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DEVELOPMENT AND USE OF SPECIFIC POLYMERASE REACTION FOR THE DETECTION OF AN ORGANISM RESEMBLING *EHRLICHIA* SP. IN WHITE-TAILED DEER

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ABSTRACT: The role of white-tailed deer (*Odocoileus virginianus*) in the epidemiology of *Ehrlichia chaffeensis* and the agent of human granulocytic ehrlichiosis (HGE) is not fully understood, and diagnostic procedures may be complicated by the recent detection of 16S rDNA sequence from an *Ehrlichia* sp.-like organism in wild deer. A specific forward primer (DGA) and an *Ehrlichia* spp. reverse primer (GA1UR) were constructed to amplify this new, distinct *Ehrlichia* sp.-like 16S rDNA. The DGA primer, a forward primer specific for *E. chaffeensis* (DCH), and a forward primer specific for the *E. phagocytophila* genogroup (GE9f) were each used with GA1UR in nested polymerase chain reactions to amplify 16S rDNA sequences from control samples containing the deer *Ehrlichia* sp.-like organism, *E. chaffeensis*, or the HGE agent. Primer pairs DGA/GA1UR and DCH/GA1UR specifically amplified 16S rDNA sequences from the corresponding target organism, whereas GE9f/GA1UR amplified 16S rDNA sequence from both the HGE agent and the deer *Ehrlichia* sp.-like organism. With a nested PCR using DGA/GA1UR and DCH/GA1UR on DNA extracted from white blood cells from 62 deer from 10 populations in four U.S. states, we observed a high prevalence (65%) of 16S rDNA sequences of the deer *Ehrlichia* sp.-like organism, and a low prevalence (5%) of the *E. chaffeensis* sequence. In this field survey, *E. chaffeensis*-reactive antibodies detected by indirect fluorescence assays were associated ($P < 0.001$) with PCR evidence of the deer *Ehrlichia* sp.-like organism, but not *E. chaffeensis*. Infestations of *Amblyomma americanum* also were associated ($P < 0.001$) with PCR evidence of the deer *Ehrlichia* sp.-like organism. The potential for serologic cross-reactions and non-specific PCR products arising from the deer *Ehrlichia* sp.-like organism should be considered when evaluating the role of deer and their ticks in the epidemiology of ehrlichial pathogens of humans.

Key words: *Ehrlichia* sp., *Ehrlichia chaffeensis*, white-tailed deer, *Odocoileus virginianus*, ehrlichiosis, serology, epizootiology.

INTRODUCTION

Human monocytic ehrlichiosis is an acute febrile illness caused by the rickettsia *Ehrlichia chaffeensis* (Dawson et al., 1991a), while human granulocytic ehrlichiosis (HGE) is a similar illness caused by an as yet unnamed *Ehrlichia* sp. more closely related to *E. phagocytophila* than *E. chaffeensis* (Chen et al., 1994). White-tailed deer (*Odocoileus virginianus*) have been implicated as a potential reservoir for *E. chaffeensis* on the basis of widespread positive indirect fluorescent antibody (IFA) test results in serum of deer from the southeastern United States (Dawson et al., 1994a). Experimental inoculations of white-tailed deer with *E. chaffeensis* showed that deer are a competent host for this organism (Dawson et al., 1994b). Nonetheless, to date, *E. chaffeensis* has not been detected in naturally infected white-tailed deer by either isolation or polymerase chain reaction (PCR) techniques.

Amblyomma americanum is suspected to be the principal vector of *E. chaffeensis*. This suspicion is based on the similarity in geographic distribution between this tick and case reports of human ehrlichiosis (Eng et al., 1990), the presence of 16S ribosomal RNA (rRNA) sequence for *E. chaffeensis* in adult *A. americanum* (An-

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derson et al., 1992, 1993), a temporal and spatial association of the presence of *A. americanum* with antibodies reactive to *E. chaffeensis* in white-tailed deer (Lockhart et al., 1995, 1996), and experimental transfer of *E. chaffeensis* among white-tailed deer by *A. americanum* (Ewing et al., 1995).

