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Ehrlichia canis-Like Agent Isolated from a Man in Venezuela: Antigenic and Genetic Characterization

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We report the first isolation and molecular and antigenic characterization of a human ehrlichial species in South America. A retrospective study was performed with serum specimens from 6 children with clinical signs suggestive of human ehrlichiosis and 43 apparently healthy adults who had a close contact with dogs exhibiting clinical signs compatible with canine ehrlichiosis. The evaluation was performed by the indirect fluorescent-antibody assay with *Ehrlichia chaffeensis* Arkansas, *Ehrlichia canis* Oklahoma, and *Ehrlichia muris* antigens. The sera from two apparently healthy humans were positive by the indirect fluorescent-antibody assay for all three antigens. Of the three antigens, samples from humans 1 and 2 showed the highest antibody titers against *E. chaffeensis* and *E. muris*, respectively. The remaining serum samples were negative for all three antigens. One year later examination of a blood sample from subject 1 revealed morulae morphologically resembling either *E. canis*, *E. chaffeensis*, or *E. muris* in monocytes in the blood smear. The microorganism, referred to here as Venezuelan human ehrlichia (VHE), was isolated from the blood of this person at 4 days after coculturing isolated blood leukocytes with a dog macrophage cell line (DH82). The organism was also isolated from mice 10 days after intraperitoneal inoculation of blood leukocytes from subject 1. Analysis by electron microscopy showed that the human isolate was ultrastructurally similar to *E. canis*, *E. chaffeensis*, and *E. muris*. When the virulence of VHE in mice was compared with those of *E. chaffeensis*, *E. canis*, and *E. muris*, only VHE and *E. muris* induced clinical signs in BALB/c mice at 4 and 10 days, respectively, after intraperitoneal inoculation. VHE was reisolated from peritoneal exudate cells of the mice. Only *E. chaffeensis*- and *E. muris*-infected mice developed significant splenomegaly. Western immunoblot analysis showed that serum from subject 1 reacted with major proteins of the VHE antigen of 110, 80, 76, 58, 43, 35, and 34 kDa. Human serum against *E. chaffeensis* reacted strongly with 58-, 54-, 52-, and 40-kDa proteins of the VHE antigen. Anti-*E. canis* dog serum reacted strongly with 26- and 24-kDa proteins of VHE. In contrast, anti-*E. sennetsu* rabbit and anti-*E. muris* mouse sera did not react with the VHE antigen. Serum from subject 1 reacted with major proteins of 90, 64, or 47 kDa of the *E. chaffeensis*, *E. canis*, and *E. muris* antigens. This reaction pattern suggests that this serum sample was similar to serum samples from *E. chaffeensis*-infected human patients in Oklahoma. The base sequence of the 16S rRNA gene of VHE was most closely related to that of *E. canis* Oklahoma. On the basis of these observations, we suggest that VHE is a new strain or a subspecies of *E. canis* which may cause asymptomatic persistent infection in humans.

Ehrlichiosis is an acute and sometimes chronic infectious disease produced by obligate intracellular bacteria of the family *Rickettsiaceae*. They are gram-negative cocci which replicate within phagosomes in the cytoplasm of mononuclear or polymorphonuclear leukocytes or thrombocytes (23). Since 1935, it has been known that ehrlichial agents affect diverse animal species including human, canine, feline, equine, ovine, and bovine species (3, 11, 17, 23, 28). *Ehrlichia sennetsu* was the first known ehrlichial agent to cause a human disease, called Sennetsu fever (12). Recently, two new ehrlichial organisms that infect humans were described. *E. chaffeensis* was isolated from three patients with human monocytic ehrlichiosis (HME) (6–8), and the human granulocytic *Ehrlichia* sp. (a strain of *E. equi* or *E. phagocytophila*) was isolated from several patients with human granulocytic ehrlichiosis (HGE) (13, 27). Clinical signs of HME are characterized by fever, headache, myalgias, chills, anorexia, rash in 20% of the patients, leukopenia, thrombocytopenia, anemia, and elevation in serum hepatic aminotransferase levels (11, 23, 30). The clinical spectrum of HME ranges from asymptomatic to severe or fatal infection (9–11, 21, 30). HGE is also an acute, severe, and sometimes fatal illness. Symptoms are similar to those in patients with HME, including fever, headache, myalgias, malaise, rigors, sweats, and less frequently, nausea, confusion, arthralgias, cough, and pulmonary infiltration (2, 5, 13, 30). *E. chaffeensis* was demonstrated in *Amblyomma americanum* ticks by PCR (1). The agent of HGE was demonstrated in *Ixodes scapularis* ticks collected in the field (19, 21).

In the United States, primarily in the southeastern and south-central states, more than 400 cases of HME have been identified within 30 states (11, 30). Serological evidence suggests the presence of human ehrlichiosis in Europe (14, 20) and Africa (29). More than 170 cases of HGE in Minnesota, Wisconsin, New York, Massachusetts, and Connecticut have recently been reported (2, 13, 30). There are no known previous reports of human monocytic ehrlichiosis in South America; however, the presence of both species of ticks and the occurrence of fevers of unknown etiology suggested the likelihood that ehrlichiosis may be present on that continent.

