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NATURAL COINFECTION OF A WHITE-TAILED DEER (*ODOCOILEUS VIRGINIANUS*) POPULATION WITH THREE *EHRLICHIA* SPP.

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ABSTRACT: The ticks *Amblyomma americanum* and *Ixodes scapularis,* strongly implicated vectors of *Ehrlichia chaffeensis* and the human granulocytic ehrlichiosis (HGE) agent, respectively, commonly are found on white-tailed deer (*Odocoileus virginianus*). As deer can be infected with *E. chaffeensis,* the HGE agent, and another *Ehrlichia*-like organism, a deer population parasitized by both tick species in coastal Georgia was tested for evidence of *Ehrlichia* spp. infection using serologic, molecular, and culture techniques. Antibodies to both *E. chaffeensis* (geometric mean titer = 111) and *Ehrlichia equi,* surrogate antigen for the HGE agent, (geometric mean titer = 1,024) were detected by indirect fluorescent antibody testing. Nested polymerase chain reaction employing species-specific primers demonstrated sequence-confirmed 16S rDNA fragments of 3 distinct *Ehrlichia* spp. in this population: *E. chaffeensis* (1/5), the HGE agent (3/5), and an *Ehrlichia*-like organism previously described from white-tailed deer (5/5). *Ehrlichia chaffeensis* was isolated in culture from the inguinal lymph node of a single deer. An *Ehrlichia*-type morula was identified in a neutrophil of 1 deer on examination of blood smears. This work provides the first evidence of the HGE agent in a nonhuman host in the southeastern United States and documents infection with both *E. chaffeensis* and the HGE agent in a single deer population, thereby supporting the importance of white-tailed deer in the natural history of the human ehrlichioses agents.

Human ehrlichioses are acute febrile tickborne illnesses, which in the United States are caused by either *Ehrlichia chaffeensis* or the human granulocytotropic ehrlichiosis (HGE) agent (Dawson et al., 1991; Goodman et al., 1996). *Ehrlichia chaffeensis,* a monocytotropic ehrlichia that can be transmitted by the lone star tick *Amblyomma americanum* (Anderson et al., 1993), is found predominantly in the southeastern, south-central, and mid-Atlantic United States (Dawson et al., 1990; McDade, 1990). The HGE agent can be transmitted by the black-legged tick *Ixodes scapularis* (Pancholi et al., 1995) and is found predominantly in the northeastern and midwestern United States (Bakken et al., 1994; Chen et al., 1994). Both organisms cause fever, headache, myalgia, nausea, and occasionally death in infected human patients (Eng et al., 1990; Bakken et al., 1994).

White-tailed deer (*Odocoileus virginianus*), which are major hosts for *A. americanum,* have been identified as a reservoir host for *E. chaffeensis* through positive serologic, polymerase chain reaction (PCR), and culture results (Dawson, Childs et al., 1994; Little et al., 1997; Lockhart, Stallknecht, Davidson, Dawson, and Howerth, 1997; Lockhart, Stallknecht, Davidson, Dawson, and Little, 1997). Serologic and PCR evidence indicates that deer, which represent a critical host for *I. scapularis,* are also infected with the HGE agent (Walker and Dumler, 1996; Belongia et al., 1997). Recently, a 16S rDNA sequence characteristic of, but distinct from, all previously described *Ehrlichia* spp. was amplified from blood of white-tailed deer in Georgia and Oklahoma (Dawson et al., 1996). Further evaluation of deer from several locations in the southeastern United States showed that this organism was very prevalent and associated with the presence of both lone star ticks and *E. chaffeensis*-reactive antibodies in deer populations (Little et al., 1997). Although not known to cause human disease, this deer *Ehrlichia*-like organism is of interest because of its potential to confound diagnostic assays. The effect of this organism on serologic tests for *E. chaffeensis* or other species of *Ehrlichia* is unknown; however, PCR primers routinely used to diagnose HGE in humans will amplify 16S rDNA of the deer *Ehrlichia*-like organism (Little et al., 1997).

