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NATURAL HISTORY OF *EHRLICHIA CHAFFEENSIS* (RICKETTSIALES: EHRLICHIEAE) IN THE PIEDMONT PHYSIOGRAPHIC PROVINCE OF GEORGIA

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ABSTRACT: The roles of wild mammals and ticks in the epidemiology of *Ehrlichia chaffeensis* at a suspected endemic site were investigated using serologic testing, culture, and polymerase chain reaction (PCR) supported by restriction endonuclease analysis and DNA sequencing. Antibodies reactive to *E. chaffeensis* (≥1:64) were detected in 92% of white-tailed deer (*Odocoileus virginianus*), 21% of raccoons (*Procyon lotor*), and 8% of opossums (*Didelphis virginianus*), but not in 8 other species of mammals. Of 7 species of ticks found by host and environmental sampling, *Amblyomma americanum* was the dominant species, accounting for greater than 99% of all ticks collected. Deer, raccoons, and opossums were the only species parasitized by all life stages of *A. americanum,* and *A. americanum* was the only tick parasitizing deer. A nested PCR protocol incorporating *E. chaffeensis*-specific primers detected *E. chaffeensis* DNA in blood, lymph nodes, or spleen from 54% of deer examined. The nested PCR detected *E. chaffeensis* DNA in 6 of 50 (12%) individual adult *A. americanum* collected from the environment, in 14 of 79 (18%) pools representing 402 adult *A. americanum* collected from the environment, and in 7 of 25 (28%) pools of mixed stages of *A. americanum* collected from deer. Although no *Ehrlichia* spp. were isolated in culture, sequencing of representative amplicons from deer and ticks confirmed PCR products as *E. chaffeensis.* These data provide strong evidence that white-tailed deer and lone star ticks are the primary reservoir and vector of *E. chaffeensis,* respectively. The same PCR protocol, incorporating primers specific for an *Ehrlichia*-like organism of white-tailed deer, detected this organism in blood, lymph nodes, or spleen from 96% of these deer. The *Ehrlichia*-like organism of deer was detected by PCR from 0 of 50 individual ticks, 7 of 79 (9%) pools, and 1 of 25 (4%) pools of *A. americanum* collected from deer. Sequencing of representative amplicons from deer and ticks confirmed PCR products as *Ehrlichia*-like organism of deer. These data suggest that the *Ehrlichia*-like organism of deer is present in both the deer and lone star ticks populations at this location.

Human ehrlichiosis in the United States is caused by 2 rickettsial pathogens in the genus *Ehrlichia.* Human monocytic ehrlichiosis (HME), caused by *Ehrlichia chaffeensis,* was first recognized in 1986 (Maeda et al., 1987). More than 400 cases of *E. chaffeensis* infection, with 12 fatalities, have been reported from 30 states. Most cases have been reported from the southeastern, mid-Atlantic, and south-central regions of the United States (Eng et al., 1990, Dawson, Childs et al., 1994). Human granulocytic ehrlichiosis (HGE) is caused by an unnamed *Ehrlichia* sp. that was recently isolated from 3 patients (Goodman et al., 1996). Approximately 170 cases of HGE, including 4 fatalities, have been reported. Human granulocytic ehrlichiosis cases are reported primarily from the northeast and upper midwest (Walker and Dumler, 1996).

White-tailed deer (*Odocoileus virginianus*) were initially implicated as a potential reservoir of *E. chaffeensis* based on a high prevalence of *E. chaffeensis*-reactive antibodies detected in deer sampled throughout the southeastern United States (Dawson, Childs et al., 1994; Lockhart et al., 1995). Subsequent experimental infections demonstrated that white-tailed deer are competent hosts for *E. chaffeensis* (Dawson, Stallknecht et al., 1994), and DNA corresponding to *E. chaffeensis* has been detected in field-collected deer tissue by polymerase chain reaction (PCR) (Little et al., 1997).

The lone star tick, *Amblyomma americanum,* is the suspected vector of *E. chaffeensis.* This suspicion is based on a geographic coincidence between cases of human ehrlichiosis and the distribution of *A. americanum* (Eng et al., 1990), temporal and geographic associations between the presence of *A. americanum,* and antibodies reactive to *E. chaffeensis* in white-tailed deer (Lockhart et al., 1995, 1996), the presence in *A. americanum* of DNA corresponding to *E. chaffeensis* (Anderson et al., 1992, 1993), and experimental transstadial transmission of *E. chaffeensis* by *A. americanum* (Ewing et al., 1995).

Recently, PCR was used to amplify a unique *Ehrlichia*-like 16S rRNA gene fragment from white blood cells from white-tailed deer in Georgia and Oklahoma (Dawson et al., 1996). Comparative analysis of this sequence with sequences of all other described *Ehrlichia* suggest that this fragment is from a previously undescribed organism, and it appears to be phylogenetically related to *Ehrlichia platys, Ehrlichia equi, Ehrlichia phagocytophila,* and the HGE agent (Dawson et al., 1996). The *Ehrlichia*-like organism of white-tailed deer appears to be widespread and prevalent among deer populations (Dawson et al., 1996; Little et al., 1997), but the organism has not been cultured. Serologic cross-reaction is known to occur among *Ehrlichia* spp. (Nyindo et al., 1991; Shankarappa et al., 1992), and it is possibile that previous serosurveys of deer for *E. chaffeensis*-reactive antibodies may have actually detected antibodies to other ehrlichiae, including the *Ehrlichia*-like organism of white-tailed deer.

