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Isolation and Characterization of *Ehrlichia chaffeensis* Strains from Patients with Fatal Ehrlichiosis

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Two new isolates of *Ehrlichia chaffeensis* (designated Jax and St. Vincent) were obtained from patients with fatal ehrlichial infections. Patients developed characteristic manifestations of severe disease due to *E. chaffeensis*, including marked thrombocytopenia, pulmonary insufficiency, and encephalopathy. Primary isolation was achieved in DH82 cells; the Jax and St. Vincent isolates were detected within 19 and 8 days postinoculation, respectively. The isolates were characterized by molecular evaluation of the 16S rRNA gene, the *groESL* heat shock operon, a 120-kDa immunodominant protein gene, and an incompletely characterized repetitive-motif sequence (variable-length PCR target [VLPT]). The sequences were compared with those of the corresponding molecular regions in the type isolate (Arkansas). St. Vincent contained one fewer repeat unit in both the 120-kDa protein gene and the VLPT compared with corresponding sequences of the Jax and Arkansas isolates. 16S rRNA gene sequences from the two new isolates had 100% identity to the corresponding sequences of the 91HE17 and Sapulpa isolates of *E. chaffeensis*, and to the corrected 16S rRNA gene sequence of the Arkansas isolate. The Jax isolate grew more slowly than the St. Vincent isolate in DH82 cells, and both of the new isolates grew more slowly than the extensively passaged Arkansas isolate. Although specific associations between ehrlichial pathogenicity and genotype were not identified from these comparisons, recovery of this organism from a spectrum of clinical presentations remains an integral step in understanding mechanisms of disease caused by *E. chaffeensis*.

In the last decade, infections with *Ehrlichia chaffeensis* (order *Rickettsiales*, family *Rickettsiaceae*) have been serologically confirmed in more than 460 people in the United States (7). This figure underestimates the true incidence of the disease, because the human ehrlichioses are reportable in only five states (Minnesota, Wisconsin, New York, Connecticut, and Florida), and the majority of cases reported from northern states result from infection with an organism that is similar or identical to *Ehrlichia equi* (4). Despite increasing awareness by clinicians and microbiologists of this emerging tick-borne pathogen, only three described isolates of *E. chaffeensis* from human patients exist (9, 10, 12), due in part to the particular growth requirements of this obligate intracellular bacterium. The limited number of isolates has restricted comparisons of phenotypic and molecular variations that may exist among isolates. The spectrum of human ehrlichiosis varies greatly, from apparently asymptomatic infection to overwhelming disease culminating in death (28). Understanding the mechanisms responsible for ehrlichial disease in humans may be facilitated by study of isolates recovered from a range of clinical situations. We report on the cultivation and initial characterization of two new isolates of *E. chaffeensis* obtained from patients with fatal ehrlichial infections.

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CASE REPORTS

Patient 1. A 51-year-old woman presented to a hospital in Jacksonville, Fla., in late April 1996 with a 7-day history of fever, nonproductive cough, nausea, vomiting, and diarrhea. Her medical history was significant only for Hashimoto's thyroiditis, treated by total thyroidectomy in 1993. Approximately 2 weeks prior to the onset of symptoms, she had received multiple tick bites while working in a heavily wooded area of Nassau County, Fla. Physical examination on admission revealed a profoundly lethargic woman with a temperature of 38.5°C and right upper quadrant tenderness. The hemoglobin level was 120 g/liter. The leukocyte count was 3.9×10^9/liter, with 64% neutrophils, 31% lymphocytes, and 4% atypical lymphocytes. The platelet count was 46×10^9/liter. The serum aspartate aminotransferase level was 399 U/liter, the alanine aminotransferase level was 319 U/liter, the alkaline phosphatase level was 507 U/liter, and the gamma-glutamyltransferase level was 673 U/level. A laparoscopic cholecystectomy with liver biopsy was performed on hospital day 2. The pathology of the gallbladder was consistent with chronic cholecystitis, and the liver appeared normal. Postoperatively, the patient became comatose and ventilator dependent. Lumbar puncture on hospital day 2 revealed an elevated cerebrospinal fluid protein level and a predominantly lymphocytic pleocytosis. Intravenous doxycycline (100 mg every 12 h) was initiated on hospital day 5. She developed marked pulmonary edema, hypotension, and anuria. The patient died on hospital day 6. Autopsy revealed pulmonary intra-alveolar hemorrhage and diffuse alveolar damage, consistent with adult respiratory distress syn-

drome. Also noted were serous pericardial and pleural effusions, diffuse lymphadenopathy, lymphoid depletion of the splenic white pulp, moderate cholestasis and perilobular microvesicular steatosis of the liver, mild lymphohistiocytic infiltrates in the leptomeninges, and diffuse intra-abdominal hemorrhages of the diaphragm, abdominal wall soft tissue, and retroperitoneum. No ehrlichial inclusions (morulae) were identified on subsequent review of multiple thin smears of peripheral blood obtained on 6 successive hospital days. Patient sera tested by an indirect immunofluorescence assay (IFA) at the Centers for Disease Control and Prevention demonstrated a diagnostic increase in reciprocal immunoglobulin G (IgG) titers to *E. chaffeensis* of 256 to 1,024 on hospital days 1 and 6, respectively.

