

EHRlichia EWINGII, A NEWLY RECOGNIZED AGENT OF HUMAN EHRlichiosis

RICHARD S. BULLER, PH.D., MAX ARENS, PH.D., S. PAUL HMIEL, M.D., PH.D., CHRISTOPHER D. PADDOCK, M.D., JOHN W. SUMNER, B.S., YASUKO RIKIHISA, PH.D., AHMET UNVER, D.V.M., M.S., MONIQUE GAUDREAU-KEENER, B.S., FARRIN A. MANIAN, M.D., ALLISON M. LIDDELL, M.D., NATHAN SCHMULEWITZ, M.D., AND GREGORY A. STORCH, M.D.

ABSTRACT

Background Human ehrlichiosis is a recently recognized tick-borne infection. Four species infect humans: *Ehrlichia chaffeensis*, *E. sennetsu*, *E. canis*, and the agent of human granulocytic ehrlichiosis.

Methods We tested peripheral-blood leukocytes from 413 patients with possible ehrlichiosis by broad-range and species-specific polymerase-chain-reaction (PCR) assays for ehrlichia. The species present were identified by species-specific PCR assays and nucleotide sequencing of the gene encoding ehrlichia 16S ribosomal RNA. Western blot analysis was used to study serologic responses.

Results In four patients, ehrlichia DNA was detected in leukocytes by a broad-range PCR assay, but not by assays specific for *E. chaffeensis* or the agent of human granulocytic ehrlichiosis. The nucleotide sequences of these PCR products matched that of *E. ewingii*, an agent previously reported as a cause of granulocytic ehrlichiosis in dogs. These four patients, all from Missouri, presented between May and August 1996, 1997, or 1998 with fever, headache, and thrombocytopenia, with or without leukopenia. All had been exposed to ticks, and three were receiving immunosuppressive therapy. Serum samples obtained from three of these patients during convalescence contained antibodies that reacted with *E. chaffeensis* and *E. canis* antigens in a pattern different from that of humans with *E. chaffeensis* infection but similar to that of a dog experimentally infected with *E. ewingii*. Morulae were identified in neutrophils from two patients. All four patients were successfully treated with doxycycline.

Conclusions These findings provide evidence of *E. ewingii* infection in humans. The associated disease may be clinically indistinguishable from infection caused by *E. chaffeensis* or the agent of human granulocytic ehrlichiosis. (N Engl J Med 1999;341:148-55.)

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HUMAN ehrlichioses are recently recognized tick-borne zoonotic infections.¹ Worldwide, four species of ehrlichia have been identified as pathogens in humans. In the United States, *Ehrlichia chaffeensis* causes human monocytic ehrlichiosis,^{2,3} and a species closely related to *E. equi* and *E. phagocytophila* causes human granulocytic ehrlichiosis.^{4,5} *E. sennetsu* causes a mononucleosis-like illness in Japan and Malaysia.⁶ Asymptomatic infection with *E. canis* has been reported in

one patient in Venezuela.⁷ The zoonotic nature of the human ehrlichioses is supported by reports of natural infections with the same ehrlichia species in dogs, deer, horses, and rodents.⁸⁻¹⁰

Human monocytic ehrlichiosis is endemic in Missouri, where 142 cases were reported from 1989 through 1997.¹¹ By comparison, 742 cases of this disease were reported from 1986 through 1997 in the entire United States.¹² Since 1994, when infection with the agent of human granulocytic ehrlichiosis was first reported,⁴ our laboratory at Washington University Medical Center has used polymerase-chain-reaction (PCR) assays for rapid confirmation of cases of acute ehrlichiosis. We used a broad-range PCR assay designed to detect all known species of ehrlichia to test all blood samples obtained from 1994 through 1998 from patients suspected of having ehrlichiosis. Unexpectedly, PCR products from four patients had sequences that matched those of *E. ewingii*, an agent previously reported as a cause of granulocytic ehrlichiosis in dogs. This report describes the clinical and laboratory characteristics of *E. ewingii* infection and the methods used to identify the infection in these four patients.

METHODS

Specimens

EDTA-treated blood samples were collected from 413 patients with possible ehrlichiosis during the period from 1994 through 1998. Leukocytes were separated by sedimentation with dextran (molecular weight, 500,000; T500, Amersham Pharmacia Biotech, Piscataway, N.J.), and alkaline lysates were prepared as previously described.¹³ DNA from 300 µl of each sample was extracted with a QIAamp blood kit (Qiagen, Valencia, Calif.) and resuspended in 70 µl of 10 mM TRIS-EDTA buffer.

