INTRODUCTION

Cytolethal distending toxin (CDT) causes distension and eventually death of cultured eukaryotic cells. The mechanism of CDT activity is reported to involve G2/M cell-cycle arrest and eventually death of cultured eukaryotic cells. The mechanism of CDT activity is reported to involve G2/M cell-cycle arrest and eventual death of cultured eukaryotic cells. The CDT toxin is encoded by three genes, cdtA, cdtB, and cdtC, all of which are required for the toxin’s function (Mayer et al., 1999; Lara-Tejero & Galañ, 2001). CdtB is proposed to be the enzymically active subunit of the holotoxin (Lara-Tejero & Galañ, 2001).

Flexispiras are a group of organisms with very similar phenotypic characteristics: a distinctive cigar-like cell shape and external fibrils surrounding the cell and bundles of sheathed flagella at both ends of the cell (Bryner et al., 1987).

The presence of the cytolethal distending toxin B gene (cdtB) was examined in eight Helicobacter sp. flexispira reference strains, Helicobacter trogontum ATCC 700114®, 12 Finnish porcine H. trogontum strains and canine flexispira isolates. Part of the cdtB gene was amplified by PCR with degenerate primers VAT2 and DHF1, cloned and sequenced. The presence/absence of the cdtB gene as determined by PCR was confirmed by Southern hybridization and toxin production by HeLa cell-line experiments. PCR amplification resulted in approximately 700 bp fragments from Helicobacter sp. flexispira taxa 2 (ATCC 49314), 3 (ATCC 49320) and 8 (ATCC 43880, ATCC 49308, ATCC 43879), from six canine isolates as well as from the control strains Helicobacter bilis and Helicobacter hepaticus. The hybridization patterns of HaellI, HindIII- and AseI-digested chromosomal DNA confirmed the results of the PCR experiments. The cdtB-positive strains had effects ranging from weak to strong on HeLa cell cultures. PCR amplification from the reference strains Helicobacter sp. flexispira taxa 1 (ATCC 43968), 4 (ATCC 49310) and 5 (ATCC 43968) and H. trogontum (ATCC 700114®) and also six of the Finnish strains, was unsuccessful. No toxic effect on HeLa cells was evident when bacterial suspensions of PCR-negative strains were used for toxicity assay. Our results are in accordance with previous observations that the cdtB gene is not present in all Helicobacter species. Further, the presence/absence of the cdtB gene in Helicobacter sp. flexispira strains was in accordance with recent taxonomic analysis of the same strains, which suggests that it could serve as a useful marker in Helicobacter taxonomy.

The taxonomy of this group is not valid, because of the limited number of isolates available for study. According to a recent proposal based on phylogenetic analysis of 16S rRNA sequences, these organisms are members of the genus Helicobacter, forming a group that includes at least ten phylogenetic groups widely interspersed among intestinal Helicobacter species (Dewhirst et al., 2000). A provisional name, Helicobacter sp. flexispira, has been proposed by Dewhirst et al. (2000) for this group of organisms until a valid taxonomic description is provided.

The cdtB gene is found to be the most conserved among the three cdt genes (Mayer et al., 1999; Pickett et al., 1996; Young et al., 2000a). With degenerate PCR primers, cdtB homologues have been identified in enteropathogenic Campylobacter species (Pickett et al., 1996) and in several enteric and enterohepatic Helicobacter species (Helicobacter pullorum, Helicobacter hepaticus, Helicobacter bilis and Helicobacter canis) (Young et al., 2000a, b; Chien et al., 2000). Neither the cdtB gene cluster nor CDT activity has been identified in

Cytotoxic lethal distending toxin B gene (cdtB) homologues in taxa 2, 3 and 8 and in six canine isolates of Helicobacter sp. flexispira

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two enterohpatic Helicobacter species, Helicobacter fenneliiae and Helicobacter cinaedi (Young et al., 2000a). The aim of this study was to determine the presence or absence of the cdtB gene in Helicobacter sp. flexispira strains including reference strains, 12 Finnish porcine Helicobacter trogontum strains (Hänninen et al., 2003) and canine flexispira isolates. Part of the cdtB gene was amplified by PCR using degenerate primers and was cloned and sequenced. The presence/absence of the cdtB gene as determined by PCR was confirmed by Southern hybridization and toxin production by cell-line experiments.