Patient 2. A 52-year-old human immunodeficiency virus-seropositive man presented to a hospital in Jacksonville, Fla., in early June 1996 with a 4-day history of fever, headache, myalgias, nausea, and vomiting. The patient had previously been well, with no known prior opportunistic infections. An absolute $CD4^+$ lymphocyte count of 164 cells/μl was documented in April 1996. Two weeks prior to admission, he had sustained a tick bite while hiking in a rural area in Bibb County, Ga. Physical examination revealed an acutely ill man with a temperature of 39.7°C and orthostatic hypotension. The hemoglobin level was 115 g/liter. The leukocyte count was 2.0×10^9/liter, with 66% neutrophils, 18% bands, 8% lymphocytes, and 8% monocytes. The platelet count was 16×10^9/liter. The alanine aminotransferase level was 73 U/liter, and the aspartate aminotransferase level was 358 U/liter. Despite the initiation of intravenous doxycycline (100 mg every 12 h) on hospital day 2, the patient remained febrile and severely thrombocytopenic and maintained markedly elevated hepatic transaminase levels. He developed a right lower lobe pneumonia and acute renal failure and died on the sixth hospital day. Peripheral blood smears obtained at admission revealed ehrlichial morulae in 2.5% of all leukocytes, with intracytoplasmic organisms in monocytes, lymphocytes, atypical lymphocytes, neutrophils, and metamyelocytes. Patient sera obtained on the first and fifth days of hospitalization were negative for IgG and IgM antibodies to *E. chaffeensis* by IFA.

MATERIALS AND METHODS

Isolation and cultivation of *E. chaffeensis* from blood samples. EDTA-anticoagulated whole blood was obtained from both patients on hospital day 1 (4 ml from patient 1, 3 ml from patient 2). Blood samples were stored at 4°C for 7 days (patient 1) and 2 days (patient 2) prior to delivery to the Centers for Disease Control and Prevention. Upon arrival, each specimen was diluted with 2 volumes of sterile 1× Hanks' balanced salt solution (HBSS), without calcium chloride, magnesium chloride, magnesium sulfate, or phenol red (GIBCO BRL, Grand Island, N.Y.). Diluted samples were layered onto 3 ml of Histopaque 1083 (Sigma Diagnostics, St. Louis, Mo.) and were centrifuged at $800 \times g$ for 20 min. The interface containing the leukocyte fraction was harvested, washed in 10 ml of HBSS, and centrifuged at $400 \times g$ for 10 min. The pelleted cells were resuspended in 4 ml of culture medium (minimum essential medium containing Earle's salts and 16 mM sodium bicarbonate [GIBCO BRL], 8.8% heat-inactivated fetal bovine serum [HyClone Laboratories, Logan, Utah], 1.8 mM L-glutamine [GIBCO BRL], 0.1 mM nonessential amino acids [GIBCO BRL], and 8.8 mM HEPES buffer [GIBCO BRL]). The cell suspensions were overlaid on a semiconfluent monolayer of DH82 cells (30) in 25-cm^2 polystyrene tissue culture flasks. The cultures were incubated at 37°C in a 5.0% CO_2-in-air atmosphere. Inocula were removed after 24 to 48 h of incubation and were replaced with 4 ml of fresh culture medium. Thereafter, the medium was changed twice weekly. Cultures were monitored for evidence of ehrlichial infection by using cytocentrifuged preparations stained with Diff-Quik stain (Baxter Scientific Products, McGaw Park, Ill.).

Extraction of *E. chaffeensis* DNA from blood samples and cultured cells. DNA was extracted from 200 μl of EDTA-treated patient blood and from pelleted DH82 cell cultures infected with *E. chaffeensis*. Samples were incubated in an equal volume of lysing buffer (10 mM Tris [pH 8.0], 10 mM NaCl, 1% sodium dodecyl sulfate, 250 μg of proteinase K [Boehringer Mannheim, Indianapolis, Ind.] per ml) for 3 h at 55°C. DNA lysates were purified by three extractions with phenol-chloroform-isoamyl alcohol (25:24:1; vol/vol). The DNA was washed three times in 2 ml of 10 mM Tris-NaCl buffer (pH 8.0) and was recovered by using Centricon-30 concentrators (Amicon, Inc., Beverly, Mass.). Extracted DNA was resuspended in 100 μl of distilled water.

TABLE 1. Characteristics of molecular markers used as PCR amplification targets for comparisons among three isolates of *E. chaffeensis*

Marker	Primer pair	Amplicon size (bp)	No. of repeats	Repeat unit size (bp)
16S rRNA gene[a]	EC12A-EC9	1,459	0	
groESL operon[b]	HS43-HS79	1,302	0	
120-kDa protein gene[c]	F1-R2	1,250 or 1,500[d]	3 or 4	240
VLPT[e]	FB3-FB5	369 or 459	3 or 4	90

[a] Data are from reference 1.
[b] Data are from reference 25.
[c] Data are from references 9 and 32.
[d] Size estimated from gel.
[e] Data are from reference 27.

