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**Occurrence and species level diagnostics of *Campylobacter* spp., enteric
Helicobacter spp. and *Anaerobiospirillum* spp. in healthy and diarrheic
dogs and cats.**

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Abstract

In order to study the occurrence and co-infection of different species of *Campylobacter*, enteric *Helicobacter* and *Anaerobiospirillum* in dogs and cats and define a possible association between these microorganisms and gastrointestinal disorders, 190 dogs and 84 cats, either healthy or with diarrhea, were sampled between 2002 and 2003. Thirty-three *C. upsaliensis*, 17 *C. jejuni*, 2 *C. helveticus*, 1 *C. lari* isolates from dogs and 14 *C. helveticus*, 7 *C. jejuni*, 6 *C. upsaliensis* isolates from cats were identified using species-specific PCR and phenotypic tests. Whole cell protein profile analysis, phenotypic tests, PCR-RFLP of *gyrB* and a phylogenetic study of partial *groEL* and 16S rRNA sequences were used to identify 37 *H. bilis*, 22 *H. canis* and 14 *H. cinaedi* in dogs and 12 *H. canis*, 5 *H. bilis* and 2 *H. cinaedi* in cats. Whole cell protein profile analysis, phenotypic tests and species-specific PCR of 16S rRNA were used to identify 14 *A. succiniciproducens*, 12 *A. thomasi* isolates and one unidentified *Anaerobiospirillum* sp. isolate in dogs and 3 *A. thomasi* isolates in cats. Fifty-two animals (19%) were positive for the isolation of more than one genus. No significant statistical correlation was found between any isolates of *Campylobacter*, *Helicobacter* or *Anaerobiospirillum* spp. or the various co-infection rates, and the presence of diarrhea in either dogs or cats. *Campylobacter* isolates were also tested for antibiotic resistance using the agar dilution method.

Introduction

Campylobacter species, in particular *Campylobacter jejuni* and *Campylobacter coli*, are considered to be the most frequent bacterial cause of human enteritis whereas their role as enteric pathogens in dogs and cats is much less evident (Burnens et al., 1992; Sandberg et al., 2002; Engvall et al., 2003; Koene et al., 2004; Wieland et al., 2005). In addition, enteric *Helicobacter* spp. have been isolated also from normal and diarrhoeic dogs and cats (Stanley et al., 1993; Kiehlbauch et al., 1995; Foley et al., 1999; Kipar et al., 2001) as well as from the liver of a dog with hepatitis (Fox et al., 1996) but their prevalence and role as pathogens in these animals have not been well-established.

Currently, the genus *Anaerobiospirillum* comprises two species, *A. succiniciproducens* and *A. thomasi*, which have been isolated from dogs and cats (Malnick, 1997) although, only few studies have related them to diarrhea (Misawa et al., 2002; De Cock et al., 2004).

Mixed infections of *Campylobacter* spp. and *Helicobacter* spp. and *Anaerobiospirillum* spp. have been described previously (Shen et al., 2001; Misawa et al., 2002) but no extensive studies have been carried out on the co-infection in dogs and cats with these three genera to verify any possible correlation with gastrointestinal disorders.

The aims of this survey were to: (i) study the occurrence and (ii) co-infection of different species of *Campylobacter*, enteric *Helicobacter* and *Anaerobiospirillum* in healthy and diarrhoeic dogs and cats; (iii) define a possible association between these microorganisms and gastrointestinal disorders; (iv) define a combination of phenotypic and genotypic tests which

could be used in diagnosing of these species. Antimicrobial susceptibility was also determined for *Campylobacter* isolates.

