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EHRLICHIA-LIKE 16S rDNA SEQUENCE FROM WILD WHITE-TAILED DEER (*ODOCOILEUS VIRGINIANUS*)

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ABSTRACT: The reservoir hosts of *Ehrlichia chaffeensis,* etiologic agent of human ehrlichiosis are unknown. Initially, white-tailed deer (WTD) were serologically implicated as possible reservoirs of *E. chaffeensis.* Subsequent studies showed that WTD were susceptible to infection with *E. chaffeensis* and that deer-to-deer transmission by a tick vector, *Amblyomma americanum,* is possible under experimental conditions. To determine if wild WTD were infected with *E. chaffeensis,* whole blood was collected from 10 deer from Oklahoma and Georgia. All 10 deer had antibodies reactive to *E. chaffeensis.* Whereas *E. chaffeensis* was not isolated, restriction enzyme mapping and sequencing of the 16S rDNA gene revealed that a unique *Ehrlichia*-like agent was present. All 10 deer appeared to be infected with the same agent. We suspect that *A. americanum* is the vector of this new agent based upon the previously published temporal association between the appearance of *E. chaffeensis* seropositive WTD and *A. americanum.* However, the taxonomic and antigenic relationships, geographic distribution, epidemiology, and zoonotic potential of this agent are yet to be determined.

Ehrlichia chaffeensis, is the causative agent of an emerging disease, human ehrlichiosis (Dawson et al., 1990; Anderson et al., 1992). Over 400 cases, including 9 fatalities in 30 states, have been laboratory confirmed in the United States. Epidemiologic evidence suggests the agent is tick-borne (Eng et al., 1990; Fishbein et al., 1994) and *E. chaffeensis* 16S rDNA has been found in adult *Amblyomma americanum* ticks by polymerase chain reaction (PCR) application (Anderson et al., 1993).

Because white-tailed deer (WTD) are a major host for all active stages of *A. americanum,* serosurveys were conducted to determine if WTD had been exposed to an *Ehrlichia* sp. (Dawson, Childs et al., 1994; Lockhart, Davidson, Dawson, and Stallknecht, 1995; Lockhart, Davidson, Stallknecht, and Dawson, 1995). These serosurveys revealed that a majority of WTD within the general range of *A. americanum* were seropositive and that the presence of seropositive WTD was associated with *A. americanum* on a temporal and site-specific geographic basis (Lockhart, Davidson, Dawson, and Stallknecht 1995; Lockhart, Davidson, Stallknecht, and Dawson, 1995.). Experimental inoculation of 2 WTD proved that this species was indeed susceptible to infection with *E. chaffeensis* (Dawson, Stallknecht et al., 1994). PCR results indicated that *E. chaffeensis* circulated in the peripheral blood for at least 2 wk without causing apparent clinical disease, clearly indicating that WTD can support *E. chaffeensis* infection and remain rickettsemic for periods sufficient to infect tick vectors. Indeed, subsequent studies proved that *A. americanum* are competent transtadial vectors transmitting *E. chaffeensis* from deer to deer (Ewing et al., 1995). The present work was initiated to determine if wild WTD are naturally infected with *E. chaffeensis.* Two geographically distant locations, 1 in east-central Oklahoma and the other in mid-central Georgia were chosen based upon the presence of human cases (Oklahoma) or seropositive WTD (Georgia).

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MATERIALS AND METHODS

Experimental design and sample collection

In 1993, 5 WTD were shot from both Cherokee Public Hunting Area, Cherokee County, Oklahoma and Whitehall Experimental Forest, Clarke County, Georgia, during June and November, respectively.

Blood samples were collected via cardiac puncture from each deer after a 95% ethanol wipe of the skin. Samples for PCR were collected in 5-ml EDTA tubes, those for culture in 10-ml heparinized tubes, and those for serologic testing in 10-ml clot tubes. Spleen samples also were collected aseptically from each animal, placed in sterile bags, and temporarily stored on ice.