METHODS

Bacterial strains and purification of DNA. Eleven Helicobacter reference strains, six Finnish porcine H. trogontum strains and six canine flexispira isolates were included in the PCR analysis of cdtB gene homologues (Table 1). The isolation procedure and cultivation of strains have been described by Hänninen et al. (2003). DNA was isolated by the method of Pitcher et al. (1989) as described previously (Hänninen et al., 1996).

PCR amplification, cloning and sequencing. The degenerate primers VAT2 (forward, 5'-GTNGCNACRGTGAAYCTNCARGG-3'), WM11 (reverse, 5'-RTTRARACTCNGCYYADATCC-3') (Pickett et al., 1996) and DHF1 (reverse, 5'-DAGNGGARRGTCRGT-3') (Chien et al., 2000) were used for PCR with genomic DNA of 23 Helicobacter strains (Table 1) as templates. Reactions (50 μl) were set up with 100 ng DNA, 20 pmol of each primer, 200 μM of each nucleotide and 2 U DyNayme DNA polymerase (Finnzymes). The cycling conditions were essentially as described previously (Young et al., 2000a).

A total of 11 PCR products of the expected size were cloned and sequenced. PCR fragments were extracted from 1:1 % NuSieve GTG low-melting agarose gel (BMA), purified with the QIAquick gel extraction kit (Qiagen), cloned into pGEM-T Easy (Promega) and transformed into E. coli DH5α or JM 109 cells. Plasmid DNA was extracted with the Qagen plasmid Mini kit and sequenced by the method of Pitcher et al., 2003).

Table 1. Strains and isolates used in this study

<table>
<thead>
<tr>
<th>Strain/isolate designation</th>
<th>Original designation</th>
<th>Source of isolation</th>
<th>cdtB*</th>
<th>Toxicity test</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H. trogontum HU 1 SU</td>
<td>Pig, faecal sample</td>
<td>--</td>
<td>ND</td>
<td>Hänninen et al. (2003)</td>
<td></td>
</tr>
<tr>
<td>H. trogontum HU 2 SU</td>
<td>Pig, faecal sample</td>
<td>--</td>
<td>ND</td>
<td>Hänninen et al. (2003)</td>
<td></td>
</tr>
<tr>
<td>H. trogontum HU 3 SU</td>
<td>Pig, faecal sample</td>
<td>--</td>
<td>ND</td>
<td>Hänninen et al. (2003)</td>
<td></td>
</tr>
<tr>
<td>H. trogontum HU A4</td>
<td>Pig, faecal sample</td>
<td>--</td>
<td>ND</td>
<td>Hänninen et al. (2003)</td>
<td></td>
</tr>
<tr>
<td>H. trogontum HU H95S</td>
<td>Pig, stomach</td>
<td>--</td>
<td>ND</td>
<td>Hänninen et al. (2003)</td>
<td></td>
</tr>
<tr>
<td>H. trogontum HU158S</td>
<td>Pig, stomach</td>
<td>--</td>
<td>ND</td>
<td>Hänninen et al. (2003)</td>
<td></td>
</tr>
<tr>
<td>H. trogontum ATCC 790114T</td>
<td>8581</td>
<td>Rat, colon</td>
<td>ND</td>
<td>Hänninen et al. (2003)</td>
<td></td>
</tr>
<tr>
<td>Helicobacter sp. taxon 1 ATCC 43968</td>
<td>Pig</td>
<td>--</td>
<td>--</td>
<td>Dewhirst et al. (2000)</td>
<td></td>
</tr>
<tr>
<td>Helicobacter sp. taxon 4 ATCC 49310</td>
<td>Sheep</td>
<td>--</td>
<td>--</td>
<td>Dewhirst et al. (2000)</td>
<td></td>
</tr>
<tr>
<td>Helicobacter sp. taxon 5 CCUG 28992</td>
<td>Aborted sheep fetus</td>
<td>--</td>
<td>--</td>
<td>Bryner et al. (1987); Kirkbride et al. (1985, 1986)</td>
<td></td>
</tr>
<tr>
<td>Helicobacter sp. taxon 2 ATCC 43914</td>
<td>Sheep</td>
<td>+</td>
<td>+(+)</td>
<td>Dewhirst et al. (2000)</td>
<td></td>
</tr>
<tr>
<td>Helicobacter sp. taxon 3 ATCC 49320</td>
<td>Pig, stomach</td>
<td>+</td>
<td>+</td>
<td>Dewhirst et al. (2000)</td>
<td></td>
</tr>
<tr>
<td>Isolate KO534B</td>
<td>Dog, faecal sample</td>
<td>+</td>
<td>ND</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Isolate KO214</td>
<td>Dog, faecal sample</td>
<td>+</td>
<td>ND</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Isolate KO114 (Alma)</td>
<td>Dog, faecal sample</td>
<td>+</td>
<td>+++</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Isolate KO115 (Etppu)</td>
<td>Dog, faecal sample</td>
<td>+</td>
<td>ND</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Isolate Hilli</td>
<td>Dog, faecal sample</td>
<td>+</td>
<td>ND</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Isolate KO220</td>
<td>Dog, faecal sample</td>
<td>+</td>
<td>+++</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Helicobacter sp. taxon 8 CCUG 38944</td>
<td>Human stool</td>
<td>+</td>
<td>ND</td>
<td>Romero et al. (1988)</td>
<td></td>
</tr>
<tr>
<td>Helicobacter sp. taxon 8 CCUG 41386</td>
<td>Dog stool</td>
<td>+</td>
<td>ND</td>
<td>Romero et al. (1988)</td>
<td></td>
</tr>
<tr>
<td>Helicobacter sp. taxon 8 CCUG 43908</td>
<td>Dog stool</td>
<td>+</td>
<td>ND</td>
<td>Romero et al. (1988)</td>
<td></td>
</tr>
<tr>
<td>Helicobacter sp. taxon 8 CCUG 223435</td>
<td>Human stool</td>
<td>+</td>
<td>+</td>
<td>Romero et al. (1988)</td>
<td></td>
</tr>
<tr>
<td>H. bilis (taxon 9) ATCC 51630T</td>
<td>Mouse faeces</td>
<td>+</td>
<td>++</td>
<td>Fox et al. (1995)</td>
<td></td>
</tr>
<tr>
<td>H. hepaticus CCUG 38637T</td>
<td>Mouse liver</td>
<td>+</td>
<td>ND</td>
<td>Fox et al. (1994)</td>
<td></td>
</tr>
</tbody>
</table>

