Sequence and phylogenetic analysis of the 16S rRNA gene of strains in dogs with clinical monocytic ehrlichiosis

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Abstract

The purpose of this study was to characterize, at the molecular level, the *Ehrlichia canis* strains involved in naturally occurring canine monocytic ehrlichiosis (CME) in Greece, and to investigate if any sequence diversity exists between the 16S rRNA genes of those involved in the mild non-myelosuppressive or the severe myelosuppressive form of CME. To this end, amplification of the ehrlichial 16S rRNA gene was attempted by nested polymerase chain reaction (PCR) assays in bone marrow (BM) aspirates from twenty dogs tentatively diagnosed as having non-myelosuppressive (*n*=10, Group A) or myelosuppressive (*n*=10, Group B) CME. PCR assay using *E. canis*-specific primers revealed that 15 BM samples, including all group A and 5 group B dogs, were positive. Using universal PCR primers, a nearly full-length 16S rRNA gene could be amplified from 13 BM samples, including 9 group A and 4 group B dogs. The 16S rDNA analysis based on secondary structure revealed that all sequences of the Greek strains were identical to each other and indicated 100% identity among some American (Venezuelan and Brazilian), European (Greek), Middle Eastern (Turkish) and Asiatic (Thailand) strains. The results of this study suggest that the *E. canis* strains involved in the non-myelosuppressive and myelosuppressive forms of CME in Greece share an identical 16S rRNA genotype.

**Keywords:** Dog; *Ehrlichia canis*; PCR, Phylogenetic analysis, 16S rRNA secondary structure
Introduction

*Ehrlichia canis* is acknowledged as the primary cause of canine monocytic ehrlichiosis (CME) worldwide (Neer et al., 2002). In natural CME, most dogs suffering from the acute disease recover spontaneously, entering a subclinical phase that lasts several months to years. Immunocompetent dogs may eliminate the bacterium during this period but some will eventually develop the chronic phase of the disease, typically characterized by severe bone marrow (BM) suppression, peripheral blood pancytopenia and a high mortality rate (Harrus et al., 1997; Harrus et al., 1998; Mylonakis et al., 2004). The conditions that may precipitate the development of the myelosuppressive form of CME have not yet been fully elucidated. Breed susceptibility and defective cell-mediated immunity are apparently associated with a more severe disease in German shepherd dogs (Nyindo et al., 1980; Harrus et al., 1997). Coinfection with other tick-borne pathogens has also been proposed as a determinant of the disease severity; however, in a recent case series of dogs with myelosuppressive CME, no substantial comorbidity was found (Mylonakis et al., 2004). Finally, strain variation may reportedly affect the spectrum and severity of the clinical and clinicopathological manifestations of the disease (Hegarty et al., 1997; Harrus et al., 1999). To the best of our knowledge, no attempt has ever been made to investigate the existence of genetic diversity among *E. canis* strains involved in CME cases with and without myelosuppression.

The purpose of the present study was to characterize the 16S rRNA genotype of *E. canis* strains naturally infecting Greek dogs and to investigate if any sequence diversity exists between the 16S rRNA genes of those involved in the non-myelosuppressive and the myelosuppressive form of CME. We analyzed the 16S rRNA gene that has been demonstrated to be a powerful tool for the identification and
2. Materials and methods

