Human monocytotropic ehrlichiosis (HME): epidemiological, clinical and laboratory diagnosis of a newly emergent infection in the United States

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This study addresses clinical, epidemiological and laboratory diagnostic issues of human monocytotropic ehrlichiosis (HME) based on results of an ongoing prospective study in Cape Girardeau, Missouri; 82 patients have been enrolled in the study. Samples were tested by immunofluorescent assay (IFA), for antibodies to *Ehrlichia chaffeensis*, and isolation was attempted in DH82, HL60 and THP-1 cells.

Twenty-two cases of HME were diagnosed during 1997 and 1998. Twelve cases were diagnosed by IFA only, four by PCR only, and six by both PCR and IFA. The target genes for amplification included the 16S rRNA gene, the 120 kDa protein gene and the *nad A* gene. DNA sequence analysis of the PCR products revealed more than 99% homology with the *E. chaffeensis* genes. Seroconversion was documented in five cases. No isolates have been obtained.

Based on our diagnostic criteria, we have 17 definite cases of HME and five probable cases. Thus, the provisional incidence of HME in Cape Girardeau was 8 cases per 100,000 population during 1997 and 14 during 1998. PCR is a sensitive and highly specific technique for diagnosis of HME in the acute phase.

Ehrlichia, nested PCR, IFA, HME, Cape Girardeau

Introduction

Ehrlichiae are obligately intracellular bacteria that reside in a phagocytic vacuole and have evolved in close association with an arthropod vector and a zoonotic host [1]. Human monocytotropic ehrlichiosis (HME) was first described in 1987 in the United States [2]. Four years later the etiologic agent, *Ehrlichia chaffeensis*, was isolated from a patient and classified in the genus *Ehrlichia* based on 16S rRNA subunit gene sequence analysis [3]. HME, has a clinical spectrum that ranges from mild to life threatening infections. More than 450 cases have been described at CDC in more than 30 of the United States, and this figure most likely represents an underestimate of the true incidence of the disease [4]. Data from a large reference laboratory (MRL Diagnostics) have added more than 1500 cases diagnosed serologically (unpublished data). The clinical and laboratory diagnosis of HME remains challenging. Clinical signs and symptoms and routine laboratory diagnostic tests are rather insensitive and non-specific for HME.

We established a prospective clinico-epidemiological study in Cape Girardeau, Missouri in order to determine the incidence and prevalence of HME in an endemic area, better characterize the clinical presentation of the disease and to evaluate diagnostically specific diagnostic tools for

Rickettsiae and rickettsial diseases at the turn of the third millenium
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HME such as detection of antibodies by immunofluorescent assay (IFA) using *E. chaffeensis* as antigen and polymerase chain reaction (PCR) for three target genes specific for *E. chaffeensis* (120 kDa protein gene, *nad A* gene and the 16S rRNA subunit gene). This work describes the preliminary results of the study being conducted in Cape Girardeau.

Materials and methods

Epidemiologic and clinical data: The total population covered by the health services offered by two multispecialty group medical practices was approximately 100,000 people. The area includes Cape Girardeau and surrounding counties in southeast Missouri and southwestern Illinois. Patients were enrolled between August 1996 and November 1998. The clinical case definition of an HME case included patients who had had fever for at least three days, possible tick exposure, and no other infectious disease diagnosis established. The patients were given two questionnaires to be completed during the acute phase and convalescent phase when a diagnosis of HME was confirmed with appropriate laboratory studies.

Laboratory case definition criteria:

Definite HME case:

Clinical signs and symptoms suggestive of HME and:

a. Serologic IgG rise from < 1:64 to ≥1:64 with a positive PCR result or
b. IgG seroconversion (four-fold rise) to ≥1:128 without positive PCR or
c. Positive PCR results in two separate laboratories or for at least two target genes or
d. Single serum IgG titer of ≥1:256 or:
e. Positive culture for *E. chaffeensis*.