The agent of HGE also is presumed to be transmitted to humans through tick bites, but the specific tick vectors have not been determined (Bakken et al., 1994). In recent work, however, *Ixodes scapularis* has been implicated as a potential vector (Magnarelli et al., 1995; Pancholi et al., 1995), and due to an association between adult *I. scapularis* and white-tailed deer (Strickland et al., 1981), the infection status of deer with the agent of HGE is of interest.

Recently, PCR was used to amplify *Ehrlichia* sp.-like 16S rRNA gene fragments from blood of wild white-tailed deer from Georgia and Oklahoma, USA (Dawson et al., 1996). The nucleotide sequence of this amplification product differed from that of *E. chaffeensis*, as well as from all other described *Ehrlichia* spp., evidence that this fragment is from a previously undescribed organism. When comparing 16S rRNA gene sequences, the white-tailed deer *Ehrlichia* sp.-like sequence most closely resembled the *E. phagocytophila* genogroup comprised of *E. phagocytophila, E. equi,* and the HGE agent (Dawson et al., 1996). To investigate the potential reservoir status of white-tailed deer for agents of human ehrlichiosis, researchers first must be able to distinguish among these closely related but genetically distinct organisms.

Polymerase chain reaction with primers specific to a variable region of the 16S rRNA gene has been employed to detect and identify *Ehrlichia* spp. (Chen et al., 1994; Anderson et al., 1992). In order to expand that technique to include the newly detected ehrlichial organism from white-tailed deer, we constructed a forward primer specific to the white-tailed deer organism. In addition, we constructed a reverse primer which was in common with the white-tailed deer organism, *E. chaffeensis,* and the HGE agent and which would amplify DNA from all of them. These primers, along with a forward primer of Chen et al. (1994) and a modified forward primer of Dawson et al. (1994b), were then used to PCR test white-tailed deer in the southeastern United States for the white-tailed deer organism, *E. chaffeensis,* and the HGE agent. Additional data collected from these deer included serologic reactivity to *E. chaffeensis* and representative potential tick vectors.

MATERIALS AND METHODS

Primers specific for the *Ehrlichia* sp.-like sequence from white-tailed deer were designed using DNAsis Mac v 2.0 (Hitachi Software Engineering Company, Ltd., San Bruno, California, USA). Ribosomal RNA gene sequences for all known *Ehrlichia* spp. were aligned, and primers were selected from within the variable region of the rRNA gene. Primer sequences and corresponding target template are shown in Table 1.

Specificity of primers was evaluated against control-positive samples from which the targeted 16S rRNA gene fragment had been previously amplified and sequenced. Positive controls consisted of blood or tissue samples that contained *E. chaffeensis,* the HGE agent, or the white-tailed deer organism. Negative controls in which no template was added to the PCR mix were used during every set of amplifications.

Nested PCR was performed as described by Dawson et al. (1994b). Briefly, outside primers ECB and ECC were used to generate a 490 base pair (bp) fragment from white blood cells of confirmed positive human patients (for *E. chaffeensis* and the HGE agent) or white-tailed deer spleen cells (for the white-tailed deer organism), then specific primers were employed to amplify an internal 405 to 412 bp fragment from 1 μl of the external product. For both external and internal reactions, components (0.2 mM dNTP, 2.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl, 0.1% Triton X-100, 0.8 μM each primer, and 20 μl or 1 μl template DNA) were combined in 50 μl reactions in 0.5 ml tubes, overlain with mineral oil, and held at 94 C. The *Taq* DNA polymerase (2.5 units; Promega, Madison, Wisconsin, USA) was added to each tube, and the reactions were allowed to

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TABLE 1. Oligonucleotide sequences used in PCR amplifications of 16S rRNA gene fragments of *Ehrlichia* spp.

