

Molecular Variation in the Variable-Length PCR Target and 120-Kilodalton Antigen Genes of *Ehrlichia chaffeensis* from White-Tailed Deer (*Odocoileus virginianus*)

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Genes encoding two surface-expressed antigens of *Ehrlichia chaffeensis*, the variable-length PCR target (VLPT) and the 120-kDa antigen, which contain variable numbers of tandem repeats, were characterized for *E. chaffeensis* from white-tailed deer (*Odocoileus virginianus*). Both genes from infected deer contained numbers of repeats similar to those reported in genes from humans and ticks, although a new variant of the 120-kDa antigen gene containing five repeat units and coinfection with multiple VLPT and 120-kDa antigen gene genetic types were detected. Sequence analysis of both genes revealed more nucleotide variation than previously reported for *E. chaffeensis* from infected humans or ticks. This is the most extensive study of *E. chaffeensis* VLPT and 120-kDa antigen gene genetic variation to date and is the first to examine genetic variation in *E. chaffeensis* from a nonhuman vertebrate host.

Ehrlichia chaffeensis, which causes human monocytotropic ehrlichiosis (HME), is an important emerging tick-borne pathogen in the southeastern and south-central United States. *E. chaffeensis* is maintained in a zoonotic cycle involving white-tailed deer (WTD; *Odocoileus virginianus*) as the principal vertebrate reservoirs and lone star ticks (*Amblyomma americanum*) as biological vectors. Severity of disease following infection varies from asymptomatic to fatal, with the most common clinical signs being fever, headache, nausea, and malaise; leucopenia and thrombocytopenia are often present (5). Since the discovery of HME in 1986, over 1,100 cases have been reported to the Centers for Disease Control and Prevention (2).

The variable-length PCR target (VLPT) and 120-kDa antigen genes of *E. chaffeensis* contain variable numbers of tandem repeats. The VLPT gene contains three to six 90-bp repeat units, and the 120-kDa antigen gene contains two to four 240-bp repeat units. In addition, the VLPT gene has variations at the nucleotide level consisting of (i) single-base substitutions at four specific locations, (ii) presence or absence of an aspartic acid codon deletion, and (iii) a 9-bp deletion. Sequencing the repeat units in the VLPT gene has revealed seven distinct amino acid repeat profiles (16). Currently, the function of the VLPT protein is unknown, but the greater number of repeats compared with the number for the 120-kDa antigen gene makes the VLPT gene useful for strain differentiation. Apparent geographic clustering of genetic types noted in a previous survey of ticks (15) led to the suggestion that there may be a geographic correlation with repeat number (11).

The 120-kDa antigen gene encodes an immunodominant surface protein that is mainly expressed on dense-core stages of *E. chaffeensis* and that is released into the intramolecular fibrillary matrix (13). Expression of this gene in *Escherichia coli* causes attachment and internalization by Vero cells, suggesting an important role for this protein in attachment and invasion of host cells by *E. chaffeensis* (13). The first repeat unit of the 120-kDa antigen gene contains four nucleotide substitutions that make it different from the other repeats, which are all identical in sequence (19).

Both the VLPT and 120-kDa antigen genes have been amplified from infected human and tick samples but have not been found in infected WTD. The objectives of this study were to (i) characterize the VLPT and 120-kDa antigen genes of *E. chaffeensis* from WTD and (ii) test the hypothesis that genetic types of *E. chaffeensis* are geographically clustered.

Sample collections. A total of 11 culture isolates and 91 infected whole-blood samples from WTD were utilized in this study. *E. chaffeensis* samples from WTD were obtained during previous studies conducted at the Southeastern Cooperative Wildlife Disease Study (8, 9, 10, 18, 18a).

Molecular analysis. DNA from 300 μ l of whole blood was extracted with the GFX genomic blood DNA purification kit (Amersham Pharmacia Biotech, Piscataway, N.J.) by following the manufacturer's protocol. PCR assays for the VLPT and 120-kDa antigen genes were performed as described previously (16, 20). Stringent protocols and controls were utilized to prevent and assay for contamination. DNA extraction, primary amplification, secondary amplification, and product analysis were performed in separate dedicated laboratory areas. Water controls were included in each set of DNA extractions, and one water control was included in each set of primary and secondary PCRs.