Probable case of HME:

Clinical signs and symptoms suggestive of HME and:

a. Single IgG titers of 1:64 or 1:128 or
b. Positive PCR in one laboratory for only one target gene.

Processing of blood samples: DNA was extracted from the acute-phase blood collected during the initial visit of the patient. The samples were collected in EDTA-containing tubes and refrigerated immediately. Samples were then sent in wet ice overnight to the Rickettsial and Ehrlichial Diseases Research Laboratory at the University of Texas Medical Branch in Galveston, Texas. The samples were placed at 4°C and processed the same day that they were received. The anticoagulated blood was first diluted 1:1 with phosphate buffered saline (PBS, pH 7.1) and the blood elements separated by using gradient centrifugation with Ficoll Hypaque [5]. The mononuclear band was then harvested and washed twice in PBS and resuspended in 2 mL of PBS; 500 µl were then inoculated into DH82, THP-1 and HL-60 cell lines. The remaining 500 µl were saved for PCR analysis. Serum samples were kept at -20°C until IFA analysis was performed.

Indirect immunofluorescent assay (IFA): Sera were diluted 1:64 in PBS with 1% BSA. All sera were screened at this dilution. Positive sera were then titrated serially in two-fold increments up to a dilution of 1:4,096. The highest dilution with a 1+ intensity of fluorescent staining was considered the end-point titer. In short, an aliquot of 10 µl of serum at a 1:64 dilution was placed on the antigen slides. Cells infected with *E. chaffeensis* (Arkansas strain) were used as antigen. The glass slides were placed in a humidified chamber and incubated for 30 minutes at 37°C. The slides were then washed three times in PBS, 10 minutes each; 10 µl of FITC-labeled goat anti-human immunoglobulin at a 1:80 dilution were placed on the glass slides and incubated for 30 minutes at 37°C in a humidified chamber. The slides were then washed three times in PBS and

counterstained with 1% Evans blue. The glass slides were then examined under ultraviolet light with excitation and barrier filters for fluorescein in a Nikon microscope.

Preparation of DNA: DNA was extracted from the harvested mononuclear band, as described previously. The pellet was first resuspended in digestion buffer (100 mM NaCl, 10 mM Tris-Cl [pH 8.0], 25 mM EDTA, 0.5% SDS and 0.1 mg/mL proteinase K) and incubated for 10-12 hours at 50°C. Proteinase K was then inactivated for 5 minutes at 95°C. An aliquot of 250 µl was then mixed with an equal amount of phenol:chloroform:isoamylalcohol (25:24:1), and the aqueous phase was then re-extracted twice. The DNA was then precipitated in the presence of 7.5 M ammonium sulfate and 100% ethanol. The DNA pellet was then washed in 70% ethanol and vacuum-dried for 30 minutes and resuspended in 50 µl of DNAase- and RNAase-free water [6].

PCR reactions:

16S rRNA subunit gene: For the outside amplification of this gene, a 100 µl reaction mixture containing 10 µl of DNA template, 75 µl of sterile H_20, 10 µl of 10X PCR buffer (Boehringer Manheim, Indianapolis, IN), 1 µl of primers ECB (5'-CGTATTACCGCGGCTGCTGGCA-3') and ECC (5'-AGAACGAACGCTGGCGGCAAGCC-3') at a final concentration of 1 µM each, 2 µl of deoxynucleotide triphosphates (final concentration, 200 µM), and 1 µl of *Taq* polymerase (Boehringer Manheim, Indianapolis, IN) final concentration 2.5 U. The cycling program consisted of 35 cycles: 1 min at 94°C, 2 min at 45°C and 60 sec at 72°C. For nested PCR, 1 µl of each outside reaction was amplified in a second 100 µl reaction tube after careful manipulation of the specimens in an AirClean 600 Workstation (AirClean Systems, Raleigh, North Carolina) and aspiration of the PCR mixture with cotton-filled tips. The conditions were essentially the same except for the use of species-specific primers *for E. chaffeensis*, HE1 (5'-CAATTGCTTATAACCTTTTCCTTATAAAT-3') and HE3 (5'-TATAGGTACCGTCATTATCTTCCCTAT-3'). The cycling program consisted of 35 cycles: 1 min at 94°C, 2 min at 55°C, and 1 min at 72°C.