PCR Assays

Four diagnostic PCR assays targeting the 16S ribosomal RNA (rRNA) gene were used: a broad-range assay designed to amplify all known ehrlichia species and assays specific for *E. chaffeensis*, *E. ewingii*, and the agent of human granulocytic ehrlichiosis. The

From the Edward Mallinckrodt Department of Pediatrics (R.S.B., M.A., S.P.H., M.G.-K., G.A.S.) and the Department of Medicine (A.M.L., N.S.), Washington University School of Medicine, St. Louis; St. Louis Children's Hospital, St. Louis (R.S.B., M.A., S.P.H., M.G.-K., G.A.S.); the Centers for Disease Control and Prevention, Atlanta (C.D.P., J.W.S.); the Department of Veterinary Biosciences, College of Veterinary Medicine, Ohio State University, Columbus (Y.R., A.U.); and St. John's Mercy Medical Center, St. Louis (F.A.M.). Address reprint requests to Dr. Storch at the Department of Pediatrics, Division of Infectious Diseases, St. Louis Children's Hospital, 1 Children's Pl., St. Louis, MO 63110, or at storch@al.kids.wustl.edu.

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following primers were used: ECA and HE3 for the broad-range assay,^{14,15} HE1 and HE3 for the *E. chaffeensis* assay,¹⁴ EHR 521 and EHR 747 for the assay for the agent of human granulocytic ehrlichiosis,¹⁶ and EWI and HE3 for the *E. ewingii* assay.¹⁷ For each patient, PCR assays were performed on lysates containing DNA from 10⁶ leukocytes. Reactions were performed in 100- μ l samples with a DNA thermal cycler (model 480, Perkin-Elmer Cetus, Norwalk, Conn.). Final concentrations of reaction mixtures and cycling protocols for the broad-range assay, the *E. chaffeensis*-specific assay, and the *E. ewingii*-specific assay were based on modifications of a previously published procedure.¹⁵

For the broad-range assay, the reaction mixture contained 10 mM TRIS (pH 9.2), 75 mM potassium chloride, 1.5 mM magnesium chloride, 200 μ M 2'-deoxyadenosine triphosphate, 200 μ M deoxycytidine triphosphate, 200 μ M deoxyguanosine triphosphate, 200 μ M deoxythymidine triphosphate, 15 percent glycerol, 40 pmol of each primer, and 2.5 U of *Taq* polymerase. Amplification was then carried out with 3 cycles at 94°C for 1 minute, 55°C for 2 minutes, and 70°C for 1.5 minutes followed by 37 cycles at 88°C for 1 minute, 52°C for 2 minutes, and 70°C for 1.5 minutes.

For the *E. chaffeensis*-specific assay and the *E. ewingii*-specific assay, the reaction mixture consisted of 10 mM TRIS-hydrochloric acid (pH 8.8), 75 mM potassium chloride, 1.5 mM magnesium chloride, 200 μ M 2'-deoxyadenosine triphosphate, 200 μ M deoxycytidine triphosphate, 200 μ M deoxyguanosine triphosphate, 200 μ M deoxythymidine triphosphate, 50 pmol of each primer, 5 percent formamide, and 2.5 U of *Taq* polymerase. Amplification was then performed according to the same protocol as that used for the broad-range assay, except that the initial phase consisted of five cycles at 94°C for 1 minute, 60°C for 2 minutes, and 70°C for 1.5 minutes. For the assay for the agent of human granulocytic ehrlichiosis, we used a final reaction mixture and cycling conditions described by Pancholi et al.¹⁶

Nucleotide Sequencing

Amplicons from the broad-range PCR assay for ehrlichia were sequenced by adding 125 ng of purified amplicon and 3.2 pmol of primer to a dye-terminator cycle-sequencing reaction (Perkin-Elmer Applied Biosystems, Foster City, Calif) with AmpliTaq FS DNA polymerase. Extension products were analyzed in an automated DNA sequencer (model 373A, Applied Biosystems). Sequence data derived from reactions initiated by at least two primers for each PCR product were processed with the Factura program (Applied Biosystems) and aligned with known sequences from GenBank and with other experimental sequences.