*cdtB positivity as determined by PCR.
†Scored as: --, no effect; (+), weak effect with undiluted sonicate; +, effect; ++, strong effect; ++++, very strong effect. ND, Not done.
uncoded cycle sequencing with Big Dye terminators (ABI 377, PE Biosystems). To exclude artefacts, at least three replicate clones were sequenced for each cloned PCR product.

**Southern hybridization.** Approximately 4 µg chromosomal DNA was digested with *AvrI*, *HaeIII* and *HindIII*, separated by 1% agarose gel electrophoresis and transferred to a Hybond-N nylon membrane according to the manufacturer’s protocol (Amersham). Procedures for pre-hybridization, hybridization, probe labelling and membrane washing have been described previously (Jalava et al., 1998, 1999). The probe was PCR-amplified from one of the recombinant clones (taxon 8, CCUG 38944/ATCC 43880). Membranes were incubated overnight at either 58°C (*HaeIII*- and *HindIII*-digested DNA) or 37°C (*AvrI*-digested DNA) for low-stringency hybridization.

**Toxicity assay in HeLa cell cultures.** Nine strains were included in a toxicity assay (Table 1). For preparation of cell sonicates, bacteria were grown on brucella blood agar plates with 7% bovine blood under micro-aerobic conditions and incubated for 2–3 days. Cells were harvested by centrifugation and the pellet was resuspended in 1 ml PBS and sonicated on ice with four 30-s pulses (30 s interval). The sonicate was centrifuged at 6000 g.