Representative products were purified with a Microcon spin

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TABLE 1. Summary of VLPT and 120-kDa antigen gene genetic types of *E. chaffeensis* detected in WTD from 12 southeastern and south central states

State	VLPT		120-kDa antigen	
	No. of WTD positive	Repeat variant(s) detected in population (no. of each repeat type)	No. of WTD positive	Repeat variant(s) detected in population (no. of each repeat type)
Arkansas	9	4 (2), 5 (7)	7	3 (2), 4 (5)
Florida	9	4 (8), 5 (1)	5	3 (2), 4 (3)
Georgia	30	4 (17), 5 (8), 3 + 5 (1), 4 + 5 (3), 3 + 4 + 5 (1)	23	3 (12), 4 (7), 5 (3), 3 + 4 (1)
Kansas	3	3 (1), 4 (1), 5 (1)	2	4 (2)
Kentucky	2	4 (1), 5 (1)	1	4 (1)
Louisiana	1	5 (1)	0	
Maryland	1	4 (1)	0	
Mississippi	1	5 (1)	1	4 (1)
Missouri	3	4 (2), 5 (1)	2	3 (1), 4 (1)
North Carolina	15	4 (12), 5 (2), 4 + 5 (1)	9	2 (1), 3 (3), 4 (5)
South Carolina	5	4 (1), 5 (3), 4 + 6 (1)	4	3 (1), 4 (2), 3 + 4 (1)
Virginia	23	4 (13), 5 (5), 4 + 5 (5)	19	3 (6), 4 (12), 3 + 4 (1)

filter (Amicon Inc., Beverly, Mass.) and sequenced at MWG-Biotech (High Point, N.C.), and the resultant sequences were compared to published sequences in the GenBank database. Samples with multiple amplicons were purified as described above and separated in 1.5% agarose, and each amplicon was separately purified with the Qiaquick gel extraction kit (Qiagen, Valencia, Calif.). Because enough template was not present in infected whole-blood samples to sequence the VLPT primary product, a heminested PCR was developed in order to obtain a larger sequence fragment of the VLPT gene. Four whole-blood samples (E937, E990, E915, and E521) were subjected to the heminested PCR using primers FB5A and FB3A in a primary reaction and FB5 and FB3A in a secondary reaction. Thermocycler conditions for the heminested PCR were 94°C for 1 min, 52°C for 90 s, and 70°C for 90 min for 36 cycles, followed by 68°C for 5 min.

VLPT antigen gene analysis. *E. chaffeensis* from WTD exhibited heterogeneity in the number of repeats ($n = 3, 4, 5,$ or 6) present in the VLPT antigen gene (Table 1). Each culture isolate of *E. chaffeensis* contained either a single four- or five-repeat amplicon except for one culture isolate (601-5; Jones County [Co.], Ga.) which had amplicons corresponding to three, four, and five repeats. All 91 infected WTD whole-blood samples were positive by VLPT PCR. Eighty of 91 (87.9%) WTD had single amplicons corresponding to three ($n = 1$ WTD), four ($n = 54$), and five repeats ($n = 25$). The remaining 11 WTD had two amplicons corresponding to three and five repeats ($n = 1$ WTD), four and five repeats ($n = 9$), or four and six repeats ($n = 1$).

Several populations of WTD were coinfecting with multiple genetic variants of the VLPT gene (e.g., two variants were detected in four infected deer from Gwinnett Co., Ga., two variants were found in seven deer from Chatham Co., Ga., and two variants were found in eight infected deer from Culpepper Co., Va.). As mentioned earlier, 11 individual deer were coinfecting with two variants. In contrast, several other locations with multiple infected deer contained the same variant (e.g., four deer with four repeats in Albemarle Co., Va., six deer with four repeats in Dare and Hyde Co., N.C., and four deer with four repeats in Wakulla Co., Fla.).

The profiles of sequence repeat units were similar to those

reported for human and tick samples (Table 2). All four-repeat-unit samples were 1,2,3,4, the single three-repeat-unit sample had a deletion of the third unit type (1,2,5), and the five-repeat-unit samples had an addition of a fifth unit type or a repeat of unit type 3 (i.e., 1,2,3,4,5 or 1,2,3,3,4). A nucleotide substitution (C for a T) at position 247 in three WTD samples (from Georgetown Co., S.C., Yazoo Co., Miss., and Culpepper Co., Va.) resulted in a substitution of a proline for a serine. This substitution created an eighth unique repeat type unit, which is most similar to the second repeat type unit. Substitutions (A or G) were noted at positions -69, 6, 27, and 487, but only 3 of 31 (9.7%) WTD contained the nucleotide guanosine at position 6. A deletion within the 9-base gap region (5'-GT TTTATAT) of a single WTD sample compared with infected human or tick samples (16) was noted; the first two thymidines (643 and 644) within the gap region were deleted from a culture isolate of *E. chaffeensis* from a deer from Greene Co., Ark.

