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TWO THEILERIA CERVI SSU rRNA GENE SEQUENCE TYPES FOUND IN ISOLATES FROM WHITE-TAILED DEER AND ELK IN NORTH AMERICA

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4 Corresponding author (e-mail: pholman@cvm.tamu.edu)

ABSTRACT: Two Theileria cervi SSU rRNA gene sequence Types, F and G, from white-tailed deer (Odocoileus virginianus) and elk (Cervus elaphus canadensis) isolates in North America were confirmed. Previously, nucleotide sequencing through a single variable (V4) region showed the presence of SSU rRNA gene Types F and G in T. cervi isolates from white-tailed deer and elk. In this study, both sequence types were found in four T. cervi isolates (two from deer and two from elk). Microheterogeneity only appeared in the Type G gene, resulting in Subtypes G1, G2 and G3. Subtype G1 was found in two elk and one white-tailed deer T. cervi isolate; Subtypes G2 and G3 were found in a white-tailed deer T. cervi isolate. The Type F SSU rRNA genes were identical in nucleotide sequence in both elk and white-tailed deer T. cervi isolates. The high degree of conservation in the Type F variable regions may be exploited to design specific oligonucleotide primers for parasite detection by the polymerase chain reaction in cervine or tick hosts.

Key words: Cervus elaphus canadensis, elk, gene amplification, nucleotide sequence, Odocoileus virginianus, small subunit ribosomal RNA gene, Theileria cervi, white-tailed deer.

INTRODUCTION

Theileria spp., the causative agents of theileriosis, are cosmopolitan hemoprotozoan parasites of domestic and wild mammals. The prevalence of the organism is dependent upon the geographic range of the vector tick. Some Theileria spp., such as Theileria parva and Theileria annulata of cattle in Africa, are highly pathogenic and infections may result in devastating losses. Other less pathogenic species, such as Theileria sergenti of cattle in the Middle East, cause mild to moderate clinical theileriosis. In the United States, the Theileria spp. infecting domestic and wild ruminants are generally considered benign. Specifically, Theileria cervi infections in deer are generally considered nonpathogenic, although clinical disease does occur in hosts debilitated by other parasitic burdens or malnutrition (Kocan and Kocan, 1991).

In 1962 the Theileria sp. found in white-tailed deer (Odocoileus virginianus) in the United States was designated Theileria cervi (Schaeffler, 1961, 1962). Theileria cervi has since been identified in white-tailed deer in Texas, Oklahoma, Missouri, Arkansas, and Alabama (reviewed by Kingston, 1981). Theileria sp. organisms indistinguishable from T. cervi also have been reported in mule deer (Odocoileus hemionus) and in exotic axis (Axis axis) and sika (Cervus nippon nippon) deer in Texas (Laird et al., 1988; Waldrup et al., 1989). The primary tick vector, Amblyomma americanum, is widespread throughout the southeastern USA, and T. cervi probably occurs throughout the range of the vector tick (Kocan and Kocan, 1991).

Identification of the benign Theileria spp. of both deer and cattle in the USA has been primarily based on the mammalian host of origin. Not only are T. cervi and the bovine Theileria sp. morphologically indistinguishable, but serologic cross-reactions occur and infections with either parasite result in mild or non-existent clin-
Table 1. Summary of Theileria cervi isolates including year of acquisition, mammalian host source, geographic origin, health status of host regarding clinical signs attributable to Theileria cervi infection, Giemsa stained blood film observations, and SSU rRNA gene types identified in each.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Year</th>
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</tr>
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<tbody>
<tr>
<td>CNElk</td>
<td>1994</td>
<td>Free-ranging elk, Canada</td>
<td>No</td>
<td>Theileria sp.</td>
<td></td>
<td>F, G, G1</td>
</tr>
<tr>
<td>OKELK1</td>
<td>1998</td>
<td>Free-ranging elk, Oklahoma</td>
<td>No</td>
<td>Theileria sp.</td>
<td></td>
<td>F, G1</td>
</tr>
<tr>
<td>USWTD1</td>
<td>1994</td>
<td>Farmed WTD*, Texas</td>
<td>Yes</td>
<td>T. cervi</td>
<td></td>
<td>F, G1</td>
</tr>
<tr>
<td>USWTD2</td>
<td>1996</td>
<td>Farmed WTD, Texas</td>
<td>Yes</td>
<td>T. cervi</td>
<td></td>
<td>F</td>
</tr>
<tr>
<td>USWTD3</td>
<td>1998</td>
<td>Wild-caught WTD, Oklahoma</td>
<td>No</td>
<td>T. cervi</td>
<td></td>
<td>F, G2, G3</td>
</tr>
</tbody>
</table>

*WTD = white-tailed deer.