Primer	Sequence	Target template	Reference
ECB	5'-CGTATTACCGCGGCTGCTGGCA-3'	*Ehrlichia* spp.	Dawson et al., 1994b
ECC	5'-AGAACGAACGCTGGCGGCGCAAGCC-3'	*Ehrlichia* spp.	Dawson et al., 1994b
DGA	5'-TTATCTCTGTAGCTTGCTACCC-3'	*Ehrlichia* sp.-like sequence of WTD	
DCH	5'-CAATTGCTTATAACCTTTTGCT-3'	*E. chaffeensis*	Anderson et al., 1992 (modified[a])
GE9f	5'-AACGGATTATTCTTTATAGCTTGCT-3'	*E. phagocytophila* genogroup	Chen et al., 1994
GA1UR	5'-GAGTTTGCCGCGGACTTCTTCT-3'	Reverse for DGA, DCH, GE9f	

[a] DCH is 22 nucleotides of the 5' end of the HE1 primer from Anderson et al. (1992).

cycle 30 times through 1 min at 94 C, 1 min at 55 C, and 2 min at 72 C. A replicate set of internal reactions were assembled as described and allowed to cycle 30 times through 1 min at 94 C, 1 min at 60 C, and 2 min at 72 C. Amplification products were separated by agarose gel electrophoresis (1.5%), and visualized with ethidium bromide and ultraviolet transillumination.

Blood samples were obtained via cardiac puncture from 62 white-tailed deer collected from 10 populations in four U.S. states (Arkansas, South Carolina, West Virginia, and Georgia). Each animal was examined visually for ticks. Representative ticks were collected, stored in 70% ethanol, and submitted to the National Veterinary Services Laboratory (U.S. Department of Agriculture, Ames, Iowa, USA) for identification by the keys of Strickland et al. (1976). 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 testing for *E. chaffeensis* was conducted on each of the serum samples as previously described (Dawson et al., 1991a). Briefly, spot slides of *E. chaffeensis* were used to screen sera at a 1:64 dilution in 0.01 M phosphate buffered saline (pH 7.2). Samples with stained ehrlichial organisms were then analyzed at two-fold serial dilutions. Results were recorded as the reciprocal of the highest dilution which produced observable specific fluorescence of *E. chaffeensis*.

For white blood cell isolation, 30 ml of lysing solution (150 mM NH_4Cl, 0.7 mM KH_2PO_4, and 3 mM EDTA-Na_2) was added to 10 ml whole blood collected in EDTA. Blood samples were allowed to lyse for 5 min at 25 C with frequent inversion, and then white blood cells were pelleted by centrifugation at 1,500 × *G* for 5 min, rinsed twice in the lysing solution, suspended in 3 ml phosphate buffered saline, and frozen at −70 C. The DNA was extracted from white blood cell preparations using InstaGene™ Purification Matrix (Bio-Rad Laboratories, Hercules, California). Each set of extraction procedures included a negative control in which phosphate buffered saline (PBS) alone was extracted and tested in subsequent nested PCR. In order to prevent cross-contamination of samples, the processes of DNA extraction, assembly of reactions, and separation of fragments on agarose gels were performed in three separate laboratories, and filter tips were used throughout.

The DNA extracted from the deer white blood cells was used as template in the nested PCR described using 20 μl of the DNA preparation in an outside reaction with primers ECC and ECB, and 1 μl of the outside reaction

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FIGURE 1a, b. Agarose gel electrophoresis (1.5%) of PCR products amplified from positive control samples. Lane M contains PCR Markers (Promega, Madison, Wisconsin) molecular size standard. Lanes 2 through 5 were amplified with DGA specific primer, lanes 6 through 9 with DCH, and lanes 10 through 13 with GE9f. Lanes W, C, and H contain template of the *Ehrlichia* sp.-like sequence from white-tailed deer, *E. chaffeensis*, and the agent of HGE, respectively. Lane N is the negative, or water, control. Figure 1a includes the reaction products obtained at an annealing temperature of 55 C, while 1b depicts reaction products obtained at an annealing temperature of 60 C.

product in an inside PCR with either DGA, DCH, or GE9f as the specific primer and GA1UR as the reverse primer.