Serology

The indirect fluorescent antibody (IFA) test was performed as previously described (Dawson et al., 1990). 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.* The conjugate used was fluorescein isothiocyanate-labeled rabbit, anti-deer immunoglobin G (Kirkegaard and Perry Laboratories, Gaithersburg, Maryland) diluted 1:100 in PBS. When distinct staining of the ehrlichial organisms was observed at this dilution, serial 2-fold dilutions were made. Serologic results were recorded as the reciprocal of the highest dilution at which specific fluorescence of *Ehrlichia* morulae or individual organisms was observed.

Attempted isolation of organisms

Samples were prepared as previously described (Dawson, Stallknecht et al., 1994). In brief, 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 3–4 times. After 5 min at room temperature, the suspension was centrifuged at 160 *g* for 5 min, the erythrocyte supernatant was removed, and the procedure repeated.

Uninfected DH82 cells (dog macrophage line) 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 hr, the supernatant was decanted, and 5 ml of fresh medium was added to each culture twice weekly. Starting 30 days after the addition of leukocytes from the deer, the cultures were examined twice weekly for evidence of infection and discarded after 67 days as previously described (Dawson and Ewing, 1992).

Two grams of spleen were soaked in 70% ethanol for 10 min, cut into small pieces with sterile scissors, and then dounce homogenized for 10 strokes before being processed with the lysing buffer as described above.

DNA extraction

DNA was extracted from 150 μl of anticoagulated whole blood (EDTA), using the DNA/RNA Isolation Kit (U.S. Biochemical Corp., Cleveland, Ohio) following the manufacturer's protocol for fast DNA extraction. The final pellet was resuspended in 100 μl of water.

DNA amplification

Each DNA template was examined by nested PCR for the presence of *Ehrlichia*-like 16S DNA. Each template was tested at 2 different concentrations because the actual template concentration was unknown.

The outside amplification consisted of either 1 μl or 10 μl of template DNA in a 50-μl reaction containing 10 mM Tris-Cl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM each dNTP, 5 μM tetramethylammonium chloride (TMAC), 1.25 units Ampli*Taq* polymerase (Perkin-Elmer/Applied Biosystems Division [PE/ABD], Foster City, California), and 0.8 μM 8F (5′-AGTTTGATCATGGCTCAG-3′) and 1448R (5′-CCATGGCGTGACGGGCAGTGTG-3′). This reaction mixture was amplified in a thermocycler (PE/ABD, Foster City, California) for 40 cycles: 1 min at 94 C, 2 min at 45 C, and 2 min at 72 C. Each 72 C cycle beginning with the third round of amplification was extended by 1 sec. This reaction allowed the amplification of a broad range of bacterial 16S genes.

For the nested PCR, 1 μl of each outside reaction product was amplified in a second 50-μl reaction identical to the 1 described for the outside reaction except for the use of primers 15F (5′-ATCATGGCTCAGAACGAACG-3′) and 1442R (5′-GTGACGGGCAGTGTGTACAAG-3′). The temperature profile employed was the same as that described above except that the annealing temperature was increased to 55 C. The primers in this reaction also allowed for the amplification of a broad range of 16S rDNA. Amplification products were analyzed by electrophoresis in 1.3% agarose gels and visualized with ethidium bromide.

Restriction endonuclease analysis

The 16S rDNAs obtained from the nested PCR amplification were analyzed by restriction endonuclease digestion. The enzymes chosen for the analysis were selected because computer mapping of known *Ehrlichia* 16S rDNA showed that these enzymes would distinguish *E. chaffeensis* from several other closely related species. The PCR products were purified on 0.6% SeaPlaque agarose (FMC BioProducts, Rockland, Maine). The DNA bands containing the PCR fragments were removed from the gel by razor blade excision and melted at 60 C. After melting, the gel pieces containing each PCR product were diluted to 250 μl with water. Each of the diluted PCR products was subdivided into 4 separate tubes. Three of these subsamples of each PCR product were digested with either *AvaI, AvaII,* or *RsaI* for 3 hr at 37 C. A fourth subsample was incubated at 37 C without enzyme under the same conditions as a negative control. The 3 restriction products and the unrestricted control were resolved by gel electrophoresis on 0.5% agarose–3.0% acrylamide gels. The fragment sizes were determined by comparison with marker DNAs (*Bst*NI-digested pBR322, New England Biolabs, Beverly, Massachusetts) run in parallel on the gels.