120-kDa antigen gene analysis. Similar to what was found for the VLPT antigen gene, various numbers of repeats (two to five) in the 120-kDa antigen gene were observed for *E. chaffeensis* from WTD (Table 1). Each culture isolate contained a single amplicon corresponding to three, four, or five repeats, with one exception, an isolate from Georgetown Co., S.C. (E886) which had amplicons corresponding to both three and four repeats. Only 62 of 91 (68.1%) infected blood samples were positive for the 120-kDa antigen gene. Single 120-kDa antigen gene amplicons corresponding to two, three, four, and five repeats were detected in 1, 24, 33, and 2 WTD, respectively. Two WTD had two amplicons corresponding to three and four repeats. One deer from Putnam Co., Ga., with two 120-kDa antigen gene amplicons was also found to have two amplicons by the VLPT PCR (four and five repeats).

Sequence analysis of a 1,311-bp fragment of the 120-kDa antigen gene (OSLN2 isolate) revealed a series of five tandem 240-bp repeat units. The five units differed by 5 nucleotides at positions 46, 138, 146, 202, and 232 from the beginning of the repeat region, resulting in two amino acid substitutions (Table 3). Each repeat unit of the OSLN2 isolate differed at between 2 and 4 nucleotides from each repeat unit of the 120-kDa antigen genes of *E. chaffeensis* in the GenBank database (ac-

TABLE 2. Summary of VLPT sequence variation for *E. chaffeensis* from WTD

Sample	Location (co., state)	No. of repeats	Repeat profile	Nucleotide (A or G) at position:				Aspartic acid deletion	Presence of 9-base gap
				-69	6	27	487		
15B ^a	Clarke, Ga.	4	1,2,3,4	A	A	G	G	No	No
40S ^a	Clarke, Ga.	4	1,2,3,4	A	A	G	G	No	No
22B ^a	Jasper, Ga.	5	1,2,3,4,5	ND ^c	A	A	ND	Yes	ND
OSLN2 ^b	Chatham, Ga.	4	1,2,3,4	A	A	G	G	No	No
604-2 ^c	Greene, Ark.	5	1,2,3,3,4	A	A	G	G	No	No
604-5	Greene, Ark.	5	1,2,3,4,5	G	G	A	A	Yes	No ^d
628-5	Phillips, Ark.	5	1,2,3,3,4	G	A	G	G	No	No
543-6	Phillips, Ark.	5	1,2,3,3,4	ND	A	G	ND	No	ND
E937	Levy, Fla.	4	1,2,3,4	ND	A	G	G	No	No
E990	Taylor, Fla.	5	1,2,3,4,5	ND	A	A	A	Yes	No
H02-66	Chatham, Ga.	5	1,2,3,4,5	ND	A	A	ND	Yes	ND
H02-68	Chatham, Ga.	4	1,2,3,4	ND	A	G	ND	No	ND
H02-68	Chatham, Ga.	3	1,2,4	ND	A	G	ND	No	ND
H02-72	Chatham, Ga.	5	1,2,3,4,5	ND	A	A	ND	Yes	ND
125B	Clarke, Ga.	4	1,2,3,4	A	A	G	A	No	No
E914	Gwinnett, Ga.	5	1,2,3,3,4	ND	A	G	ND	No	ND
E915	Gwinnett, Ga.	4	1,2,3,4	ND	A	G	A	No	No
601-5	Jones, Ga.	3	1,2,4	A	A	G	G	No	No
601-5	Jones, Ga.	5	1,2,3,3,4	A	A	G	G	No	No
E905	Lowndes, Ga.	4	1,2,3,4	ND	A	G	ND	No	ND
E897	Washington, Ga.	4	1,2,3,4	ND	A	G	ND	No	ND
E521	Jefferson, Kans.	5	1,2,3,4,5	ND	A	A	A	Yes	Yes
529-3	Madison, La.	5	1,2,3,3,4	ND	ND	G	ND	No	ND
E890	Kent, Md.	4	1,2,3,4	ND	A	G	ND	No	ND
606-1	Yazoo, Miss.	5	1,8,3,4,5	ND	ND	A	ND	Yes	ND
623-4	Anson, N.C.	5	1,2,3,4,5	ND	G	A	A	Yes	Yes
590-4	Dare, N.C.	4	1,2,3,4	ND	A	G	ND	No	ND
CC377-02	Johnston, N.C.	4	1,2,3,4	ND	A	G	ND	No	ND
E886	Georgetown, S.C.	5	1,8,3,4,5	G	G	A	A	No	Yes
CC228-02	Buckingham, Va.	5	1,2,3,4,5	ND	A	A	ND	Yes	ND
E1058	Culpepper, Va.	4	1,2,3,4	ND	A	G	ND	No	ND
E1059	Culpepper, Va.	5	1,8,3,4,5	ND	A	A	ND	Yes	ND
CC343-02	Cumberland, Va.	4	1,2,3,4	ND	A	G	ND	No	ND