120 kD protein gene: The outside amplification reactions contained the same reagents as described above with the exception of *E. chaffeensis* species-specific primers for the 120 kD protein gene: PXCF3 (5'-GAGAATTGATTGTGGAGTTGG-3') and PXAR4 (5'-ACATAACATTCCACTTTCAAA-3'). The temperature profile for this cycling program was as follows: 1 min at 94°C, 2 min at 48°C and 1 min at 72°C. One µl was then amplified with nested primers for the 120 kD protein gene with primers PXCF3b (5'-CAGCAAGAGCAAGAAGATGAC-3') and PXAR5 (5'-ATCTTTCTCTACAACAACCGG-3'). The cycling program for this nested reaction of the 120 kD protein gene was as follows: 1 min at 94°C, 2 min at 54°C and 1 min at 72°C using the same reagent concentrations as described above.

nad A gene: The outside amplification was done under the same conditions as described for the other genes with the following primers: ECHNADA1 (5'-TCATTTCGTGCTTTCTTATTG-3') and PXCR6(5'-CAAACGCATATG TGGGCA-3'). The cycling program was as follows: 35 cycles, 1 min at 94°C, 2 min at 48°C and 1 min at 72°C each cycle. One µl was then amplified in a second 100 µl-reaction tube with nested primers specific for the *nad A* gene of *E. chaffeensis*: NADPCR (5'-ACGTCATTTGGCTCAGGA-3') and PXCR7 (5'-TGTCGATCCAATGAAAT GAGC-3'). The cycling program was as follows: 35 cycles, 1 min at 94°C, 2 min at 48°C and 1 min at 72 min.

All reactions were performed in a PowerBlock II™ System (Ericomp Inc., San Diego, California). The PCR products were then separated electrophoretically in a 1.5% agarose gel at 100V for 30-40 minutes. The gel was then stained with ethidium bromide and examined under ultraviolet light.

Sequence analysis: The PCR products were purified by QIAquick, (QIAgen, Santa Clarita, CA). The nucleotide sequence was then determined by the dideoxynucleotide method of cycle sequencing with *Taq* polymerase (ABI Prism 377 DNA sequencer, Perkin-Elmer Corp., Foster City, CA).

The sequencing reaction was carried out for each strand of DNA to avoid possible errors of incorporation of nucleotides by *Taq* polymerase. The sequences were then analyzed by Genetics Computer Group, Wisconsin Package software and by Lasergene software (DNA Star, Inc., Madison, Wisconsin).

Results

A total of 82 cases met the clinical case definition. Acute and convalescent serum samples were obtained from 33 patients. Based on the laboratory case definition criteria previously described, 22 cases were diagnosed with HME. Seventeen cases (77%) were considered as definite and five cases (23%) were probable. Eight cases were diagnosed in 1997 and 14 cases during 1998. The total population of the area included in the study is approximately 100,000 people. Therefore the estimated incidence was 8 cases per 100,000 population for 1997 and 14 per 100,000 population for 1998.

Clinical and epidemiological data have been obtained in 10 of the 22 cases diagnosed to date. The average age was 45.8 years (range: 22-70); 70% of cases were males and 30% were females. All of the cases came from rural areas, and history of tick bites was elicited in 80% of patients. All cases occurred between April and September. The clinical signs and symptoms associated with HME included fever, headache, chills, weakness, nausea, vomiting, diarrhea, abdominal pain, dizziness, dyspnea, cough, sore throat, stiff neck and cutaneous rash (Table 1).