2.1. Dogs and BM samples

Twenty dogs with a tentative diagnosis of CME due to E. canis admitted to the Companion Animal Clinic (Medicine), from September 2001 to December 2003, were included in the study. This study population was comprised of 15 males and 5 females (17 purebreds and 3 mongrels), with an age ranging from 3.5 months to 8 years (median: 1.7 years). The inclusion criteria for these dogs were the clinical and clinicopathological compatibility to CME, a positive IgG antibody titre against E. canis and/or the observation of presumed E. canis morulae in Giemsa-stained buffy coat smears (Table 1). Good quality BM Giemsa-stained smears, frozen (-20°C) EDTA-anticoagulated BM aspirate material, lack of historical evidence of previous anti-ehrlichial treatment, and negative serology against Leishmania infantum (Snap Leishmania Test Kit, IDEXX, USA), were also considered. Ten dogs (numbered sequentially from #1 to #10) that were assigned to group A, suffered from the non-myelosuppressive CME, on the basis of normocellular BM on aspiration cytology and full response to treatment with doxycycline (Ronaxan, Merial, France). The remaining 10 dogs (numbered sequentially from #11 to #20) that were assigned to group B, demonstrated the myelosuppressive CME because of profound BM hypoplasia documented by evaluating numerous BM unit particles in at least 4 aspiration smears, and poor clinical outcome (death or euthanasia due to CME-related complications) despite doxycycline treatment.
2.2. DNA extraction

DNA was extracted from 25-30mg of BM aspirate material with a NucleoSpin Tissue kit (Macherey-Nagel, Germany) according to the manufacturer’s recommendations. DNA concentrations and purity were determined by spectrophotometry (A₂₆₀ & A₂₈₀) and the samples were stored at -20°C until further processing. Each DNA sample was used as template for PCR.

2.3. Nested PCR amplification of the ehrlichial 16S rRNA gene

Two nested PCR amplifications were applied in this study and performed as previously described with minor modifications (Table 2).

The nested PCR intended for detecting *E. canis* 16S rDNA was performed by using the primers ECC and ECB for the primary amplification, and “canis” and HE3 for the secondary amplification. In order to amplify the entire length of 16S rDNA for comparative sequence analysis, another nested PCR was performed, by using the primers ER5-3 and ER-R1 for the primary amplification, and Ap-F1 and Ap-R1 for the secondary amplification. The expected sizes of the “canis”/HE3 and Ap-F1/Ap-R1 amplicons were approximately 389 bp (Wen et al., 1997; Harrus et al., 1998) and 1.4 kb (Inayoshi et al., 2004), respectively. In each set of amplifications, a positive (BM DNA from a molecularly confirmed *E. canis*-infected clinical case) (Mylonakis et al., 2004) and a negative (without DNA template) control were included.

2.4. Sequencing of PCR products

The Ap-F1/Ap-R1 PCR amplicons that represented nearly the entire length of 16S rRNA gene (approximately 1.4 kb) were purified for DNA sequencing with the aid of a QIAquick PCR purification kit (Qiagen, Hilden, Germany) following the
manufacturer’s instructions. Aliquots of purified products were separated in 1% agarose gel to determine the template quantity required for subsequent sequencing, which was performed by Macrogen Inc., Seoul, Korea. Both forward and reverse strands of each PCR amplicon were sequenced with the primers used for PCR (Ap-F1 and Ap-R1). In case “canis”/HE3 PCR amplicons were also sequenced, the representative PCR primers (“canis” and HE3) were used.

2.5. Computer analyses of DNA sequences

Sequences of 16S rDNA were initially matched with those from both a SeqMatch search of the Ribosomal Database Project (RDP-II Release 9) (http://rdp.cme.msu.edu) and from a BLAST search of the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov). Multiple sequence alignments with the reference sequences downloaded from the EMBL (http://www.ebi.ac.uk/emb1) and GenBank were initially performed with the Clustal W program (Thompson et al., 1994). Subsequent phylogenetic and molecular evolutionary analysis was inferred from alignments based on secondary structure model of Robin Gutell and colleagues (Cannone et al., 2002) and made with RDP’s aligner (Cole et al., 2006). Weighbor weighted neighbor-joining analysis under Jukes-Cantor corrected distance model was conducted with RDP’s Tree Builder (Cole et al., 2006). The ATV version 4.00 ALPHA 4 program (Zmasek and Eddy, 2001) was used to display and manipulate the downloaded phylogenetic trees.