A male white-tailed deer housed at Oklahoma State University (Stillwater, Oklahoma, USA) was found by routine examination of a Giemsa-stained blood smear to be infected with *T. cervi* (designated USWTD3). The animal originated from Payne County (35°50'N, 97°00'W), Oklahoma, and was 1-yr-old when the blood sample was drawn. During a routine epidemiological survey, a 2-yr-old bull elk (*Cervus elaphus canadensis*) residing at the Spavinaw Game Management Area (Spavinaw, Oklahoma; 36°20'N, 95°60'W) was found infected with *Theileria* sp. organisms (OKELK1). Two additional *T. cervi* isolates (USWTD1 and USWTD2) from Texas white-tailed deer and a Canadian elk *Theileria* sp. isolate (CNElk) used in this study have been previously described (Chae et al., 1998). A summary of the *Theileria* spp. isolates used in this study is given in Table 1.

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Plasmid DNA for each selected clone was purified from overnight broth cultures by a modified alkaline lysis procedure (QIAPrep Spin Miniprep Kit; Qiagen®, Valencia, California, USA).

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nia, USA). The plasmid DNA samples were then used to identify both Type F and Type G clones for further study. Sequencing reactions for each plasmid DNA sample (Dye Terminator Cycle Sequencing Ready Reaction; PE Applied Biosystem, Norwalk, Connecticut, USA) used primer 528F to determine the SSU rRNA gene V4 variable region nucleotide sequence as previously described (Chae et al., 1998). Sequencing was carried out in either an ABI PRISM Model 373A or ABA Model 377 automated sequencer with Version 1.2.2 or Version 2.1.1 software, respectively (Gene Technologies Laboratory, Institute of Developmental and Molecular Biology, Department of Biology, Texas A&M University, College Station, Texas, USA).

Plasmid clones containing the entire SSU rRNA genes from USWTD1, USWTD2, and CNELK from previous work (Chae et al., 1998) were used for complete gene sequencing for these isolates. Since previously only one plasmid clone containing the WTD2 SSU rRNA gene insert was obtained (Chae et al., 1998), transformation of the original ligated plasmid into INVaeF' One Shot® E. coli was repeated. Putative transformed colonies were selected and tested by colony amplification as described above. Those with the correct-sized insert were then checked by sequence analysis of the SSU rRNA gene V4 variable region.

The complete forward and reverse strands of clones of SSU rRNA gene Type F in USWTD1, USWTD2, and CNELK were sequenced. The Type G genes from CNELK and USWTD1, Subtype G1 (USWTD1 and CNELK) and Subtype G2 genes (USWTD3) also were sequenced. A primer complementary to the T7 promotor region of the plasmid vector (Stratagene®, La Jolla, California, USA) and a series of previously described internal primers (Elwood et al., 1989) were used for automated sequencing reactions and sequencing was carried out as described above. The CLUSTAL W (Version 1.60) multiple sequence alignment program (Thompson et al., 1994) and MACAW multiple alignment construction and analysis workbench (Version 2.05 Win 32I; Schuler et al., 1991) were used to facilitate sequence alignment and comparison. Sequence Types F and G and Subtype G1 also were aligned with corresponding Theileria spp. SSU rRNA gene sequences from the GenBank database including T. buffeli (GenBank Accession No. Z15106; Allsopp et al., 1994), T. annulata (GenBank Accession No. M64243, M34845; Gajadhar et al., 1991), T. parva 18S rRNA gene (GenBank Accession No. L02366; Allsopp et al., 1993), T. parva 16S rRNA gene (GenBank Accession No. L28999; Kibe et al., 1994), T. taurotragi (GenBank Accession No. L19082; Allsopp et al., 1994) and identity values were obtained by the ALIGN program (GeneStream, Centre de Recherche en Biochimie Macromoleculaire, Montpelier, France).

Nucleotide sequence data reported in this paper have been submitted to the GenBank® database with the accession numbers U97054, U97055, U97056, AF86804, AF86805.