Past studies on the epidemiology of *E. chaffeensis* have focused on individual vector and host components, and most wild mammal serum samples tested were from existing research projects being conducted for other purposes. Although providing clues to potential mammalian reservoir hosts and vectors, these studies have not been geographically or temporally synchronized to evaluate various species as potential reservoirs. In order to better determine the epidemiology of *E. chaffeensis,* we selected a study site where there was a high prevalence of *E. chaffeensis*-reactive antibodies among white-tailed deer in an effort to evaluate the entire mammal and tick communities.

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MATERIALS AND METHODS

Site description

Whitehall Experimental Forest (WHEF), Clarke County, Georgia (33°54′N, 83°22′W) is a 325-ha property owned by the D. B. Warnell School of Forest Resources, The University of Georgia, Athens, Georgia, and is located in the Piedmont physiographic province (Fenneman, 1946) at the confluence of the North Oconee and Middle Oconee Rivers. The property has a diversity of habitat types including riparian hardwood forests, pine and mixed pine–hardwood upland forests, old fields, and pastures, all of which include a history of human disturbance. Past studies from this location (Lockhart et al., 1995; Dawson et al., 1996) indicated a high prevalence of *E. chaffeensis*-reactive antibodies in the white-tailed deer population.

Animal procedures

Mammals representing most major terrestrial species present at WHEF were collected for serologic testing for antibodies reactive to *E. chaffeensis*. Small rodents were collected using Sherman traps (Sherman Traps, Inc., Tallahassee, Florida) baited with corn, peanut butter, or birdseed. Larger mammals (raccoons [*Procyon lotor*] and opossums [*Didelphis virginianus*]) were trapped using Tomahawk live traps (Tomahawk Live Trap Company, Tomahawk, Wisconsin) baited with cat food or sardines. White-tailed deer, Eastern gray squirrels (*Sciurus carolinensis*), and rabbits (*Sylvilagus floridanus*) were collected by shooting.

Live-trapped animals were anesthetized with ketamine/xylazine (5:1 ratio, 1.2 ml/4.5 kg body weight) prior to bleeding. Blood was collected either retro-orbitally (for rodents) or through cardiac puncture and was allowed to clot. Serum was decanted and stored at −20 C until use. A limited number of existing serum samples from gray squirrels (6 collected in 1992), opossums (2 collected in 1992, 1 collected in 1993), and raccoons (14 collected in 1992, 10 collected in 1993) were available from previous studies and were included in the serologic portion of this study.

Tick sampling

Each animal collected was examined visually for ticks. Samples of ticks collected from white-tailed deer were pooled and frozen in phosphate-buffered saline (PBS) for PCR testing. Representative specimens of ticks were stored in 70% ethanol and submitted to the National Veterinary Services Laboratories (NVSL, U.S. Department of Agriculture, Ames, Iowa) for identification and archiving (representative accession numbers: 93-36292, 93-39912, 94-30609, 94-35383–35388, 94-36879, 95-2934, 95-5461, 95-10694, 95-13955, and 96-2487). Skins were removed from all animals except white-tailed deer and stored frozen for future digestion. Animal skins were digested using 10% potassium hydroxide (Beer and Cook, 1954); the digestion products were washed with water in a 100-mesh screen; and retained digestion products were examined at 7–30× for ectoparasites. All ticks were counted and identified. Representative specimens of ticks were stored in 70% ethanol and submitted to NVSL for confirmation of identifications.

An environmental survey to determine species composition and relative abundance of ticks at WHEF was conducted by sampling 6 sites within 2-wk periods during May and September 1993; March, May, and September 1994; and March, May, July, and September 1995. Carbon dioxide-baited (dry ice) cardboard panels, with masking tape applied to the perimeters, were used to collect adult and nymph ticks (Koch and McNew, 1981) and cloth drags were used to capture all life stages, particularly larvae (Gladney, 1978). Fifteen carbon dioxide traps were set and 30, 25-m cloth drags were performed at each of the 6 sites during each sampling period. All ticks collected were identified and counted. Samples of ticks were retained and processed for PCR analysis as described below. Representative samples of ticks collected were submitted to NVSL for confirmation of identifications.

Serology

A human *E. chaffeensis* isolate (Arkansas strain) obtained from the Centers for Disease Control and Prevention (CDC) in Atlanta, Georgia was grown in DH82-canine macrophage cells and was used as antigen for the indirect immunofluorescent antibody (IFA) test. The IFA test was accomplished as described by Dawson et al. (1991). Serum samples were tested at serial dilutions beginning at 1:64 in 0.01 M PBS. Commercial antibody conjugates used in this study were obtained from Kirkegaard & Perry Laboratories Inc., Gaithersburg, Maryland, and included fluorescein isothiocyanate (FITC)-labeled rabbit anti-deer immunoglobulin G (IgG) used for white-tailed deer; FITC-labeled goat anti-raccoon IgG used for raccoons; FITC-labeled goat anti-rat IgG used for squirrels, chipmunks, and cotton rats; FITC-labeled goat anti-mouse IgG used for house mice, golden mice, and Eastern harvest mice; and FITC-labeled goat anti-*Peromyscus leucopus* IgG for white-footed mice. FITC-labeled rabbit anti-opossum IgG was obtained from the CDC, Atlanta, Georgia. All conjugates were used at a 1:100 dilution in 0.01 M PBS (pH 7.2). Positive results were reported as the reciprocal of the highest dilution at which specific binding was observed.

