers of 16, for whom second samples were available, showed titer increases to 32, 256, and 512 (Table 1). Five patients demonstrated class-specific IgG seroconversion to *E. chaffeensis* antigen from initial titers of <64 to titers ranging from 128 to 4,096 (Table 1). Two patients with IgG antibody titers of ≥64 at initial presentation had greater-than-fourfold increases in their endpoint titers at the second sampling, 6 and 36 days after their first blood samples were drawn (patients 1 and 4; Table 1). Overall, the number of patients with detectable IgG at either the first or second testing of sera was eight of nine. The IgG class-specific GMT for the seven patients with positive second serum samples was 1,261 (range, 256 to 8,192). One patient concomitantly infected with the human immunodeficiency virus (patient 2; Table 1) had IgM titers of <16 and IgG titers of <64 at days 4 and 8 after onset of illness, at which time he died of HME.

EDTA-anticoagulated whole blood samples obtained at the time of initial presentation and leading to the isolation of *E. chaffeensis* were positive for ehrlichial DNA by PCR amplification of regions of the 16S rDNA, the VLPT gene, and the *groESL* operon for all patients (Table 1).

The number of repeat units present in the PCR amplicons of the VLPT were identical when the results from whole blood samples and *E. chaffeensis* isolates obtained in cell culture were compared. Samples analyzed separately at Vanderbilt University and CDC gave identical results. None of the three paired samples from non-HME cases submitted by Vanderbilt University was positive by any test.

DISCUSSION

The results reported here, although based on a small series of HME patients, are a first attempt to compare diagnostic outcomes from various laboratory tests by using specimens obtained from patients with culture-confirmed *E. chaffeensis* infection. These comparisons permitted a preliminary assessment of different methods for inferring an infectious process either through detection of antibody or through identification of DNA from organisms of undetermined viability. The validity of laboratory methods, such as serologic testing, in determining disease status early in infection is frequently assumed and relied upon by the public health community interested in surveilling for HME (8). The data reported here indicate that early serologic testing by the most widely available test, an IFA for IgM and IgG antibody detection using *E. chaffeensis* antigen, missed 56 and 67% of the isolation-confirmed HME patients, respectively, at initial clinical presentation and first-sample collection. Detection of morulae in blood smears (22%) was also insensitive for HME diagnosis. As most case definitions of HME require a serologic response with compatible illness for categorization of even probable disease (2), many, if not most, cases of HME in patients who present early in the course of the disease may be missed. The significance of these findings is corroborated by results from epidemiologic studies of HME indicating that the median time from disease onset to the first visit to a physician was 4 days (8), similar to the 3-day interval described in this case series.

It is widely understood that initiation of antibiotic treatment for potentially fatal tick-borne diseases, such as the ehrlichioses or Rocky Mountain spotted fever (RMSF), rests on clinical suspicion and not laboratory test results that may require weeks to obtain (7, 10). As laboratory confirmation of the diagnosis is not directly linked to initiation of treatment, obtaining second serum samples during convalescence, typically 3 to 6 weeks after onset of disease, may become a low priority for physicians. However, case definitions for the confirmation of a diagnosis of a tick-borne rickettsial disease, promulgated by the Council of State and Territorial Epidemiologists and CDC and in use by many states, require testing of paired serologic specimens to demonstrate a fourfold-or-greater change in antibody titer. Although seven of the eight patients for whom paired samples were available seroconverted by IgG or IgM with a fourfold-or-greater increase in antibody titer, most serologic evaluations for the ehrlichioses and RMSF are based on analysis of a single acute-phase serum sample (2a, 4). Secondary testing of whole blood or serum by PCR would have been required to confirm the diagnosis of HME in all nine of these patients, as only three or four would have been classified as probable on the basis of their clinical disease and a single positive serologic titer (IgM or IgG).

Although results from molecular diagnostic methods correlated well with the culture-positive samples, PCR testing for HME is not a universally available or necessarily desirable confirmatory test for routine application. The PCR assays conducted in this study were performed on whole blood, not serum, and in many instances serum or coagulated whole blood will be the only sample submitted to laboratories. Although potentially useful in certain applications, serum-based PCR assays for the human ehrlichioses are still under assessment (3,- 6, 11, 15). In addition, there remain questions in interpreting a single positive PCR result in the absence of serologic or other corroborating laboratory results. Culture confirmation of HME, as with HGE (9), must be regarded as the gold standard for investigation of the parameters of other diagnostic tests, although this method may not ultimately be the most sensitive measure of infection.

The results we present here for HME cases are not unique among the tick-borne rickettsioses. In analyses comparing culture-proven and culture-negative cases of HGE, nested PCR assays for the 16S rDNA showed a concordance of 100% with eight culture-proven cases, as did detection of morulae for seven available samples (9). However, at initial presentation, only two of eight culture-positive cases had antibodies to HGE antigen that were detectable by IFAs designed to detect multiple classes of antibody, although six of the eight eventually showed fourfold-or-greater titer changes at the second sampling. In an additional study of HGE, two of four culture-positive patients had "equivocal" IgM or IgG titers (1:80) at the first IFA 2 days after onset of disease (serologically negative samples from the other culture-confirmed cases were collected 1 and 5 days after disease onset), although enzyme immunoassay and Western immunoblotting results were negative for these four culture-positive patients at initial presentation (16). Similarly, approximately half of all fatalities due to RMSF occur within the first week of illness in persons lacking diagnostic antibody titers at the time of death. These deaths would not be classified as even probable RMSF cases without subsequent autopsy and immunohistochemical demonstration of rickettsiae in tissues by pathologists (13a).

A survey of state health departments conducted by CDC indicated that in 1997, ehrlichiosis (HGE and/or HME) was a

reportable condition in 19 states, of which 13 offered some type of serologic testing for antibody (12). Of these 13 state laboratories, 7 offered molecular diagnostic testing, although at least two depended entirely on university or private laboratories for PCR testing. Less than one-third of the states that mandate ehrlichiosis reporting have the capacity to offer additional laboratory testing beyond serology, and it seems certain that despite the recognized limitations, serologic testing will remain the standard for evaluation of suspected HME cases into the near future. The current standards and limitations of laboratory methods for diagnosing HME indicate that if additional efforts are to be made to improve the sensitivity and specificity of available diagnostic procedures, then encouraging the collection of convalescent-phase blood specimens may be the most reasonable recourse for the immediate future. Additional efforts among research institutions, state health departments, and CDC are required to help assess and define the analytical and diagnostic sensitivities and specificities of the myriad PCR assays currently in use. When the distinction between confirmed and probable HME cases is required for specific studies, arrangements should be made for the collection of anticoagulated whole blood specimens for confirmatory testing by PCR or isolation at a site performing these activities. As a complement to current surveillance efforts, studies that estimate errors in calculating HME case numbers by using various laboratory assays and limited specimens will be of continued importance.

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