Two other methods were used to sequence large portions of the 16S rRNA gene. At Washington University School of Medicine, a 1360-bp product of a nested PCR assay amplified from blood samples from two of the four patients with samples positive for *E. ewingii* (Patients 3 and 4 in Table 1) was sequenced with the use of 8F and 1448R as outside primers and 15F and 1442R as inside primers.¹⁷ At the Centers for Disease Control and Prevention (CDC), amplification of 16S rDNA from samples of whole blood was performed according to the method of Anderson et al.³ with two exceptions: primer EC12A (5'TGATCCTGGCT-CAGAACGAACG) was substituted for EC12, and the PCR product was sequenced directly. For Patient 3, a 2- μ l sample of the reaction mixture was used as a template in a heminested PCR with primers EC12A and EC11.³ The product was cloned into the TA cloning vector pGEM-T (Promega, Madison, Wis.) and sequenced with a dRhodamine kit (Perkin-Elmer) and an automated sequencer (model 377, Applied Biosystems).

Serologic Analysis

Serum and plasma samples obtained from the patients during the acute and convalescent phases were evaluated at the CDC with an indirect immunofluorescence assay for IgG antibodies reactive with *E. chaffeensis*.¹⁸ End-point titers were recorded as the reciprocal of the highest dilution exhibiting specific fluorescence.

Serum samples from Patients 1, 2, 3, and 4 and from two dogs

belonging to Patient 3 and one dog experimentally infected with *E. ewingii* were tested by Western blot analysis with preparations of *E. chaffeensis* and *E. canis* that were free of host cells (chromatography-purified) as antigen, as previously described¹⁹ but with the following modification. The assay membranes were incubated with the human or canine serum at a 1:1000 dilution and then with a preparation of peroxidase-conjugated, affinity-purified antihuman IgG, IgM, and IgA (Kirkegaard & Perry Laboratories, Gaithersburg, Md.) or with antidog IgG at 1:2000, followed by bathing in developing and stop solutions.

RESULTS

Patients

From 1994 through 1998, samples from 413 patients with possible ehrlichiosis were tested by PCR. A total of 60 samples (15 percent) were positive for an ehrlichia species: 56 for *E. chaffeensis* and 4 for *E. ewingii*. All four patients with *E. ewingii* were male, lived in Missouri, and presented with a febrile illness between May and August 1996, 1997, or 1998. They ranged in age from 11 to 65 years, and all reported having been exposed to ticks before their illness (Table 1). Three of the four patients were receiving immunosuppressive therapy for underlying conditions. All four had fever and headache, prompting lumbar puncture in three. All four patients had thrombocytopenia (<150,000 platelets per cubic millimeter), and two had leukopenia (<4500 white cells per cubic millimeter). All four patients responded to treatment with doxycycline.

Clinical data for all four patients are summarized in Table 1. Patients 1 and 4 are described in detail below.

Patient 1 was an 11-year-old boy from southern Missouri who had received a kidney graft from a living related donor at 27 months of age. In May 1996, fever, headache, nasal congestion, myalgia, and a stiff neck developed. The patient had a pet dog and had been exposed to ticks on multiple occasions during the month preceding his illness. Physical examination revealed acute illness with mild cervical, axillary, and inguinal lymphadenopathy. The white-cell count was 4500 per cubic millimeter, the hemoglobin level was 9.7 g per deciliter (6.0 mmol per liter), the platelet count was 105,000 per cubic millimeter, and the creatinine level was 1.3 mg per deciliter (115 μ mol per liter; previous base-line value during regular follow-up after transplantation, 1.0 mg per deciliter [88.4 μ mol per liter]). Routine bacterial cultures of blood and PCR assays of blood were negative for cytomegalovirus and Epstein-Barr virus. The boy was treated with intravenous vancomycin, ceftazidime, and doxycycline. A blood sample obtained on the day of admission was negative for *E. chaffeensis* on a PCR assay. No morulae were seen on a peripheral-blood smear obtained on the third hospital day. Doxycycline treatment was discontinued after five days.

One week after discharge from the hospital, the boy's condition had improved. A broad-range PCR assay of the blood sample initially tested for *E. chaffeensis* subsequently tested positive for ehrlichia, and

