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Susceptibility of White-Tailed Deer (*Odocoileus virginianus*) to Infection with *Ehrlichia chaffeensis*, the Etiologic Agent of Human Ehrlichiosis

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Although more than 320 cases of human ehrlichiosis have been diagnosed in 27 states since 1986, the reservoir host or hosts remain unknown. Since antibodies reactive to *Ehrlichia chaffeensis*, the etiologic agent of human ehrlichiosis, have been found in white-tailed deer (*Odocoileus virginianus*), we experimentally evaluated the susceptibilities of four white-tailed deer to infection with *E. chaffeensis* and *Ehrlichia canis*, a closely related species. A fifth deer served as a negative control. Isolation and nested PCR amplification results from peripheral blood indicated that *E. chaffeensis* circulated for at least 2 weeks. The deer developed antibodies to *E. chaffeensis* by day 10 after inoculation, but there was no indication of clinical disease. Immunohistochemical staining identified *E. chaffeensis* within macrophage-type cells in lymph nodes. The deer inoculated with *E. canis* did not become infected and did not seroconvert. These results indicate that white-tailed deer can support an *E. chaffeensis* infection with resulting rickettsemia of at least 2 weeks. The resistance to infection and the absence of seroconversion upon exposure to *E. canis* indicate that antibody responses previously detected among wild deer are not *E. canis* cross-reactions. The role of deer as competent reservoirs in the life cycle of *E. chaffeensis* remains to be explored with suspected tick vectors.

More than 320 cases of human ehrlichiosis have been diagnosed in 27 states in the United States since 1986, when the disease was first recognized (3a). In 1990, the etiologic agent, *Ehrlichia chaffeensis*, was first isolated from a patient suspected of having ehrlichiosis (5). Human ehrlichiosis is characterized by fever, malaise, headache, myalgia, rigor, arthralgia, and nausea and/or vomiting. Over 93% of the 237 ehrlichiosis patients reported by Fishbein et al. (9) had onsets of illness in the 6-month period between April and September. Tick bites or attachments were reported by 67.9% of the patients; another 15.5% reported being in a tick-infested area but did not recall a tick bite or attachment. *Amblyomma americanum* has been implicated as a possible vector of *E. chaffeensis*, on the basis of the geographic distribution of cases and the identification of DNA amplified from tick pools (1).

Although the reservoir host or hosts of human ehrlichiosis remain unknown, a recent serologic study found that 43% of 1,269 white-tailed deer, from 17 states, were positive for antibodies reactive to *E. chaffeensis* (6), implicating this species in the epidemiology of human ehrlichiosis. Since serologic cross-reactions to different *Ehrlichia* spp. are known to occur, it is unclear if detected antibodies resulted from infection by *E. chaffeensis* or a related rickettsia. The purpose of this study was to evaluate experimentally the susceptibility of white-tailed deer to infection with *E. chaffeensis* and to compare the results with those for infection with *Ehrlichia canis*, a closely related species.

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MATERIALS AND METHODS

Experimental design and sample collection. Five white-tailed deer (three males and two females, approximately 9 months old, orphaned and hand reared in North Carolina) were housed in separate climate-controlled rooms at the College of Veterinary Medicine, University of Georgia. Before initiation of the experiment, all deer were tested by the indirect fluorescent-antibody test and found to be seronegative for *E. chaffeensis* and *E. canis* (8). Deer were tranquilized by intramuscular injection of approximately 2 mg of xylazine per kg (Butler Co., Columbus, Ohio) for experimental inoculations, determination of rectal temperatures, and blood sample collection.

After the jugular groove was wiped with 70% isopropanol, 5 ml of blood was collected from each deer via jugular venipuncture 1 day before inoculation and on days 4, 10, 13, 17, 20, 24, 27, and 31 after inoculation. Blood samples were collected for isolation of *Ehrlichia* spp. (heparin), PCR (EDTA), and serologic testing at all intervals commencing on day 10 postinoculation.

On all sampling dates blood was analyzed for the following parameters: hematocrit, erythrocyte count, hemoglobin levels, platelet count, total and differential leukocyte counts, and levels of total protein, urea nitrogen, creatinine, albumin, sodium, chloride, calcium, phosphorus, glucose, and aspartate amino transferase. Blood smears from each bleeding and buffy

coat smears from days 24, 27, and 31 postinoculation were stained with Wright stain and examined for organisms.