PCR analyses of *E. chaffeensis* isolates. To characterize each isolate, four genomic regions were amplified by PCR. Ten microliters of purified DNA (from patient whole blood or cell culture material) was used in each 100-μl reaction mixture. Each primer was used at a final concentration of 1.0 μM. The other components of the PCR, supplied in GeneAmp kits (Roche Molecular Systems, Inc., Branchburg, N.J.), were 10 mM Tris (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 200 μmol of each deoxynucleoside triphosphate, and 2.5 U of *Taq* polymerase. All reactions were performed in a Perkin-Elmer DNA thermal cycler. Two thermocycler profiles were used. For the broad-range 16S rRNA gene primers (primers EC12A and EC9, described below), the profile consisted of three cycles of 94°C for 1 min, 48°C for 2 min, and 70°C for 90 s, followed by 37 cycles of 88°C for 1 min, 52°C for 2 min, and 70°C for 90 s, followed by an extension period of 5 min at 68°C. For all other primer pairs, the annealing temperature was set at 55°C throughout the profile, and the other parameters remained unchanged. Primer sequences, PCR targets, and amplicon sizes are summarized in Table 1 and are outlined below.

(i) 16S rRNA gene. Broad-range primers EC12A (5′-TGATCCTGGCTC AGAACGAACG-3′) and EC9 (1) were used to amplify a 1,459-bp fragment representing nearly the entire length of the 16S rRNA gene. The primer pair HE1 and HE3 (2) was used to amplify a 389-bp product of the 16S rRNA gene specific for *E. chaffeensis.*

(ii) *groESL* operon. The primers HS43 and HS79 (25) were used to amplify a 1,302-bp segment of the *groESL* heat shock operon of *E. chaffeensis* (26) that includes the 100-base intergenic spacer region between the *groES* and *groEL* reading frames and approximately 75% of the *groEL*-coding sequence.

(iii) 120-kDa protein gene. Primers F1 and R2 (32) were used to amplify a genomic sequence coding for a 120-kDa immunodominant protein of *E. chaffeensis*. This gene contains a series of tandem, 240-bp repeats, the number of which may vary among isolates (9, 32).

(iv) Variable-length PCR target (VLPT). A clone selected from an *E. chaffeensis* Arkansas genomic library with hyperimmune mouse ascitic fluid revealed an open reading frame containing four imperfect tandem repeats (27). Primers FB3 (5′-GCCTAATTCAGATAAACTAAC-3′) and FB5 (5′-AAATAGGGTATAA ATATGTCAC-3′) amplify the repeat region and define a 459-bp product in the Arkansas isolate.

Nucleotide sequencing of PCR products. Fifty microliters of the PCR mixture was electrophoresed in a 1.2% gel made with low-melting-point agarose (Boehringer Mannheim). Fragments of the expected size were excised and purified with Wizard PCR Preps (Promega, Madison, Wis.) according to the manufacturer's recommendations. Alternatively, if nonspecific bands were absent from the reaction mixture, 70 μl of the PCR sample was directly purified with Wizard PCR Preps. Aliquots of purified products were electrophoresed in 1.4% agarose gels to determine the template quantity required for subsequent sequencing. Dye-Terminator Ready Reaction Cycle Sequencing kits (Applied Biosystems, Foster City, Calif.) were used for the sequencing reactions. All reactions were performed in a Perkin-Elmer 9600 thermocycler for 25 cycles, with each cycle having the following profile: 96°C for 10 s, 50°C for 5 s, and 60°C for 4 min. Unincorporated fluorescein-labeled dideoxynucleotides were removed with Centri-Sep columns (Princeton Separations, Inc., Adelphia, N.J.) according to the manufacturer's recommendations. Samples were electrophoresed in 4.25% polyacrylamide gels and sequenced with an ABI 377 automated sequencer (Applied Biosystems, Inc., Foster City, Calif.). Both strands of each product were sequenced by using established primer sets or by primer walking. Nucleotide sequences were edited and assembled with the TED and XBAP programs of the STADEN sequence analysis package. Sequence homology comparisons were

made with the GAP program of the GCG package (Genetics Computer Group, Madison, Wis.).

Comparative growth of *E. chaffeensis* isolates in DH82 cells. Uninfected DH82 cells were seeded into 24-well polystyrene plates (Corning Glass Works, Corning, N.Y.) at a concentration of 4.4×10^5 cells per well and were allowed to grow to confluency. *E. chaffeensis* isolates (Jax, third passage in DH82 cells; St. Vincent, third passage in DH82 cells; Arkansas, unknown multiple passages in DH82 cells) were grown in DH82 cells in separate 25-cm^2 tissue culture flasks as described above. Infected cells were harvested by removing the medium, washing the cell layer with 5 ml of sterile HBSS, and adding 2 ml of sterile trypsin-EDTA (GIBCO BRL). After a 5-min incubation at 37°C, 3 ml of culture medium was added to the flasks and the flasks were gently swirled to dislodge the cells. Cell counts were determined for each cell suspension with a hemocytometer, and the percentage of cells infected was obtained by examining cytocentrifuged preparations stained with Diff-Quik stain. The volumes of the inocula were adjusted with culture medium to achieve the desired concentration of infected cells. Five wells in each plate were inoculated with 5×10^3 cells infected with each isolate. Inoculated DH82 monolayers were grown in 1 ml of culture medium per well, and the plates were incubated at 37°C in a 5% CO_2-in-air atmosphere. Medium was changed after the first 24 h and every 3 days thereafter. Individual wells from one plate were sampled every 2 days over 14 days to determine the percentage of cells infected per well. The medium was removed, the wells were washed with 1 ml of sterile HBSS, and the monolayers were incubated with 200 μl of trypsin-EDTA. After a 5-min incubation, the volumes were adjusted to 1 ml with culture medium and gently aspirated with a micropipette to dislodge all remaining cells. One hundred microliters of cell suspension from each well (five replicates per isolate) was cytocentrifuged and stained with Diff-Quik stain. The stained slides were examined by oil immersion microscopy at ×1,000 magnification, and percent infection was determined by examining 500 cells for characteristic intracellular ehrlichial inclusions. For comparisons of growth characteristics, only the values for the inner three wells were used, because the outer two wells consistently gave values 2 to 5 times greater for each isolate at each sampling period. Statistical differences in percentage of cells infected by isolate, day postinoculation, and isolate-day interaction were determined by both one-way and two-way analyses of variance (ANOVAs) after transforming the values by taking the arcsine of the square root of the proportion (24). *P* values of less than 0.05 were considered significant, and all values reported were for two-tailed tests of significance.