Sequencing was performed on both strands of DNA generated by PCR amplification of positive control samples at 55 C annealing temperature. Both strands of products were sequenced for each of three deer that were PCR-positive for *E. chaffeensis*, and for five deer that were PCR-positive for the white-tailed deer organism. Amplicons were purified with a Microcon® spin filter (Amicon Inc., Beverely, Massachusetts, USA) and submitted with corresponding primers to the Molecular Genetics Facility at The University of Georgia (Athens, 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 v 2.0 and compared to published sequence data for *E. chaffeensis*, the HGE agent, and the white-tailed deer organism.

Prevalence and geometric mean titers of serum antibodies reactive to *E. chaffeensis* were calculated for each deer population. The prevalence of positive PCR results for *E. chaffeensis* and for the *Ehrlichia* sp.-like white-tailed deer organism as well as the prevalence of tick infestation were calculated for each population. The chi-square test with Yate's correction factor (Ott, 1993) was used to test if results of PCR for *E. chaffeensis* or the white-tailed deer organism were associated with *E. chaffeensis*-reactive antibodies or tick infestation.

RESULTS

The primers described here to amplify 16S rRNA gene targets from the *Ehrlichia* sp.-like deer organism and *E. chaffeensis* were specific to their respective templates within this system (Fig. 1a, b). The GE9f primer described by Chen et al. (1994) not only amplified the HGE agent, but also amplified the 16S rRNA gene targets from the white-tailed deer organism at 55 C annealing temperature (Fig. 1a). The sequence of each product from positive control amplifications was identical to its respective starting template DNA. The sequence of DNA from the deer organism amplified with GE9f was identical to the deer organism sequence as previously described by Dawson et al. (1996). The GE9f amplification of *Ehrlichia* sp.-like sequence from white-tailed deer was often, but not always, lost when the annealing temperature was raised to a more restrictive 60 C (Fig. 1b). The 3′ end of the GE9f primer has a nine bp region of identity to the 16S rDNA sequence of the *Ehrlichia* sp.-like deer organism. Of the 25 nucleotides in GE9f, 21 (84%) are available for annealing to the deer agent sequence (data not shown).

Due to generation of sequence-confirmed, non-specific product by the GE9f/GA1UR primers on deer infected with the *Ehrlichia* sp.-like deer organism, the use of this primer combination on field samples was suspended after testing five deer (Fig. 2a, b). Using PCR on DNA preparations of deer white blood cells, we demonstrated the presence of 16S rRNA gene

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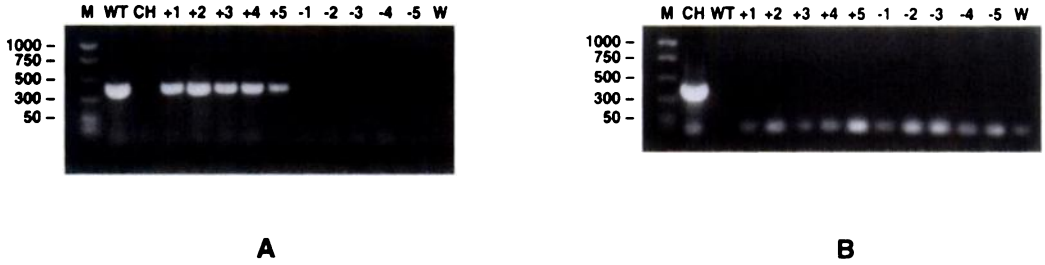

FIGURE 2a, b. Representative agarose gel (1.5%) electrophoresis of PCR products obtained by amplification of DNA extracted from sample deer white blood cells. Lane M contains PCR Markers (Promega, Madison, Wisconsin) molecular size standard. Lanes WT and CH are amplification products from positive control DNA of the *Ehrlichia* sp.-like sequence from white-tailed deer and *E. chaffeensis*, respectively. Lanes +1 through +5 are amplification products from DNA extractions of white blood cell samples from seropositive deer, and lanes −1 through −5 from seronegative deer. Lane W is the negative or water control. Amplifications in 2a were performed using the *Ehrlichia*-like sequence from white-tailed deer specific primer DGA, and 2b the *E. chaffeensis* specific primer DCH.