We report here the isolation of a monocytic *Ehrlichia* sp. from an apparently healthy person in Venezuela and the antigenic, genetic, and biologic characterization of this organism.

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MATERIALS AND METHODS

Serum samples. One retrospective study was made of serum specimens collected in the summer of 1994 in Barquisimeto, Venezuela, from 43 apparently healthy adults of different ages and sexes who were in contact with dogs exhibiting clinical signs compatible with canine ehrlichiosis. Serum samples from 11 dogs were tested by the *E. canis* indirect-fluorescent-antibody assay (IFA), and 6 were found to be seropositive. Buffy coat specimens from one of the six dogs was positive also by the *E. canis* PCR test (21a). Serum samples from six febrile 1- to 9-year-old children were included. Some of them were hospitalized with possible dengue hemorrhagic fever, a viral infection which manifests clinical signs similar to those of human ehrlichiosis (fever, headache, rash, lymphadenopathy, leukopenia, and thrombocytopenia). A 10-ml blood sample was collected from each patient and was centrifuged at 1,500 × *g* for 10 min. Serum samples were harvested and stored at −80°C until they were analyzed.

Leukocyte isolation. After 1 year, a 20-ml heparinized blood sample was collected from the individual who had shown a positive serologic response against *E. chaffeensis*, *E. canis*, or *E. muris*. The blood specimen was kept at ambient temperature for 1 week and was centrifuged at 1,500 × *g* for 5 min. The plasma was saved for serologic analysis. The buffy coat was aspirated and overlaid on Histopaque 1077, 1083, and 1119 (Sigma Chemical Co., St. Louis, Mo.) to separate monocytes, granulocytes, and erythrocytes, respectively. Each interface containing the leukocytes was harvested, washed in Dulbecco's minimum essential medium (DMEM; GIBCO, Grand Island, N.Y.), and centrifuged at 1,500 × *g* for 5 min for culture use.

Isolation of human ehrlichia. After leukocyte isolation, the cell pellet was resuspended in 3 ml of DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS; Atlanta Biologicals, Norcross, Ga.)–2 mM L-glutamine (GIBCO). For culture, 1 ml of the suspension was overlaid on a monolayer of a dog macrophage cell line (DH82), and the mixture was incubated in 12-well plates at 37°C in 5% CO_2–95% air. The cells were examined for infectivity every 2 days by microscopic examination of a Diff-Quik (American Scientific Product, Obetz, Ohio)-stained cytocentrifuged preparation. The remaining 2 ml of the cell suspension was centrifuged, and the pellet was resuspended in 2 ml of the freezing medium which consisted of RPMI 1640 (GIBCO), 30% FBS, 2 mM L-glutamine, and 15% dimethyl sulfoxide (Sigma). The cell suspension was stored at −80°C. Flasks containing *E. canis* were not concurrently maintained in the same incubator while attempts were made to isolate it from the clinical samples. DH82 cells were negative for any ehrlichial organisms before isolation. Maximum precautions were taken to prevent cross-contamination.

Culturing and purification of organisms. *E. chaffeensis* Arkansas, *E. canis* Oklahoma, *E. muris* AS145, *E. sennetsu* Miyayama, and *Neorickettsia helmintho-eca* (26) were propagated as described previously (24) in a dog macrophage cell line (DH82). Briefly, 10^6 frozen cells per ml were overlaid on uninfected DH82 cells, and the mixture was incubated at 37°C in 5% CO_2–95% air with DMEM supplemented with 10% FBS–2 mM L-glutamine. The medium was changed every 2 days until infectivity was at least 80%, and then purification of the microorganisms and preparation of slides containing antigen for IFA were carried out.

Purification was performed as described previously (24, 25), and heavily infected cells from eight flasks (150 cm^2) were pooled and centrifuged at 1,500 × *g* for 10 min at 4°C. The cell pellet was resuspended in DMEM and was disrupted by sonication for 2 to 5 s. The supernatant was collected and kept on ice. The pellet was resuspended in DMEM, and the procedure was repeated twice. The pooled supernatant was centrifuged at 10,000 × *g* for 15 min at 4°C, and the pellet containing free microorganisms was resuspended in phosphate-buffered saline (PBS; 2 mM KH_2PO_4, 6 mM Na_2HPO_4, 2 mM KCl, 136 mM NaCl [pH 7.4]) and placed on top of a packed chromatography column of Sephacryl-S-1,000 (Pharmacia, Uppsala, Sweden). After elution with 2× PBS, the flowthrough fraction recovered was centrifuged in a microcentrifuge at 10,000 × *g* for 10 min, and the pellet containing ehrlichial microorganisms was frozen at −80°C until use for Western immunoblotting.