DNA sequencing

The PCR products from the 16S rDNA were labeled for sequencing using the PRISM® Taq DyeDeoxy® Terminator Cycle Sequencing Kit (PE/ABD, Foster City, California). The unincorporated fluorescent nucleotides were separated from labeled products using Centri-Sep columns (Princeton Diagnostics, Princeton, New Jersey). The sequence was collected with an Applied Biosystems Inc. 373A automated sequencer and edited using Sequence Navigator (PE/ABD, Foster City, California).

DNA analysis

The *Pileup* program was used to create a multiple sequence file (msf), using progressive pairwise alignments of the sequences. This msf file was then entered into the program *Olddistances* to create the pairwise distance matrix. The msf file was also used in the program *Growtree* to create the phylogenetic tree. The sequence of the WTD 16S rDNA agent was examined in the GCG program *Mapplot* to identify the presence of *AvaI, AvaII,* and *RsaI* sites. Computer analysis, including restriction site mapping and phylogenetic comparisons were made using GCG Sequence Analysis Software programs (Madison, Wisconsin).

RESULTS

Serology

Antibodies reactive to *E. chaffeensis* were detected in all 10 of the WTD from Georgia and Oklahoma. Negative control sera was obtained from a pen-reared WTD. Positive control sera was obtained from a deer experimentally inoculated with *E. chaffeensis.* Reciprocal antibody titers for the Georgia deer ranged from 256 to 2,048 (deer 1, 2,048; deer 2, 1,024; deer 3, 2,048; deer 4, 2,048; deer 5, 256). Reciprocal antibody titers from the Oklahoma deer showed similar levels ranging from 512 to 2,048 (deer 1, 1,024; deer 2, 512; deer 3, 2,048; deer 4, 1,024; deer 5, 512).

Attempted isolation of organisms

All attempts to isolate the *Ehrlichia*-like agent from whole blood and spleen were unsuccessful. The spleen culture from Georgia deer 1 and WB cultures from Georgia deer 2 and Oklahoma deer 2 were contaminated and therefore discarded. All other whole blood and spleen cultures were negative at the end of the 67-day retention period.

PCR results

The nested PCR results are shown in Figure 1. A 1,357-base pair product was observed from all Georgia and Oklahoma WTD.

Sequencing of PCR products

Approximately 1,100 nucleotides of sequence were determined from PCR-amplified material from Oklahoma WTD 1 and 3 and Georgia WTD 2 and 4. The 4 sequences do not differ from each other.

Anaylsis of 16S DNA sequence

The results of the analysis of restriction enzyme sites are shown in Figure 2. Restriction digest of the WTD 16S rDNA is shown in Figure 3. All of the predicted fragments (except for the 2 smallest ones) are evident in these digests. Properly identifying relatively large DNA fragments (1,268-base pair [bp] *AvaI* fragment) and extremely small ones (89-bp fragment in an *AvaI* digest and a 16-bp fragment in an *RsaI* digest) in a single gel is not feasible. Restriction analysis of all 10 WTD PCR products gave similar results, indicating that the agent detected by PCR in each of these deer was the same.

To position the WTD agent 16S rDNA sequence among other closely related members of the order Rickettsiales, 3 programs of GCG DNA analysis software were used. Because the sequences from each of the 10 animals did not differ from each other, the GAWTD #2 sequence was used. The 16S rDNA sequences (and their GenBank accession numbers) used in the comparisons were: *Rickettsia montana* (U11016), *Rickettsia rickettsii* (M21293), *Rickettsia bellii* (U11014), *Bartonella bacilliformis* (M65249), *Coxiella burnetii* (M21291), *Ehrlichia canis* (M73221), *E. chaffeensis* (M73222), *Ehrlichia ewingii* (M73227),