TABLE 1. CLINICAL CHARACTERISTICS OF THE FOUR PATIENTS WITH *EHRLICHIA EWINGII* INFECTION.*

CHARACTERISTIC	PATIENT 1	PATIENT 2	PATIENT 3	PATIENT 4
Age (yr)	11	49	60	65
Underlying condition	Kidney transplantation	Rheumatoid arthritis	None	Chronic obstructive pulmonary disease
Immunosuppressive therapy	Prednisone, azathioprine	Methotrexate	None	Prednisone
Maximal temperature (°C)	38.9	40.0	39.1	39.5
Myalgia	Yes	No	No	No
Headache	Yes	Yes	Yes	Yes
Stiff neck	Yes	No	No	No
Exposure to ticks	Yes	Yes	Yes	Yes
Morulae on blood smear	No	No	Yes	Yes
Minimal white-cell count (per mm ³)	4,500	1,400	4,000	6,800
Minimal platelet count (per mm ³)	105,000	88,000	130,000	29,000
Abnormal liver-function tests†	No	No	No	Yes
Cerebrospinal fluid leukocyte count (per mm ³)	0	0	ND	774
<i>E. chaffeensis</i> titer during convalescence‡	≥2,048	ND	256	4,096

*All four patients were male. ND denotes not done.

†Liver-function tests included measurements of aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, and bilirubin.

‡The titer was determined by indirect immunofluorescence assay.

treatment with doxycycline was resumed and continued for 10 days. A serum specimen obtained during convalescence and tested by an indirect immunofluorescence assay contained antibodies reactive with *E. chaffeensis* at a titer of at least 2048. No antibodies to the agent of human granulocytic ehrlichiosis or rickettsia species were detected.

Patient 4 was a 65-year-old man with a history of chronic obstructive pulmonary disease and hypertension. In August 1998, a respiratory tract infection developed and was treated with amoxicillin and 60 mg of prednisone per day, with subsequent tapering of the dose. Twelve days later a severe frontal headache developed. The patient had regular contact with a dog and had hiked in the woods and removed a tick from his left shoulder several days before the onset of symptoms. On examination, his temperature was 39.3°C and his pulse rate was 100 beats per minute. His neck was supple, and breath sounds on the right were decreased. The liver and spleen were not palpable, and there was no lymphadenopathy. The white-cell count was 6800 per cubic millimeter, with 79 percent neutrophils, 9 percent lymphocytes, 9 percent monocytes, 1 percent eosinophils, and 2 percent basophils; the hemoglobin level was 9.4 g per deciliter (5.8 mmol per liter), and the platelet count was 54,000 per cubic millimeter.

A lumbar puncture revealed an opening pressure

of 20 cm of water. The cerebrospinal fluid contained 774 white cells per cubic millimeter, with 95 percent neutrophils, and was initially reported to contain intracellular gram-negative coccobacilli. Ceftriaxone therapy was begun. Subsequently, examination of the peripheral-blood smear revealed morulae in approximately 50 percent of neutrophils and in a few eosinophils (Fig. 1A), and on reinspection the cerebrospinal fluid smear was found to contain morulae in neutrophils (Fig. 1B). Treatment with doxycycline was begun for possible ehrlichiosis. The broad-range ehrlichia PCR assay revealed ehrlichia DNA in blood and cerebrospinal fluid, and serologic studies indicated that seroconversion to *E. chaffeensis* had taken place (Table 1). The patient received a two-week course of doxycycline and had an uneventful recovery.

Molecular Testing of Specimens

PCR analyses of specimens from Patients 1, 2, 3, and 4 yielded positive results on the broad-range PCR assay and negative results on the assays specific for *E. chaffeensis* and the agent of human granulocytic ehrlichiosis.

Sequencing of PCR Products

To identify the organism responsible for the positive results on the broad-range PCR test for the four patients, the products of this assay were sequenced.