**Sequence analysis.** The DNA sequences of the partial *cdtB* genes from 11 flexispira strains obtained from this study were compared with homologous sequences available in public databases. Partial *H. canis* sequences from *H. bilis* (AF243076), Campylobacter jejuni (AJ281209) and *H. hepaticus* (AF243078), *H. pullorum* (AF220766) and *H. bicornis* (H053077), Campylobacter jejuni (US1121) and two novel Helicobacter spp. isolates 96-1001 (AF243080) and 98-6070 (AF243079) (Chien et al., 2000) were included in the analysis. Nucleotide sequences were translated to amino acid sequences by the TRANSEQ program included in the EMBOSS software analysis package. Percentage similarity values were calculated by BioNumerics version 2.5 (Applied Maths). The most parsimonious phylogenetic trees were constructed and bootstrap support (Felsenstein, 1985), performing 1000 replicates with 10 random additions for each node, was assessed using PAUP 4.0 (Swofford, 1996).

**RESULTS**

**PCR amplification with degenerate primers, cloning and sequencing**

PCR amplification with primer pair VAT2+WM1I was not unsuccessful from any of the 22 templates. Another pair of degenerate primers, VAT2 and DHF1, amplified approximately 700 bp fragments from Helicobacter sp. flexispira taxa 2, 3 and 8 and from six canine isolates as well as from *H. bilis* and *H. hepaticus* (Table 1). PCR amplification with this pair of primers from six porcine *H. trogontum* strains and the reference taxa 1, 4 and 5 was unsuccessful (Table 1).

The cloned PCR fragments were sequenced from at least three recombinant plasmids. The inserts were 708 (taxa 2 and 3), 702 (Hilli) and 699 bp [taxon 8 and canine isolates 534b, KO214, KO220, KO114 (Alma) and KO115 (Eppu)] in length (excluding primer sequences). The lengths of the respective amino acid sequences were 256, 234 and 233 residues. The partial *cdtB* sequences of taxa 2 and 3 included an insertion of 9 bp, of which the third codon was the translation termination codon UAA/TAA in the coding frame (Fig. 1). The *cdtB* sequence of isolate Hilli had an insertion of 3 bp (Fig. 1).

Three taxon 8 reference strains were included in the PCR and sequence analysis (Table 1). The partial *cdtB* sequences of these strains were identical, and only one DNA sequence, PCR-amplified from strain CCUG 38944/ATCC 43880, has been entered in the GenBank database under the accession number AF467241. The GenBank accession numbers for the *cdtB* gene homologues in Helicobacter were derived from: 1, *H. bilis*; 2, KO115 (Eppu); 3, KO220; 4, KO534B; 5, taxon 8; 6, KO214; 7, KO114 (Alma); 8, Hilli; 9, taxon 2; 10, taxon 3.

**Fig. 1.** Alignment of predicted amino acid sequences of partial *cdtB* proteins of the Helicobacter sp. flexispira canine isolates and *H. bilis*. Amino acid sites that differ between strains are in bold. Sequences were derived from: 1, *H. bilis*; 2, KO115 (Eppu); 3, KO220; 4, KO534B; 5, taxon 8; 6, KO214; 7, KO114 (Alma); 8, Hilli; 9, taxon 2; 10, taxon 3.
other new partial cdtB sequences are AF467235 (isolate Hilli), AF467236 (isolate KO214), AF467237 (isolate KO534B), AF467238 (isolate KO220), AF467239 [isolate KO114 (Alma)], AF467240 [isolate KO115 (Eppu)], AF467242 (taxon 2) and AF467243 (taxon 3).

**Sequence comparisons**

The partial cdtB DNA sequences of the *Helicobacter* sp. flexispira reference strains of taxa 2, 3 and 8 and six canine isolates (Hilli, KO114, KO115, KO214, KO534B and KO220) showed approximately 70% similarity to homologues from *H. pullorum* (AF220065) and *H. hepaticus* (AF243076) and 76% similarity to the *H. canis* homologue (AF243078). Comparison of amino acid sequences of the same *Helicobacter* strains resulted in 80–84% identity. Comparisons of flexispira cdtB DNA sequences to *C. jejuni* (U51121) and to *Helicobacter* spp. isolates 96-1001 (AF243080) and 98-6070 (AF243079) (Chien et al., 2000) revealed 56–61% similarity.

The amino acid sequences of *C. jejuni* and isolate 98-6070 revealed 66–70% identity and that of isolate 96-1001 less than 40% identity to flexispira sequences.