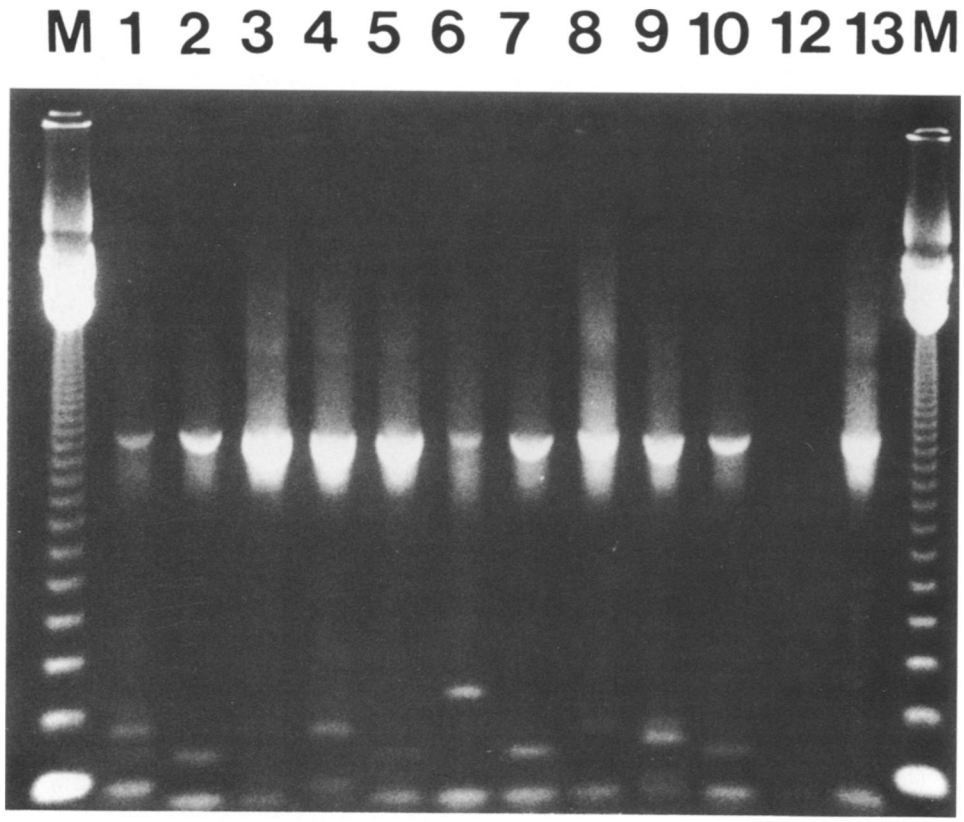

FIGURE 1. A nested PCR was used to detect DNA targets from naturally infected blood from wild white-tailed deer. A 1,357-bp product was obtained from all deer collected in Georgia (lanes 1–5) and Oklahoma (lanes 6–10). No specific product was observed from uninfected dog blood processed at the same time (lanes 12). Lane 13 contains a positive control consisting of *E. chaffeensis* obtained from tissue culture. Lanes marked M contain a 123-bp molecular weight ladder (Life Technologies, Gaithersburg, Maryland).