Positive control serum for the deer IFA test was obtained from an experimentally infected deer with a titer of 1:512. Negative control serum was obtained from a hunter-killed fawn from Lake Russell Wildlife Management Area, Stephens County, Georgia, an area where *E. chaffeensis* antibodies in white-tailed deer have not been detected. Positive control serum samples for raccoons, opossums, and mice were obtained from the CDC.

Culture procedures

Isolation of *E. chaffeensis* from white-tailed deer was attempted as described by Dawson, Stallknecht et al. (1994). Approximately 10 ml of whole blood collected in ethylenediaminetetraacetic acid (EDTA) was mixed 1:3 with ACE lysing buffer (150 mM NH_4Cl, 0.7 mM KH_2PO_4, and 3 mM EDTA-Na_2) and incubated at room temperature for 5 min with frequent inversion. The suspension was centrifuged at 1,500 *g* for 5 min to concentrate leukocytes, and the supernatant was discarded. This procedure was repeated 2 more times as a wash. Uninfected DH82 canine macrophage cells from a 25-cm^2 flask were suspended in 5 ml of media (MEM with 5% fetal bovine serum) and were added to the pellet. The entire suspension was then put back into the flask. Twice weekly, the supernatant from the culture flask was decanted and 5 ml of media was added.

Cells were harvested from deer prescapular lymph nodes and processed in a similar manner. A small section was removed aseptically from isolated tissue, crushed with the edge of a scalpel blade, and mixed with sterile PBS. Erythrocytes were lysed with ACE lysing buffer and cultures were established as described above.

Starting 30 days after culture, inoculated DH82 cells were examined twice weekly for evidence of infection using direct fluorescent antibody (FA) staining as previously described (Dawson and Ewing, 1992). Culture supernatant was collected and centrifuged at 1,500 *g* for 5 min. Supernatant was decanted and the cells were resuspended in 1 ml of fresh media. Approximately 0.1 ml of the suspension was cytocentrifuged (Shandon Upshaw, Pittsburgh, Pennsylvania) onto glass slides. Slides were allowed to air dry for 1 hr, fixed in acetone for 15 min, and dried for 30 min. Conjugate for the direct FA consisted of a 1:50 dilution of FITC-labeled human IgG obtained from a patient diagnosed with *E. chaffeensis* infection.

PCR

Isolated peripheral blood leukocytes, lymph node cells, and spleen cells collected from deer as described above were concentrated by centrifugation and resuspended in 0.5 ml PBS (pH 7.2). DNA was extracted from cell samples using InstaGene™ Purification Matrix (Bio-Rad, Hercules, California) according to manufacturer's directions. Nested PCR was performed as described by Dawson, Stallknecht et al. (1994). For the outside amplification, a 100-µl reaction mixture containing 10 µl of DNA template, 10 mM Tris-Cl (pH 8.3), 50 mM KCl, 1.4 mM $MgCl_2$, 0.2 mM each deoxynucleoside triphosphate, 5 µM tetramethylammonium chloride, 2.5 U of *Taq* DNA polymerase (Promega, Madison, Wisconsin), and 2 µM of primers ECB (5′-CGTATTACCGCGGCTGCTGGCA-3′) and ECC (5′-AGAACGAACGCTGGCGGCAAGCC-3′) was assembled and overlain with mineral oil. 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 sec at 72 C, with 1 sec added to each successive 72 C extension step.

For the nested PCR, 1 µl of each outside reaction was amplified in a second 100-µl reaction mixture assembled as described above except using *E. chaffeensis*-specific primers HE1 (5′-CAATTGCTTATAACCTTTT-CCTTATAAAT-3′) and HE3 (5′-TATAGGTACCGTCATTATCTTCCCT-

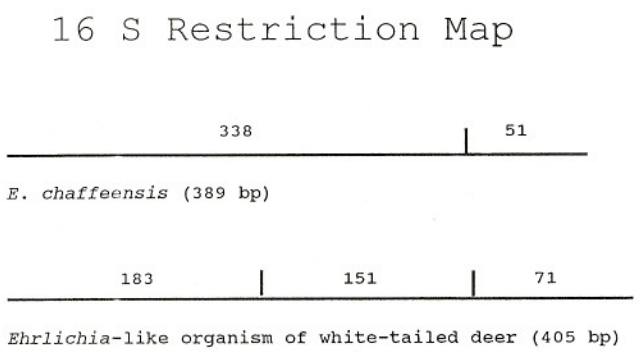

FIGURE 1. Map of *Hae*III restriction sites on the 16S rDNA fragment of *Ehrlichia chaffeensis* amplified by primers HE1 and HE3 and of the *Ehrlichia*-like organism of white-tailed deer amplified by primers DGA and GA1UR.