Deer were examined daily for clinical signs of infection, and temperatures were recorded just before inoculation and on days 10, 13, 17, 20, 24, 27, and 31 after inoculation.

Inocula and culture of challenge organisms. *E. chaffeensis* and *E. canis* were cultured in the continuous canine macrophage cell line, DH82, as previously described (7). Normal DH82 cell cultures served as the source for the negative control inoculum.

The *E. chaffeensis* inoculum was prepared in one 150-cm^2 culture flask. Cells were harvested, when 90% infected, by decanting the old medium, adding 4 ml of fresh medium, and subsequently detaching the monolayer with a cell scraper (GIBCO Laboratories, Grand Island, N.Y.). The infected cell suspension was Dounce homogenized (100 strokes), liberating the organisms from host cells, and then centrifuged at 160 × *g* for 5 min. The same procedure was used for *E. canis* inocula, except that the *E. canis* inocula originated from two 75-cm^2 culture flasks, one containing 50% infected cells and the other containing 90% infected cells. The noninfected DH82 control cells were grown in a 75-cm^2 flask and were treated in the same manner. The number of *E. chaffeensis* or *E. canis* organisms per milliliter was calculated as previously described (4).

Deer were inoculated, via the jugular vein, with 2 ml of infected or normal control supernatant. Deer 1 and 2 were inoculated with 2.9×10^6 *E. chaffeensis*-infected cells. Deer 3 and 4 were inoculated with 1.7×10^6 and 9×10^5 *E. canis*-infected cells, respectively. Deer 5 served as a control. Unused fractions of all inocula were cultured (see below) to confirm organism viability.

Serology. The indirect fluorescent-antibody test was performed as previously described (8). In brief, sera were screened at a dilution of 1:64 in 0.01 M phosphate-buffered saline (PBS) on spot slides of *E. chaffeensis* and *E. canis*. The conjugate used was fluorescein isothiocyanate-labelled rabbit anti-deer immunoglobulin G (Kirkegaard & Perry Laboratories, Gaithersburg, Md.), diluted 1:100 in PBS. When distinct staining of the ehrlichial organisms was observed at this dilution, serial twofold dilutions were made. Serologic results were reported as reciprocals of the highest dilution at which specific fluorescence of *Ehrlichia* morulae or individual organisms was observed.

Isolation of organisms. After the heparin tubes were rinsed with 70% ethanol, the blood was transferred into sterile 50-ml plastic tubes containing 25 ml of Ace lysing buffer (150 mM NH_4Cl, 0.7 mM KH_2PO_4, 3 mM EDTA-Na_2), and the tubes were gently inverted three to four times. After 5 min at room temperature, the suspension was centrifuged at 160 × *g* for 5 min, the disrupted erythrocyte supernatant was removed, and the procedure was repeated.

Uninfected cells from a 25-cm^2 flask were suspended in 5 ml of fresh medium and added to each pellet, and the mixture was returned to the 25-cm^2 flask. After 72 h, the supernatant was decanted, and 5 ml of fresh medium was added to each culture. Starting 30 days after the addition of leukocytes from the five deer, the cultures were examined twice weekly for evidence of infection, as previously described (7).

Bone marrow samples, obtained from the heads of the femurs of deer 1, 2, and 5, were processed as described above. Two grams of spleen was soaked in 70% ethanol for 10 min, cut into small pieces with sterile scissors, and finally Dounce homogenized for 10 strokes before being processed with the lysing buffer as described above.

DNA template preparation. A modification of the method described by Casareale et al. was used to extract DNA (3). All samples were processed by adding 1.5 ml of lysing solution (150 mM NH_4Cl, 0.7 mM KH_2PO_4, and 3 mM EDTA-Na_2) and inverting the vials several times over a 5-min period at room temperature. The mixture was centrifuged at 7,000 × *g* for 5 min, and the supernatant was aspirated. An additional 1.5 ml of lysing solution was added to each pellet, and the process was repeated. The resulting pellets were washed once in 1.5 ml of water and centrifuged as described above. The pellets were resuspended in 100 μl of heat-detergent extraction buffer (10 mM Tris-Cl [pH 8.3], 50 mM KCl, 7.5 mM $MgCl_2$, 1.2% Nonidet P-40, and 1.2% Tween 20) and 0.01 mg of glycogen. After vortexing, the suspension was heated at 100°C for 30 min.