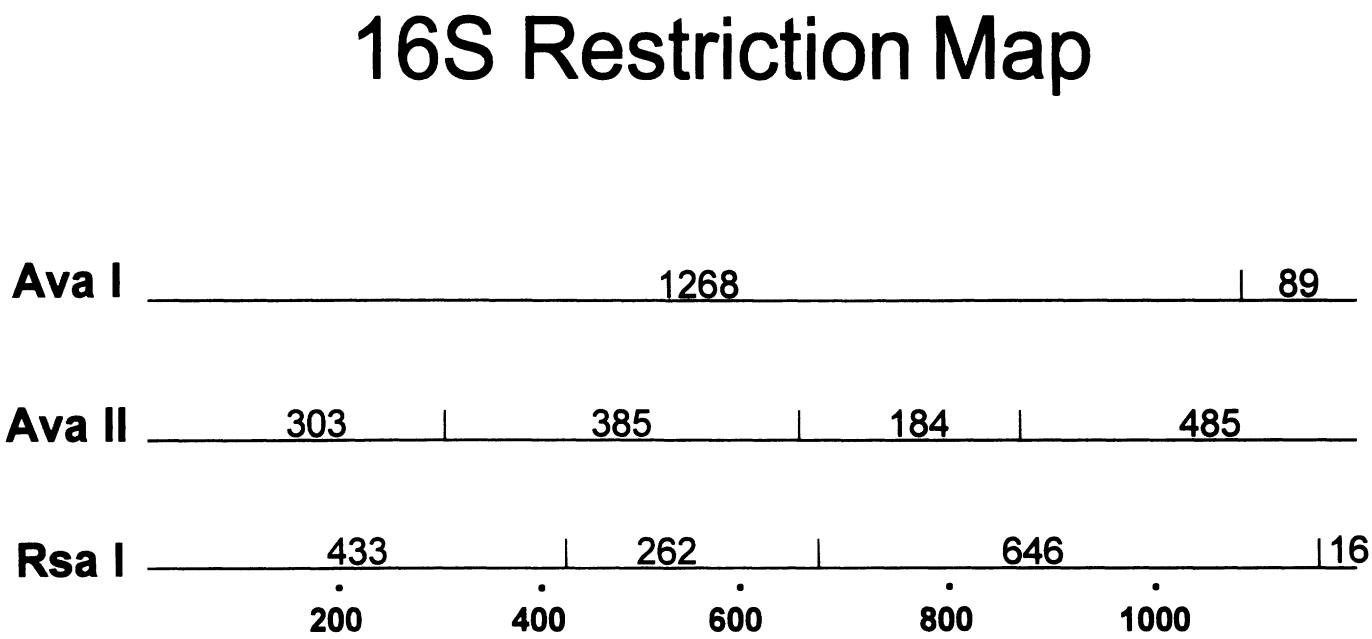

FIGURE 2. After the 16S rDNA sequence of the WTD agent was determined, GCG Mapplot was used to generate this theoretical restriction map.

Ehrlichia muris (U15527), *Cowdria ruminatium* (X61659), *Anaplasma marginale* (M60313), *Ehrlichia platys* (M82801), human granulocytic *Ehrlichia* (HGE) (U02521), *Ehrlichia equi* (M73223), and *Ehrlichia phagocytophila* (M73224). In Table I, the distance values are the number of matches between each pair of sequences (including gaps) divided by the length of the shorter member of the pair. For this data set, there was no difference in the distance values when the denominator for each value was the average length of the pair (data not shown). The distances show that the WTD agent is most closely related to the ehrlichial agents, *E. platys* (0.9376), *E. phagocytophila* (0.9384), *E. equi* (0.9376), and to the newly recognized (but unnamed) agent HGE (0.9400).

The phylogenetic tree is shown in Figure 4. In this display, the WTD agent groups in the branch of the tree containing the *Ehrlichia* to which it is most closely related phylogenetically. This display of the sequence information clearly shows that the WTD agent is as distant from the agents to which it is most closely related as those agents are from *Anaplasma marginale.* The WTD agent is considerably more distant from its nearest phylogentic neighbors than they are from each other.

Nucleotide sequence accession numbers

The nucleotide sequences determined in these experiments have been assigned the following Genbank Accession numbers: OK WTD #1 U27102; OK WTD #3 U27101; GA WTD #2 U27103; GA WTD #4 U27104.

DISCUSSION

In order to substantiate whether WTD in nature are infected with *E. chaffeensis* or a closely related ehrlichial species, PCR sequences were determined for samples recovered from seropositive wild deer. The sequence results from 4 of 10 PCR-positive deer and restriction enzyme digest analyses of the remaining deer indicate that these animals, from 2 geographic regions approximately 1,000 miles distant from each other, were infected with a unique agent rather than with *E. chaffeensis.* The WTD agent appears to be related to several ehrlichial species, namely *E. platys, E. equi, E. phagocytophila,* and the newly recognized but unnamed human agent known as HGE. These findings suggest that the agent is geographically widespread in WTD but does not eliminate the possibility that some WTD may be infected with *E. chaffeensis* or even other ehrlichial agents.