fragments of the *Ehrlichia* sp.-like deer organism in 33 of 40 seropositive deer but in only seven of 22 seronegative deer (Table 2). Seven seropositive deer were negative by PCR for both organisms. *Ehrlichia chaffeensis* 16S rDNA sequence was detected in three of the 62 samples analyzed. All three of these deer, two from the Beaufort County, South Carolina population and one from the Jasper/Jones County, Georgia population, were seropositive for *E. chaffeensis* and also were PCR-positive for the white-tailed deer *Ehrlichia* sp.-like organism. Sequences of products

TABLE 2. Serologica analysis, PCR test results, and tick prevalence data from 62 white-tailed deer from the southeastern United States.

Location State County	Number examined	Serology			Specific polymerase chain reactions for				
					E. chaffeensis		*Ehrlichia* sp.-like of WTD		
		Prevalence	Geometric mean titer	Maximum titer	Number positive	Prevalence	Number positive	Prevalence	*A. americanum* prevalence
Georgia									
Clarke[a]	5	100	358	512	0	0	5	100	100
Bartow/Cherokee	5	0	<64	<64	0	0	0	0	0
Chatham	14	79	90	128	0	0	14	100	N/A[b]
Jasper/Jones	5	100	384	512	1	20	5	100	100
West Virginia									
Tucker	5	0	<64	<64	0	0	0	0	0
Tyler	5	0	<64	<64	0	0	0	0	0
South Carolina									
Beaufort	5	100	525	1,024	2	40	3	60	N/A[b]
Arkansas									
Pike	5	60	128	256	0	0	5	100	100
Conway	5	100	128	256	0	0	4	80	100
Howard/Polk/Sevier	8	75	128	512	0	0	4	50	100

[a] Site from which deer blood samples were positive with GE9f/GA1UR but sequencing showed the products were due to non-specific amplification of the *Ehrlichia* sp.-like deer organism.

[b] Data not available for analysis.

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from three deer that were PCR-positive to *E. chaffeensis* and five deer that were PCR-positive for the white-tailed deer organism were identical to their respective published sequences. None of the negative control samples had evidence of contamination at any point in these experiments.

Ticks were present on all 28 deer from five populations with *E. chaffeensis*-reactive antibodies, but on none of 15 deer from three populations with no evidence of serologic reactivity. Twenty-three of the 28 deer with ticks also had PCR evidence of the deer *Ehrlichia* sp.-like organism, and three of the 28 had PCR evidence of *E. chaffeensis*. *Amblyomma americanum* was the only tick identified from these deer and was present on all of the 28 tick-infested deer examined (Table 2).

A significant association (chi-square = 13.79, $P < 0.001$) existed between the presence of *E. chaffeensis*-reactive antibodies and the presence of the PCR derived DNA sequences identical to those of the white-tailed deer organism. A significant association (chi-square = 24.07, $P < 0.001$) also was detected between presence of *Amblyomma americanum* and PCR evidence of the deer organism. There was no significant association (chi-square = 0.1, $P = 0.75$) between PCR results for *E. chaffeensis* and serologic findings.

DISCUSSION

Amplification of 16S rRNA gene fragments of the *Ehrlichia* sp.-like deer organism with a primer designed to detect the *E. phagocytophila* genogroup is problematic, but not altogether surprising. The organisms comprising the *E. phagocytophila* genogroup share 99.8% 16S rDNA sequence identity (Chen et al., 1994), and some authors suggest they may represent the same species (Dumler et al., 1995; Madigan et al., 1995). The 16S rDNA sequence of the *E. phagocytophila* genogroup has 93.8 to 94.0% identity with that of the *Ehrlichia* sp.-like deer organism. Using phylogenetic analyses, Dawson et al. (1996) placed sequence of the deer organism closer to the granulocytic *E. phagocytophila* genogroup than the monocytic *Ehrlichia chaffeensis*. Sufficient identity exists between the *E. phagocytophila* genogroup primer GE9f and the *Ehrlichia* sp.-like deer organism to generate product from both positive control samples and field samples, using annealing temperatures higher than the 52 C described by Chen et al. (1994).