Materials and methods

Sampling and isolation. Between 2002 and 2003, 274 samples of fresh faeces from pet dogs and cats were collected by the owners and delivered to private veterinary clinics located in province of Bologna. The study included 52 healthy dogs, 21 healthy cats as well as 138 dogs and 63 cats with clinically diagnosed diarrhea. A maximum of one pet per household was included in the study. The anamnesis revealed that all the animals had been vaccinated against canine or feline *Parvovirus*, were negative for enteric parasites and had not been treated with antibiotics for two months before sampling. Moreover, all the animals in the study suffering from diarrhea had been tested for *Salmonella* by direct plating faeces in different selective media and also by enrichment methods, resulting negative. The faeces were transported to the laboratory in BBL-Cary and Blair Transport Medium (Becton, Dickinson and Co., Sparks, MD 21152, USA) and processed within 12 h after collection. Approximately 5g of faeces were homogenized in 5 ml of sterile saline and ten microlitres of each sample were streaked directly by a loop onto three different selective media: Blaser-Wang's agar plate (Oxoid LTD, Basingstoke, Hampshire, UK), Skirrow's agar plate (Oxoid) and modified Charcoal Cefoperazone Deoxycholate Agar (Oxoid). The plates were incubated in a jar at $37^{\circ}\text{C}\pm 1$ under a microaerobic atmosphere with hydrogen obtained by the gas replacement method with anaerobic gas mixture (H_2 10%, CO_2 10%, N_2 80%) (Bolton et al., 1992) and examined daily for growth for up to one week. Colonies of Gram negative, curved, spiral or fusiform rod organisms were subcultivated on Difco-Brucella Agar (Becton,

Dickinson and Co.) supplemented with 10% sheep blood. After cloning, on the basis of colony morphology and microscopic observation, the isolates were roughly grouped into campylobacters, spiral-shaped helicobacters, fusiform-shaped or cigar-like helicobacters and *Anaerobiospirillum*.

DNA extraction. The chromosomal DNA of bacteria was extracted using DNeasy Tissue kit (Qiagen, GmbH D-40724, Hilden, Germany) according to the manufacturer's instructions.

Genus identification. The isolates underwent genus specific PCRs for *Campylobacter* (Linton et al. 1996), *Helicobacter* (Bohr et al. 2002), and *Anaerobiospirillum* for which we developed a new PCR primer set. Forward primer anasF437 5'-ACGTTACCCACAGAAGAAG-3' and reverse primer anasR937 5'-CCTACGATGTCAAGGTCAG-3', designed from regions of the 16S rRNA gene of *A. succiniciproducens* ATCC 29305^T (Accession number U96412) and *A. thomasi* DSM 11806^T (Accession number AJ420985), were used to amplify a 500 bp specific fragment. The specificity of anasF437 and anasR937 primers was confirmed by comparing the sequences of the purified amplicons of four different positive isolates with deposited 16S rRNA sequences in non-redundant databases using BLASTN (<http://www.ncbi.nlm.nih.gov/BLAST/>). The sequence similarity among all four isolates and the sequences of *Anaerobiospirillum* deposited was always >92%.

Identification of *Campylobacter* species. The isolates were identified at the species level by *C. jejuni* - *C. coli* specific multiplex PCR (Denis et al., 1999), *C. upsaliensis* - *C. helveticus* specific duplex PCR (Lawson et al., 1997) and *C. lari* PCR (Linton et al., 1996). The isolates also underwent biochemical

tests using the methods, media and quality control strains recommended by On et al. (1996) (see Table 2).

Identification of *Helicobacter* species. The isolates were identified at the species level using whole cell protein profile analysis in 1D SDS-PAGE (Zanoni et al., 2007) and biochemical characterization (see Table 2). All the tests were performed according to On et al. (1996), except for the Gamma-glutamyltranspeptidase production test which was carried out as recommended by Zanoni et al. (2007). Further identification of urease-negative spiral-shaped *Helicobacter* isolates was carried out by sequence analysis of *groEL* (Mikkonen et al., 2004). Moreover, as an additional identification tool for urease-positive fusiform shaped *Helicobacter* isolates, a PCR-RFLP based on *gyrB* was used. Briefly, forward primer HBILISF 5'-AATGGTGGCACACATGAAG-3' and reverse primer HBILISR 5'-CGCAACCAAAGGCAGTAATC-3' were used to amplify a 600 bp fragment. A specific restriction pattern (324, 224 and 52 bp) for *H. bilis* was obtained using *HindIII* (New England Biolabs, Inc., 240 County Road, Ipswich, MA 01938-2723 USA). Fusiform-shaped *Helicobacter* also underwent morphological examination by Transmission Electron Microscopy (TEM; ZEISS E 900) using 1% (w/v) phosphotungstate acid (Sigma Chemical Co., St. Luis, MO63178, USA) as negative staining.