AT-3′). The following temperature profile was run for 40 cycles: 1 min at 94 C, 2 min at 55 C, and 15 sec at 72 C with 1 sec added to each successive 72 C extension step. A replicate set of nested reactions for the newly recognized *Ehrlichia*-like organism of white-tailed deer (Little et al., 1997) was performed using primers DGA (5′-TTATCTCTGTAGCTTGCTACG-3′) and GA1UR (5′-GAGTTTGCCGGGACTTCTTCT-3′). The reaction was run for 30 cycles of 1 min at 94 C, 2 min at 55 C, and 2 min at 72 C. All products from the nested reactions were analyzed by electrophoresis in 1.5% agarose, stained with ethidium bromide, and visualized by ultraviolet transillumination.

Samples of adult *A. americanum* collected from the environment were evaluated by PCR for the presence of *E. chaffeensis* or the white-tailed deer *Ehrlichia*-like organism in pool sizes of 1–10. Single adults (N = 50) and 79 pools of 4–10 adults were placed in individual sterile Whirl-pak bags with 0.5 ml of sterile PBS (pH 7.2) and were pulverized by striking with a hammer (Anderson et al., 1993). The tick internal contents and PBS were pipetted from the bag, taking care to leave exoskeleton parts, and were retained frozen until PCR could be performed. Twenty-five mixed pools of engorged and partially engorged nymph and adult *A. americanum* collected from white-tailed deer were prepared in the same manner. DNA isolation was performed using InstaGene™ Purification Matrix as described above, and the resulting DNA templates used in nested PCRs were as described above.

Identification of PCR products

Restriction endonuclease digestion using *Hae*III (Promega, Madison, Wisconsin) was performed on representative amplicons to confirm their identity. *Hae*III was selected because predicted restriction sites would distinguish between the *E. chaffeensis* product and the *Ehrlichia*-like organism of white-tailed deer product (Fig. 1). Five microliters of the PCR product was directly digested from the nested reaction products when a strong band was present. When bands were weak, the rest of the remaining PCR reaction product was ethanol precipitated to concentrate DNA prior to restriction analysis. The PCR reactions were digested with *Hae*III for 75 min at 37 C and digestion products were resolved on a 3% agarose gel stained with ethidium bromide. The fragment sizes were determined by comparison with PCR marker DNA (Promega Corp., Madison, Wisconsin). Products tested by restriction endonuclease digestion included 20 with detectable 389-basepair *E. chaffeensis* products; 10 from white-tailed deer tissue (blood, lymph node, and spleen) and 10 from adult *A. americanum* (5 from environmental pools, 3 from environmental individual ticks, and 2 from pools collected from deer). Products from 17 reactions positive for the 405-basepair *Ehrlichia*-like organism of deer were also analyzed by *Hae*III restriction enzyme analysis, including 10 from deer tissues (blood, lymph node, and spleen) and 7 from adult *A. americanum* (all from environmentally collected pools).

Both strands of DNA generated by PCR amplification were sequenced for 8 samples: 2 samples positive for *E. chaffeensis* from white-tailed deer, 2 positive for *E. chaffeensis* from ticks, 2 positive for the *Ehrlichia*-like organism of deer from white-tailed deer, and 2 positive for the *Ehrlichia*-like organism of deer from ticks. Amplicons were purified with a Microcon™ spin filter (Amicon Inc., Beverly, Massachusetts) and submitted with corresponding PCR amplification primers to the Molecular Genetics Instrumentation Facility at The University of Georgia (Athens, Georgia) for sequencing following the Applied Biosystems Inc. protocol for the ABI 373A automated sequencer. Resulting sequences were aligned using DNAsis Mac V. 2.0 and compared to published sequence data for *E. chaffeensis* and the white-tailed deer organism.

TABLE I. Prevalence of antibodies reactive to *Ehrlichia chaffeensis* in seropositive animal species collected from Whitehall Experimental Forest, Clarke County, Georgia, 1993–1995.

Species	N	Prevalence (%)	GMT*	Titer range
White-tailed deer	94	92	172.5	64–2,048
Raccoon	43	21	128	64–512
Opossum	38	8	128	64–512

* Geometric mean titer of seropositive animals.

Statistical analysis

Chi-square analyses using Yates correction factor were performed using Epi Info, V. 6 software (Dean et al., 1994) to compare antibody prevalence among seropositive species.

Pools of ticks that were PCR positive were assumed to contain at least 1 positive tick, and a minimum infection frequency (MIF) was calculated for pools of environmentally collected ticks.