This is the first evidence documenting the presence of an ehrlichial agent in wild WTD. Additional wild deer from other locations should be tested in order to determine distribution in the deer population. Because all 10 of the deer sampled in this study were positive for this agent, high prevalences of infection may occur among free-ranging deer. Indeed, all 14 deer recently sampled from Skidaway Island, Chatham County, Georgia were determined to be infected with this agent using PCR primers designed to amplify the deer agent specifically (unpubl. obs.).

The potential of this ehrlichial agent to cause disease and the mechanism(s) for spread should be studied. An additional important implication of finding this new agent at a high prevalence in wild deer is the possibility that previous serosurveys of deer for *E. chaffeensis*-reactive antibodies have actually detected antibodies to this new *Ehrlichia*-like agent. Serologic cross-reaction is known to occur among *Ehrlichia* spp. (Dawson, Childs et. al, 1994; Lockhart, Davidson, Dawson, and Stallknecht, 1995; Lockhart, Davidson, Stallknecht, and Dawson, 1995).

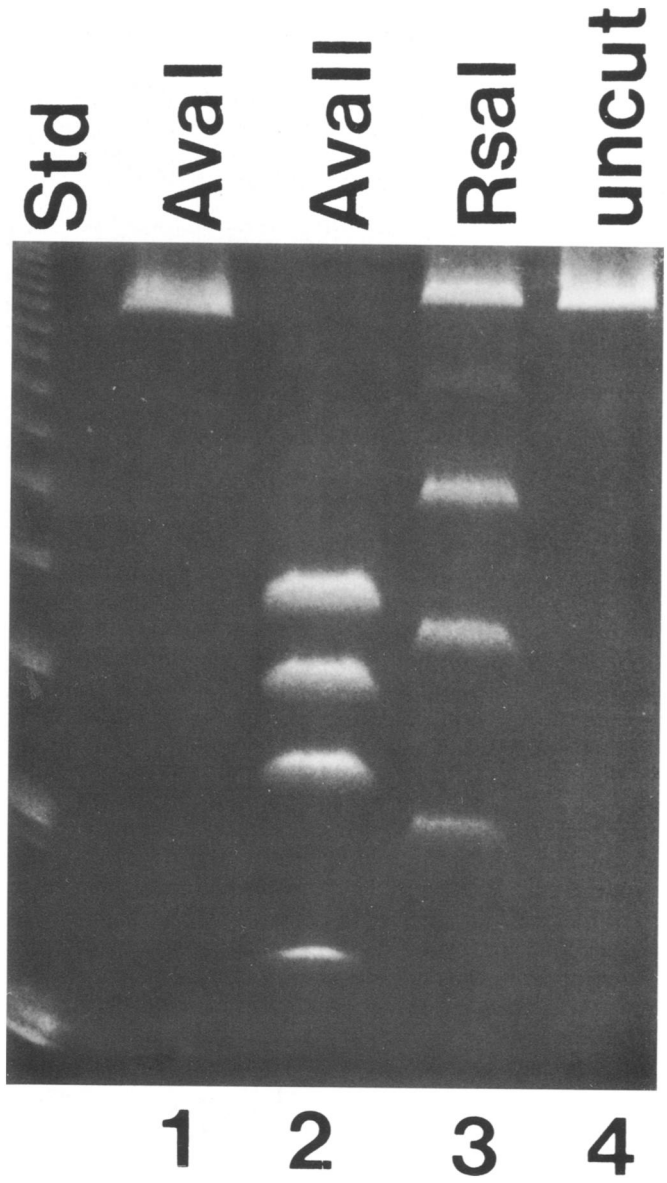

FIGURE 3. With the exception of 2 low molecular weight fragments (*RsaI* 16 kDa; *AvaI* 89 kDa) all predicted fragments for the WTD agent were present. These results represent a reasonable confirmation of the theoretical restriction map.