Table 1: Clinical manifestations of HME, Cape Girardeau, MO, 1997 and 1998.

Fever	100%
Headache	70%
Chills	80%
Nausea	60%
Vomiting	20%
Abdominal Pain	10%
Cough	20%
Dyspnea	10%
Rash	40%
Sore throat	40%
Myalgia	80%
Arthralgias	20%
Stiff neck	10%

Hemoglobin and hematocrit values were normal in 90% of the patients. Leukopenia was found in 56% of the patients, thrombocytopenia in 44% of patients, and elevated liver enzymes in 22% of cases.

A total of 18 cases were diagnosed by IFA. Seroconversions were documented in five cases. Of all cases diagnosed by serology, 6 cases were also positive by PCR. Of the 12 cases that were negative by PCR and positive by IFA, seven cases had titers ≥1:1024 and one case had a titer of 1:256. The four remaining cases had titers of 1:64, and no convalescent serum samples were available for IFA testing. The end-point titers ranged from 1:64 to ≥ 1:4096. Five cases (28%) had titers of 1:64 to 1:128, 3 cases had titers between 1:256 to 1:512 and 10 cases had titers ≥1:1024 (geometric mean titer, 492.6).

Ehrlichia chaffeensis DNA was detected by PCR amplification in ten cases. Six of these cases were also positive by IFA, and seroconversion was demonstrated in five cases. One case had a

single titer of 1:256 by IFA. Of all the cases diagnosed by PCR, five cases were positive for one target gene, four cases for two target genes and one case for all three target genes.

The overall sensitivity and specificity of PCR when using seroconversion as the gold standard for diagnosis of HME were 80% and 100%, respectively. The 120 kDa protein gene and the *nad A* gene targets had a sensitivity of 60% whereas the sensitivity of the 16S rRNA subunit gene was 40%. When we calculated the sensitivity of PCR by using the number of confirmed cases as the denominator, the results were as follows: The overall PCR sensitivity was 41%. Sensitivity for each of the three target genes was 24%. The specificity was 100%. Of all 17 definite cases, 7 cases had titers of ≥1:1024, and according to clinical information obtained from the patients, they had been sick for more than 3 weeks. The sensitivity of PCR based on 10 definite cases with samples collected early in the disease course would then be 70% for all targets and 40% for each of the individual target genes. The specificity was still 100%. The positive likelihood ratio for PCR was theoretically infinite since the specificity was 100%. In a hypothetical situation of one false positive PCR result in 100 tests performed, the positive likelihood ratio would have been 80. The negative likelihood ratios were 0.17, 0.3 and 0.59 for sensitivity values of 80%, 70% and 41%, respectively. The post-test probability of a positive PCR test for the clinical case definition was greater than 99.7% and for a negative PCR result was 6% when calculated at a sensitivity value of 80%

DNA sequencing analysis of PCR products was performed on samples from three patients that yielded PCR products for the *nad A* and 120 kDa protein genes. The sequences revealed more than 99% homology with the published sequences of *E. chaffeensis* genes.

No isolates have been obtained during the period of the study.

Discussion

HME is a prevalent disease in southeast Missouri. Based on our clinical case definition, we were able to enroll 82 patients in the study during a two-year period, and 22 (27%) patients had either definite or probable HME. For 1997 and 1998 the calculated incidence for HME was 8 and 14 per 100,000 population, respectively. These figures are higher than expected even for an endemic area such as Missouri [4].

The spectrum of illness in our study ranges from mild to life-threatening disease requiring hospitalization and intensive care; 43% of the patients in our study were hospitalized. Since we are detecting cases based on a clinical case definition that includes fever for three days or more, we are probably excluding the mildest cases of the disease that possibly develop a self-limited illness that resolves spontaneously or patients that even seroconvert asymptomatically after being inoculated with *E. chaffeensis* during a tick feeding. In fact, asymptomatic seroconversion has been documented in soldiers who underwent field training and were exposed to ticks [7]. The clinical syndrome of HME that has emerged from this study is similar to that described in other series published in the literature [8-10]. Signs and symptoms are not specific, and routine laboratory data are insensitive and non-specific for the diagnosis of HME. It is worth mentioning the presence of a cutaneous rash that developed in 30% of the patients. Most of them were focal in nature and limited to either the thoracic area or the upper or lower extremities.