RESULTS

Amplification with SSU rRNA gene primers A and B resulted in a single band of approximately 1.8 kb for the Theileria spp. isolates USWTD3 and OKELK1 as observed by ethidium bromide stained agarose gel electrophoresis (not shown). Theileria cervi SSU rRNA gene sequence Types F (GenBank Accession No. U97054) and G Subtypes G1, G2 and G3 (GenBank Accession Nos. U97056, AF086804, and AF086805, respectively) were identified from these isolates (Table 1). Three additional plasmid clones from USWTD2 were identified with the correct-sized insert. These inserts were determined to be Type F by SSU rRNA gene V4 variable region sequence analysis.

Entire SSU rRNA gene sequences for Types F (USWTD1, USWTD2, and CNELK) and G (GenBank Accession No. U97055) (USWTD1 and CNELK) and Subtypes G1 (CNELK and USWTD1) and G2 (USWTD3) were obtained (Fig. 1). Partial gene sequences were obtained for Type F (USWTD3 and OKELK1) and Subtype G3 (USWTD3). USWTD3 and OKELK1 Type F sequences (3 clones checked for each) were identical to the V4 variable region sequences of USWTD1, USWTD2, and CNELK. Subtype G3 was found in three clones from the USWTD3 isolate, but as it differed from G2 only in one nucleotide position (635) in the V4 region (Fig. 1) the entire gene was not sequenced. The T. cervi Type F sequence, based on alignment of the V4 variable region, was found in all isolates tested (USWTD1, USWTD2, USWTD3, CNELK and OKELK1); T. cervi Type G was found in USWTD1 and CNELK (Table 1). Sub-
FIGURE 1. Nucleotide sequence of Theileria cervi SSU rRNA gene types and subtypes aligned with the Theileria parva SSU rRNA gene nucleotide sequence. Gaps represented by dashes (-) were introduced into the aligned sequences to maximize homology. Nucleotide positions differing from those of T. parva are designated by bold type. The V4 variable region is delineated by arrows (nucleotide positions 621-661). Gaps where the sequence is identical in all genes are designated by dotted lines (-----). The unsequenced portion of Subtype G3 is shown by a broken line (---). The SSU rRNA gene sequence shown for T. parva was obtained from the GenBank database, Accession Number M67476. Theileria cervi SSU rRNA gene GenBank Accession Numbers are as follows: Type F, U97054; Type G, U97055; Subtype G1, U97056; Subtype G2, AF86804; Subtype G3, AF86805.

Type G1 was found in both deer and elk isolates, USWTD1 and OKELK1. Subtypes G2 and G3 were identified in USWTD3. The total SSU rRNA gene lengths were: Type F, 1748 bp; Type G, 1750 bp; Subtype G1, 1750 bp; Subtype G2, 1748. G1, G2, and G3 are considered subtypes of...
Table 2. Number of nucleotide position differences among the *Theileria cervi* SSU rRNA gene types. Top matrix shows differences found in the entire gene sequence; bottom matrix shows differences found only in the V4 variable region of the gene (nucleotide positions 621–661).

<table>
<thead>
<tr>
<th>Nucleotide differences among <em>Theileria cervi</em> SSU rRNA gene types</th>
<th>Type</th>
<th>G</th>
<th>G1</th>
<th>G2</th>
<th>G3</th>
<th>F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type G</td>
<td>0</td>
<td>23</td>
<td>19</td>
<td>ND</td>
<td>34</td>
<td></td>
</tr>
<tr>
<td>G1</td>
<td>2</td>
<td>0</td>
<td>11</td>
<td>ND</td>
<td>45</td>
<td></td>
</tr>
<tr>
<td>G2</td>
<td>1</td>
<td>3</td>
<td>0</td>
<td>ND</td>
<td>54</td>
<td></td>
</tr>
<tr>
<td>G3</td>
<td>2</td>
<td>4</td>
<td>1</td>
<td>0</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>10</td>
<td>10</td>
<td>11</td>
<td>11</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

*ND = not done. Entire *Theileria cervi* G3 SSU rRNA gene not sequenced.*

Type G because they differ from G in only one or two nucleotide positions through the V4 region (nucleotide positions 623–663) (Fig. 1 and Table 2) and share sequence identity in nucleotide positions 1171–1174, 1199–1204, 1307–1309, 1319–1328, and 1334–1339 (Fig. 1). In contrast, Type F does not share sequence identity in these positions (Fig. 1) and also differs from Type G in 10 nucleotide positions through the V4 region (Fig. 1 and Table 2).