Electron microscopy of *E. chaffeensis* isolates. Aspirated bone marrow from patient 2 and DH82 cells infected with the Jax and St. Vincent isolates were examined by electron microscopy. Infected DH82 monolayers were gently disrupted with sterile glass beads and were pelleted by centrifugation at $400 \times g$ for 5 min. The supernatant was removed and the pellet was resuspended in 0.2 M phosphate buffer and collected by centrifugation. The samples were fixed at 4°C for 1.5 h in 2.5% glutaraldehyde in 0.2 M phosphate buffer, postfixed for 30 min in buffered 1% osmium tetroxide, dehydrated in a series of graded ethanol concentrations and propylene oxide, and embedded in an Epon substitute-Araldite mixture. Ultrathin sections were stained with lead citrate and uranyl acetate and were examined with a Philips EM410-LS electron microscope at 60 kV.

Nucleotide sequence accession numbers. The following sequences from *E. chaffeensis* were submitted to GenBank and were given the indicated accession numbers: 16S rRNA gene (Jax isolate), U86664; 16S rRNA gene (St. Vincent isolate), U86665; VLPT (Jax isolate), U87409; and VLPT (St. Vincent isolate), U87410. The corrected sequences of the 16S rRNA gene and the *groESL* operon of the Arkansas isolate have been submitted to GenBank, where they appear under the original accession numbers (M73222 and L10917, respectively).

RESULTS

Isolation and culture from patient blood. Nineteen days after inoculation with leukocytes from patient 1, ehrlichial morulae were observed within the cytoplasms of 0.1% of the DH82 cells. By day 34, approximately 27% of the cells were infected, and the isolate was subcultured. This isolate, referred to as the Jax isolate, grew relatively slowly, although serial passages consistently displayed >80% infection of the cells in inoculated subcultures. Leukocytes harvested from patient 2 produced detectable infection in 76% of the DH82 cells by day 8 postinoculation. Infected cells containing this isolate, referred to as the St. Vincent isolate, were immediately subcultured and maintained uniformly high (>80%) infection rates in all subsequent passages. The Jax and St. Vincent isolates of *E. chaffeensis* have been deposited in the American Type Culture Collection (ATCC; Rockville, Md; strains ATCC 1455-VR and ATCC 1454-VR, respectively).

Molecular characterization of *E. chaffeensis* isolates. (i) 16S rRNA gene. DNA extracts of whole blood and cell culture isolates obtained from both patients yielded the characteristic 389-bp product from the 16S rRNA gene when the DNAs were amplified by using the *E. chaffeensis*-specific primers HE1 and HE3 (data not shown). The nucleotide sequences of the amplified products from the blood of each patient and from the Arkansas, Jax, and St. Vincent isolates were identical. A 1,435-bp sequence representing the amplified product between the 16S rRNA broad-range primers was obtained from each isolate and was fully sequenced. The nucleotide sequences of the products from the Jax and St. Vincent isolates were identical to each other and to the sequences reported for the 91HE17 and Sapulpa isolates of *E. chaffeensis*. However, when compared with the reported sequence of the Arkansas isolate (1), nucleotide differences were observed at two positions: a G-to-T substitution at position 744 and a G insertion at position 883. These differences have previously been noted in comparisons of the sequences of the 91HE17 and Sapulpa isolates with that of the Arkansas isolate (9, 12). The region of the 16S rRNA gene of the Arkansas isolate containing the apparent variations was subsequently analyzed by automated sequencing. It was determined that the originally reported sequence, obtained by manual sequencing methods, contained errors at positions 744 and 883; the sequence of the resequenced region of the Arkansas isolate was in fact identical to the 16S rRNA gene sequences reported for the Jax, St. Vincent, 91HE17, and Sapulpa isolates of *E. chaffeensis.*

(ii) *groESL* operon. Amplicons of the expected size (1,302 bp) were obtained from the Jax and St. Vincent isolates and from corresponding patient blood samples (data not shown). A 1,256-bp region from each product was sequenced and was found to be identical between the two new isolates. However, the sequence of each amplicon differed from that of the corresponding region of a *groESL* clone from the Arkansas isolate by a G-to-T substitution at position 1212. When the sequences of the regions from the Arkansas isolate and from the original *groESL* clone (26) containing the apparent base difference were analyzed by automated sequencing, these sequences were found to be identical to the corresponding heat shock sequences from the Jax and St. Vincent isolates. The *groEL* sequences of the Jax and St. Vincent isolates were identical to the sequence of a 425-bp region of the *groEL* gene of the 91HE17 isolate except for a single ambiguous base described for the latter isolate (12).