DNA amplification. Each DNA template was examined by PCR at two different concentrations. For the outside amplification, 1 and 10 μl of each template sample were amplified in a 50-μl reaction mixture containing 10 mM Tris-Cl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM each deoxynucleoside triphosphate, 5 μM tetramethylammonium chloride, 1.25 U of Ampli*Taq* polymerase (Perkin-Elmer Cetus), and 0.8 μM (each) primers ECB (5′-CGTATTACCGCGGCTGCTGG CA-3′) and ECC (5′-AGAACGAACGCTGGCGGCAAG CC-3′). These primers amplify all known *Ehrlichia* spp. and a few other bacteria. The following temperature profile was run for 40 cycles: 1 min at 94°C, 2 min at 45°C, and 30 s at 72°C (with a 1-s extension on 72°C cycles, beginning with the third).

For the nested PCR, 1 μl of each outside reaction product was amplified in a second 50-μl reaction mixture with *E. chaffeensis*-specific primers HE1 (5′-CAATTGCTTATAAC CTTTTGGTTATAAAT-3′) and HE3 (5′-TATAGGTACC GTCATTATCTTCCCTAT-3′), as previously described (2). Nested amplification was found to be necessary because of the low sensitivity of the initial reaction. Amplification products were analyzed by electrophoresis in 1.3% agarose.

Pathology. All animals were killed with an overdose of sodium pentobarbital, and necropsies were performed. Spleens and bone marrow were aseptically removed for culture. Pieces of brain, lung, spleen, lymph nodes (prescapular, mesenteric, retropharyngeal, and bronchial), heart, skin, kidney, bone marrow, eye, liver, urinary bladder, rumen, abomasum, small intestine, and adrenal gland were fixed in 10% buffered formalin, embedded in paraffin within 48 h, cut into 4-μm-thick sections, and stained with hematoxylin and eosin. Selected tissues were also stained by the Kinyoun acid-fast method.

For immunohistochemistry, a method using the commercially available avidin-biotin-peroxidase complex was used (Vectastain ABC kit; Vector Laboratories, Burlingame, Calif.). Formalin-fixed, paraffin-embedded tissue sections and formalin-fixed, paraffin-embedded pellets of uninfected and *E. chaffeensis*-infected DH82 cells were deparaffinized in xylene and rehydrated in an ethanol series; endogenous peroxidase activity was blocked by incubation with 3% hydrogen peroxide. Sections were pretreated with 0.1% trypsin (Sigma Chemical Company, St. Louis, Mo.) for 4 h, washed in 0.01 M PBS (pH 7.4), and incubated with normal goat serum (Vectastain ABC kit). Goat serum was blotted off before incubation with the primary antibody, human anti-*E. chaffeensis* serum (single donor; titer, 1:1,024) diluted 1:800 with 0.01 M PBS (pH 7.4). Sections were incubated at 4°C overnight, washed in 0.01 M PBS (pH 7.4), and then incubated at 25°C with biotinylated goat anti-human immunoglobulin G (Vectastain ABC kit) for 30 min. After being washed in 0.01 M PBS (pH 7.4), sections were incubated for 50 min at 25°C with avidin-biotin complexes (Vectastain ABC kit) at the suggested dilution but with the addition of 0.21 g of NaCl per 10 ml. The sections were then washed in 0.01 M PBS (pH 7.4), incubated with peroxidase