2.6. Nucleotide sequences accession numbers

GenBank accession numbers of the 16S rRNA gene reference sequences are given in Table 3 and Figure 1.
3. Results

3.1. Detection of ehrlichial 16S rRNA gene by nested PCR

Nested PCR using the *E. canis* specific primers “canis” and HE3 yielded a single approximately 389 bp DNA amplicon in 15 out of 20 (75%) BM samples, including all group A and 5 group B (#11, #16, #18, #19 and #20) dogs. When the 20 BM specimens were amplified by nested PCR with the use of Ap-F1 and Ap-R1 primers, a single amplicon of approximately 1.4 kb was produced in 13 (65%) BM samples, representing 9 group A (with the exception of #7) and 4 group B (#11, #16, #19 and #20) dogs.

3.2. Comparison of 16S rRNA gene sequences and phylogenetic analysis

All thirteen Ap-F1/Ap-R1 amplicons were sequenced, and a nearly complete sequence of approximately 1,380 bp of the 16S rDNA was obtained for each one. Furthermore, the two “canis”/HE3 PCR amplicons that were negative by Ap-F1/Ap-R1 nested PCR (samples #7 and #18) were sequenced and 389 bp was obtained for each one. All 15 sequences, which were confirmed as being sequences of *E. canis*, were aligned and compared to each other as well as to 16S rDNA sequences of 91 strains belonging to *E. canis* and other closely related species (Table 3). The 16S rDNA sequences of all non-myelosuppressive (group A) and myelosuppressive (group B) samples were identical to each other. Two sequences derived from samples of the present study and designated as GR21 (dog #2 of group A) and GR78 (dog #19 of group B), were deposited in GenBank under accession numbers EF011110 and EF011111, respectively.

In the alignment of the 16S rDNA sequences, GR21 and GR78 were identical to the *E. canis* VDE, VHE, VTE, Brazil-CO1 and Bangkok strains, as well as to the *E.
ovina Turkey strain. On the other hand, the Greek *E. canis* sequences deviated by one nucleotide from those of the Jake, 95E10, Brazil-CO2, Germishuys, Gdt3 and Kagoshima 1 strains, by two nucleotides from the Oklahoma\(^T\), Madrid, 611 and Gzh982 strains, by three nucleotides from the Florida, and by five nucleotides from the Gxht67 strain. The nucleotide differences of 16S rDNA sequences among GR21 and GR78 and other *E. canis* strains from different geographical areas as well as closely related species are shown in Table 3.

Phylogenetic analysis based on secondary structure of 16S rDNA was performed using the largest available fragment for most sequences. In particular, a 1,279 to 1,292 bp fragment of 15 *E. canis* (Oklahoma\(^T\), Florida, Jake, VDE, VHE, Brazil-CO1, Brazil-CO2, Madrid, GR21, GR78, 611, Germishuys, Gzh982, Bangkok and Kagoshima 1) and 17 other closely related species (type or reference strains) sequences were employed to validate subsequent comparisons using a smaller fragment that included 965 to 970 bp of the larger fragment, so that two additional *E. canis* (Gdt3 and Gxht67) sequences could also be assessed (Figure 1 A & B). Five groups were consistently identified in both the large and small fragment comparisons including the genera *Ehrlichia*, *Anaplasma*, *Neorickettsia*, *Wolbachia* and the recently established species ‘*Candidatus* Neoehrlichia mikurensis’ (Kawahara et al., 2004). The GR21 and GR78 strains were positioned in the *E. canis* cluster among the most of *E. canis* strains. The Oklahoma\(^T\) and Florida strains by themselves or with Gdt3 and Gxht67 strains formed a distinct clade (bootstrap values 85% and 61% in Figure 1 A & B, respectively). Another clade was formed from the 611 and Kagoshima 1 strains (bootstrap values 61% and 67% in Figure 1 A & B, respectively). The Brazil-CO2 strain formed a distinct clade but without significant branching (bootstrap values < 60%).
4. Discussion