Pairwise comparisons of partial cdtB DNA sequences of taxa 2, 3 and 8 and the six Finnish canine isolates revealed 90–99% similarity. Among the amino acid sequences of the same strains, identity was 94–100%. Comparison of the sequences of taxa 2 and 3 revealed one synonymous substitution at the DNA level, i.e. the predicted amino acid sequences were identical. The cdtB DNA sequences of two isolates, KO115 and KO220, differed at only six sites and by only one amino acid. The DNA sequence of another dog isolate, 534B, differed from KO115 and KO220 at 14 sites and by 4 and 5 amino acids, respectively. These three sequences, as well as KO214, differed from the DNA sequence of ATCC 43880 (taxon 8) at 17–19 sites and by 2–6 amino acids. Isolate KO114 showed most similarity to isolate KO214 (respectively 27 and 6 different sites in the DNA and amino acid sequences) and isolate Hilli to isolate KO114 (Alma) and KO214 (in addition to a 3 bp insertion, respectively 36 and 12 different sites in the DNA and amino acid sequences). Parsimony analysis was done for both DNA and amino acid sequences of cdtB. The consensus tree of the two most-parsimonious trees of the cdtB DNA sequences and the bootstrap values are presented in Fig. 2.

**Southern hybridization**

Chromosomal DNA of flexispira strains was digested with three different restriction enzymes. No hybridization signal was observed in *Helicobacter* sp. flexispira taxon 1, 4 or 5, nor in any of the porcine isolates. A clear hybridization signal was apparent in digested DNA of taxa 2, 3 and 8, as well as in the canine isolates, *H. bilis* and *H. hepaticus* in all three hybridization experiments. The hybridization pattern of HindIII-digested DNA revealed a fragment of over 20 kb from all the strains studied. Hybridization of *Hae* III-digested DNA resulted in a fragment of >20 kb from taxon 8 and from the canine isolates, *H. bilis* and *H. hepaticus* and an approximately 15 kb fragment from the reference taxa 2 and 3. The hybridization pattern of *Ase*I-digested DNA revealed a fragment of approximately 2 kb from the canine isolates, taxa 2, 3 and 8 and *H. bilis* and an approximately 1·2 kb fragment from *H. hepaticus*.

**Toxicity assay**

The reference strain for the CDT-producing bacterium *H. bilis* caused a clear reduction in cell growth at dilutions of 1:1 to 1:4. Long, fibroblast-like cells and, finally, cell death occurred (Table 1). The cell layer was only 40% confluent, compared with 90% of the cells of control wells. Three strains, taxa 2, 3 and 8, had a weak toxic effect, with specific cell alteration in 1:1 or 1:2 dilutions (Table 1). Bacterial suspensions of taxa 1, 4 and 5 were negative; the cells multiplied, with no toxic effect detected on cells. Two isolates, KO114 and KO220, showed a clear, strong effect in all dilutions between 1:1 and 1:16. During the first day of observation, fibroblast-like cells and multinucleate cells were evident. From day 2 to day 4, the cells became rounded and they detached from the bottoms of wells.

**DISCUSSION**

For six reference strains of *Helicobacter* sp. flexispira taxa 2, 3 and 8 and six canine flexispira isolates, a PCR amplification product of approximately 700 bp suggests that these organisms have the cdtB gene identified in earlier studies from several Gram-negative pathogens including *H. pullorum*, *H. hepaticus* and *H. bilis* (Young et al., 2000a, b; Chien et al., 2000). That the amplification was unsuccessful for three of the reference strains of taxa 1, 4 and 5 and *H. trogontum* and for Finnish porcine strains suggests that these organisms lack a cdtB gene. All these strains are, according to our recent
results, members of *H. trogontum* (Hänninen et al., 2003). Our results are in accordance with previous observations that the *cdtB* gene is not present in all *Helicobacter* species (Young et al., 2000a, b). Southern hybridization of the digested chromosomal DNA with a 700 bp partial *cdtB* gene amplified from *Helicobacter* sp. flexispira reference strain ATCC 43880 (taxon 8) as a probe confirmed the results of the PCR experiments. No hybridization signal appeared in taxon 1, 4 or 5, in *H. trogontum* ATCC 700114T or in porcine *H. trogontum* strains, whereas a clear hybridization signal appeared in taxa 2, 3 and 8, the canine isolates and *H. bilis* and *H. hepaticus*. Chien et al. (2000) have reported that the primers VAT2, WM11 and DHF1 failed to produce an amplicon of the expected size from the reference strain ATCC 43879 (referred to as *Helicobacter rappini* by Chien et al., 2000). We succeeded in amplifying a *cdtB* gene fragment from this strain (taxon 8; see Table 1) with the primer pair VAT2 and DHF1. In accordance with Chien et al. (2000), we were also unsuccessful in amplification of the expected PCR product from *H. bilis* with primers VAT2 and WM11. Primers VAT2 and WM11 are designed for the *cdtB* gene of *C. jejuni* and, due to divergence of the gene, use of these degenerate primers may not amplify the *cdtB* fragment from species that possess the gene.