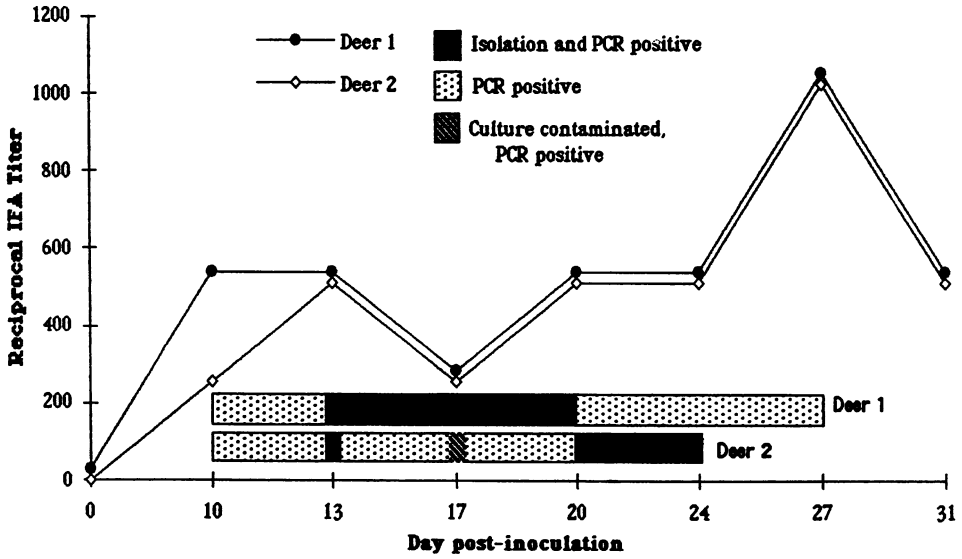

FIG. 1. Nested-PCR and isolation results and reciprocal indirect fluorescent-antibody (IFA) titers for two deer inoculated with *E. chaffeensis*.

substrate solution (Vector VIP; Vector Laboratories) for 8 min at 25°C, washed in tap water, counterstained with Harris hematoxylin, and dehydrated, and a coverslip was applied to each.

RESULTS

Serology. Both deer infected with *E. chaffeensis* seroconverted by day 10 after inoculation (Fig. 1). The peak antibody response (1:1,024) was observed, for both deer, at day 27 postinoculation, and both were positive (1:512) at the end of the 31-day study period. Neither deer inoculated with *E. canis* seroconverted. Both *E. chaffeensis* and *E. canis*, however, were successfully cultured from a fraction of the original inoculum, verifying organism viability. The control deer remained seronegative throughout the study period.

Isolation of organisms. *E. chaffeensis* was isolated from leukocytes from deer 1 on days 13, 17, and 20 postinoculation and from deer 2 on days 13, 20, and 24 (Fig. 1). The culture from deer 2 on day 17 was contaminated. Isolation attempts were negative for the bone marrow and spleen cultures. Cultures from both *E. canis*-inoculated deer and from the control deer were negative at the end of the 60-day retention period.

PCR results. The initial amplification with the outside (ECC and ECB) primers failed to yield a detectable product (data not shown). The nested-PCR results, using the primers specific for *E. chaffeensis*, are shown in Fig. 2. The specific 389-bp product was observed from day 10 to 27 postinoculation in the leukocytes of deer 1 and from day 10 to 24 in the leukocytes of deer 2. While in most cases the amplified product was observed with a 10-μl template volume, on day 27 a specific product was obtained only with the 1-μl template volume (deer 1).

Clinical and hematologic results. Neither *E. chaffeensis*- nor *E. canis*-inoculated deer showed any clinical signs of infection. Values for erythrocyte parameters and platelet counts, as well as levels of urea nitrogen, creatinine, total serum protein, albumin, glucose, sodium, calcium, phosphorus, and aspartate amino transferase, were within normal limits, as determined by our laboratory, for normal deer of similar ages (10). *E. chaffeensis*-infected deer 1 and *E. canis*-inoculated deer 4 each had a mild physiologic leukocytosis, characterized by mature neutrophilia and lymphocytosis, prior to inoculation and on several postinoculation days. No ehrlichial organisms were observed in blood or buffy coat smears but *Theileria* spp. were seen in *E. chaffeensis*-infected deer 1 and in both *E. canis*-inoculated deer, 3 and 4.

Pathology. Gross pathologic findings at necropsy were confined to incidental findings. These consisted of cysticercosis and unilateral hydronephrosis in the control deer and nasal bots (a *Cephenemyia* sp.) in two deer. Microscopically, changes seen in the inoculated deer that were not seen in the control animal consisted of lymphoid hyperplasia in the two deer infected with *E. chaffeensis* and granulomatous lymphadenitis caused by acid-fast bacilli in *E. chaffeensis*-infected deer 2 and in *E. canis*-inoculated deer 3. Expansion of the paracortex and severe sinus histiocytosis were present in all the examined lymph nodes of deer 1 and in the prescapular and mesenteric