Identification of *Anaerobiospirillum* species. The isolates were first identified by whole cell protein profile analysis in 1D SDS PAGE (Zanoni et al., 2007). Moreover, phenotypic identification (see Table 3) was carried out by employing enzymatic reactions using rapid ID 32 A (BioMérieux SA, 69280, Marcy l'etoile, France) and carbohydrate fermentation tests as described by Malnick et al. (1990). The isolates were also identified as *A.*

thomasi by a PCR primer set thomF419 5'-GAGGAAATCGCAAGAGTG-3' and thomR919 5'-GTAAGGTTCTTCGCGTTG-3', designed from the 16S rRNA gene of *A. thomasi* DSM 11806^T (Accession number AJ420985) developed in the present study. The specificity of this PCR was confirmed by comparing the results obtained to those achieved by whole cell protein profile analysis and phenotypic tests.

16S rRNA gene sequence analysis. The nearly complete 16S rRNA gene of *Helicobacter* and *Anaerobiospirillum* isolates was amplified by using universal primers p27f (5'-AGAGTTTGATCCTGGCTCAG-3') and p1492r (5'-TACGGCTACCTTGTACGACT-5') and the PCR-amplified template was sequenced by primer walking strategy (Primm s.r.l., Milan, Italy). All sequences were compared directly with the NCBI GenBank nonredundant nucleotide database using MEGABLAST (<http://www.ncbi.nlm.nih.gov/BLAST/>). Subsequently, the nucleotide sequences were aligned in BioEdit (<http://www.mbio.ncsu.edu/BioEdit/bioedit.html>) with reference sequences obtained from GenBank using ClustalW and the alignment was corrected visually removing IVS regions. A phylogenetic tree was constructed in MEGA3 (<http://www.megasoftware.net/>) using the neighbour-joining method. Data were corrected for multiple base changes using the method of Jukes and Cantor (Jukes and Cantor, 1969), and bootstrap analysis was performed with 1000 resembled data sets.

Antimicrobial susceptibility. Minimum Inhibitory Concentration (MIC) values of eight antimicrobial agents were determined on 78 *Campylobacter* isolates (see Table 4). MICs values for *C. jejuni* isolates were determined by the agar dilution method recommended by the Clinical and Laboratory

Standards Institute (CLSI, formerly NCCLS; NCCLS, 2002). Since not all strains of *C. upsaliensis* and *C. helveticus* grew on Muller Hinton Agar supplement with 5% sheep blood, the original method was modified as described by Zanoni et al. (2007) for *Helicobacter pullorum*. The following breakpoints for resistance were used: erythromycin $\geq 8 \mu\text{g}\cdot\text{ml}^{-1}$; chloramphenicol $\geq 32 \mu\text{g}\cdot\text{ml}^{-1}$; gentamicin $\geq 16 \mu\text{g}\cdot\text{ml}^{-1}$; ampicillin $\geq 32 \mu\text{g}\cdot\text{ml}^{-1}$; tetracycline $\geq 16 \mu\text{g}\cdot\text{ml}^{-1}$; nalidixic acid $\geq 32 \mu\text{g}\cdot\text{ml}^{-1}$; ciprofloxacin $\geq 4 \mu\text{g}\cdot\text{ml}^{-1}$; enrofloxacin $\geq 4 \mu\text{g}\cdot\text{ml}^{-1}$.

Statistical analysis. The results were analyzed using SPSS/PC+ statistical package for logistic regression. The Yates-corrected χ^2 test or 2-tailed Fisher exact test ($\alpha = 0.05$) were used for testing the correlation between the presence of *Campylobacter*, *Helicobacter* or *Anaerobiospirillum* and gastrointestinal disorders.