Differences in the V4 region sequences among the subtypes range from one nucleotide substitution between G and G2, and G2 and G3 to four substitutions between G1 and G3 (Table 2). Subtypes G2 and G3 share substitutions distinguishing them from G1: G2 and G3 have guanine (G) instead of thymidine (T) at position 657; G2 and G3 have cytosine (C) instead of thymidine (T) at 926 (Fig. 1).

Differences found in the entire gene sequences among the various *T. cervi* SSU rRNA genes are summarized in Table 2. Total Type G and Subtype G1 genes showed variation in 23 positions; Type G and Subtype G2 showed variation in 19 positions. Variation was only found in 11 positions between Subtypes G1 and G2. In contrast, differences at 33 positions were noted between Types F and G. No microheterogeneity was found among the Type F sequences.

Identity values determined among the *Theileria* spp. and Types F and G and Subtype G1 showed Type G and Subtype G1 to share the highest identity among the obtained sequences, 98.8% (Table 3). The percent identity between *T. cervi* Type F and Type G was 97.8. When compared to SSU rRNA gene sequences reported for other *Theileria* spp., identity values for Type F ranged from 96.7 to 97.5%, and for Type G from 95 to 95.6%. These percentages reflect 43–57 position differences in Type F and 76–96 position differences in Type G when compared to other *Theileria* spp. (Table 3). *Theileria cervi* SSU rRNA sequence Types F and G (and subtypes) were closest in sequence homology to that of *T. parva* (GenBank Accession Nos. L02366 and L28999).

**DISCUSSION**

SSU rRNA data indicate that both white-tailed deer and elk harbor *T. cervi*, as supported by this study and previous work (Chae et al., 1998). Complete nucleotide sequences from SSU rRNA genes amplified from cervine blood infected with *Theileria* spp. confirmed that two sequence Types, F and G, were present in the same isolate population. Microheterogeneity in Type G was observed in isolates from two white-tailed deer and two elk, and subtypes G1, G2, and G3 were designated. Considering that Type G SSU rRNA gene heterogeneity was the norm rather than the exception among the small number of isolates in this study, it is likely that additional divergent Type G sequences will be found among other *T. cervi* isolates. Thus, the Type G gene and G subtypes likely comprise a polymorphic family of G SSU rRNA genes.

SSU rRNA Type G or a G subtype gene was not found in one isolate, USWTD2. Amplification of the SSU rRNA gene from this isolate was very difficult and the original ligation and transformation yielded only one plasmid clone with the SSU
TABLE 3. ALIGN program sequence homology of *Theileria* spp. SSU rRNA genes. SSU rRNA gene sequences from the bovine *Theileria* spp., *Theileria annulata*, *Theileria parva*, and *Theileria taurotragi*, and from the cervine *Theileria* spp., *Theileria* sp. from sable antelope and *Theileria cervi* SSU rRNA gene Types F and G and Subtype G1. Upper matrix shows percent identity between sequences. Lower matrix shows the number of nucleotide differences in bold print and the number of overlapping nucleotides in parentheses.