RESULTS

Mammals

Eleven species of mammals including 94 white-tailed deer, 43 raccoons (24 from previous studies), 38 opossums (3 from previous studies), 38 white-footed mice (*P. leucopus*), 20 cotton rats (*Sigmodon hispidus*), 10 Eastern gray squirrels (6 from previous studies), 8 house mice (*Mus musculus*), 4 Eastern cottontail rabbits, 4 Eastern harvest mice (*Reithrodontomys humulis*), 4 Golden mice (*Ochrotomys nuttalli*), and 3 Eastern chipmunks (*Tamias striatus*) were examined for ticks and serologically tested. Of these, white-tailed deer had the highest prevalence of antibodies reactive to *E. chaffeensis,* followed by raccoons and opossums (Table I). All other mammal species were negative for antibodies reactive to *E. chaffeensis.* The prevalence of antibodies reactive to *E. chaffeensis* was significantly higher in white-tailed deer than in either raccoons or opossums (χ^2 = 65.82, $P > 0.01$, χ^2 = 82.33, $P > 0.01$, respectively). There was no significant difference in the prevalence of antibodies between raccoons and opossums (χ^2 = 1.78, $P > 0.18$).

Cultures were attempted on lymph node cells and peripheral blood leukocytes from 26 deer. No isolates of *E. chaffeensis* or the *Ehrlichia*-like organism of deer were obtained. Of the 52 cultures, 20 were carried out to 60 days. Because of contamination by other organisms (bacteria, fungi, and trypanosomes), 1 culture was discontinued at 1 wk, 1 at 2 wk, 7 at 3 wk, 7 at 4 wk, 5 at 5 wk, 4 at 6 wk, and 7 at 7 wk. All 242 direct FA tests were negative.

Seventy-two samples from 28 white-tailed deer were tested by PCR for the presence of *E. chaffeensis* and the *Ehrlichia*-like organism of white-tailed deer (Table II). PCR testing disclosed a higher prevalence of the *Ehrlichia*-like organism of deer than *E. chaffeensis* and apparent differences in tissue tro-

TABLE II. Prevalence of 16S rDNA fragments of *Ehrlichia chaffeensis* and *Ehrlichia*-like organism of deer in tissues collected from white-tailed deer from Whitehall Experimental Forest, Clarke County, Georgia, 1993–1995.

Tissue	N*	PCR product of *Ehrlichia chaffeensis* (%)	PCR product of *Ehrlichia*-like organism of deer (%)
Peripheral blood leukocytes	22	18	86
Lymph nodes	28	32	36
Spleen	22	41	91
At least one tissue	28	54	96

* Number of deer examined.

pism between these organisms. Representative PCR results are depicted in Figure 2.

Results of the restriction enzyme analysis of *E. chaffeensis* and the *Ehrlichia*-like organism of deer using *Hae*III are shown in Figure 2. All of the predicted fragments for both *E. chaffeensis* and the *Ehrlichia*-like organism of white-tailed deer were present in the positive controls. Restriction analysis of representative *E. chaffeensis* PCR products obtained from deer also gave predicted fragment patterns indicating that the products generated by PCR in all of these samples were identical to those generated by digestion of known-positive *E. chaffeensis* rDNA fragments (Fig. 2). Restriction analysis of all *Ehrlichia*-like organisms of deer PCR products obtained from deer tissue resulted in the predicted fragment patterns for that sequence (Fig. 2). Sequencing results (data not shown) for representative PCR products obtained from deer using HE1/HE3 or DGA/GA1UR demonstrated identical sequences to those published for *E. chaffeensis* (Anderson et al., 1991) or the *Ehrlichia*-like organism of deer (Dawson et al., 1996).