If *A. americanum* is the vector of this agent, as implied by the temporal association between the appearance of *A. americanum* and seropositive WTD (Lockhart, Davidson, Dawson, and Stallknecht 1995), the possibility of a zoonotic infection should be examined because larval, nymphal, and adult *A. americanum* readily bite humans. Although the vector of *E. chaffeensis* has not been determined with certainty, all epidemiologic, experimental tick transmission studies and PCR evidence point to *A. americanum.* Therefore, it may be determined in the future that *E. chaffeensis* and the deer agent are concurrent or even cotransmitted.

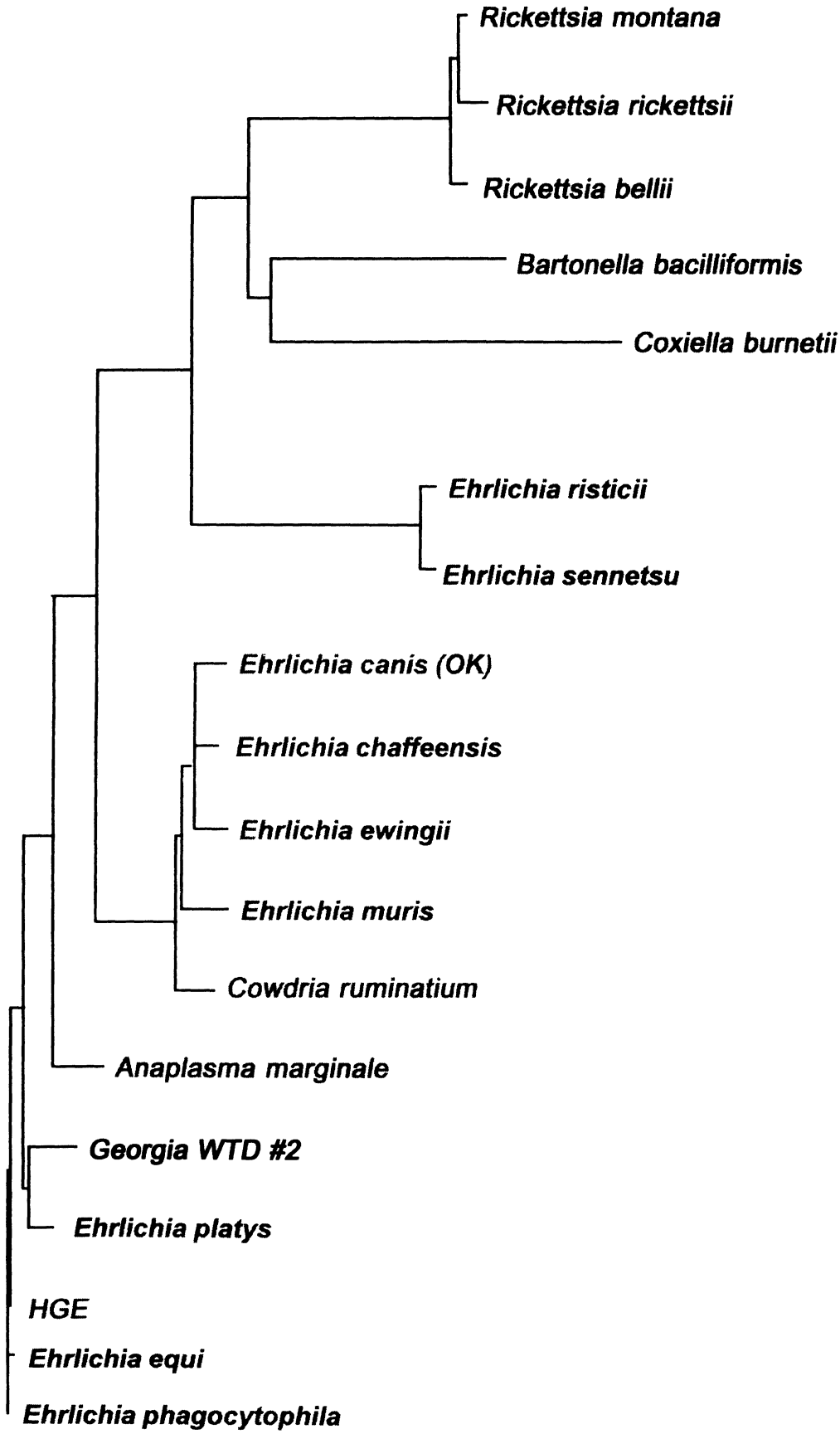

FIGURE 4. A phylogenetic tree constructed using GCG Growtree program depicts the relationship between the WTD agent and other closely related rickettsiae.

TABLE I. Distances between the nucleic acid sequences of various species in the order Rickettsiales were made using GCG Distances program.

	Ehrlichia risticii	*Ehrlichia sennetsu*	*Ehrlichia canis* (OK)	*Ehrlichia chaffeensis*	*Ehrlichia ewingii*	*Ehrlichia muris*	*Cowdria ruminatium*	*Ehrlichia equi*	*Ehrlichia phagocytophila*	Human granulocytic ehrlichiosis	Georgia WTD#2	*Ehrlichia platys*	*Anaplasma marginale*	*Rickettsia montana*	*Rickettsia rickettsii*	*Rickettsia bellii*	*Bartonella bacilliformis*	*Coxiella burnetii*
E. risticii	1.0000	0.9441	0.7987	0.8003	0.7979	0.8003	0.8028	0.8102	0.8110	0.8118	0.8118	0.8094	0.8085	0.7872	0.7880	0.7888	0.7576	0.7502
E. sennetsu		1.0000	0.8003	0.8012	0.7979	0.8020	0.8069	0.8102	0.8110	0.8118	0.8118	0.8077	0.8085	0.7880	0.7872	0.7896	0.7584	0.7527