Although CME was first reported in Greece in the late 1980s (Koutinas et al., 1989), no molecular characterization of *E. canis* strains originating from native dogs has ever been attempted. The 16S rDNA sequences of the strains presented in this study demonstrated 100% identity to the *E. canis* VDE, VHE and VTE strains isolated from dog, human and *Rhipicephalus sanguineus* ticks, respectively, in Venezuela (Unver et al., 2001), to the *E. canis* Brazil-CO1 and Bangkok canine strains isolated in Brazil and Thailand respectively, as well as to the *E. ovina* Turkey strain, isolated from a sheep in Turkey (Bekker et al., 2002). Interestingly, the 16S rDNA sequences of the human *E. canis* VHE strain and the recently reported human strains from Venezuela are identical to the canine *E. canis* VDE strain from the same area (Unver et al., 2001; Perez et al., 2006). Also, the 16S rDNA sequence of the *E. ovina* Turkey strain was recently reported to be identical to an *E. canis* strain (Kutahya) isolated from a Turkish dog and to the VDE strain (Unver et al., 2005; Yu et al., 2006). In South Africa, the *E. canis* Germishuys strain was also reported to infect sheep with clinical signs similar to heartwater disease (Allsopp et al., 1997). These data may suggest that the host range of *E. canis* strains is broader than previously thought, not excluding a zoonotic potential (Unver et al., 2001; Perez et al., 2006).

Phylogenetic analysis based on secondary structure of 16S rRNA suggests that the gene in most *E. canis* strains appear to be highly conserved. Sequences of the 16S rRNA gene bear a 99.4-100% similarity among *E. canis* strains from North America (Oklahoma T, Florida and Jake), South America (VDE, VHE, Brazil-CO1 and Brazil-CO2), Europe (Madrid, GR21 and GR78), Middle East (Turkey and 611), South Africa (Germishuys), and Asia (Czh982, Gdt3, Gxht67, Bangkok and Kagoshima 1). Our results indicate 100% identity between some American (Venezuelan and...
Brazilian), European (Greek), Middle Eastern (Turkish) and Asiatic (Thailand) strains and that the 16S rDNA sequence divergence observed in certain *E. canis* strains does not correlate with geographical distribution (Table 3, Figure 1A & B); in some cases, strains from the same geographical area were found to present 16S rDNA sequence divergence (i.e. strains Oklahoma\(^T\), Florida and Jake which were from North America and strains Brazil-CO1 and Brazil-CO2 from Brazil), contrary to what is reported with *E. chaffeensis* strains (Yu et al., 2006).

Importantly, sequence differences among strains do not always represent true divergence, but may be sequencing artefacts (Suksawat et al., 2001). Resequencing of the 16S rDNA of Oklahoma\(^T\) strain (Unver et al., 2001) demonstrated that at position 941 (*E. coli* J01695 numbering system) there is an additional guanine nucleotide (G), identical to the majority of of *E. canis* strains and closely related species (Table 3). By this correction GR21 and GR78 sequences deviate by one nucleotide from the Oklahoma\(^T\) strain, located at position 230 (*E. coli* J01695 numbering system) (Table 3).