In the toxicity test with bacterial sonicates from taxa 1, 4 and 5, no indication of CDT production was evident in the cultured HeLa cells, which was expected and further confirmed the results from the DNA analysis. An insertion with a translation termination codon was detected in sequences from taxa 2 and 3. The presumed ribosome-binding sites for the *cdtB* ORF, as well as the probable initiation codon, are approximately 70 bp upstream from the binding site for VAT2. It is quite obvious that most of the *cdtB* gene is not translated in taxa 2 and 3. The possibility that this nonsense mutation represents a PCR artefact is unlikely, since the same hybridization signal appeared in taxon 1, 4 or 5 and differ from the strains of taxa 2, 3 and 8, as well as from *H. bilis* (Hänninen et al., 2003). Taxa 2, 3 and 8 are presently unnamed and their taxonomic characterization awaits further study (Dewhirst et al., 2000). Young et al. (2000b) have also proposed that the presence/absence of the *cdtB* gene may prove useful in the classification of microaerobic spiral bacteria. Their suggestion was based on experiments that included eight clinical isolates and three reference strains of *H. pullorum*. The two isolates that were *cdtB*-negative actually represented a novel *Helicobacter* species closely related to, but distinct from, *H. pullorum*; the name *Helicobacter canadensis* has been proposed for these *cdtB*-negative strains (Fox et al., 2000).

The *cdt*-positive *Helicobacter* sp. flexispira strains were analysed further by phylogenetic methods. According to phylogenetic analysis of 16S rRNA sequences, *Helicobacter* sp. flexispira taxa 2 and 3 cluster together and may represent different species (Dewhirst et al., 2000). The partial *cdtB* genes of these two strains were almost identical and probably represent a pseudogene. The *cdtB* DNA sequences of taxa 2 and 3 showed 91–93 % similarity to the canine isolates, taxon 8 strains and *H. bilis* and they clustered together in the phylogenetic tree, with strong bootstrap support (Fig. 2). DNA sequences of the *cdtB* gene of the three reference strains for *Helicobacter* sp. flexispira taxon 8 were identical. These strains were isolated from stool samples of a human patient, his daughter and the family dog during a diarrhoeal episode in the family (Romero et al., 1988). Our *cdtB* sequence findings further support the hypothesis that the flexispira strain was transmitted to the human patient and his daughter from the dog. The six canine flexispira isolates included in this study all cluster with *Helicobacter* sp. flexispira taxon 8, as shown by 16S rRNA sequence analysis (F. E. Dewhirst, personal communication). Partial DNA sequences of the *cdtB* gene of isolates KO115, KO220 and KOS34B differed from each other and from sequences of reference strains of *Helicobacter* sp. flexispira taxon 8 by only 1–3 %. Phylogenetic analysis supports the idea that isolates KO115, KO220 and KOS34B may represent taxon 8 strains, but support for isolates KO214, KO114 and Hiili is weak (Fig. 2). Because *cdtB* is not present in all *Helicobacter* species, or can represent a pseudogene in some species, the sequence diversity of this gene is not necessarily a good phylogenetic marker for taxonomic purposes.

**ACKNOWLEDGEMENTS**

We thank Dr Floyd Dewhirst for providing 16S rRNA information about the Finnish canine isolates, Mikko Koikkala, MSc, for help in phylogenetic analysis and Päivi Ronkainen, MSc, for technical assistance. This work was supported financially by the University of Helsinki Foundation.

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