The goal of the present study was to determine if a white-tailed deer population naturally exposed to both *A. americanum* and *I. scapularis* could be infected with *E. chaffeensis,* the HGE agent, and the deer *Ehrlichia*-like organism, either alone or in combination. Ossabaw Island, Chatham County, Georgia, was chosen as the study site because of the presence of both tick species. A secondary goal was to evaluate the suitability of testing tissues other than blood for evidence of *Ehrlichia* spp. infection because both experimental and field studies had demonstrated PCR-negative blood concurrent with serologic or culture evidence of infection.

MATERIALS AND METHODS

Five deer from Ossabaw Island, a barrier island off the coast of Georgia, were collected by cervical gunshot. Blood samples were obtained via cardiac puncture from each deer after a 95% ethanol drench of the skin. Whole blood was collected into 7-ml EDTA tubes, 10-ml heparinized tubes, and 10-ml clot tubes. Samples of spleen, skeletal muscle, and inguinal, mandibular, and prescapular lymph nodes were excised aseptically from each deer and immediately placed on ice. Each deer was visually examined for ticks. Representative ticks were removed from each deer, stored in 70% ethanol, and submitted to the National Veterinary Services Laboratory (U.S. Department of Agriculture, Ames, Iowa) for identification.

Whole blood collected in vacuum tubes was allowed to clot, centrifuged for 5 min at 25 C, and the serum harvested and held at −20 C. Indirect fluorescent antibody (IFA) testing for *E. chaffeensis* and *Ehrlichia equi* antigen was performed on each of the samples as previously described (Dawson et al., 1990). Sera were screened at a dilution of 1:64 in 0.01 M phosphate-buffered saline (PBS) on spot slides of *E. chaffeensis,* prepared as previously described (Dawson et al., 1990), and

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TABLE I. Summary of polymerase chain reaction (PCR) test results, serology, culture, and tick infestation data on 5 individual white-tailed deer from Ossabaw Island, Chatham County, Georgia (September 1995).

	Ehrlichia chaffeensis			HGE agent		Deer *Ehrlichia*-like organism		Ticks
Deer no.	PCR*	IFA	Culture	PCR†	IFA‡	PCR*	IFA§	present
1	−	1:128	−	−	1:128	+	N/D	*Amblyomma americanum* *Ixodes affinis*
2	+	1:128	+	−	Negative	+	N/D	*A. americanum* *I. affinis*
3	−	1:64	−	+	1:2,048	+	N/D	*A. americanum*
4	−	1:128	−	+	Negative	+	N/D	*A. americanum* *I. affinis*
5	−	1:128	−	+	1:4,096	+	N/D	*A. americanum*

* Summary data from 7 tissues tested.
† Only blood sample tested.
‡ Using *Ehrlichia equi* antigen.
§ N/D = not done, no antigen available.

commercially available *E. equi* slides (Fuller Laboratories, Fullerton, California). A fluorescein isothiocyanate-labeled rabbit anti-deer immunoglobulin G (Kirkegaard and Perry Laboratories Inc., Gaithersburg, Maryland) was used as the conjugate in each test. The conjugate was diluted 1:100 in PBS for the *E. chaffeensis* IFA and 1:50 for the *E. equi* IFA.

White blood cells were isolated from EDTA whole-blood samples as previously described (Little et al., 1997). Cell suspensions were prepared from spleen and lymph node samples by scraping the cut edge of the tissue with a scalpel blade and transferring the tissue to a 1.5-ml microcentrifuge tube containing sterile PBS. The concentration of cells was adjusted to approximately 3,000 cells/ml, and the cell suspensions were frozen at −20 C. Skeletal muscle fibers were teased apart with sterile scalpel blades, suspended in PBS, and held frozen until digested as described below.

Nucleic acid extraction of white blood cells, serum samples, spleen, and lymph node cell suspensions was performed using Instagene purification matrix (Bio-Rad Laboratories, Hercules, California) according to the manufacturer's directions. For extraction of nucleic acid from skeletal muscle, the fibers were incubated at 37 C overnight in a digestion buffer (1% sodium dodecyl sulfate, 50 μg/ml proteinase K). Nucleic acid was purified from the digested muscle by phenol/chloroform extraction followed by ethanol precipitation. The resulting pellet was resuspended in 100 μl of distilled water and stored at −20 C.