The results of the present study indicate that the *E. canis* strains responsible for the mild acute as well as the severe chronic CME in Greece have identical 16S rRNA gene sequences. Since the distinction between the acute and chronic clinical stage is not straightforward in spontaneous cases, the terms “non-myelosuppressive” and “myelosuppressive”, respectively, may better reflect the clinical severity of the disease irrespective of its time progression. Our data provide further evidence, that despite the worldwide distribution of *E. canis*, the already investigated part of the genetic profile (i.e. 16S rRNA, groEL, gltA, *p28* family, *gp140* and *virB9* gene sequences) is conserved and that no isolate is predictive of a particular clinical outcome (Suksawat et al., 2001; Unver et al., 2005; Yu et al., 2006; Perez et al.,
Of comparative interest, some variants of *E. ruminantium* that share common pCS20 and 16S sequences with highly pathogenic isolates do not cause heartwater (Allsopp et al. 2006), whilst there is currently no convincing evidence associating molecular features of *E. chaffeensis* with disease severity in humans, despite the fact that many isolates exhibit substantial divergence in some well characterized immunoreactive genes such as the *p28* and *gp120* genes, the variable length PCR target gene and the *gp47* gene (Paddock and Childs, 2003; Yu et al., 2006). From a medical perspective, further study of possible genetic polymorphisms of other genomic regions (e.g. *gp36* or *gp19* genes in *E. canis*) would be interesting in order to determine if they substantially affect strain virulence and clinical outcome (Dumler et al., 1995; Paddock et al., 1997; Standaert et al., 2000; Suksawat et al., 2001; Yu et al. 2006). Interestingly, recent evidence in experimental studies with dogs, suggests that clinical severity in CME may be related to the nature of the patient's immunological response (i.e. Th1 versus Th2) and to the cytokine profile induced post-inoculation; high levels of IFN-γ have been associated with the mild disease, while persistently elevated IL-1β and IL-8 were detected in the severely-affected dogs (Tajima and Rikihisa, 2005; Unver et al., 2006). Similarly, upregulation of myelosuppressive chemokines including IL-8, macrophage inflammatory protein-1α and monocytic chemotactic protein-1 may account for the clinical severity of human granulocytic ehrlichiosis (Klein et al., 2000). Collectively, the aforementioned data lend support to the concept that in CME (and probably in human monocytic and granulocytic ehrlichioses) the clinical severity is primarily mediated by the host immune response rather than the sequence divergence of 16S rRNA gene of the *E. canis* strains. Additional sequence information from other genes will be necessary to determine the role of genetic variation of strains in pathogenicity of CME.
5. Conclusion

The present study describes the first molecular characterization of *E. canis* strains amplified from Greek dogs with monocytic ehrlichiosis. Sequence and phylogenetic analyses of the 16S rRNA genes indicate no substantial genetic diversity between the Greek and the GenBank deposited *E. canis* strains, the former being identical to Venezuelan canine and human strains, to Brazilian and Thai canine strains, as well as to the Turkish ovine strain. These data provide further evidence, that despite the worldwide distribution of *E. canis*, the 16S rRNA genotype is conserved. To our knowledge, this study provides the first molecular evidence that the clinical severity of canine monocytic ehrlichiosis does not correlate with 16S rRNA genotype.

Acknowledgements

The authors are grateful to Professor Michael Day, School of Clinical Veterinary Science, University of Bristol, UK, for editing the manuscript, and acknowledge the expertise of Stelios Gargas and Ioannis Psathas for technical support and software configuration.

References


Table 1. Clinical, hematological, biochemical, serologic and buffy coat (BC) cytology findings in 20 dogs with presumptive canine monocytic ehrlichiosis (CME)

<table>
<thead>
<tr>
<th>Clinical findings</th>
<th>Non-myelosuppressive CME</th>
<th>Myelosuppressive CME</th>
</tr>
</thead>
<tbody>
<tr>
<td>Depression</td>
<td>10/10 (100%)</td>
<td>10/10 (100%)</td>
</tr>
<tr>
<td>Anorexia</td>
<td>9/10 (90%)</td>
<td>9/10 (90%)</td>
</tr>
<tr>
<td>Body weight loss</td>
<td>6/10 (60%)</td>
<td>10/10 (100%)</td>
</tr>
<tr>
<td>Fever</td>
<td>10/10 (100%)</td>
<td>5/10 (50%)</td>
</tr>
<tr>
<td>Bleeding tendency</td>
<td>3/10 (30%)</td>
<td>10/10 (100%)</td>
</tr>
<tr>
<td>Mucosal pallor</td>
<td>3/10 (30%)</td>
<td>10/10 (100%)</td>
</tr>
<tr>
<td>Lymphadenomegaly</td>
<td>7/10 (70%)</td>
<td>4/10 (40%)</td>
</tr>
<tr>
<td>Splenomegaly</td>
<td>1/10 (10%)</td>
<td>3/10 (30%)</td>
</tr>
<tr>
<td>Dyspnea</td>
<td>1/10 (10%)</td>
<td>3/10 (30%)</td>
</tr>
<tr>
<td>Hypothermia</td>
<td>0</td>
<td>3/10 (30%)</td>
</tr>
</tbody>
</table>