^a Culture isolates from reference 9.

^b Culture isolate from reference 8.

^c Remaining samples from reference 18a.

^d The first two thymidines (T₆₄₃ and T₆₄₄) of the 9-base region were deleted.

^e ND, not determined.

cession numbers U49426, U74670, and AF474890 to AF47499).

This study reports the first molecular characterization of *E. chaffeensis* from WTD, the principal vertebrate reservoir host. Similar to what was found in previous studies of human and tick samples of *E. chaffeensis*, we detected variable numbers of repeat units in both the VLPT and 120-kDa antigen genes and also additional sequence variations in the VLPT antigen gene. *E. chaffeensis* has been isolated from only two other hosts, a

domestic goat (*Capra capra*) from Clarke Co., Ga. (4), and a red ruffed lemur (*Varecia variegata rubra*) from Durham Co., N.C. (17), although molecular evidence of infection has been detected in domestic dogs and coyotes (*Canis latrans*) (3, 6). The genetic types of *E. chaffeensis* infecting the coyotes, domestic dogs, and lemur are unknown, but a fragment of the VLPT gene from a goat isolate from Clarke Co., Ga., was identical in sequence to those from WTD isolates from Clarke Co. tested during this study (E. J. Sims and M. J. Yabsley,

TABLE 3. Nucleotide and amino acid differences for the units of the novel five-repeat genetic variant of *E. chaffeensis*

Repeat unit	Nucleotide at position ^a :					Amino acid at position ^b :				
	46	138	146	202	232	15	46	49	67	77
1	G	A	A	C	T	K	K	M	L	N
2	A	— ^c	—	—	—	—	—	—	—	—
3	—	—	—	—	—	—	—	—	—	—
4	—	G	G	A	C	—	R	V	—	—
5	—	G	G	—	C	—	R	V	—	—

^a Nucleotide numbering begins at first nucleotide of repeat unit 1 of OSLN2.

^b Deduced amino acid sequence numbers based on open reading frame of U49426; the deduced sequence begins at the second nucleotide of repeat unit 1.

^c —, sequence identity to the first repeat.

unpublished data). In contrast, the 120-kDa antigen gene of the goat isolate differed from WTD *E. chaffeensis* samples collected from Clarke Co.; the goat isolate contained four repeats (M. J. Yabsley, unpublished data), while the WTD samples contained three repeats ($n = 1$) or four repeats ($n = 2$).

For both the VLPT and 120-kDa antigen genes, only a single genetic type has been detected in individual infected humans or in individual ticks. A single tick pool containing 10 ticks tested by Sumner et al. (16) had two amplicons, a strong one corresponding to three repeat units and a faint one for four repeat units; however, because of pooling, coinfection of a single tick could not be confirmed. In this study, 11.8 and 2.7% of individual WTD were infected with at least two genetic variants of the VLPT and 120-kDa antigen genes, respectively.

The four- and five-repeat types of the VLPT gene were the most commonly detected genetic variant in WTD, similar to findings in other studies of humans and ticks (11, 14, 16). The three- and six-repeat types of the VLPT gene were the least common variants observed in this study; these variants have been found in infected humans on five occasions and one occasion, respectively. The three-repeat VLPT type has been found in humans only in Georgia, Tennessee, and Nebraska (1, 12); however, in the present study this genetic variant was found in WTD from Kansas, thereby extending the geographic distribution of this genetic type. Similarly, the six-repeat VLPT profile has been found only once, in a human from Wakulla Co., Fla. (16). In this study, four infected WTD from Wakulla Co. were infected with *E. chaffeensis* having the four-repeat variant and none were infected with the six-repeat variant; however, we detected a WTD infected with the six-repeat variant in Florence County, S.C. Because the four- and five-repeat variants were detected in all states examined in this study and because multiple genetic variants were detected in single populations and single deer, it appears unlikely that there is a specific geographic distribution of the VLPT genetic variants. Additional studies are needed to fully understand the importance of the VLPT protein and any corresponding distribution of genetic variants.