Results

Isolation. A total of 131 out of 274 animals sampled (51% of the dogs and 41.6% of the cats) were positive for at least one genus considered in our study. The isolation rates of the different species are shown in Table 1. *Campylobacter* spp. were isolated from 53 dogs and from 27 cats, *Helicobacter* spp. from 65 dogs and 18 cats and *Anaerobiospirillum* spp. from 27 dogs and 3 cats. From the canine samples, the highest isolation rate was for *H. bilis* (19.5%) followed by *C. upsaliensis* (17.3%), *H. canis* (11%), *C. jejuni* (8.9%), *H. cinaedi* (7.9%), *A. succiniciproducens* (7.4%) and *A. thomasi* (6.3%). In cats, the highest isolation rate was for *C. helveticus* (16.7%) followed by *H. canis* (14.3%), *C. jejuni* (8.3%), *C. upsaliensis* (7.2%), *H. bilis* (5.9%), *A. thomasi* (3.6%) and *H. cinaedi* (2.4%). An unidentified *Anaerobiospirillum* sp. was isolated from a dog. Fifty-two animals (19%)

were positive for the isolation of more than one genus. Co-infection with *Campylobacter* and *Helicobacter*, observed in about 10% of both dog and cat samples, was the most frequent while only 8 dogs (4 healthy and 4 with diarrhea) and 2 cats with diarrhea were simultaneously infected by all three genera. Co-infection with *Campylobacter* and *Anaerobiospirillum* was found in 6 dogs (3.1%) and 1 cat (1.2%) whereas a mixed co-infection of *Helicobacter* and *Anaerobiospirillum* was found in 8 dogs (4.2%).

Identification of *Campylobacter* spp. Eighty strains of *Campylobacter* spp. were isolated in this study: 24 *C. jejuni*, 39 *C. upsaliensis*, 16 *C. helveticus* and 1 *C. lari*. The results of the biochemical characterization of *Campylobacter* spp. are shown in Table 2. Genotypic identification using species-specific PCR was concordant with biochemical characterization.

Identification of *Helicobacter* spp. A total of 92 strains of *Helicobacter* spp. were isolated: 42 *H. bilis*, 33 *H. canis* and 17 *H. cinaedi*. Biochemical characterization results of *Helicobacter* spp. are shown in Table 2. The numerical analysis of the protein electrophoregrams pointed out 42 urease-positive fusiform-shaped *Helicobacter* isolates in one cluster with an 82% of similarity to *H. bilis* ATCC 51630^T (data not shown). All these isolates, according to TEM analysis, had a net-like ultrastructure with periplasmic fibers on the surface and bipolar tufts of sheated flagella; moreover, they showed a *HindIII* pattern of the *gyrB* fragment (324, 224 and 52 bp - data not shown) identical to *H. bilis*. On the basis of both phenotypic and genotypic results, we found that all 42 urease-positive isolates were *H. bilis*. Fifty urease-negative spiral-shaped *Helicobacter* isolates were divided, by whole cell protein profile analysis, into 3 different groups with more than 82% of similarity within the clusters: (i) *H. canis* ATCC 51401 cluster; (ii) *H. cinaedi*

CCUG 18818 cluster and (iii) a group including ten isolates which did not cluster with any of the reference strains. Visual analysis of the protein profiles of these ten isolates showed a high level of similarity with *H. canis*, except for two dense low-molecular weight bands absent in the *H. canis* reference strain (data not shown). A phylogenetic tree based on the partial *groEL* sequences of the isolates referring to the group mentioned above, along with randomly selected 4 putative *H. canis* and 3 putative *H. cinaedi* isolates, is presented in Figure 1a. Six out of the ten isolates were closely related with the four putative and reference *H. canis* strains, while four isolates (isolate numbers 192, 163, 170, 162) clustered together in a distinct clade separated from the other *H. canis* strains with high bootstrap value (100). The sequence similarity between these two distinct clusters was 94% and within the ten sequences ranged from 93,1% to 100%. A similar divergence in nucleotide sequences was observed also for *H. cinaedi* strains. This divergence was not supported by the phylogenetic tree based on deduced partial GroEL protein sequences (Figure 1b), which showed a high similarity within all the *H. canis* or *H. cinaedi* sequences. MEGABLAST analysis of partial 16S rRNA sequences indicated that there was a very high degree of sequence similarity among isolates 192, 163, 170 and 162 and *H. canis*. Phylogenetic analysis showed a robust clade including *H. canis* strains (data not shown). For all four 16S rRNA gene sequences, the similarity with the type strain of *H. canis* (strain NCTC 12739) was >99%. Analysis of the partial *groEL* and 16S rRNA sequences demonstrated that these ten isolates were *H. canis* and supported the whole cell protein profile results.