IFA. Slides containing cultured cells infected with *E. canis* and *E. chaffeensis* as antigens were made as described previously (24). Slides containing *E. muris* antigen were made with peritoneal cells from experimentally infected BALB/c mice as described by Kawahara et al. (16). Titration of sera was made by using 10 serial twofold dilutions in PBS, starting at a 1:20 dilution, as described previously (24). Diluted serum (10 μl) was placed into each well of 12-well antigen-coated slides. A positive control serum and PBS as a negative control were placed in one well each. After incubation at 37°C for 1 h, the slide was washed three times with PBS. Ten microliters of fluorescein isothiocyanate-conjugated goat anti-human, anti-dog, and anti-mouse immunoglobulin G (Cappel-Laboratories Inc., Durham, N.C.) diluted at 1:100 in PBS was placed into each well, and the plates were incubated at 37°C and rinsed as described above. When *E. muris* antigen was used, the secondary antibody was preadsorbed with the murine $P388D_1$ cell line (10^6 cells per ml of serum) for 1 h at 37°C to remove nonspecific binding to the murine uninfected macrophages. Counterstaining was performed with Evans blue diluted 1:1,000 with PBS for 5 min. A coverslip was placed on the slide with mounting medium (Sigma), and the slide was examined under an epifluorescent microscope.

Western immunoblot analysis. Purified Venezuelan human ehrlichia (VHE), *E. chaffeensis*, *E. canis*, *E. muris*, *N. helminthoeca*, *E. sennetsu*, and uninfected DH82 cells (40 μg of protein per lane) were separated by sodium dodecyl sulfate–12% polyacrylamide gel electrophoresis, and Western immunoblotting was performed with dog, human, mouse, or rabbit sera against various ehrlichial spp. as described previously (25). Antigens electroblotted onto nitrocellulose membranes were reacted with the primary antibody at a 1:25 dilution and were then incubated with alkaline phosphatase-conjugated affinity-purified anti-dog, anti-human, anti-mouse, or anti-rabbit immunoglobulin G (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Md.) at a 1:1,000 or a 1:500 dilution. The enzyme reaction was followed for color development as described previously (25).

Mouse virulence test. For the mouse virulence tests, groups of three male BALB/c mice each were injected intraperitoneally with 10^6 infected cells per 0.1 ml of RPMI. The mice were treated as follows: group 1, inoculated with blood leukocytes isolated from human subject 1; group 2, inoculated with human isolate-infected DH82 cells; group 3, inoculated with *E. canis*-infected DH82 cells (to verify the presence of the ehrlichial organisms in the peritoneal cells, a direct immunofluorescent test was performed with a positive anti-*E. canis* control serum for labeling the organisms); group 4, inoculated with *E. chaffeensis*-infected DH82 cells; group 5, inoculated with spleen cells of an *E. muris*-infected mouse; group 6, uninoculated controls; and group 7, controls inoculated with uninfected DH82 cells. The mice were monitored once a day for clinical signs of illness. All of the mice were killed at day 10 postinoculation (p.i.). Spleens and livers were weighed, and the relative weights (grams per 100 g of body weight) were compared with that of the control by Student's *t* test, and a *P* value of <0.05 was considered significant. Blood samples were taken from the mice by intracardiac puncture, and the mononuclear cell fraction was separated by centrifugation over Histopaque 1077. Peritoneal fluid was aspirated after intraperitoneal injection of 5 ml of RPMI. The fluid was centrifuged at 1,500 × *g* for 5 min. The pellet was washed with DMEM, resuspended in 1 ml of the same medium, and cocultured with DH82 cells. The spleens were removed aseptically, homogenized in 5 ml of RPMI, and centrifuged at 1,500 × *g* for 5 min. The pellet was resuspended in 2 ml of RPMI placed over Histopaque 1077 for separation of leukocytes. All of the cell preparations were washed with DMEM and were cultured as described previously to evaluate the preparations for the presence of live ehrlichial organisms.

Amplification of the 16S rRNA gene of VHE. The DNA of VHE-infected DH82 cells was extracted with a QIAamp tissue kit (QIAGEN, Inc., Chatsworth, Calif.) by using the protocol recommended by the manufacturer. This DNA was used as the template in the PCR. The 16S rRNA gene was amplified with 5′-end primer A-17 (5′-GTTTGATCATGGCTCAG-3′) and 3′-end primer 3-17 (5′-AAGGAGGTAATCCAGCC-3′), which are universal primers for the 16S rRNA gene sequences of prokaryotic cells. The PCR was performed as described previously (31). The amplified DNA fragment, which was the expected size (approximately 1.5 kb), was purified from a 1% low-melting-temperature agarose gel (GIBCO BRL) by using a PCR DNA purification system (Promega, Madison, Wis.). This DNA was used as the template for cycle sequencing.