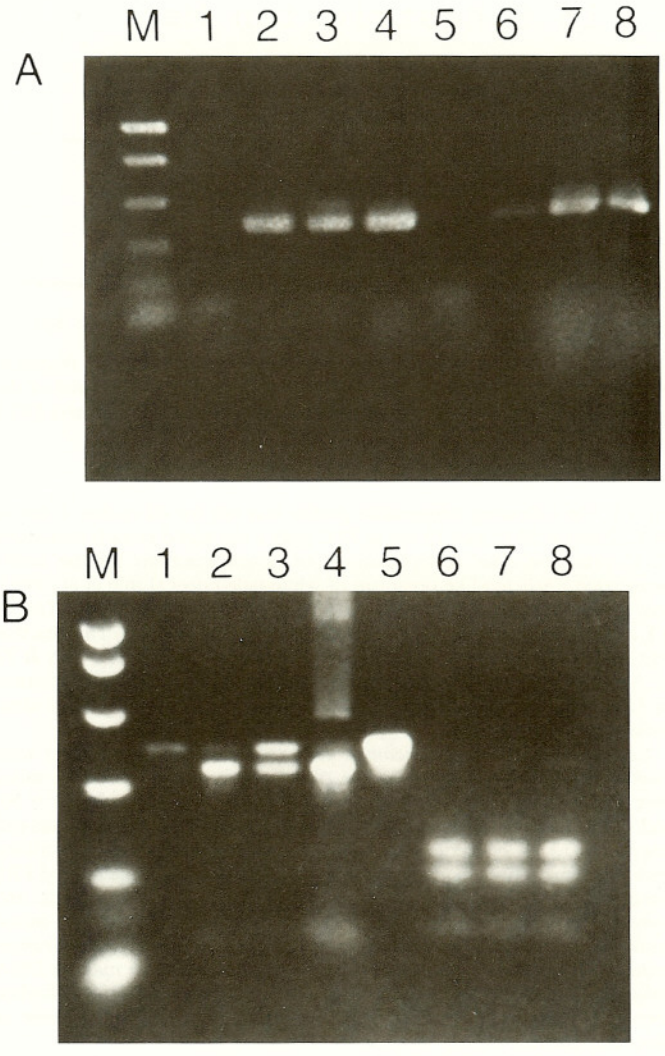

FIGURE 2. A nested PCR/restriction enzyme combination was used to detect and differentiate DNA targets from naturally infected white-tailed deer and adult lone star ticks. **A.** Polymerase chain reaction results using HE1/HE3 and DGA/GA1UR primer pairs. Lanes 1–4, representative reactions for *E. chaffeensis,* 1—negative control, 2—positive control, 3—white-tailed deer #D2-53 peripheral blood leukocytes, 4—lone star tick (pool #T34) preparation. Lanes 5–8, representative reactions for *Ehrlichia*-like agent of white-tailed deer, 5—negative control, 6—positive control, 7—white-tailed deer #D2-35 spleen, 8—lone star tick (pool #T91) preparation. **B.** Restriction enzyme analysis using *Hae*III. Lanes 1–4, representative reactions for *E. chaffeensis,* 1—positive control (undigested), 2—positive control (digested), 3—white-tailed deer #D2-53 peripheral blood leukocytes (top band represents residual undigested product), 4—lone star tick (pool #T34) preparation. Lanes 5–8, representative reactions for *Ehrlichia*-like agent of white-tailed deer, 5—positive control (undigested), 6—positive control (digested), 7—white-tailed deer #D2-35 spleen, 8—lone star tick (pool #T91) preparation. M = Molecular weight markers of 1,000, 750, 500, 300, 150, and 50 bp (Promega, Madison, Wisconsin).

Ticks

Seven species of ticks were collected from the 11 species of hosts during the study period (Table III). *Amblyomma americanum* and *Dermacentor variabilis* had the broadest host diversity and were present on 6 and 4 different host species, respectively. In contrast, *Haemaphysalis leporispalustris, Ixodes scapularis, Ixodes texanus,* and *Ixodes cookei* each occurred on only 1 host species, and *Ixodes dentatus* occurred on 2 host species.

Environmental monitoring at WHEF from 1993 to 1995 disclosed an abundance of ticks. *Amblyomma americanum* was the dominant tick species collected, comprising >99.9% of the 52,924 ticks recovered. A total of 786 carbon dioxide trapping sessions and 1,530 25-m cloth drags produced overall mean capture rates for *A. americanum* of 6.5 adults, 6.2 nymphs, and 0.6 larvae per hr of CO_2 sampling and 0.25 adults, 1.7 nymphs, and 25.8 larvae per 25-m drag. The only other ticks collected by environmental sampling were 8 *Dermacentor variabilis* and 4 *Ixodes* sp.

A total of 129 samples representing 452 adult *A. americanum* ticks collected from the environment were evaluated by PCR for the presence of *E. chaffeensis* or the *Ehrlichia*-like agent of white-tailed deer (Table IV). The prevalence of *E. chaffeensis* DNA detected by PCR was much higher among ticks tested individually than among pools of ticks. Representative PCR reaction products are presented in Figure 2.

Representative results of restriction analysis of PCR products generated from amplification of tick material are shown in Figure 2. Restriction analysis of 10 *E. chaffeensis* PCR products

TABLE III. Ticks recovered from mammals collected at Whitehall Experimental Forest, Clarke County, Georgia, 1993–1995.

	Animal species						
	White-tailed deer	Raccoon	Opossum	Gray squirrel	Cottontail rabbit	Cotton rat	White-footed mouse
Number examined*	44	19	35	4	4	20	38
Tick species							
Amblyomma americanum	L, N, A† (68)‡	L, N, A (74)	L, N, A (60)	L, N (25)	L, N (25)	L (5)	—
Dermacentor variabilis	—	A (53)	L, N, A (49)	—	—	N (15)	L, N (13)
Haemaphysalis leporispalustris	—	—	—	—	L, N, A (75)	—	—
Ixodes scapularis	—	—	—	—	—	L (5)	—
I. texanus	—	L, N, A (42)	—	—	—	—	—
I. cookei	—	L, N, A (32)	—	—	—	—	—
I. dentatus	—	—	L, N (11)	—	L, N, A (50)	—	—

* No ticks were collected from 8 house mice, three Eastern chipmunks, four Eastern harvest mice, or four golden mice.
† L = larvae, N = nymphs, A = adults.
‡ Prevalence of infestation for the combination of all life stages.

obtained from adult ticks generated fragment patterns compatible with *E. chaffeensis*. Restriction analysis of the *Ehrlichia*-like organism of deer PCR products obtained from adult ticks produced predicted fragment patterns for 1 of 7 PCR products. The other 6 products were lost in processing and multiple attempts to reamplify from original template material and from original outside reactions were unsuccessful. Sequences of representative PCR products amplified by HE1/HE3 or DGA/GA1UR from ticks were identical to those published for *E. chaffeensis* or the *Ehrlichia*-like organism of deer, respectively.