PCR testing for species-specific fragments of 16S rDNA of *E. chaffeensis,* the HGE agent, and the deer *Ehrlichia*-like organism was performed using a previously described nested protocol (Dawson, Stallknecht et al., 1994; Little et al., 1997). Briefly, a primary amplification with *Ehrlichia*-wide primers ECB (5′-CGTATTACCGCG GCTGCTGGCA-3′)/ECC (5′-AGAACGAACGCTGGCGGCAAGCC-3′) is followed by 3, individual, species-specific reactions using HE1 (5′-CAATTGCTTATAACCTTTTGGTTATAAAT-3′) and HE3 (5′-TATAGGTACCGTCATTATCTTCCC-TAT-3′) for *E. chaffeensis,* GE9f (5′-AACGGATTATTCTTTATAGCTTGCT-3′) and GA1UR (5′-GAGTTTGCCGGGACTTCTTCT-3′) for the HGE agent, and DGA (5′-TTATCTCTGTAGCTTG-CTACG-3′) and GA1UR for the deer *Ehrlichia*-like organism, according to previously described reaction conditions. The identity of all amplification products generated from blood samples was confirmed by sequencing both strands of individual PCR products. Internal amplification products were purified with a Microcon spin filter (Amicon Inc., Beverley, Massachusetts) and submitted with corresponding primers to the Molecular Genetics Facility at The University of Georgia for sequencing following the Applied Biosystems Inc. protocol for the ABI 373A automated sequencer (Perkin-Elmer, Foster City, California). Resulting sequences were aligned using DNAsis Mac version 2.0 and compared with published sequence data for *E. chaffeensis,* the HGE agent, and the deer *Ehrlichia*-like organism.

Samples were prepared for and maintained in tissue culture as previously described (Dawson, Stallknecht et al., 1994). For culture of lymph nodes and spleen, an approximately 3-mm cube of tissue was excised from the interior of the organ, minced, and crushed with a sterile scalpel blade, and the cells suspended in 1 ml of PBS. Fresh (not frozen) white blood cells isolated from whole blood or from tissue samples were suspended in DH82 growth medium (minimal essential medium with 5% fetal bovine serum) and inoculated into a 25-cm^2 tissue culture flask containing uninfected DH82 cells. Cultures were monitored weekly for evidence of infection by direct fluorescent antibody staining with fluorescein-labeled human anti-*E. chaffeensis* antibody as described by Dawson and Ewing (1992).

Three thin blood smears were prepared from whole blood samples collected from each deer. Smears were allowed to air dry, fixed with methanol, and 1 slide from each deer was stained with Wright's stain, Giemsa stain, or Gimenez stain. Each slide was examined microscopically, in its entirety, at 400× and/or 1,000× oil immersion.

RESULTS

Antibodies to both *E. chaffeensis* (5/5) and *E. equi* (3/5) antigen were detected (Table I). PCR evidence of *E. chaffeensis* was found in 1 of the deer, with both inguinal and prescapular lymph nodes testing positive (Table I; Fig. 1). PCR-positive results for the deer *Ehrlichia*-like organism were obtained for all 5 blood samples (Table I; Fig. 2) but also could be found in 6 of the 7 tissues tested, including spleen (3/5), serum (2/5),

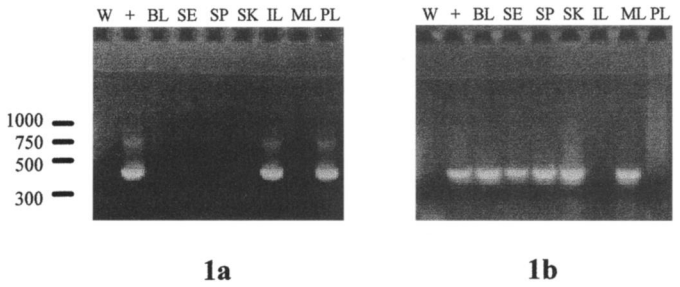

FIGURE 1. Amplification products from blood (BL), serum (SE), spleen (SP), skeletal muscle (SK), and inguinal (IL), mandibular (ML), and prescapular (PL) lymph nodes from WTD no. 2. Products in 1a were amplified with primers HE1/HE3 (*Ehrlichia chaffeensis*) and those in 1b with primers DCH/GA1UR (*Ehrlichia*-like deer organism). Positive (+) control and water (w) or negative control are run with each set of reactions.