DNA sequencing of the 16S rRNA PCR product. The sequences of both DNA strands were determined by using the dideoxynucleotide method, a double-stranded DNA cycle sequencing system (GIBCO BRL), and the protocol recommended by the manufacturer. The forward and reverse sequencing primers used were derived from the conserved sequences of known ehrlichial 16S rRNA genes on the basis of the results of an analysis in which we used the program OLIGO (National Biosciences, Inc., Hamel, Mich.). The sequencing primers used were primers A-17, 3-17, ER323-341 (5′-CTACGGGAGGCAGCAGCAGTC-3′), ER757-775 (5′-TAGTCCACGCTGTAAACG-3′), ER1043-1062 (5′-TAAGTCCCGACAACGAGC-3′), ER864-846 (5′-GAGTTTTAGTCTTGCGACC-3′), and ER493-476 (5′-GTATTACCGCGGCGCTG-3′). The primer positions were designated on the basis of the 16S rRNA sequence of *E. risticii* Illinois. A 5% Long Ranger gel (AT Biochem, Malvern, Pa.) was used for sequencing in order to determine more than 400 bases with each primer.

Electron microscopy. VHE-infected DH82 cells were fixed overnight at 4°C in a mixture containing 5% glutaraldehyde, 2.5% paraformaldehyde, and 0.03% trinitrophenol in 0.1 M sodium cacodylate buffer (pH 7.4). The cells were washed twice with 0.1 M sodium cacodylate buffer, postfixed with 1% osmium tetroxide in 1.5% potassium ferrocyanide for 1 h, block stained with 1% uranyl acetate, dehydrated in a graded ethanol-propylene oxide series, and embedded in Poly/Bed 812 resin (Polysciences, Inc., Warrington, Pa.). Ultrathin sections were stained with uranyl acetate and lead citrate, and the stained sections were examined with a Philips model 300 electron microscope at 60 kV.

RESULTS

In the retrospective study performed with serum samples from 6 febrile children and 43 adults, sera from 2 asymptomatic females, 27 and 18 years of age, were seropositive for *E. chaffeensis*, *E. muris*, and *E. canis* antigens by IFA. The serum of subject 1 had a higher titer against *E. chaffeensis* antigen (1:160) than against the other antigens. The serum of subject 2 had a higher titer against all of the antigens than the serum of

TABLE 1. Serologic cross-reactivity among VHE and other *Ehrlichia* spp.

Serum specimen	IFA antibody titer against the following antigens:			
	VHE sp.	*E. chaffeensis*	*E. canis*	*E. muris*
Subject 1	1:1,280/1:2,560[a]	1:160/1:320	1:10/1:10	Negative/1:80
Subject 2	1:640	1:2,560	1:1,280	1:10,000
Anti-*E. chaffeensis* (human)	1:1,280	1:2,560	1:320	1:2,560
Anti-*E. canis* (dog)	1:1,280	1:320	1:5,120	1:5,120
Anti-*E. muris* (mouse)	1:80	Negative	Negative	1:1,280
Anti-*E. ewingii* (dog)	1:160	1:20	Negative	1:1,280

[a] Antibody titer of the first serum sample/antibody titer of the second serum sample obtained 1 year later.

subject 1, and the titer against *E. muris* antigen (1:10,000) was the highest (Table 1).

Only subject 1 could be contacted again 1 year later, and a blood sample was obtained. Throughout this period she had remained asymptomatic and no antibiotic treatment had been given. Hematological findings were as follows: hemoglobin level, 12.7 g% (normal range, 12 to 14 g%); leukocytes, 5,500/mm^3 (normal range, 5,000 to 10,000/mm^3), thrombocytes, 288,000/mm^3 (normal range, 200,000 to 500,000/mm^3), and relative lymphocytosis, 50% by differential counting. The serum sample collected 1 year later had a 1:320 titer against *E. chaffeensis* antigen (Table 1).

Morulae resembling those of ehrlichial organisms were seen in the cytoplasms of a few blood leukocytes. Subsequently, the leukocytes were overlaid on a monolayer of DH82 cells, and intracytoplasmic morulae were seen in DH82 cells after 4 days in the culture (Fig. 1). Electron microscopy of the infected DH82 cells revealed that the VHE isolate had an ultrastructural morphology compatible with that of the *E. canis*, *E. chaffeensis*, and *E. muris* gene (antigen) group. (Fig. 2). IFA with the VHE sp. as the antigen was performed. In a serum sample from subject 1, a higher titer (1:1,280) to anti-*E. canis* and anti-*E. chaffeensis* was recorded. The paired serum sample from subject 1 had a twofold increase in antibody titer against the homologous antigens (Table 1).

Ehrlichial organisms were also reisolated from BALB/c mice after intraperitoneal inoculation of leukocytes isolated from subject 1 (group 1) and infected cultured cells (group 2) (Table 2). Ehrlichiae were recovered from both peritoneal and spleen cells after they were cocultured with DH82 cells. Mice from group 1 developed clinical signs 4 days after the human leukocytes were inoculated, which included inactivity, ruffled fur, and squinty eyes, but they recovered after 6 days p.i. When VHE-infected cultured cells were inoculated (group 2), the mice did not develop clinical signs (Table 2).