(iii) 120-kDa protein gene. Primers F1 and R2 produced amplicons of the same apparent size (approximately 1,500 bp) from the Arkansas and Jax isolates and yielded a smaller, approximately 1,250-bp amplicon from the St. Vincent isolate. The identities of these products were confirmed by partial sequencing (data not shown). Amplification products obtained from the blood of patients 1 and 2 were the same apparent size as products obtained from the Jax isolate and the St. Vincent isolate, respectively (Fig. 1). The size difference noted between these products suggests that the 120-kDa antigen gene of the St. Vincent isolate contains one less 240-bp repeat than the Arkansas and Jax isolates.

(iv) VLPT. Primers FB3 and FB5 were used to amplify a 459-bp product from the Arkansas isolate, from the Jax isolate, and from the blood of patient 1. In contrast, 369-bp amplicons were obtained from the St. Vincent isolate and from the blood of patient 2 (Fig. 2). Sequence analysis of VLPT amplicons from each isolate revealed identical sequences in the Arkansas and the Jax isolates characterized by three imperfect tandem 90-bp repeats, followed by a fourth repeat with numerous substitutions at the 3′ end. The nucleotide sequence of the St.

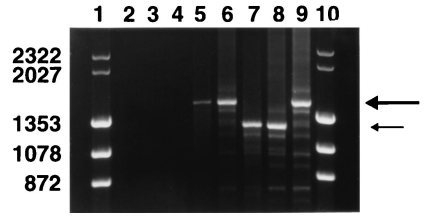

FIG. 1. Agarose gel electrophoresis of PCR products (1.4% gel stained with ethidium bromide) obtained after amplification of patient blood and cell culture templates with primers F1 and R2. These primers amplify the 120-kDa protein gene of *E. chaffeensis*, which is characterized by a variable number of repeats. Amplicons of approximately 1,500 bp (large arrow) were observed in the Jax and Arkansas isolates. An approximately 1,250-bp product (small arrow) was observed in the St. Vincent isolate, a size difference consistent with a missing 240-bp repeat unit. The ladder of faint bands represents nonspecific annealing of the primers to other regions in the repeat sequence. Lanes 1 and 10, the molecular size standards (*Hin*dIII-digested bacteriophage λ DNA and *Hae*III-digested ϕX174 DNA); lanes 2 to 9, reactions with templates obtained from the following sources: water (no template) (lane 2), control (uninfected DH82 cells) (lane 3), control (uninfected whole blood) (lane 4), whole blood from patient 1 (lane 5), Jax isolate cultivated in DH82 cells (lane 6), whole blood from patient 2 (lane 7), St. Vincent isolate cultivated in DH82 cells (lane 8), and Arkansas isolate cultivated in DH82 cells (lane 9). Numbers to the left are in base pairs.

Vincent isolate differed from those of the Arkansas and Jax isolates by the absence of the second 90-bp repeat region. The nucleotide sequences of the corresponding repeat units were identical among all three isolates.

Comparative growth in DH82 cells. Two-way ANOVA indicated significant differences in the percentage of infected cells by day postinoculation ($P < 0.001$), by isolate ($P < 0.001$), and by isolate-day postinoculation interactions ($P < 0.001$), demonstrating that the isolates behaved differently over the period studied. To further investigate these differences, a one-way ANOVA was conducted for each sampling point. One-way ANOVA indicated no significant differences in percentage of cells infected among the three isolates for the first 6 days postinoculation (Fig. 3). After 8 days, the mean percentage of cells infected observed for the Arkansas isolate was significantly higher ($P < 0.0001$) than the mean percent infected cells observed for the Jax and St. Vincent isolates. By 12 days, virtually all DH82 cells were heavily infected with the Arkansas isolate, compared with mean infection prevalences of 14.4 and 46.3% with the Jax and St. Vincent isolates, respectively. At day 14, near-complete obliteration of the DH82 cells infected with the Arkansas isolate precluded an adequate cell count, and results for this isolate on that day were excluded from comparisons. By days 12 and 14, the mean percentage of cells infected with the St. Vincent isolate was significantly higher ($P < 0.0001$ and $P < 0.003$, respectively) than the corresponding percentages observed for the Jax isolate.

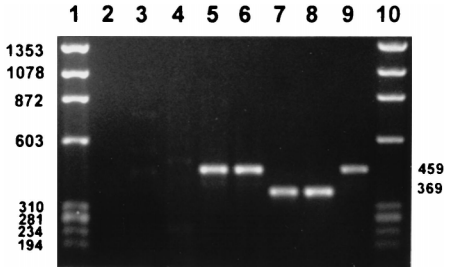

FIG. 2. Agarose gel electrophoresis of PCR products (1.4% gel stained with ethidium bromide) obtained after amplification of patient blood and cell culture templates with primers FB3 and FB5. These primers amplify an incompletely characterized repetitive genomic sequence of *E. chaffeensis* (VLPT), which exhibits a variable number of repeats. Amplicons containing four repeat units were represented by a 459-bp product, while those containing three repeat units were identified by a 369-bp product. Lanes 1 and 10, molecular size standards (*Hae*III-digested ϕX174 DNA); lanes 2 to 9, reactions with templates obtained from the following sources: water (no template) (lane 2), control (uninfected DH82 cells) (lane 3), control (uninfected whole blood) (lane 4), whole blood from patient 1 (lane 5), Jax isolate cultivated in DH82 cells (lane 6), whole blood from patient 2 (lane 7), St. Vincent isolate cultivated in DH82 cells (lane 8), and Arkansas isolate cultivated in DH82 cells (lane 9). Numbers to the left and right are in base pairs.