skeletal muscle (2/5), inguinal lymph node (2/5), and mandibular lymph node (2/5). Three of the 5 deer were PCR positive for the HGE agent in blood samples (Fig. 2). The sequences of all PCR products from blood samples were identical to their respective target templates upon comparison to published sequence data. However, PCR tests of additional tissues for the HGE agent were not pursued because, although occasionally reliable, as was seen in the blood samples tested here, these primers do not consistently distinguish between the deer *Ehrlichia*-like organism and the HGE agent (Little et al., 1997).

Both *A. americanum* and *Ixodes affinis* were present on the deer at the time of collection (Table I). *Ixodes scapularis* was not identified; however, ectoparasite records collected over the last 20 yr demonstrate this tick is present on deer collected from this area in winter months (W. R. Davidson, unpubl. obs.).

Ehrlichia chaffeensis was isolated from the prescapular lymph node from 1 deer, the same deer that was positive for *E. chaffeensis* by sequence-confirmed diagnostic PCR of both prescapular and inguinal lymph node. Organisms were not present in cultures from any other deer tissues. An *Ehrlichia*-like morula was detected in a neutrophil of WTD no. 3 by microscopic examination of blood smears (Fig. 3).

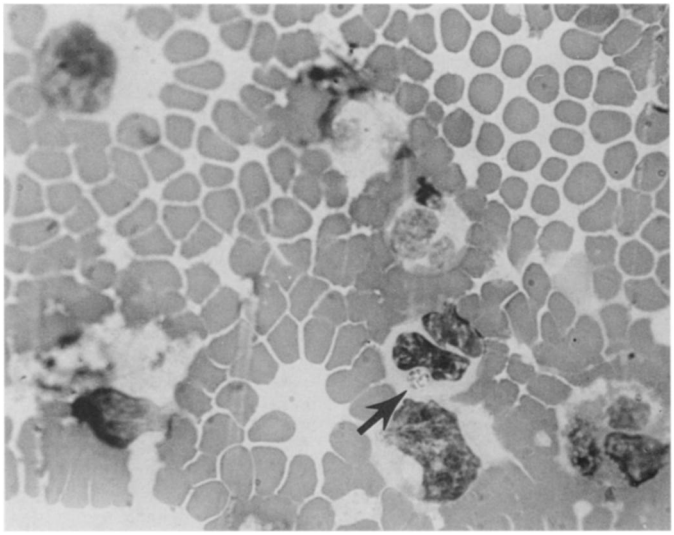

FIGURE 3. Photomicrograph of *Ehrlichia* inclusion (arrow) in neutrophil of a white-tailed deer (WTD no. 3). Giemsa. Bar = 4 μm.

DISCUSSION

Template for PCR tests for *Ehrlichia* spp. is commonly prepared from whole blood (Dawson et al., 1996; Belongia et al., 1997; Little et al., 1997). Our data show that this approach is useful for detecting the HGE agent and the deer *Ehrlichia*-like organism; however, evidence of *E. chaffeensis* infection would have been missed in 1 deer had lymph nodes not been tested as well. In other studies, incorporation of spleen and prescapular lymph node into PCR-based field surveys of deer for *E. chaffeensis* increased prevalence estimates from that of blood alone by 2- and 3-fold (Lockhart, Stallknecht, Davidson, Dawson, and Howerth, 1997; Lockhart, Stallknecht, Davidson, Dawson, and Little, 1997), thus providing a more reliable estimate than testing a single tissue. Further comparisons of PCR test results from multiple tissues of individual deer, as was done in this study, should aid in selecting the most appropriate tissue(s) for use in epidemiologic surveys.