E. canis (OK)			1.0000	0.9417	0.9367	0.9269	0.9269	0.8817	0.8825	0.8833	0.8718	0.8710	0.8792	0.7954	0.7946	0.7954	0.7707	0.7576
E. chaffeensis				1.0000	0.9400	0.9351	0.9293	0.8858	0.8866	0.8874	0.8718	0.8767	0.8784	0.7962	0.7954	0.7962	0.7732	0.7601
E. ewingii					1.0000	0.9302	0.9293	0.8809	0.8817	0.8825	0.8702	0.8710	0.8759	0.7921	0.7913	0.7921	0.7749	0.7601
E. muris						1.0000	0.9269	0.8792	0.8800	0.8800	0.8726	0.8751	0.8825	0.7913	0.7905	0.7913	0.7716	0.7609
C. ruminatium							1.0000	0.8833	0.8841	0.8858	0.8759	0.8751	0.8767	0.8003	0.7995	0.8012	0.7740	0.7576
E. equi								1.0000	0.9589	0.9581	0.9376	0.9408	0.9195	0.7970	0.7987	0.7962	0.7864	0.7658
E. phagocytophila									1.0000	0.9589	0.9384	0.9417	0.9203	0.7979	0.7995	0.7970	0.7872	0.7666
HGE										1.0000	0.9400	0.9417	0.9219	0.7995	0.8012	0.7987	0.7888	0.7683
GA WTD#2											1.0000	0.9376	0.9195	0.7896	0.7913	0.7913	0.7880	0.7650
E. platys												1.0000	0.9170	0.7888	0.7905	0.7880	0.7888	0.7642
A. marginale													1.0000	0.7979	0.7995	0.7970	0.7814	0.7724
R. montana														1.0000	0.9507	0.9441	0.7962	0.7642
R. rickettsii															1.0000	0.9466	0.7987	0.7658
R. bellii																1.0000	0.7995	0.7617
B. bacilliformis																	1.0000	0.7683
C. burnetii																		1.0000

The fact that WTD are seroreactive to the human pathogen, *E. chaffeensis,* is especially noteworthy. In the future individuals who are seropositive for *E. chaffeensis* but PCR negative should be examined for this *Ehrlichia* agent.

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