At the nucleotide level, the VLPT antigen gene of *E. chaffeensis* from WTD was similar to isolates from humans or ticks, but some differences were noted. A single nucleotide substitution resulted in a unique VLPT repeat type unit; thus there are now eight described repeat type units, with two types (types 6 and 7) having been reported only once each from an infected human and tick, respectively (16). Single adenosine and guanosine substitutions were detected at four previously reported A or G variable positions; however, only 3 of 31 (9.7%) WTD samples had a G at position 6 while 41% of human isolates (9, 19) and 25% of tick samples contained a G at this position (16). Previously in human isolates, the presence of the aspartic acid deletion was associated with the presence of the 9-base gap; however, no association was noted for infected-tick pools (16). Similar to findings for *E. chaffeensis* from ticks, there was no association for *E. chaffeensis* from WTD between the aspartic acid deletion and the presence of the 9-base gap. The 9-base gap region of the *E. chaffeensis* VLPT gene, located 45 nucleotides downstream from the putative stop codon, from a single infected WTD contained a 2-nucleotide deletion not previously reported. The significance

of these variations is unknown, but more variation is present in this gene than previously reported.

As with the VLPT antigen gene, variable numbers of repeats were detected in the 120-kDa antigen gene of *E. chaffeensis* from WTD. A new variant of the 120-kDa antigen gene with five repeats was detected, indicating that there also is a broader range of variation in this gene than previously reported. Numerous nucleotide substitutions were noted in the repeat region of a single novel five-repeat variant, resulting in several amino acid substitutions, the importance of which is unknown. This novel 120-kDa five-repeat variant was detected only in a single population of WTD located on Ossabaw Island in Chatham Co., Ga. Two genetic variants of the 120-kDa antigen gene were detected on Ossabaw Island, where two deer were infected with the novel five-repeat variant and two other deer were infected with the three-repeat variant. This new 120-kDa antigen gene five-repeat variant was not detected in 36 other populations, including other coastal populations. Additional testing of WTD from barrier islands may reveal additional populations infected with this genetic variant or possibly additional new variants.

The finding of multiple genetic variants of the VLPT and 120-kDa antigen genes in individual deer is interesting because it suggests that, for *E. chaffeensis*, infection of individuals with multiple genetic variants is not excluded. The finding of only one genetic variant of *E. chaffeensis* in humans but not in WTD may be related simply to the number of potentially infectious ticks parasitizing an individual. Deer commonly are infested with hundreds to thousands of ticks, while a human generally is only infested with one or a few ticks, which decreases the chance of exposure to multiple genetic types of *E. chaffeensis*. Sequential infections in humans are possible based on a report of reinfection. Two years after a liver transplant patient recovered from an infection with *E. chaffeensis*, a second infection with a genetically distinct variant of *E. chaffeensis* was detected (7). The above data suggest that either the immune response to one genetic type of *E. chaffeensis* is insufficient to prevent infection with another genetic variant or that neither the VLPT nor 120-kDa antigen is involved in clearance or prevention of reinfection with *E. chaffeensis*.

Although this study was not conducted to examine the sensitivity of the primer sets used, amplification of *E. chaffeensis* was more often successful with the VLPT primers than with the 120-kDa antigen gene primers, possibly because of the increased size of this 120-kDa antigen gene PCR target or sample degradation. Inability to amplify multiple gene targets from single samples has been reported in a survey of tick vectors (15).

The significance of the 120-kDa and VLPT proteins to the relative virulence of different strains of *E. chaffeensis* infecting humans is not understood. However, inclusion of reservoir source organisms in the comparison of *E. chaffeensis* isolates provides a more comprehensive view of the intraspecific variation present in this pathogen. Further studies of the genetic variations of the VLPT, 120-kDa antigen, and other genes in human, tick, and reservoir populations will help in understanding the epizootiology and pathogenicity of *E. chaffeensis*.

Nucleotide sequence accession numbers. The 33 VLPT sequences obtained in this study have been submitted to the GenBank database under accession numbers AY307329 to

AY307353. The accession number for the OSLN2 isolate 120-kDa antigen gene sequence is AY307354.

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