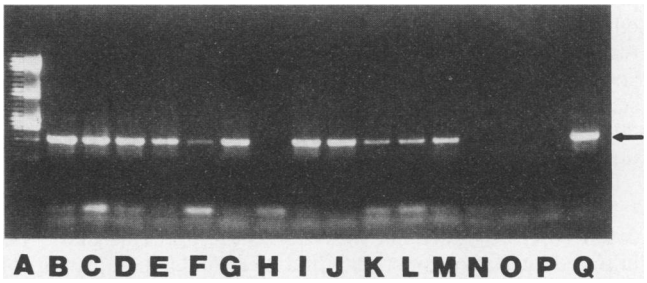

FIG. 2. Primers HE1 and HE3 were used in a nested PCR to detect *E. chaffeensis*-specific DNA targets in experimentally infected blood from deer 1 and 2. A 389-bp product (arrow) was obtained from deer 1 on days 10, 13, 17, 20, 24, and 27 postinoculation (lanes B to G) and from deer 2 on days 10, 13, 17, 20, and 24 (lanes I to M). No specific product was observed for deer 1 on day 31 (lane H) or for deer 2 on day 27 or 31 (lanes N and O). Lane P contains a no-target control, and lane Q contains a positive control consisting of *E. chaffeensis* obtained from tissue culture. Lane A contains pBR322 DNA digested with *Bst*NI as molecular size standards (1,857, 1,058, 929, 383, 121, and 13 bp top to bottom).

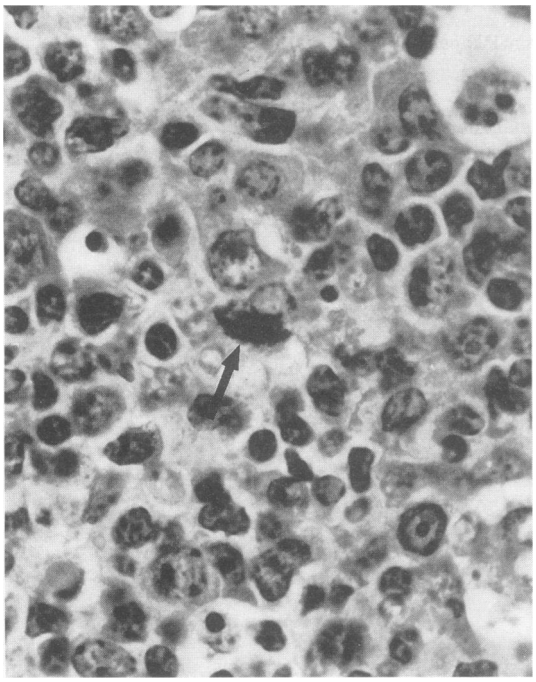

FIG. 3. Photomicrograph of a germinal center in a mesenteric lymph node from a white-tailed deer experimentally infected with *E. chaffeensis*. Note the single dendritic reticulum-like cell (arrow) with granular cytoplasmic immunoreactivity to *E. chaffeensis*. The preparation of sections included incubation with avidin-biotin-peroxidase complexes and Vector VIP substrate and use of the Harris hematoxylin counterstain. Magnification, ×700.

lymph nodes of deer 2. Multifocal granulomatous inflammation with a few intracellular acid-fast bacilli was seen in the retropharyngeal and bronchial lymph nodes of deer 2 and in a retropharyngeal lymph node of deer 3. *Mycobacterium avium* complex organisms were isolated from the lymph nodes of deer 2.

Sinus histiocytes in the prescapular and retropharyngeal lymph nodes of deer 1 contained one to several intracytoplasmic, small spherical structures that were positive for *E. chaffeensis* antigen by the method using the avidin-biotin-peroxidase complex. Immunohistochemical staining of a mesenteric lymph node of deer 2 with antibodies to *E. chaffeensis* revealed that macrophage-type cells in the center of follicles contained similar positively staining structures that formed small clusters similar to morulae (Fig. 3).

DISCUSSION

This study was undertaken to determine if white-tailed deer could support infections with *Ehrlichia* spp. This effort was initiated because prior studies had identified a high proportion of white-tailed deer with antibodies reactive to *E. chaffeensis* (6). Both analyses are necessary to implicate white-tailed deer as reservoirs of *E. chaffeensis* in nature, although our study stopped short of evaluating the infectivity for putative tick vectors such as *A. americanum*.

The reisolation of *E. chaffeensis* from both deer 1 and 2 on three different postinoculation days and the positive PCR results indicated that *E. chaffeensis* circulated in the peripheral blood for at least 2 weeks. This rickettsemia should provide sufficient time for ticks to attach and become infected, if the titer of circulating organisms is sufficiently high.