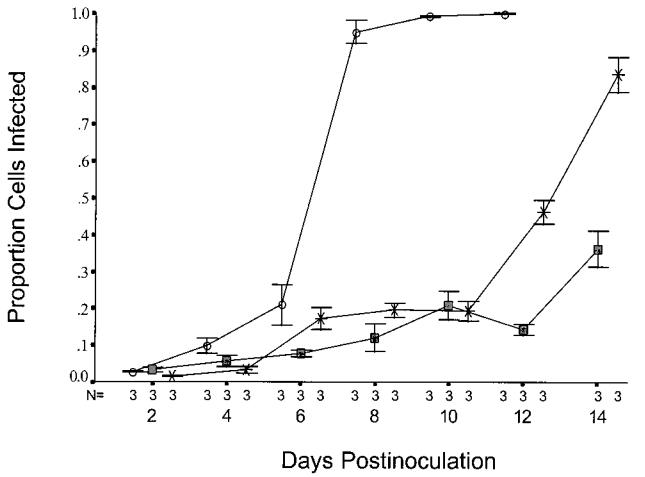

FIG. 3. Growth of three isolates of *E. chaffeensis* in DH82 cells. Datum points represent percentage of cells infected (mean ± standard error of the mean; $n = 3$). The isolates (Arkansas [○], Jax [▩], and St. Vincent [∗]) were examined at 2-day intervals over 14 days. By one-way ANOVA, percentage of cells infected between isolates were not significantly different for the first 6 days postinoculation. Significant differences in percentage of cells infected were noted between the Arkansas isolate and the Jax and St. Vincent isolates beginning on day 8 ($P < 0.0001$) and between the Jax and St. Vincent isolates beginning on day 12 ($P < 0.0001$).

Ultrastructure of Jax and St. Vincent isolates of *E. chaffeensis*. Morulae and individual organisms of *E. chaffeensis* in bone marrow mononuclear cells and within infected DH82 cells (Fig. 4) resembled previous descriptions of species ultrastructure in size, morphology, and distribution within the host cell (9, 12, 22). Intracytoplasmic vacuoles contained reticulate and dense-cored forms in the patient sample and in the cell culture material, although reticulate forms appeared to predominate in the bone marrow cells. In DH82 cells, aggregates of dense-cored and reticulate forms were occasionally found within the same vacuole. Reticulate organisms displayed prominently ruffled outer membranes and wide periplasmic spaces and appeared to be slightly larger (mean greatest dimension, 516 ± 241 nm) than the dense-cored forms (mean greatest dimension, 358 ± 71 nm). Intramorular tubules arising from the cell walls of individual bacteria and giant ehrlichiae as large as 1,370 nm were observed in infected bone marrow leukocytes and DH82 cells. Fine, striated fibrils were visualized in the morular matrix of both new isolates in DH82 cells, but not in morulae found in patient's cells. Mitochondria were frequently identified at the margins of morulae from bone marrow leukocytes and in DH82 cells, as reported for the Arkansas isolate (22). No discrete ultrastructural differences were identified between the cultured isolates.

DISCUSSION

The isolates described in this report were identified as *E. chaffeensis* on the basis of the following evidence: (i) recov-