<table>
<thead>
<tr>
<th>SSU rRNA Type</th>
<th>T. annulata</th>
<th>T. parva</th>
<th>T. taurotragi</th>
<th>Theileria sp.</th>
<th>T. buffeli</th>
<th>T. cervi-F</th>
<th>T. cervi-G</th>
<th>T. cervi-G1</th>
</tr>
</thead>
<tbody>
<tr>
<td>T. annulata</td>
<td>100 (1,744)</td>
<td>98.4</td>
<td>97.8</td>
<td>96.1</td>
<td>96.3</td>
<td>96.8</td>
<td>95.2</td>
<td>94.7</td>
</tr>
<tr>
<td>T. parva</td>
<td>28 (1,744)</td>
<td>100</td>
<td>98.5</td>
<td>96.6</td>
<td>96.6</td>
<td>97.5</td>
<td>95.6</td>
<td>95.2</td>
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<tr>
<td>T. taurotragi</td>
<td>39 (1,744)</td>
<td>27 (1,742)</td>
<td>100</td>
<td>96.6</td>
<td>96.4</td>
<td>97.0</td>
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<tr>
<td>Theileria sp.</td>
<td>68 (1,746)</td>
<td>59 (1,746)</td>
<td>62 (1,746)</td>
<td>100</td>
<td>96.8</td>
<td>96.7</td>
<td>95.2</td>
<td>94.8</td>
</tr>
<tr>
<td>T. buffeli</td>
<td>65 (1,746)</td>
<td>59</td>
<td>62 (1,746)</td>
<td>55</td>
<td>100</td>
<td>96.9</td>
<td>95.0</td>
<td>94.7</td>
</tr>
<tr>
<td>T. cervi-F</td>
<td>56 (1,724)</td>
<td>43 (1,740)</td>
<td>52 (1,746)</td>
<td>57 (1,746)</td>
<td>54 (1,740)</td>
<td>100</td>
<td>97.8</td>
<td>96.8</td>
</tr>
<tr>
<td>T. cervi-G</td>
<td>84 (1,748)</td>
<td>76 (1,748)</td>
<td>80 (1,748)</td>
<td>84 (1,748)</td>
<td>87 (1,748)</td>
<td>38 (1,748)</td>
<td>100</td>
<td>98.6</td>
</tr>
<tr>
<td>T. cervi-G1</td>
<td>92 (1,750)</td>
<td>83 (1,750)</td>
<td>88 (1,750)</td>
<td>91 (1,750)</td>
<td>93 (1,750)</td>
<td>56 (1,750)</td>
<td>24 (1,750)</td>
<td>100</td>
</tr>
</tbody>
</table>

* GenBank Accession Numbers for SSU rRNA gene sequences: *Theileria annulata*, M64243 and M34845; *Theileria parva*, L02366 and L28999; *Theileria taurotragi*, L19082, *Theileria* sp., L19082, *Theileria buffeli*, Z15106; *Theileria cervi* Type F, U97054; *Theileria cervi* Type G, U97055; *Theileria cervi* Subtype G1, U97056.

rRNA gene insert (Chae et al., 1998). In this study, bacterial transformation by the original ligated plasmid preparation was repeated in an effort to obtain additional plasmid clones to sequence. Only three more clones containing the gene were obtained and all three contained inserts of the Type F sequence. Unfortunately, additional DNA was not available to repeat the entire protocol. Clearly, there is a possibility that this isolate possessed other SSU rRNA gene types that we did not find.

The existence of multiple *T. cervi* SSU rRNA gene sequence types may represent genes from mixed populations of parasites or multiple copy units within a parasite as shown in *T. parva* (Kibe et al., 1994). In-as-much as both Types F and G (or G subtypes) were found in all isolates, with the exception of USWTD2, it appears likely that individual *T. cervi* parasites possess both gene types. It is not unlikely that different SSU rRNA genes may be expressed during different developmental stages of the *Theileria* life cycle. Transcript regulation of distinct stage specific SSU rRNAs has been shown in *Plasmodium* spp. (Li et al., 1997; Waters et al., 1989; Corredor and Enea, 1994). Further studies are needed to determine if both gene types are represented in multiple gene clusters in *T. cervi*, whether both types produce functional rRNAs, and whether transcription of different SSU rRNA gene types is stage dependent in *T. cervi*. It is also possible that the microheterogeneity observed in the Type G sequence reflects the presence of pseudogenes.

SSU rRNA Type G microheterogeneity previously reported based on V4 region data (Chae et al., 1998) was confirmed by the present study. Subtype G1 was found in both white-tailed deer and elk isolates, USWTD1, CNELK, and OKELK1. Subtypes G2 and G3, which are nearly identical through the V4 region, were found in a white-tailed deer isolate, USWTD3. Alternatively, no microheterogeneity was found among the Type F SSU rRNA gene sequences regardless of host or geographic origin. Thus, the Type F gene appears...
highly conserved among the *T. cervi*. Although a single base substitution in the V4 region has been reported (Chae et al., 1998), the Type F genes identified in this study did not show this substitution.

The conservation of the Type F SSU rRNA gene sequence among *T. cervi* isolates provides a base of reference when investigating new isolates and presents an opportunity to develop specific DNA primer sequences which could be used in amplification-based assays for detecting *T. cervi* infections in deer or vector ticks. Such assays could be used to screen ticks for the presence of the parasite to identify potential vectors aside from the known vector, *A. americanum*, although ultimately transmission experiments would be necessary to confirm the ability of a tick species to vector the parasite. Sensitive molecular methods could facilitate epidemiological studies in ticks, deer, and other suspected hosts of the parasite.

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**LITERATURE CITED**


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