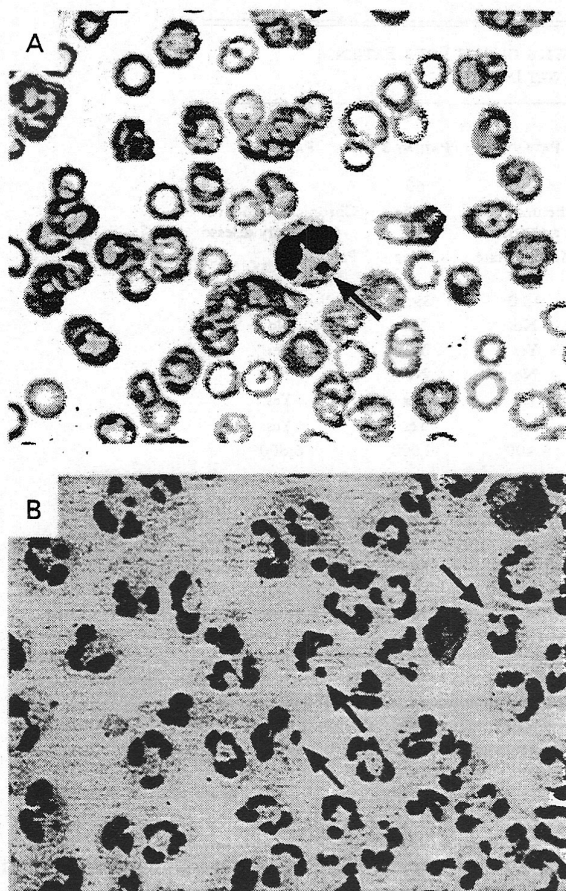


Figure 1. Detection of Morulae in Specimens from Patient 4. In Panel A, a peripheral-blood smear reveals a morula (arrow) in a neutrophil (Wright's stain, $\times 1000$). In Panel B, a cytospin preparation of cerebrospinal fluid shows morulae (arrows), which were originally identified as gram-negative coccobacilli (Gram's stain, $\times 1000$).

Sequences from all four patients were identical: all differed at 12 nucleotide positions from the known sequence of *E. chaffeensis* (GenBank accession number, M73222) but matched the known sequence of the 16S rRNA gene of *E. ewingii* (GenBank accession number, U96436) (Table 2). In addition, the four sequences contained a 2-bp insert not present in the *E. chaffeensis* sequence. All these differences were within a 28-bp segment from positions 54 through 81 of the *E. ewingii* 16S rRNA gene. Sequences from 12 patients in whom *E. chaffeensis* infection had been diagnosed on the basis of positive results with *E. chaffeensis*-specific PCR were identical to one another and to the sequence of *E. chaffeensis*.

Sequencing of the 16S rRNA Gene

Partial and nearly full-length 16S rRNA gene sequences were obtained from blood specimens from

Patients 3 and 4. A 750-bp segment from the 5' end of the 16S gene from Patient 3 and 951-bp and 1284-bp segments from Patient 4 were amplified and sequenced. All the sequences were identical to the corresponding 16S rRNA sequence of *E. ewingii*.

PCR Assay for *E. ewingii*

To confirm the presence of *E. ewingii*, a PCR assay specific for *E. ewingii* was performed on the product of the broad-range PCR assay from Patient 1 (for whom no blood specimen was available) and on blood samples from Patients 2, 3, and 4, as well as on blood samples from 12 of the 56 patients with PCR-confirmed *E. chaffeensis* infection. Positive results were obtained only in specimens from Patients 1, 2, 3, and 4 (Fig. 2). *E. ewingii* DNA was also detected in blood samples from two dogs owned by Patient 3. Neither dog was ill at the time the blood samples were obtained.

Serologic Studies

Serum samples from Patients 1, 3, and 4 contained IgG antibody that reacted with *E. chaffeensis* at titers of at least 2048, 256, and 4096, respectively, on an indirect immunofluorescence assay. Western blot analysis of samples obtained from these three patients during convalescence, with the use of *E. chaffeensis* and *E. canis* as antigens, revealed patterns indicative of infection with *E. ewingii*: the samples reacted with antigens of at least 40 kd but not with the 28-kd major antigen of *E. chaffeensis* or the 30-kd major antigen of *E. canis*¹⁹⁻²¹ (Fig. 3). A serum sample from one of the two dogs belonging to Patient 3 and from the dog experimentally infected with *E. ewingii* showed the same pattern. The serum sample from the other dog belonging to Patient 3 had a pattern suggestive of infection with multiple ehrlichia species: it showed reactivity with high-molecular-weight antigens as well as with the 28-kd and 30-kd antigens of *E. chaffeensis* and *E. canis*, respectively. Serum samples from two patients with *E. chaffeensis* were strongly reactive to the 28-kd and 30-kd antigens, indicating infection with either *E. chaffeensis* or *E. canis* (Fig. 3).