Our criteria for laboratory diagnosis of HME are rather strict in the sense that we considered definite HME serologically confirmed cases only those which had high end-point titers of antibodies against *E. chaffeensis* by IFA (≥1:256). Likewise, definite cases diagnosed by PCR were considered definite only if ehrlichial DNA was amplified for at least two target genes or the results were confirmed by a separate laboratory. Therefore, we are lowering the chances of including cases with marginally positive IFA titers or cases diagnosed by PCR for only one target gene. IFA

seroconversion has been considered the gold standard for the diagnosis of HME. However, samples with high end-point titers by IFA (≥1:256) are highly suggestive of acute HME unless the patient is recovering from an acute infection and the titers are returning back to normal levels. If we consider the latter as a gold standard, the overall sensitivity and specificity of PCR would be 70% and 100%, respectively. Most of our patients developed good immune responses with high titers against *E. chaffeensis* as evidenced by the IFA geometric mean titer and the distribution of the different titers. 72% of patients had titers ≥1:256. Unfortunately we were not able to collect convalescent serum samples from all patients. Four cases that were diagnosed with HME by PCR were not confirmed by IFA. This finding has been reported in another case series [10], and one is left wondering if these are either false positive cases or the patients were treated so early in the disease process that the antigenic stimulation was not enough to trigger an immune response detectable by IFA.

The number of cases diagnosed by PCR is rather low for powerful statistical analysis. However preliminary estimates can be calculated. The specificity is very encouraging. Whether the denominator used to calculate the specificity was the number of definite cases or the number of cases confirmed by seroconversions, the specificity was 100% for all target genes. We used nested PCR for all three target genes, increasing our sensitivity without compromising our specificity. In fact, all cases diagnosed by the *nad A* and the 120 kDa protein genes were detected by nested reactions except for one case. The sensitivity of PCR ranged from 41% to 80% depending on the denominator used for the calculations (definite cases diagnosed by seroconversion or definite cases diagnosed by single IFA values). If the latter value was used as denominator, it is worth mentioning that seven of the 17 cases diagnosed by single IFA titers had end-point titers of ≥1:1024. These cases were all patients that presented for medical care late in the disease process, and one could hypothesize that the patients might have mounted a strong immune response against the circulating ehrlichiae, lowering their numbers in circulating blood to be detected by PCR. If the PCR sensitivity is calculated based on the ten definite cases that presented earlier in the course of illness instead of all 17 definite cases, the sensitivity would rise to 70% for all target genes.

Likelihood ratios both positive and negative are useful parameters for evaluating the usefulness of diagnostic tests in clinical settings. The likelihood ratio for a positive PCR test is very high in our series due to our high specificity. Positive likelihood ratio values over 20 are considered excellent diagnostic tools for a given disease. Conversely, negative likelihood ratios below 0.2 are considered adequate for diagnostic tests used in the clinical setting [11,12]. Negative likelihood ratios obtained at sensitivity levels of 70 and 80% were adequate. Likewise, post-test probabilities based on the prevalence, PCR sensitivity, specificity and likelihood ratios are excellent for diagnostic purposes as evidenced by the values obtained for post-test probabilities for a positive result. In case of a negative result the post-test probabilities are still acceptable to rule out a diagnosis confidently.

In summary, HME is a prevalent disease in southeast Missouri. The clinical and epidemiological picture emerging from this study is similar to other published HME series. We think that PCR and serology are two diagnostic tools that have a place in the clinical setting to diagnose cases of HME. PCR is valuable as a diagnostic tool in the acute phase of the disease.

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