TABLE IV. Polymerase chain reactions on *Amblyomma americanum* collected from the environment and from white-tailed deer at Whitehall Experimental Forest, Clarke County, Georgia.*

			Ehrlichia chaffeensis		*Ehrlichia*-like organism of deer	
Sample origin and type	N	Pools	Positive pools	MIF† (%)	Positive pools	MIF (%)
Environment						
Individual adults	50	—	6	12.0	0	0.0
Pooled adults	402	79	14	3.5	7	1.7
Deer						
Mixed stages‡	—	25	7	—	1	—

* *Ehrlichia chaffeensis* reactions were performed using primers HE1 and HE3 *Ehrlichia*-like organism of deer reactions were performed using primers DGA and GA1UR.
† MIF = minimum infection frequency.
‡ Mixed stages consisted of engorged and partially engorged nymph and adult ticks.

DISCUSSION

This study is the first geographically and temporally synchronized survey of a wild mammalian community for serologic evidence of exposure to *E. chaffeensis* and, therefore, allows comparison of the relative frequency of exposure among potential host species. Previous serologic surveys of wildlife have disclosed *E. chaffeensis*-reactive antibodies among white-tailed deer (Dawson, Childs et al., 1994; Dawson et al., 1996; Lockhart et al., 1996), raccoons, red foxes (*Vulpes vulpes*), gray foxes (*Urocyon cinereoargenteus*), and rabbits (J. E. Dawson, unpubl. obs.), but not in several species of rodents (J. E. Dawson, unpubl. obs.; Lockhart, 1996). However, the specimens tested in these previous surveys were obtained fortuitously at different locations and times, including sites with no evidence to indicate that *E. chaffeensis* was present. Although the high prevalence and wide geographic distribution of *E. chaffeensis*-reactive antibodies among white-tailed deer in these surveys have suggested that deer may play an important role in the epidemiology of *E. chaffeensis,* the significance of antibodies among other wild mammals and the relative importance of these species as potential hosts for *E. chaffeensis* have been unclear. Our serotesting of the mammalian community at WHEF disclosed that only 3 species, principally deer and, to a lesser extent, raccoons and opossums, potentially had been infected with *E. chaffeensis.* These findings substantiate the hypothesis that deer may serve as an important reservoir host (Dawson, Childs et al. 1994; Lockhart et al., 1995, 1996), but also indicate that certain other species should not be excluded as potential hosts, although they appear to be less important than deer. Because serologic cross-reactions are known to occur frequently among *Ehrlichia* spp. (Nyindo et al., 1991; Shankarappa et al., 1992; Dawson, Childs et al., 1994; Dawson, Stallknecht et al., 1994),

antibodies can only be taken as presumptive evidence of infection.

The detection of a 16S rDNA PCR product corresponding to that of the type strain of *E. chaffeensis* (Arkansas isolate) (Anderson et al., 1991) from over half of the deer tested provides strong evidence supporting the concept that white-tailed deer are an important vertebrate reservoir for this organism. The presence of *E. chaffeensis* DNA in multiple tissues suggests a disseminated infection, and its detection in the blood of approximately 1 of every 5 deer suggests that a substantial portion of the deer population was potentially rickettsemic. Previous experimental inoculations of white-tailed deer with *E. chaffeensis* have confirmed that deer are susceptible to infection and circulate viable organisms in peripheral blood for at least 2 wk (Dawson, Stallknecht et al., 1994; Ewing et al., 1995). Experimentally infected deer served as a source of infection for *A. americanum* larvae and nymphs that fed to repletion within a 7-day period beginning 11 days after the deer were needle-inoculated (Ewing et al., 1995). Although these experimental studies may not be representative of naturally obtained *E. chaffeensis* infection among wild deer, a rickettsemia of 2 wk duration would appear to provide sufficient time for immature ticks to feed and become infected. This may be especially true in areas such as WHEF, which support dense populations of both deer and ticks.

The failure to isolate *E. chaffeensis* in tissue culture is problematic because the possibility cannot be excluded that the *E. chaffeensis* DNA detected by PCR was from nonviable organisms. However, our inability to isolate *E. chaffeensis* was not unexpected because many ehrlichiae, including *E. chaffeensis,* are difficult to culture (Walker and Dumler, 1996). Furthermore, although the DH82 cell culture system has been successfully applied with human cases (Dawson et al., 1991; Dumler et al., 1995) and with experimentally infected deer (Dawson, Stallknecht et al., 1994; Ewing et al., 1995) and dogs (Dawson and Ewing, 1992), its sensitivity for detection of natural infections among wild deer is not known. Inconsistency in isolation of *E. chaffeensis* has been described using DH82 cell culture with experimentally inoculated deer (Dawson, Stallknecht et al., 1994; Ewing et al., 1995). We also encountered problems inherent with antibiotic-free culture systems such as contamination from exogenous bacteria and fungi; especially troublesome, however, were trypanosomes (*Trypanosoma cervi*). These protozoa are common in wild white-tailed deer (Kingston, 1981), and future attempts to isolate *E. chaffeensis* from wild deer should incorporate specific steps to minimize contamination by *T. cervi.*