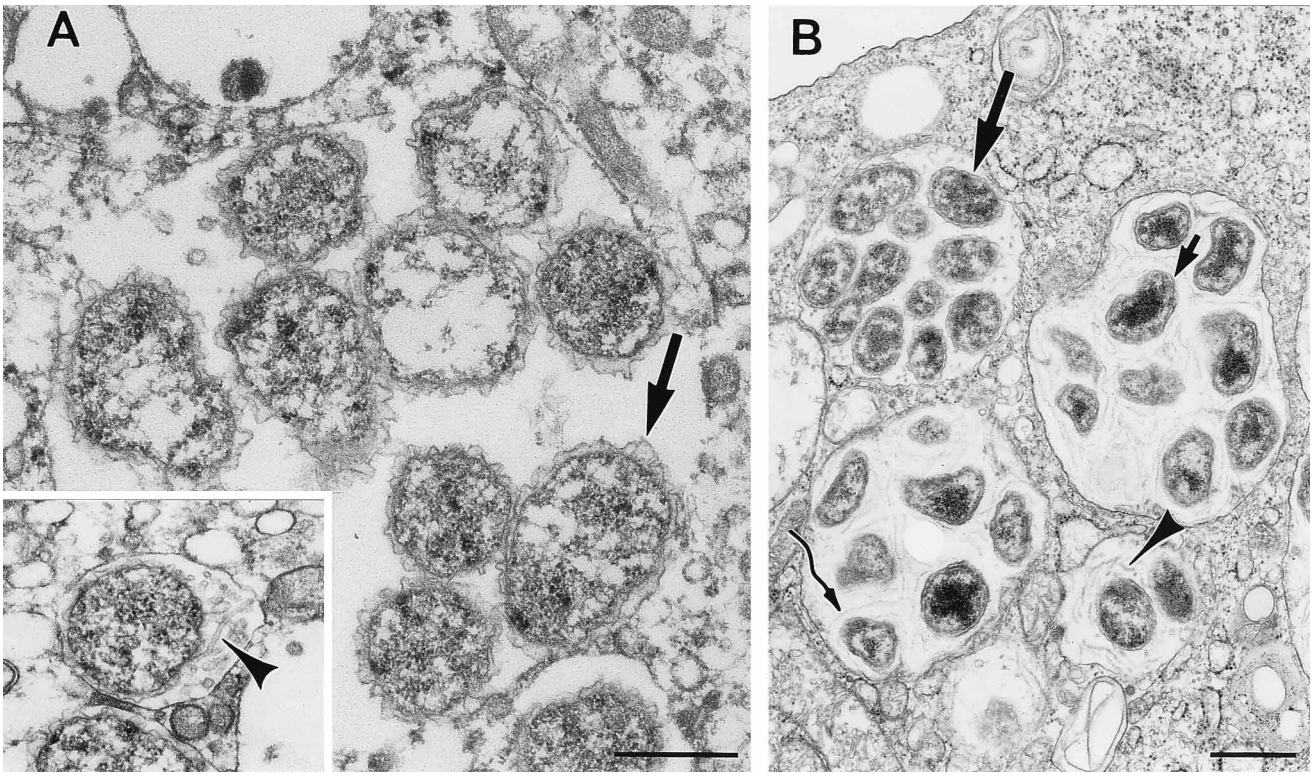

FIG. 4. Transmission electron photomicrographs of *E. chaffeensis* in the bone marrow of a patient with fatal ehrlichiosis (A) and the organism after isolation in DH82 cells (B). Reticulate forms predominate in a morula within a bone marrow mononuclear cell (A, large arrow). Individual ehrlichiae display markedly ruffled cell walls, wide periplasmic spaces, and occasional intramorular tubules (arrowhead in the inset to panel A). Morulae of the St. Vincent isolate (B) demonstrate reticulate forms (large arrow) and dense-cored forms (small arrow), intramorular tubules (arrowhead), and fine, striated matrical fibrils (wavy arrow). Lead citrate-uranyl acetate stain was used. Bars, 0.5 μm.

ery of the organism from patients with well-defined, clinically compatible illnesses; (ii) the light microscopic and ultrastructural appearances of the bacteria in patient leukocytes and in cultured cells; and (iii) sequence comparisons with known *E. chaffeensis* sequences. The Jax and St. Vincent isolates originated from patients who presumably acquired their ehrlichial infections in northeastern Florida and central Georgia, respectively. Jax and St. Vincent represent the first isolates recovered from patients with fatal *E. chaffeensis* infections, demonstrate intraspecific genetic diversity in two molecular markers, and display phenotypic variation in their growth in DH82 cells.

Attempts to isolate *E. chaffeensis* from patient samples have met with limited success. However, several laboratories have recently identified susceptible cell lines and appropriate growth conditions which may facilitate more frequent recovery of this organism. The described isolates of *E. chaffeensis* have been obtained in primary culture by using a continuous canine histiocytoma cell line (DH82 cells) (9, 10, 12, 17) and by using human embryonic lung fibroblasts (HEL 299 cells) (9). The Arkansas isolate has been grown in a number of additional cell lines, including human microvascular endothelial cells (HMEC-1 cells) (11), African green monkey kidney cells (Vero cells) (6, 8), human cervical epithelioid carcinoma cells (HeLa cells) (6), human monocytic leukemia cells (THP-1 cells) (5), HEL 299 cells (6, 8), mouse embryo cells (8), buffalo green monkey cells (8), and murine fibroblasts (L929 cells) (8). The demonstrated susceptibilities of a wide range of cell lines to infection with *E. chaffeensis* suggests that multiple lines might be used for primary isolation of this organism from patient specimens.

The three previously described human isolates of *E. chaffeensis* were obtained from patients in Arkansas (10, 12) and Oklahoma (9) with relatively mild to severe disease who survived their infections. In the previous studies, detection of the organism in cell culture required 26 to 36 days of incubation. In the present study, ehrlichiae were identified in primary culture by as early as 8 days. In addition to the selection of an appropriate cell line, the success and speed of isolation of *E. chaffeensis* depend on factors related to the quality of the inoculum (including the age and volume of the blood specimen), the level of bacteremia in the patient, and the efficiency of the leukocyte separation techniques. Optimal samples are obtained during the acute febrile stage of the illness prior to the initiation of antimicrobial therapy. The duration of ehrlichial viability in blood outside of the host is unknown, although the organism was successfully isolated from EDTA-anticoagulated blood refrigerated at 4°C for 1 week in the present study. However, sample age should be considered when using Ficoll-Isopaque gradient separations, because the success of this technique depends on the availability of intact leukocytes. Similar methods have been successfully applied in the recovery of the Sapulpa isolate of *E. chaffeensis* (9) and in the isolation of the agent of human granulocytic ehrlichiosis (23). This technique appears particularly well-suited for isolating ehrlichia from the blood of hosts with low-level bacteremia and has been used to recover *Ehrlichia canis* from dogs with subclinical infections and from animals previously treated with doxycycline (16). The use of a Ficoll-Isopaque preparation with a higher specific gravity concentrates mononuclear cells and granulocytes, and both of these cell series may be infected by *E. chaffeensis*. Subsequently, this method allows one to process a

relatively large volume of blood into a heterogeneous collection of leukocytes and may improve the success of organism isolation. In addition, removal of the erythrocytes from the inoculum reduces the marked disruption of the DH82 monolayer that may occur when whole blood is used to inoculate cell cultures (12).