Identification of *Anaerobiospirillum* spp. A total of 30 *Anaerobiospirillum* were isolated: 15 *A. thomasi*, 14 *A. succiniciproducens* and 1 unidentified

Anaerobiospirillum. Numerical analysis of the protein profiles divided the isolates at 83% of similarity in two major clusters, *A. succiniciproducens* and *A. thomasi*, and into a single profile concerning the isolate 78/9/02 (data not shown). Divergence between the 78/9/02 isolate and the other *Anaerobiospirillum* spp. was also observed by biochemical characterization (Table 3): the isolate was positive for α -galactosidase and produced acid from fructose, raffinose and adonitol. For *A. succiniciproducens* and *A. thomasi* isolates the biochemical results were consistent with those published previously by Malnick (1997), except for two isolates (44/6/02 and 78/3/02) of *A. succiniciproducens* which were not able to produced acid from raffinose. In order to obtain further taxonomic data, an analysis of the partial 16S rRNA sequence of the 78/9/02 isolate was carried out. A high degree of sequence similarity (>99%) between 78/9/02 and the 3J102 strain of *Anaerobiospirillum* sp. isolated in a puppy with hemorrhagic diarrhea (Misawa et al., 2002) was found by MEGABLAST analysis. Phylogenetic analysis indicated a close genetic relationship between the 78/9/02 and 3J102 strains as well as their clear separation from the other *Anaerobiospirillum* spp. (Figure 2). The genotypic identification of *A. thomasi* isolates by thomF419 and thomR919 primer set was always concordant with biochemical and 1D SDS PAGE data.

Antibiotic susceptibility testing. The distribution of MIC values and the overall resistance rates of the 78 *Campylobacter* strains tested are presented in Table 4. The fluoroquinolones resistance were most common resistance among isolates. One out of 24 *C. jejuni* isolates showed multidrug resistance to nalidixic acid, ciprofloxacin, tetracycline and ampicillin; while other five *C. jejuni* strains were resistant to nalidixic acid, ciprofloxacin and tetracycline at the same time.

Statistical analysis. Based on the results of χ^2 tests or the 2-tailed Fisher exact test ($\alpha = 0.05$), no statistically significant correlation was found between the isolation of *Campylobacter*, *Helicobacter*, *Anaerobiospirillum* or different co-infection rates, and the presence of gastroenteric disorders, in either dogs or cats. Moreover, no positive association between the presence of diarrhea and *Campylobacter*, *Helicobacter* or *Anaerobiospirillum* isolation in dogs and cats was found when matching the healthy and ill animals on sex and age.

Nucleotide sequence accession numbers. Partial sequence of 16S rRNA gene of the *H. canis* isolates 162, 163, 170, 192 and the *Anaerobiospirillum* isolate 78/9/02 were submitted to GenBank with the follow accession numbers: EF569185, EU144017, EU144018, EU144019 and EF428122. Partial sequence of *groEL* of the *H. canis* isolates 142, 143, 148, 149, 155, 162, 163, 168, 170, 171, 185, 188, 192, 194 and *H. cinaedi* isolates 139, 161 and 190 were submitted to GenBank with the follow accession numbers: EU233438, EU233439, EU233440, EU233441, EU233442, EU233443, EU233444, EU233445, EU233446, EU233447, EU233448, EU233449, EU233450, EU233451 and EU233452, EU233453, EU233454.