The use of serum as a PCR template proved an effective means of identifying some animals that were infected with the deer *Ehrlichia*-like organism in this study, and archived deer sera from Ossabaw Island dating back to 1982 have tested positive by PCR for the *Ehrlichia*-like deer organism (S. E. Little, unpubl. obs.). HGE-diagnostic amplicons can be generated from human serum samples (Wormser et al., 1995), but this remains to be validated in white-tailed deer. However, PCR tests for *E. chaffeensis* on the serum samples in this study and in several other trials of experimentally infected and field-collect-

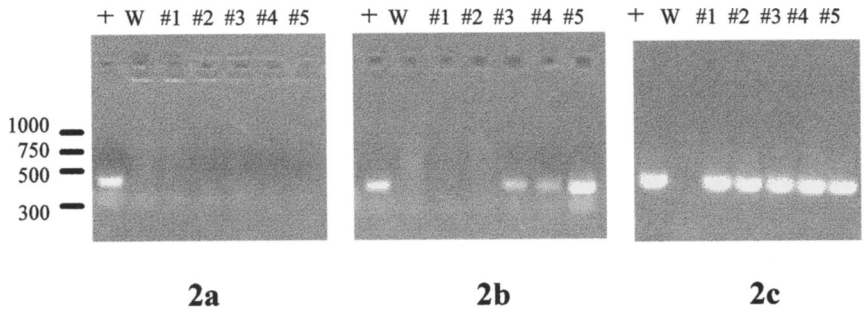

FIGURE 2. Amplification products from blood samples from individual white-tailed deer (WTD nos. 1–5). Products in 2a were amplified with primers HE1/HE3 (*Ehrlichia chaffeensis*), 2b with primers GE9f /GA1UR (*Ehrlichia phagocytophila* genogroup), and 2c with primers DCH/GA1UR (*Ehrlichia*-like deer organism). Positive (+) control and water (w) or negative control are run with each set of reactions.

ed deer are consistently negative (S. E. Little, unpubl. obs.), suggesting serum may not be a useful sample for PCR-based identification of infection with this organism.

The *Ehrlichia*-like morula seen in a neutrophil on examination of a blood smear from one of the deer was not definitively identified. Although this morula may be the granulocytic HGE agent, it could also represent the deer *Ehrlichia*-like organism, the cell preference of which is not yet known, or some other as yet undescribed *Ehrlichia* sp. The deer in which this morula was seen was PCR positive for both the HGE agent and the deer *Ehrlichia*-like organism.

The coinfection of a wild white-tailed deer population with 3 distinct *Ehrlichia* spp., 2 of which are known to cause disease in humans, further underscores the importance of this cosmopolitan vertebrate in the epidemiology of ehrlichial organisms. The widespread occurrence of *E. chaffeensis* and the deer *Ehrlichia*-like organism in deer parasitized by *A. americanum* has been established (Dawson, Childs et al., 1994; Lockhart et al., 1996; Little et al., 1997; Lockhart, Stallknecht, Davidson, Dawson, and Howerth, 1997). Although the role of white-tailed deer in the epidemiology of HGE has not been established, evidence of the HGE agent has been previously reported from deer in areas of the north-central United States where deer are parasitized by *I. scapularis* (Belongia et al., 1997). The present work is the first indication that deer in the southeast are also infected with the HGE agent. Interestingly, *Borrelia burgdorferi,* which also is vectored by *I. scapularis,* has been associated with the barrier island habitats in this region (Oliver et al., 1993).

No single deer in this limited study had direct (PCR or culture) evidence of active infection with all 3 organisms. If serology is considered as indirect evidence of exposure, then 4 of the deer (80%) had active infection with or previous exposure to all 3 organisms. The presence of multiple, distinct *Ehrlichia* spp. within the same wildlife reservoir population can present challenges in the conduct of epidemiologic surveys, particularly in the interpretation of indirect evidence of infection such as serologic status. Clearly, experimental infections in deer are needed to better evaluate the degree of serologic cross reactions between these and other *Ehrlichia* spp. and improved molecular diagnostic techniques required to accurately determine the infection status of vector and reservoir hosts with the human ehrlichiosis agents. However, detection of both agents associated with human ehrlichiosis, as well as the *Ehrlichia*-like organism, from a single population suggests that white-tailed deer may prove to be the ideal sentinel species for determining the geographic distribution of these organisms.

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