Ultrastructural evaluation of the bacteria in cell culture and sequence analyses of amplified ehrlichial genes confirmed the identities of the two new isolates as *E. chaffeensis*. Evaluation of genetic variability among these isolates was investigated with four molecular markers. Analysis of the 16S rRNA gene revealed identical sequences among the two new isolates and the three previously described isolates. Similarly, the *groESL* operon sequences of the Arkansas, Jax, and St. Vincent isolates were identical and had at least 99.8% sequence identity with a 425-bp region of the *groEL* gene of the 91HE17 isolate (12). However, distinct molecular differences between the St. Vincent isolate and the Arkansas and Jax isolates were identified in a 120-kDa protein gene and a smaller repetitive-domain nucleotide sequence (VLPT).

Molecular variability in the 120-kDa protein gene has previously been documented among isolates of *E. chaffeensis* (9, 32). The size of this amplicon in the St. Vincent isolate was smaller than those observed in the Arkansas and Jax isolates, suggesting the absence of one tandem repeat. A variant size of the 120-kDa protein gene corresponding to a missing 240-bp repeat has also been reported for the Sapulpa isolate, which contains a repeat region composed of three tandem repeat units, in contrast to four units observed in the Arkansas isolate (9). Analysis of VLPT sequences confirmed four repeats in the Jax and Arkansas isolates, compared with three repeats in the St. Vincent isolate. Additional studies are necessary to define the molecular and biologic significance of the VLPT. This sequence is characterized by an open reading frame and was initially detected in an expression library by using hyperimmune serum, suggesting that the VLPT represents a functional gene and codes for an antigenic protein (27).

The function and biologic consequences of the repetitive sequences in *E. chaffeensis* await elucidation. Genes with repeat domains are widely recognized among pathogenic protozoa and bacteria, including *Rickettsia* spp., in which they may code for surface proteins involved in antigenic variation, opsonization, and ligand binding (15). A tandem repeat domain within the rickettsial outer membrane protein A gene (*rompA*) represents an important determinant of interspecific (15) and intraspecific (29) genetic diversity among the spotted fever group rickettsiae. By example, *Rickettsia conorii* isolates from different continents exhibit marked variation in the number of *rompA* repeat units, which correlate with the antigenic heterogeneity exhibited among different isolates of this species (29).

Collectively, these data further illustrate genetic diversity among isolates of *E. chaffeensis*, although the biologic and epidemiologic implications of the individual molecular variations are unknown. On the basis of the results obtained with a limited number of examples, the pathogenicity and specific geographic distribution of this organism do not appear to be associated with a particular arrangement of repeats in either gene. Both of the described versions of the 120-kDa protein gene and the VLPT were identified in isolates from patients with fatal infections, and repetitive-motif patterns in the 120-kDa protein gene and the VLPT were identical between an isolate producing relatively mild infection (Arkansas) and an isolate responsible for fatal disease (Jax). Discovery and characterization of additional ehrlichial genes may provide better predictors of pathogenicity, particularly if this feature is represented by composites of many genotypic markers.

Except for immunoblot analyses with monoclonal antibodies (9, 12), few phenotypic assays assess variability among isolates of *E. chaffeensis*. Although plaque isolation or plaque assay with *E. chaffeensis* leads to reproducible differences in the appearance and timing of plaques between different cell lines (8), variation in the kinetics of growth among isolates has not been described. Significant differences in ehrlichial growth in DH82 cells were observed between the Jax and St. Vincent isolates after several days in culture, although the limitations of this assay must be considered. Quantification of the inoculum by cell count represents an estimate, because infected cells contain variable numbers of morulae and individual ehrlichiae. However, because no significant differences in percentage of cells infected were observed among the isolates for the first week postinoculation, we believe that for the purposes of this study, comparable inocula were obtained for each isolate. Multiple passages of the Arkansas isolate in DH82 cells limits comparisons with the Jax and St. Vincent isolates, except to note that the recently obtained, early-passage isolates grew more slowly than the extensively passaged Arkansas isolate in a conventionally used cell line. A slower growth rate in DH82 cells relative to that for the Arkansas isolate has also been described for early passages of five new isolates of *E. chaffeensis* obtained from white-tailed deer (*Odocoileus virginianus*) in Georgia (17).

In a broad sense, our knowledge of the clinical manifestations of *E. chaffeensis* infection and the factors responsible for these manifestations is nascent and is defined primarily through case reports for patients with the most severe and devastating presentations of this illness. Paradoxically, seroconversion studies of persons at risk for acquiring *E. chaffeensis* suggest that as many as two-thirds of patients with acute ehrlichial infections demonstrate minimal or clinically silent signs and symptoms (21, 31). Morbidity and mortality resulting from this infection have often been observed in patients with underlying immunosuppressive conditions (3, 13, 14, 18, 20), illustrating a central role for host factors as determinants of disease severity. However, recognition of profound disease with multiorgan system involvement and potentially fatal outcomes in otherwise healthy young adults or children (14, 19) suggests that virulence factors peculiar to the bacterial isolate may contribute to the dynamics between infection and disease. Cultivation of *E. chaffeensis* from a variety of clinical settings remains a primary step toward understanding the variations and mechanism of pathogenicity observed with this organism.

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