Discussion and conclusion

There is a paucity data regarding the occurrence of mixed infections of *Campylobacter* spp., enteric *Helicobacter* spp. and *Anaerobiospirillum* spp. in dogs and cats. To our knowledge, only two studies have been published. Shen et al. (2001) reported a high prevalence (26%) of co-infections by *Campylobacter* spp. and enteric *Helicobacter* spp. in a large number of clinically healthy cats using species-specific PCR-RFLP. Furthermore, Misawa et al. (2002) described a mixed infection with *Campylobacter* spp., enteric *Helicobacter* spp. and *Anaerobiospirillum* spp. in a puppy with

hemorrhagic diarrhea. In the present study, which represents the first extensive investigation on co-infection by *Campylobacter* spp., enteric *Helicobacter* spp. and *Anaerobiospirillum* spp. in healthy or diarrhoeic dogs and cats, 27 animals (10%) were found to be infected with *Campylobacter* spp. and *Helicobacter* spp., and eight dogs and two cats were positive for all three genera.

The isolation rates of different *Campylobacter* spp. observed in our study were similar to those described in other countries (Burnens et al., 1992; Sandberg et al., 2002; Hald et al., 2004). *C. upsaliensis* was the most frequently isolated species in dogs and *C. helveticus* from cats while the isolation rates of *C. jejuni* were similar in both animals.

Concerning *Helicobacter*, in 1993 Stanley et al. first described a prevalence of about 1.5% of *H. canis* in one thousand healthy and diarrhoeic domestic pets. Then, *H. canis* was isolated by Fox et al. (1996) in a dog liver with multifocal necrotizing hepatitis, by Foley et al. (1999) in four bengal cats with endemic diarrhea and by Shen et al. (2001) in one healthy cat. Canine and feline *H. cinaedi* strains were reported by different authors (Kiehlbauch et al., 1995; Vandamme et al. 2000; Misawa et al. 2002) but prevalences of this species in dogs or cats are not currently available. *Helicobacter* sp. flexispira taxon 8, recently included in the *H. bilis* species (Hänninen et al., 2005), was first isolated from an asymptomatic young dog and from its owners by Romero et al. (1988). Further descriptions of these isolates were reported by Dewhirst et al. (2000), Shen et al. (2001) and Hänninen et al. (2005) in healthy pets, but no data about prevalence are available in these studies. In our study, the most frequent isolates were *H. bilis* (15,3%) followed by *H. canis* (12%) and finally *H. cinaedi* (6,2%).

Studies regarding the prevalence of different *Anaerobiospirillum* species in dogs and cats have not previously been published, even if Malnick et al. (1990) reported that these bacteria may belong to the normal faecal flora of these animals. We detected *A. succiniciproducens* in 7.4% of animals and *A. thomasi* in 5.5%.

On the basis of our results, no statistically significant correlation between isolation of single or mixed infections and the presence of diarrhea was observed. Concerning *Campylobacter* spp., these data are in agreement with those described by Sandberg et al. (2002) in an analogous study.

To overcome the difficult identification of *Helicobacter* spp., in our study it was indispensable to use a polyphasic approach, in order to define a real frequency of these species and subsequently to evaluate their pathogenic role in the animal hosts. Actually, misidentification of *Helicobacter* spp. is frequent, due to their taxonomic complexity and the morphological similarity of some species with *Campylobacter*. 16S rRNA sequence analysis, which is a common tool for bacterial species identification, is not suitable for all enteric *Helicobacter* spp. inasmuch as sequence variation within a species can be as high as 4.5% and, on other hand, the sequences of two species can be 99% similar (Vandamme et al., 2000; Hänninen et al., 2003).

This identification strategy, applied to *Anaerobiospirillum* spp., also allowed the detection of a strain from a dog (isolate 78/9/02) which was not identifiable at the species level. In particular, the nearly complete 16S rRNA gene revealed a close genetic relationship between this isolate and the strain 3J102 isolated by Misawa et al. (2002) from a puppy as well as its clear separation from the other *Anaerobiospirillum* spp., indicating that both isolates probably belong to a new taxon.