Although both deer 1 and 2 were infected with *E. chaffeensis*, clinical disease was not apparent. Microscopic findings may have been confounded by concurrent lymph node mycobacteriosis in deer 2 and 3. Paracortical lymphoid hyperplasia, however, was present in the lymph nodes of the two deer inoculated with *E. chaffeensis* and probably was due to antigenic stimulation by that organism. Results of immunohistochemical staining with antibodies to *E. chaffeensis* suggest that the organism may be harbored in macrophage-type cells in the lymph nodes. The cells containing structures that stained positively for the *E. chaffeensis* antigen in the center of lymph node follicles in deer 2 probably were dendritic reticular cells.

In contrast to the deer's responses to infection with *E. chaffeensis*, deer were refractory to infection with *E. canis* and did not seroconvert or develop lymphoid hyperplasia upon exposure. These findings suggest that this organism did not replicate in the deer and that *Ehrlichia*-reactive antibody responses in wild deer are not *E. canis* cross-reactions.

Since serologic data indicate that more than 40% of a deer population can be seropositive for *Ehrlichia*-reactive antibodies, we propose that a substantial fraction of the wild deer population may have circulating organisms at any given time. Several isolates or PCR sequences from field-sampled white-tailed deer will be required to determine if they are naturally infected with *E. chaffeensis* or a closely related ehrlichial species. However, the present work clearly indicated that white-tailed deer can support *E. chaffeensis* infection and remain rickettsemic for periods sufficient to infect tick vectors.

REFERENCES

1. **Anderson, B. E., K. G. Sims, J. G. Olson, J. E. Childs, J. F. Piesman, C. M. Happ, G. O. Maupin, and J. B. Johnson.** 1993. *Amblyomma americanum*: a potential vector of human ehrlichiosis. Am. J. Trop. Med. Hyg. **49**:239–244.
2. **Anderson, B. E., J. W. Sumner, J. E. Dawson, T. Tzianabos, C. R. Greene, J. G. Olson, D. B. Fishbein, M. Olsen-Rasmussen, B. P. Holloway, E. H. George, and A. F. Azad.** 1992. Detection of the etiologic agent of human ehrlichiosis by polymerase chain reaction. J. Clin. Microbiol. **30**:775–780.
3. **Casareale, D., R. Pottahil, and R. Diaco.** 1992. Improved blood sample processing for PCR. PCR Methods Applications **2**:149–153.

3a. **Centers for Disease Control and Prevention.** Unpublished data.

4. **Dawson, J. E., I. Abeygunawardena, C. J. Holland, M. M. Buese, and M. Ristic.** 1988. Susceptibility of cats to infection with *Ehrlichia risticii*, causative agent of equine monocytic ehrlichiosis. Am. J. Vet. Res. **49**:2096–2100.
5. **Dawson, J. E., B. E. Anderson, D. B. Fishbein, J. L. Sanchez, C. S. Goldsmith, K. H. Wilson, and C. W. Duntley.** 1991. Isolation and characterization of an *Ehrlichia* sp. from a patient diagnosed with human ehrlichiosis. J. Clin. Microbiol. **29**:2741–2745.
6. **Dawson, J. E., J. E. Childs, K. L. Biggie, C. Moore, D. Stallknecht, J. Shaddock, J. Bouseman, E. Hofmeister, and J. G. Olson.** 1994. White-tailed deer as a potential reservoir of *Ehrlichia spp.* J. Wildl. Dis. **30**:162–167.
7. **Dawson, J. E., and S. A. Ewing.** 1992. Susceptibility of dogs to infection with *Ehrlichia chaffeensis*, causative agent of human ehrlichiosis. Am. J. Vet. Res. **53**:1322–1327.
8. **Dawson, J. E., Y. Rikihisa, S. A. Ewing, and D. B. Fishbein.** 1991. Serologic diagnosis of human ehrlichiosis using two *Ehrlichia canis* isolates. J. Infect. Dis. **163**:91–95.
9. **Fishbein, D. B., J. E. Dawson, and L. Robinson.** 1994. Human ehrlichiosis in the United States, 1985–1990. Ann. Intern. Med. **120**:736–743.
10. **Howerth, E.** Unpublished data.