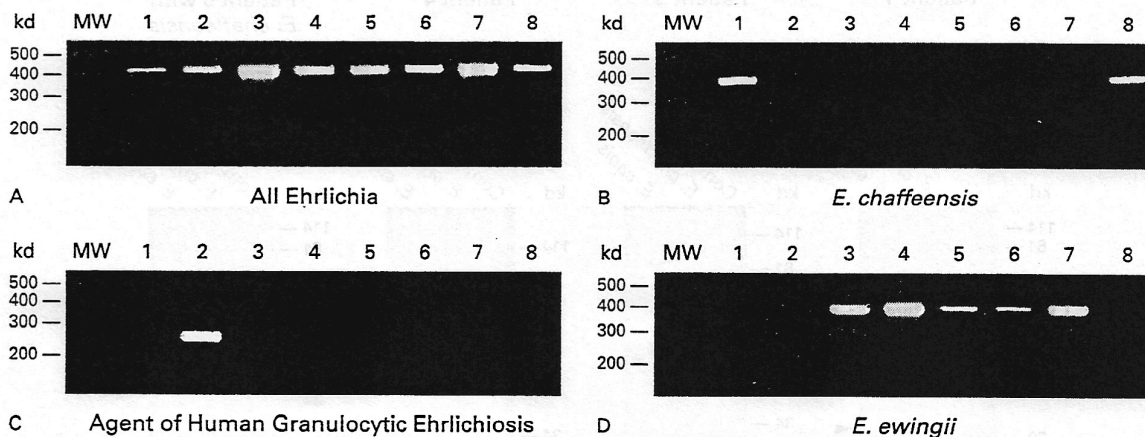
DISCUSSION

Using evidence obtained from molecular and serologic testing, we confirmed that *E. ewingii* caused disease in four patients from Missouri, an area where *E. chaffeensis* infection is also endemic. Findings that supported the diagnosis of *E. ewingii* infection included the detection of *E. ewingii* DNA sequences in the patients' blood specimens, serologic responses similar to those of dogs infected with *E. ewingii*, and the presence of morulae in granulocytes from two of the patients, both of whom were negative on PCR assays for the agent of human granulocytic ehrlichiosis. The finding of morulae in these patients' granulocytes

TABLE 2. NUCLEOTIDE SEQUENCES OF THE PCR PRODUCTS FROM 4 PATIENTS WITH *EHRLICHIA EWINGII* INFECTION AND 12 WITH *E. CHAFFEENSIS* INFECTION.*

SOURCE	16S rRNA NUCLEOTIDE SEQUENCE					
<i>E. chaffeensis</i> (GenBank accession no. M73222)	ACAATTGCTT	ATAACCTTTT	GTTAT--AA	ATAATTGTTA	GTGGCAGA	
<i>E. ewingii</i> (GenBank accession no. U96436)C..A	.AT.GTC.C.	.AC...TT.G	...G.....	
Patients with <i>E. ewingii</i> infectionC..A	.AT.GTC.C.	.AC...TT.G	...G.....	
Patients with <i>E. chaffeensis</i> infection	

*The sequence shown is the variable region (positions 48 through 95 of *E. ewingii* sequence) of the 16S rRNA gene of *E. chaffeensis*. Dots indicate positions that are identical to those of the *E. chaffeensis* sequence, and dashes the *E. chaffeensis* sequence that corresponds to the 2-bp insert in *E. ewingii*.

**Figure 2.** Ethidium Bromide–Stained Gels of Products of PCR Assays for Ehrlichia.

Panel A shows the results of broad-range assays for ehrlichia; Panel B, assays specific for *E. chaffeensis*; Panel C, assays specific for the agent of human granulocytic ehrlichiosis; and Panel D, assays specific for *E. ewingii*. MW denotes the molecular-weight markers. In each panel, lane 1 shows an *E. chaffeensis*-positive control from a patient; lane 2 a control positive for the agent of human granulocytic ehrlichiosis (plasmid pDGE-2, containing the 16S rRNA gene of the agent of human granulocytic ehrlichiosis [kindly provided by Dr. R. Massung, CDC]); lane 3 an *E. ewingii*-positive control (leukocytes from an experimentally infected dog [kindly provided by Dr. S. Stockham, University of Missouri, Columbia]); lanes 4, 5, 6, and 7 leukocytes from Patients 1, 2, 3, and 4, respectively; and lane 8 leukocytes from a patient with *E. chaffeensis* infection, as confirmed by nucleotide sequencing of the product of 16S rRNA gene amplification. Gels were photographed under ultraviolet illumination with a digital imaging system (model IS-1000, Alpha Innotech, San Leandro, Calif.). PCR assays also included negative controls, in which water was substituted for specimen; all consistently yielded negative results (data not shown).

parallels the finding of morulae in the granulocytes of dogs infected with *E. ewingii* and is in contrast to the presence of morulae in mononuclear cells in patients infected with the closely related *E. chaffeensis*. The four patients, all of whom were male and had a recent history of exposure to ticks, presented with an illness indistinguishable from the other human ehrlichioses recognized in the United States. Indeed, on the basis of the clinical features, ehrlichial infection was included in the initial differential diagnoses.