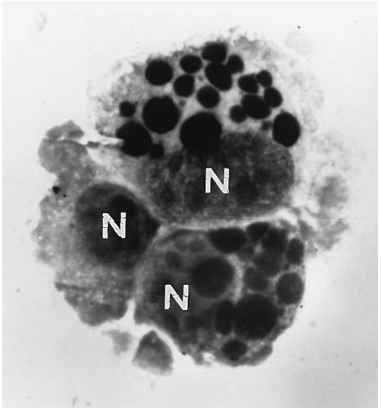

FIG. 1. Ehrlichial organisms isolated from the blood leukocytes of a human in Venezuela. Note the multiple round, large morulae in the cytoplasms of DH82 cells. N, nucleus of DH82 cells. Diff-Quik staining was used. Magnification, ×714.

Since the clinical signs of VHE-infected mice were similar to those of the *E. muris*-infected mice (16, 17) and comparative studies of the pathologies of ehrlichial species in mice have not previously been performed, we performed a comparative mouse virulence study by inoculating BALB/c mice with *E. canis*, *E. chaffeensis*, or *E. muris*. No pathologic changes were observed in *E. canis*-infected mice, but the organism was seen in low numbers in blood and spleen cells after they were stained with Diff-Quik or when IFA was performed with a dog anti-*E. canis* serum sample to label intracytoplasmic ehrlichiae. *E. canis* was also isolated when spleen or peritoneal cells were cocultured with DH82 cells. *E. chaffeensis*-inoculated mice developed profound splenomegaly; however, organisms were not recovered from peritoneal or spleen cells (Table 2). The findings for *E. muris*-infected mice were similar to those reported previously (16, 17). Results of Western immunoblot analysis of both samples of human sera using the five purified ehrlichial antigens and the uninfected DH82 cell lysate as a control are provided in Fig. 3 and Table 3. The protein profiles of ehrlichial antigens stained with Coomassie blue are provided in Fig. 3. Sera from both human subjects reacted to *E. chaffeensis*, *E. canis*, and *E. muris* antigens; however, the reaction patterns of the sera were slightly different. Serum from subject 1 bound to 64-kDa proteins of the *E. chaffeensis* antigen. This serum sample also reacted with 64- and 47-kDa proteins of the *E. canis* antigen, 90- and 64-kDa proteins of the *E. muris* antigen, and the 64-kDa protein of *E. sennetsu* antigen. Serum from subject 2 recognized 120-, 90-, and 74-kDa proteins of the *E. chaffeensis* antigen and 120- and 90-kDa proteins of the *E. muris* antigen. This serum sample reacted weakly with the 74-kDa protein of the *E. muris* antigen. The molecular sizes of the major proteins that reacted with *E. canis* antigen were 175 and 120 kDa. Neither of the serum samples reacted to major protein bands of less than 47 kDa. Neither serum sample developed strong antibody reactions with *N. helminthoeca* antigen or uninfected DH82 cell lysates (Fig. 3).

By using purified VHE as the antigen, the reaction of serum from subject 1 was compared with those of other positive control sera (Fig. 4; Table 3). Serum from subject 1 reacted with major proteins of 110, 80, 76, 58, 43, 35, and 34 kDa. Positive control anti-*E. chaffeensis* Arkansas serum from a patient in Oklahoma (24) reacted with major proteins of 58, 54, 52, 40, 38, and 34 kDa. Dog anti-*E. chaffeensis* serum showed major protein bands of 71, 58, 50, 42, 32 and 14 kDa. Dog anti-*E. canis* serum reacted strongly with proteins of 70, 58, 54, 26, and 24 kDa. Dog anti-*E. ewingii* serum reacted strongly with proteins of 76, 48, and 46 kDa. Mouse anti-*E. muris* and