Several previous studies have implicated *A. americanum* as a probable natural vector of *E. chaffeensis* (Eng et al., 1990; Anderson et al., 1992, 1993; Lockhart et al., 1996), including 1 that demonstrated a temporal relationship between establishment of the *A. americanum* population at WHEF and the appearance of *E. chaffeensis*-reactive antibodies among deer (Lockhart et al., 1995). Experimental studies later confirmed that *A. americanum* is capable of transstadial transmission of *E. chaffeensis* among deer (Ewing et al., 1995). Several findings from the current study reinforce the concept that *A. americanum* is an important natural vector. These include: (1) *A. americanum* was the predominant tick species collected from the environment at WHEF accounting for over 99% of all ticks collected; (2) *A. americanum* was the only species of tick detected on white-tailed deer; (3) the 3 species of mammals with *E. chaffeensis*-reactive antibodies were the only species parasitized by adult *A. americanum* and were the predominant hosts for nymphs; and (4) *E. chaffeensis* DNA was detected in adult *A. americanum* collected from the environment at WHEF. The absence or low prevalence of *E. chaffeensis*-reactive antibodies among the hosts parasitized by the 6 other species of ticks suggests that these species of ticks have minimal, if any, involvement in transmission of *E. chaffeensis* at WHEF. These findings have important public health significance because a recent study found that *A. americanum* represented 83% of ticks parasitizing humans in Georgia and South Carolina (Felz et al., 1996).

Detection of *E. chaffeensis* DNA in field-collected *A. americanum* in this and earlier studies (Anderson et al., 1992, 1993) is the strongest evidence that this species of tick is infected in nature. The 3.5% MIF among pooled adult ticks was comparable to the 1.1 to 3.6% MIF reported by Anderson et al. (1993); however, adult ticks that we tested individually had a higher (12%) prevalence of *E. chaffeensis* DNA. This difference may be related to the presence of PCR-inhibiting substances in ticks that hinder amplification of the *E. chaffeensis* 16S gene (Ewing et al., 1995). Consequently, future quantitative PCR-based surveys of ticks for *E. chaffeensis* DNA should include analysis of at least some individual ticks in order to estimate prevalence more accurately.

Dawson et al. (1996) recently reported a novel *Ehrlichia*-like 16S rDNA sequence from each of 10 wild white-tailed deer from Georgia and Oklahoma, including 5 collected from WHEF in November 1993. Comparison of the 16S rDNA sequences showed that this *Ehrlichia*-like gene fragment was most closely related to the *E. phagocytophila* genogroup comprised of *E. phagocytophila, E. platys, E. equi,* and the agent of HGE (Dawson et al., 1996). Subsequently, Little et al. (1997) detected the same 16S rDNA sequence in 50–100% of the deer from 7 populations in Arkansas, Georgia, and South Carolina. Our detection of this 16S *Ehrlichia*-like gene fragment in 96% of the deer from WHEF corroborates that this organism is ubiquitous in certain deer populations.

Dawson et al. (1996) suggested that this *Ehrlichia*-like organism might be transmitted by ticks, and Little et al. (1997) found an association between the presence of this 16S rDNA sequence within 10 deer populations and infestation of these populations by *A. americanum.* Our detection of an identical 16S rDNA sequence in adult *A. americanum* supports the suggestion by Little et al. (1997) that *A. americanum* is a possible vector of this novel *Ehrlichia*-like organism. Only 1 of 7 PCR products obtained from amplification of tick material could be confirmed by restriction analysis. Our inability to reamplify DNA from many ticks suggests that substances in ticks also may inhibit detection of the *Ehrlichia*-like organism as postulated for the *E. chaffeensis* 16S rDNA gene (Ewing et al., 1995).

One reason the presence of this *Ehrlichia*-like organism among deer and ticks is important is that it potentially could complicate interpretation of serologic or PCR-based epidemiologic investigations on human ehrlichiosis due to both *E. chaffeensis* and the HGE agent. Little et al. (1997) demonstrated that PCR primers often used to detect the HGE agent (Chen et al., 1994) would generate product when the *Ehrlichia*-like or-

ganism of deer was used as template; the potential for serologic cross-reactions has been recognized, but has not been investigated (Dawson et al., 1996; Little et al., 1997) due to the inability to culture the deer *Ehrlichia*-like agent. Another reason is that it may have zoonotic potential (Dawson et al., 1996).

Collectively, our data provide strong support for the hypothesis that *E. chaffeensis* is maintained in nature by a tick vector and vertebrate reservoir system consisting principally of lone star ticks and white-tailed deer. Although the possibility of transmission by other ticks or infection among other hosts presently cannot, and should not, be excluded, the relative importance of other species in the epidemiology of *E. chaffeensis* appears minimal compared to that of lone star ticks and white-tailed deer. Our data also indicate that an undescribed *Ehrlichia*-like organism, which is common among southeastern white-tailed deer populations, also occurs in and may be transmitted by lone star ticks. This apparently novel ehrlichial agent of deer merits further research because of its potential relationships to human ehrlichiosis.

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Addendum: Research subsequent to the present report has been successful in isolating *Ehrlichia chaffeensis,* confirming the role of white-tailed deer as natural reservoir hosts (*Journal of Clinical Microbiology.* Volume 35, No. 7).