The observation that three of these patients were immunocompromised raises the question whether *E. ewingii* infection is symptomatic primarily in such patients. Nonetheless, the fourth patient had moderate disease despite an apparently normal immune system. All four patients had dramatic responses to treatment with doxycycline, as is seen in cases of infection caused by *E. chaffeensis* or the agent of human granulocytic ehrlichiosis.^{1,4,5}

Canine granulocytic ehrlichiosis was first described

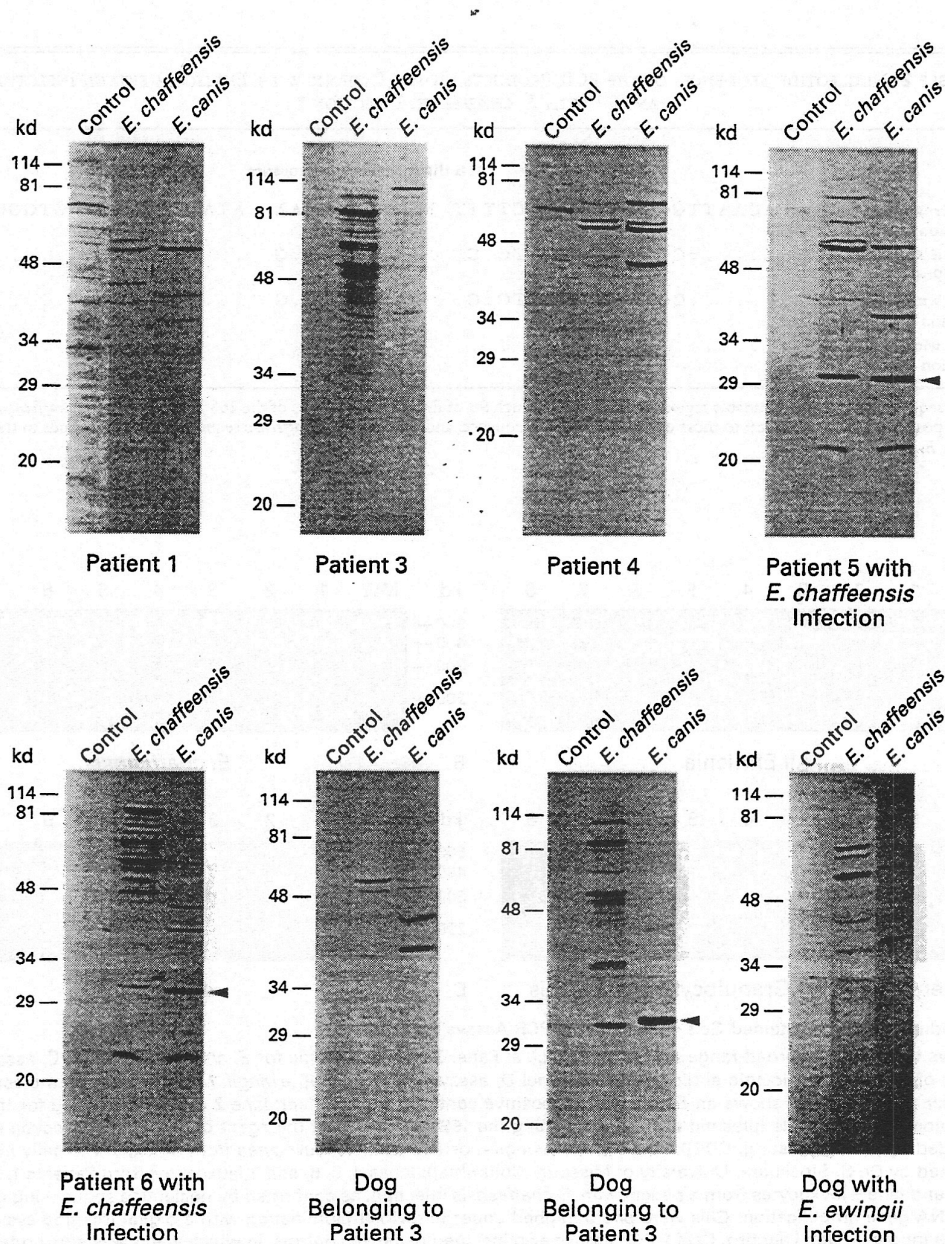


Figure 3. Western Blot Analysis of Serum Samples for Reactivity with *Ehrlichia chaffeensis* and *E. canis* Antigens. Serum specimens were analyzed with the use of antigen prepared from cultures of DH82 canine macrophages infected with *E. chaffeensis* or *E. canis*. Uninfected DH82 cells were used as a control. Serum from patients with *E. chaffeensis* infection is expected to show reactivity with the 28-kd major antigen of *E. chaffeensis*, the 30-kd major antigen of *E. canis* (indicated by arrowheads), or both; serum from dogs infected with *E. ewingii* has shown reactivity with antigens of 40 kd or greater but not with the 28-kd or 30-kd major antigens of *E. chaffeensis* or *E. canis*. Shown are results for Patients 1, 3, and 4; results for two patients known to be infected with *E. chaffeensis* (Patients 5 and 6); two dogs belonging to Patient 3; and a third dog experimentally infected with *E. ewingii*.

in a dog from Arkansas in 1971,²² and subsequently in dogs from several other states, including Missouri.²³ *E. ewingii* was identified as an etiologic agent of this syndrome in 1992.²⁴ Since then, canine infection with *E. ewingii* has been reported in Oklahoma, North Carolina, and Virginia.^{8,24,25} *E. ewingii* is one member of a group of closely related species that also includes *E. chaffeensis*, *E. muris*, *E. canis*, and *Cowdria ruminantium*. Members of this group exhibit antigenic similarities and closely related 16S rRNA gene sequences (≥ 98 percent homology between *E. ewingii* and *E. chaffeensis* and between *E. ewingii* and *E. canis*).²⁴ In dogs, *E. ewingii* infection is usually milder than *E. canis* infection and responds to treatment with tetracycline.²³

In experiments in dogs, *E. ewingii* infection has been transmitted by the Lone Star tick (*Amblyomma americanum*),²⁶ the tick that also transmits *E. chaffeensis*.²⁷ All four of our patients had been exposed to ticks and had had contact with dogs shortly before the onset of symptoms. Dogs belonging to one of the patients had serologic and molecular evidence of asymptomatic *E. ewingii* infection, suggesting that dogs may act as a reservoir for this agent. Human and canine infections with identical ehrlichia species have also been described in Minnesota, Wisconsin, and Sweden, where the agent of human granulocytic ehrlichiosis has been implicated as a cause of both human and canine granulocytic ehrlichiosis.^{28,29}