**Hematology**

- Anemia: 10/10 (100%) / 10/10 (100%)
- Thrombocytopenia: 10/10 (100%) / 10/10 (100%)
- Eosinopenia: 7/10 (70%) / 10/10 (100%)
- Lymphopenia: 5/10 (50%) / 8/10 (80%)
- Neutropenia: 0 / 8/10 (80%)
- Monocytopenia: 8/10 (80%) / 0
- Aplastic pancytopenia: 0 / 8/10 (80%)

**Biochemistry**

- Hypoalbuminemia: 8/10 (80%) / 7/10 (70%)
- Alanine aminotransferase (T): 6/9 (66.5%) / 5/8 (62.5%)
- Hypoproteinemia: 5/10 (50%) / 3/10 (30%)
- Hypercreatinemia: 3/9 (33.5%) / 2/9 (22%)
- Hyperglobulinemia: 1/10 (10%) / 2/10 (20%)
- Glomerular proteinuria*: 6/9 (66.5%) / 4/8 (50%)
- Seropositivity to *E. canis**: 8/10 (80%) / 10/10 (100%)
- *Ehrlichia* sp. morulae in BC***: 7/10 (70%) / 0

†: increased activity

---

1. Table 1. Clinical, hematological, biochemical, serologic and buffy coat (BC) cytology findings in 20 dogs with presumptive canine monocytic ehrlichiosis (CME)

2. **Hematology**

3. **Biochemistry**
Proteinuria was confirmed with the aid of a semiquantitative turbidometric method (Heller’s test) in the context of inactive sediment.

**Imunocomb®, Biogal, Israel.

***In all 20 dogs, 1000 oil immersion fields were screened. Morulae were found in the two group A seronegative dogs.
Table 2. Characteristics of primers and nested PCR amplification protocols

<table>
<thead>
<tr>
<th>Target</th>
<th>Primer</th>
<th>Sequence (5’→3’)</th>
<th>Annealing temperature</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hypervariable region</td>
<td>ECC</td>
<td>AGAACGAACGCTGGCGGCAAGCC</td>
<td>60°C</td>
<td>Wen et al. (1997) &amp; Harrus et al. (1998)</td>
</tr>
<tr>
<td>&quot;canis&quot;</td>
<td>ECB</td>
<td>CGTATTACCGCGGCTGGCGGCA</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>HE3</td>
<td>CAATTATTTATAGCCTCAGGTATAGGA</td>
<td>60°C</td>
<td></td>
</tr>
<tr>
<td>Hypervariable region</td>
<td>HE3</td>
<td>TATAGGTACCGTCATTATCTTCCCTAT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Entire length of</td>
<td>ER5-3</td>
<td>TTGAGAGTTTGATCCTGG</td>
<td>58°C</td>
<td>Inayoshi et al. (2004)</td>
</tr>
<tr>
<td>Ehrlichia 16S rDNA</td>
<td>ER-R1</td>
<td>GGAGGTAATCCACCGCGCA</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ap-F1</td>
<td>TCCTGGCTCAGAACEGCAAGC</td>
<td>58°C</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ap-R1</td>
<td>CCTACAGCTACCCTTTGACG</td>
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<td></td>
</tr>
</tbody>
</table>
Table 3. Nucleotide differences of the 16S rDNA sequences among *E. canis* strains originating from different geographical areas and closely related species