Inspection of other primers reportedly specific for members of the *E. phagocytophila* genogroup including GE10r (Chen et al., 1994; Magnarelli et al., 1995), Ehr521/Ehr747 (Pancholi et al., 1995), and GER3/GER4 (Goodman et al., 1996) shows that they are identical in sequence to the newly described white-tailed deer organism. The demonstrated ability of one *E. phagocytophila* genogroup specific primer, GE9f, to amplify DNA of the deer organism and the predicted ability of other described primers to do so based on sequence identity (Chen et al., 1994; Magnarelli et al., 1995; Pancholi et al., 1995; Goodman et al., 1996) indicate that sequencing of products to confirm their identity should be implemented in PCR-based surveys of ticks or wildlife for the presence of these agents.

White-tailed deer were first implicated as a potential reservoir host for *E. chaffeensis* when Dawson et al. (1994a) reported widespread serologic evidence for the presence of this organism in deer. This potential role subsequently was supported by demonstrating that experimentally infected deer are capable of supporting replication of this organism (Dawson et al., 1994b) and that *A. americanum*, the lone star tick, can transmit the organism to naive deer (Ewing et al., 1995). This report is the first PCR-based evidence of naturally occurring *E. chaffeensis* infection in white-tailed deer; however, results of PCR on circulating white blood cells are evidence that infection of white-tailed deer by *E. chaffeensis* may not be as common as infection with the *Ehrlichia* sp.-like deer organism.

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Blood of white-tailed deer from Arkansas, Georgia, and South Carolina often contained 16S rRNA gene sequence characteristic of the newly detected *Ehrlichia* sp.-like white-tailed deer organism. Dawson et al. (1996) also reported a high prevalence of this organism in a small number of deer from Georgia and Oklahoma. One possible explanation for the association of the *Ehrlichia* sp.-like deer organism with the presence of *E. chaffeensis*-reactive antibodies is serologic cross-reaction with antibodies against the white-tailed deer organism. Alternatively, these deer actually may have been previously infected by *E. chaffeensis* but are no longer rickettsemic. The latter is supported by our data which suggest that, in addition to transmitting *E. chaffeensis* (Ewing et al., 1995), *A. americanum* is also a likely vector of the deer *Ehrlichia* sp.-like organism.

Serologic cross-reactivity is common among the *Ehrlichia* spp. (Nyindo et al., 1991; Shankarappa et al., 1992) and has been the basis for diagnostic tests in humans when target antigen is not available (Dawson et al., 1991b; Bakken et al., 1994; Pancholi et al., 1995). *Ehrlichia equi* antigen is currently used for testing for antibodies to members of the *E. phagocytophila* genogroup, including the HGE agent. Since serologic cross-reactions readily occur within the *E. phagocytophila* genogroup (Dumler et al., 1995), deer may harbor antibodies reactive to *E. equi* antigen. Consequently, epizootiologic studies of deer evaluating the presence of antibodies reactive to *E. equi* antigen should consider the potential for cross-reactions with antibodies to the *Ehrlichia* sp.-like white-tailed deer organism.

The results of this study do not exclude white-tailed deer as a significant reservoir host for *E. chaffeensis*. Rather, they may provide an explanation for some of the serologic evidence for *E. chaffeensis* infection in white-tailed deer in the absence of corresponding PCR or culture data. Furthermore, it is important to note that the *Ehrlichia* sp.-like organism of white-tailed deer has only been found in wild deer; this rDNA sequence has not been reported in humans with either monocytic or granulocytic ehrlichiosis. The data presented here also are evidence that investigating the status of ticks, deer, and other wildlife in the epidemiology of HGE will require refining current detection techniques and underscore the difficulties encountered in interpreting indirect evidence of infection such as serology and unsequenced PCR products. Culture of many *Ehrlichia* spp. can be difficult; however, isolation of the causative agent by *in vitro* culture techniques may be necessary before definitive conclusions can be drawn regarding the role of white-tailed deer in the epidemiology of human ehrlichiosis.

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