We do not know whether *E. ewingii* infection in humans is a new phenomenon or merely a newly recognized phenomenon. As illustrated by the cases of Patients 1, 3, and 4, *E. ewingii* and *E. chaffeensis* are antigenically related and stimulate the production of cross-reacting antibodies, as measured by indirect immunofluorescence assay.¹⁹ Differences between *E. ewingii* infection and *E. chaffeensis* infection in the pattern of serologic reactivity were apparent on Western blot analyses: in specimens from the patients and from dogs infected with *E. ewingii*, there was no reactivity to the 28-kd antigen of *E. chaffeensis* or to the 30-kd antigen of *E. ewingii*. This pattern suggests that the cross-reactivity observed on the indirect immunofluorescence assay is due to other antigens, such as those of higher molecular weight that were seen on the Western blots. Since the indirect immunofluorescence assay is the most widely used serologic method for detecting antibodies to ehrlichia, the use of this method could lead to erroneous identification of *E. ewingii* infection as *E. chaffeensis* infection.

Since *E. ewingii* has not been cultivated, laboratory confirmation of infection currently requires molecular techniques. Because of the variation between *E. ewingii* and *E. chaffeensis* in the 16S rRNA gene segment corresponding to the binding region of published *E. chaffeensis* primers,¹⁴ PCR testing with *E. chaffeensis* 16S rRNA primers would fail to detect

E. ewingii. Thus, it is possible that some previously reported cases of ehrlichiosis with a serologic response to *E. canis* or *E. chaffeensis* antigens but negative results on PCR assay for *E. chaffeensis* may have been due to *E. ewingii*.³⁰ Human infection with *E. ewingii* is also suggested by the report of a patient from Missouri who had granulocytic ehrlichiosis and positive results on a broad-range PCR assay but negative results on a *E. chaffeensis*-specific assay.³¹

On the basis of our findings, *E. ewingii* should be added to the list of ehrlichia pathogens of humans. In our laboratory, *E. ewingii* accounted for 7 percent of all specimens that were positive for ehrlichia. Further study is required to refine this estimate and to define more precisely the magnitude, geographic distribution, ecologic features, and clinical manifestations of *E. ewingii* infection in humans.

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