<table>
<thead>
<tr>
<th>Organisms</th>
<th>Nucleotide position&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>230</td>
</tr>
<tr>
<td><em>Ehrlichia canis</em> Oklahoma&lt;sup&gt;b&lt;/sup&gt;, USA (M75221)</td>
<td>A'</td>
</tr>
<tr>
<td><em>E. canis</em> Florida, USA (M73226)</td>
<td>A</td>
</tr>
<tr>
<td><em>E. canis</em> Jake, USA (CP000107)</td>
<td>G</td>
</tr>
<tr>
<td><em>E. canis</em> 95E10-26039, USA (U96437)</td>
<td>G</td>
</tr>
<tr>
<td><em>E. canis</em> VHE/VDE Venezuela (AF37162-3)</td>
<td>G</td>
</tr>
<tr>
<td><em>E. canis</em> CO1/CO2 Brazil (EF195134-5)</td>
<td>G</td>
</tr>
<tr>
<td><em>E. canis</em> Madrid, Spain (AY394465)</td>
<td>G</td>
</tr>
<tr>
<td><em>E. canis</em> GR23/GR78, Greece (EF011110-1)</td>
<td>G</td>
</tr>
<tr>
<td><em>E. canis</em> 611, Israel (JU26740)</td>
<td>G</td>
</tr>
<tr>
<td><em>E. canis</em> Gzh982, China (AF162860)</td>
<td>G</td>
</tr>
<tr>
<td><em>E. canis</em> Gd t3, China (AF156785)</td>
<td>G</td>
</tr>
<tr>
<td><em>E. canis</em> Mad, Thailand (AF318946)</td>
<td>G</td>
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</tr>
<tr>
<td><em>E. canis</em> 611, Israel (JU26740)</td>
<td>G</td>
</tr>
</tbody>
</table>

<sup>a</sup> Nucleotide position of 16S rDNA following the *E. coli* J01695 numbering system (Cannone et al., 2002).

<sup>b</sup> Changes from the *E. canis* consensus sequence and similar nucleotide bases in related species sequences are indicated in bold. Dashes indicate nucleotide gaps in 16S rDNA sequence comparison; between positions 934 and 935 (*E. coli* J01695 numbering system) the A represents an “insertion” base.

The “nd” indicates the sequences were not determined.

* An additional guanine nucleotide (G) at position 941 (*E. coli* J01695 numbering system) was demonstrated after resequencing by Unver et al. (2001).

<sup>b</sup> More than three accession numbers for each species were analyzed (accession numbers of type or reference strains are given in Figure 1).
Figure 1. Phylogenetic trees based on secondary structure of 16S rRNA gene analysis of Ehrlichia canis and closely related species, using in A: the large fragment (1,279 to 1,292 bp) of 15 E. canis and 17 other species sequences, and in B: the small fragment (965 to 970 bp) of sequences including two additional E. canis strains; only part of the B phylogenetic tree (Ehrlichia group) is shown. The trees were constructed with RDP’s Tree Builder using the Neighbor weighted neighbor-joining analysis (Cole et al., 2006). The numbers at the nodes indicate the percentage of 100 bootstrap replicates that supported the branch; only bootstrap values equal or greater than 50% and 30% are shown in A and B trees, respectively. Accession numbers, source and origin are in parentheses; NA: not available designation. The E. canis sequences identified in this study are boxed and in boldface.
**Figure 1**

E. canis Oklahoma\(^T\) (M73221) [Dog, USA]
E. canis Florida (M73226) [Dog, USA]
E. canis Germishuys (US4805) [Sheep, South Africa]
E. canis Gzh982 (AF162860) [Dog, China]
E. canis Madrid (AY394465) [Dog, Spain]
E. canis GR78 (EF011111) [Dog, Greece]
E. canis GR21 (EF011110) [Dog, Greece]
E. ovina Turkey (AF318946) [Sheep, Turkey]
E. canis 611 (U26740) [Dog, Israel]
E. canis Kagoshima 1 (AF536827) [Dog, Japan]
E. canis Jake (CP000107) [Dog, USA]
E. canis Brazil-CO1 (EF195134) [Dog, Brazil]
E. ovina Turkey (AF318946) [Sheep, Turkey]
E. canis VHE (AF373612) [Human, Venezuela]
E. canis Bangkok (EF139458) [Dog, Thailand]
E. chaffeensis Arkansas\(^T\) (M73222)
E. muris AS145\(^T\) (U15527)
Ehrlichia sp. Anan (AB028319)
Ehrlichia sp. Tibet (AF414399)
E. ewingii Stillwater\(^T\) (M73227)
E. ruminantium Crystal Springs (X61659)

**Scale:** 0.1