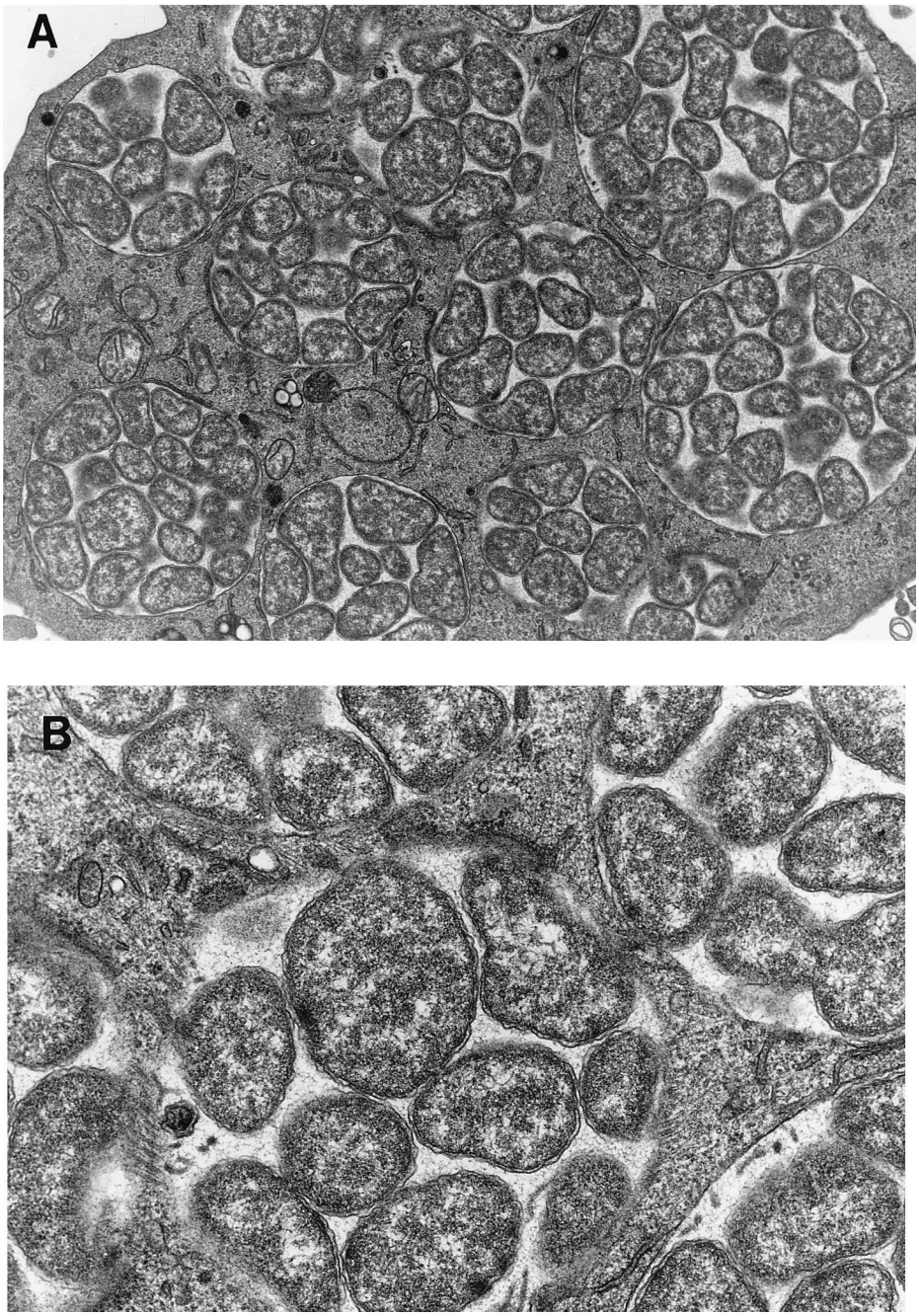

FIG. 2. Transmission electron micrographs of ehrlichial organisms isolated from the blood of a human in Venezuela. Note the multiple round organisms in the membrane-bound inclusions in the cytoplasm of DH82 cells (A). The matrix of the inclusion is filled with filamentous ground substance (B). Magnifications ×10,000 (A) and ×22,400 (B).

rabbit anti-*E. sennetsu* serum gave minimum reactions with the VHE antigen.

The 16S rRNA gene was amplified by PCR and was sequenced. Of 1,434 bases, the isolate was only one and two bases different from *E. canis* Florida and Oklahoma, respectively (level of sequence similarity, 99.9%). VHE, *E. canis* Oklahoma, and *E. canis* Florida had G, A, and A residues at position 199 of the 16S rRNA sequence, respectively, and they had C, C, and T residues at position 1264, respectively.

DISCUSSION

Human monocytic ehrlichiosis was first described in the United States in 1986 in a 56-year-old man. The spectrum of clinical manifestations of the infection varies (8–11). In many cases, ehrlichiosis is associated with nonspecific findings and rarely is sufficiently severe to result in death (11). The pathogenic mechanisms in human ehrlichiosis are unknown. The severity of illness, however, might also be related to certain host factors such as the presence of underlying immunosup-

TABLE 2. Clinicopathologic findings of ehrlichia-inoculated mice and reisolation of *Ehrlichia* spp.

Group	Mouse inoculum	% of Body wt (mean ± SD)		Clinical signs[a]	Peritoneal exudate cells	Reisolation of ehrlichiae
		Spleen	Liver			
1	Leukocytes of subject 1	0.54 ± 0.01	5.97 ± 0.01	±	+	+
2	Venezuelan isolate	0.42 ± 0.03	7.76 ± 2.64	−	+	+
3	*E. canis*	0.43 ± 0.08	4.90 ± 0.30	−	+	+
4	*E. chaffeensis*	0.90 ± 0.84[b]	5.99 ± 0.12	−	−	−
5	*E. muris*	0.81 ± 0.09[c]	5.09 ± 0.96	+	+	+
6	None	0.42 ± 0.08	5.47 ± 0.47	−	−	−
7	DH82	0.43 ± 0.10	5.20 ± 0.15	−	−	−

[a] Ruffled fur coat, squinty eyes, and inactivity lasting more than 1 day.
[b] $P < 0.01$ compared with controls inoculated with uninfected DH82 cells or noninoculated by Student's *t* test.
[c] $P < 0.001$.

pression, advanced age, and other factors, such as differences in ehrlichial strains, virulence, and delays in diagnosis and the initiation of therapy (8, 10, 11).

Human monocytic ehrlichiosis is primarily reported in the southeastern and south-central regions of the United States. Venezuela is a tropical country with a high frequency of ticks. Febrile illnesses of unknown etiology are quite common there. Since both of these are important factors in finding ehrlichial infections, we made a serological evaluation of sera from febrile children and healthy adults who had contact with dogs exhibiting clinical signs of canine ehrlichiosis. We report here the first isolation and antigenic and genetic characterization of an *Ehrlichia* sp. from a human being in South America. By 16S rRNA base sequence comparison, the VHE sp. was found to be most closely related to *E. canis.*

The VHE sp. was isolated from a 27-year-old woman who did not show clinical signs of illness during the 1-year period between collection of the paired blood samples. She is a veterinarian and, as a consequence, is frequently exposed to animals and ticks. She did not, however, recall a tick bite prior to either sample collection. Tick exposure is the common factor among human ehrlichiosis patients in the United States (10, 11, 22). Laboratory findings for the subject described here showed borderline low hematological values, including those for thrombocytes and leukocytes. Since ehrlichiosis is characterized by the reduction of hematological parameters during the acute stage of infection and mildly decreased values in the subclinical stage of infection (4), these slightly low values could have been related to the asymptomatic infection. Relative lymphocytosis caused by relatively low numbers of neutrophils was also present. Leukopenia is a common feature reported in human ehrlichiosis (4, 8). In one study describing patients with human ehrlichiosis, lymphocytosis with high levels of γδ T cells were found 2 or 3 days after the initiation of doxycycline treatment (4). These types of cells have been implicated in the immune responses and clearance of microorganisms in some intracellular infections and may participate in the eradication of ehrlichiae from patients (4). On the other hand, as mentioned earlier, previous reports have pointed out other factors that may enhance the severity of the infection. Thus, the absence of clinical signs in subjects 1 and 2 could have been due to their young age, the relatively low virulence of the strain, or to the low level of parasitemia present in subject 1. Moreover, the possible frequent exposure to infected ticks by subject 1 could have induced some resistance in this individual.

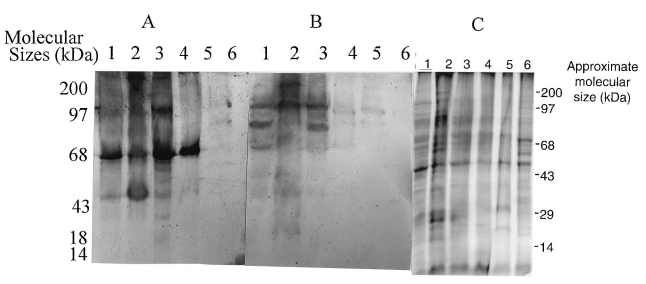

FIG. 3. Western immunoblot analysis of sera from subject 1 (A) and subject 2 (B) with various ehrlichial antigens. The ehrlichial antigens were as follows: *E. chaffeensis* (lane 1), *E. canis* (lane 2), *E. muris* (lane 3), *E. sennetsu* (lane 4), *N. helminthoeca* (lane 5), uninfected DH82 cell lysate (lane 6). (C) Coomassie-stained antigens.

The morphology of the isolated microorganism was similar to those of *E. chaffeensis*, *E. canis*, and *E. muris*, but was distinct from those of the *E. risticii*, *E. sennetsu*, and *N. helminthoeca* gene group or the HGE agent, *E. equi*, and *E. phagocytophila* gene group. Similar to *E. chaffeensis*, *E. canis*, and *E. muris*, VHE was isolated by coculturing blood leukocytes with the DH82 cell line (23). However, the morulae of VHE could be seen earlier in culture, after only 4 days instead of 14 to 36 days, as was reported previously for *E. canis* or *E. chaffeensis* (7, 8, 15). Keysary et al. (18), however, reported the isolation of *E. canis* Israel using primary canine peripheral blood monocyte culture after 7 days.

Paired serum samples were collected from subject 1 at a 1-year interval. At the later time, the antibody titer against *E. chaffeensis* antigen had increased only twofold (Table 1), while a fourfold or greater change in titer or seroconversion between the acute and the convalescent phases is usually considered to be a definitive diagnosis of ehrlichial infection (10, 11, 22, 23). Such a criterion may not be useful for the apparent persistent infection that occurred in this subject. Although subject 2 showed high antibody titers against *E. chaffeensis* and *E. canis*, the strongest reaction was observed against *E. muris* antigen (1:10,000). Because we were unable to obtain a paired serum sample from this person, this result only suggests an infection with ehrlichial organisms that were closely related to *E. muris*. Interestingly, the positive control sera against *E. chaffeensis*, *E. canis*, and *E. ewingii* also had strong reactions against *E. muris* antigen, suggesting the possibility that the *E. muris* antigen prepared with peritoneal macrophages infected with *E. muris* in vivo may be stronger than the ehrlichia-infected DH82 cell antigen (Table 1).

In the present study, VHE could be isolated from BALB/c mice. *E. sennetsu*, *E. risticii*, and *E. muris* are the only ehrlichial species previously reported to infect and cause diseases in mice and to be reisolated from mice (16, 23). Furthermore, VHE revealed some differences in pathogenicity from those of these other ehrlichial species. VHE induced mild clinical signs in

TABLE 3. Molecular sizes of VHE sp., *E. chaffeensis*, *E. canis*, and *E. muris* proteins reacting with sera containing antibodies against various *Ehrlichia* spp. by Western immunoblotting

Serum specimen	Molecular size(s) (kDa) of reacting antigens			
	VHE sp.	*E. chaffeensis*	*E. canis*	*E. muris*
Subject 1	110, 80, 76, 58, 43, 35, 34	64	64, 47	90, 64
Subject 2	ND[a]	120, 90, 74	175, 120	120, 90
Anti-*E. chaffeensis* (human)	58, 54, 52, 40, 38, 34	74, 64, 47, 40[b]	74, 64, 47, 45, 40[b]	ND
Anti-*E. chaffeensis* (dog)	71, 58, 50, 42, 32, 14	74, 70, 64, 47, 31, 29[b]	ND	ND
Anti-*E. canis* (dog)	70, 58, 54, 26, 24	ND	78, 64, 47, 30, 21[c]	ND
Anti-*E. ewingii* (dog)	76, 48, 46	74, 64, 47[b]	74, 64, 47[b]	ND
Anti-*E. muris* (mouse)	Negative	74, 68, 65, 47, 26[d]	30[d]	74, 47, 30[d]

[a] ND, not done.
[b] Data are from Rikihisa et al. (25).
[c] Data are from Iqbal et al. (15).
[d] Data are from Wen et al. (31).

infected mice 4 days p.i., with spontaneous and rapid recovery (2 days). When we made one subpassage of infected cultured cells in mice, VHE lost its virulence and mice developed neither clinical signs nor pathologic changes. In contrast, *E. sennetsu* and *E. risticii* induced progressive and more severe clinical signs at days 7 to 14 p.i., including death in infected mice (23). *E. muris* infection, on the other hand, causes severe clinical signs with spontaneous recovery (16). Whether loss of virulence after subpassage in vitro is a universal phenomenon among all ehrlichial species has not been investigated.

As far as we know, there have been no comparative studies on experimental infection of mice with *E. chaffeensis* and *E. canis*. In our comparative mouse virulence study *E. chaffeensis* apparently did not induce disease. The mice did develop marked splenomegaly. However, the organism could not be reisolated. Kawahara et al. (16) reported that in *E. muris*-infected mice, maximal splenomegaly was observed on day 15 p.i., but the maximum infective titer was observed on day 10 p.i. Therefore, it would seem to be necessary to follow the time course of infectivity in *E. chaffeensis*-infected mice to understand the apparently rapid clearance of the organism. As was reported previously (23), *E. canis* did not induce disease in mice; however, we could isolate *E. canis* after 10 days p.i.

By Western immunoblot analysis, VHE had a reaction pattern related to that of serum from a patient in Oklahoma who was determined to have been infected with *E. chaffeensis* on the basis of IFA with an *E. canis* antigen. However, several antigenic differences were present. Protein bands of 54 and 52 kDa were not present when autologous serum was reacted with the VHE antigen and were present when serum from the patient in Oklahoma was used. Additionally, an approximately 30-kDa protein common among *E. chaffeensis*, *E. canis*, and *E. muris* had only a weak reaction when the VHE antigen was incubated with sera containing antibody against *E. chaffeensis* or VHE. Since anti-*E. canis* serum reacted to a protein with a molecular mass similar to that of the protein of the VHE antigen, VHE must have this antigen, but the immune system of subject did not recognize this antigen well. The 58-kDa protein of VHE is most likely heat shock protein 60 because we demonstrated the antigenic cross-reactivity of heat shock proteins 60 from various *Ehrlichia* spp. (32).

In conclusion, our results indicate that VHE is a new strain or subspecies of *E. canis* which causes subclinical chronic infection in humans. A study of the infectivity and pathogenesis of VHE in the dog is in progress.

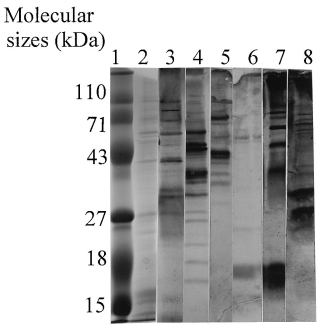

FIG. 4. Western immunoblot analysis of Venezuelan ehrlichial isolate antigen with sera containing antibodies against various ehrlichial species. Lane 1, molecular size standard; lane 2, Coomassie blue staining of VHE; lanes 3 to 8, Western immunoblot analysis of VHE: lane 3, serum from subject 1; lane 4, human anti-*E. chaffeensis* serum; lane 5, dog anti-*E. ewingii* serum; lane 6, dog anti-*E. muris* serum; lane 7, dog anti-*E. chaffeensis* serum; lane 8, dog anti-*E. canis* serum.

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