Moreover, the polyphasic approach has permitted the development of novel genomic diagnostic tools for the identification of *Helicobacter* spp. and *Anaerobiospirillum* spp.. Recently, Hannula and Hänninen (2007) showed that partial *gyrB* gene sequence analysis proved to be a particularly well-suited tool for phylogenetic analysis of *Helicobacter* and, in particular, for distinguishing closely related species which belong to the ‘flexispira-like’ group. In this study, we developed a new PCR-RFLP analysis of *gyrB* in order to differentiate *H. bilis* from the other members of this group. Likewise, new PCR approaches were described for rapid and simple identification both at the genus level of *Anaerobiospirillum* and the species level of *A. thomasi*; the previously used conventional phenotypic methods for this identification were difficult and time consuming. Some studies showed that approximately 6% of human enteric campylobacteriosis is transmitted from pets (Tenkate and Stafford, 2001) and that these animals represent potential sources of the spread of antimicrobial resistance due to their close contact with humans (Guardabassi et al., 2004). Direct evidence of the transmission of fluoroquinolone resistant *C. jejuni* between humans and pets living in the same households has also been shown (Damborg et al., 2004). Currently, macrolides and fluoroquinolone are the antibiotics of choice when therapeutic intervention is warranted in human campylobacteriosis. Our results showed the absence of resistance to erythromycin in *Campylobacter* isolates but a high percentage of *C. jejuni* isolates were resistant to nalidixic acid and ciprofloxacin, with values higher than those described by Sanberg et al. (2002) in Norway. These data probably reflect the extensive use of fluoroquinolones in dogs and cats in Italy.

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Table 1. Isolation rates (%) of *Campylobacter* spp., enteric *Helicobacter* spp. and *Anaerobiospirillum* spp. from healthy and diarrhoeic dogs and cats.

Species	Dogs				Cats			
	Total (n=190)		Healthy (n=52)	Diarrhoeic (n=138)	Total (n=84)		Healthy (n=21)	Diarrhoeic (n=63)
<i>C. jejuni</i>	8,9	[17] ^a	11,5	7,9	8,3	[7]	4,8	9,5
<i>C. upsaliensis</i>	17,3	[33]	30,8	12,3	7,2	[6]	14,3	4,8
<i>C. helveticus</i>	1	[2]	1,9	0,7	16,7	[14]	19	15,9
<i>C. lari</i>	0,5	[1]	1,9	-	-	-	-	-
<i>H. canis</i>	11	[21]	21,2	7,3	14,3	[12]	28,6	9,5
<i>H. cinaedi</i>	7,9	[15]	5,8	8,7	2,4	[2]	4,8	1,6
<i>H. bilis</i>	19,5	[37]	36,5	13,1	5,9	[5]	4,8	6,3
<i>A. succiniciproducens</i>	7,4	[14]	7,7	7,3	-	-	-	-
<i>A. thomasi</i>	6,3	[12]	17,3	2,2	3,6	[3]	-	4,8
Unidentified	0,5	[1]	-	1,9	-	-	-	-
<i>Anaerobiospirillum</i>								

^a In square bracket the number of isolates

505 Table 2. Phenotypic characteristics of the 80 isolates of *Campylobacter* spp. and 92

506 *Helicobacter* spp

Phenotypic tests ^a	<i>C. jejuni</i> (n=24)	<i>C. upsaliensis</i> (n=39)	<i>C. helveticus</i> (n=16)	<i>C. lari</i> (n=1)	<i>H. canis</i> (n=33)	<i>H. cinaedi</i> (n=17)	<i>H. bilis</i> (n=42)
Positive reaction to							
Oxidase	24	39	16	1	33	17	42
Catalase	24	4	6	1	1	4	2
Urease	0	0	0	0	0	0	42
Hippurate hydrolysis	24	0	0	0	ND	ND	ND
Indoxyl acetate hydrolysis	24	39	16	0	31	8	3
γ -Glutamyltranspeptidase	ND	ND	ND	ND	32	0	42
Alkaline phosphatase	ND	ND	ND	ND	20	10	42
Trace H ₂ S in TSI	0	0	0	0	ND	ND	ND
Nitrate reduction	24	39	16	1	0	15	0
Growth on special media							
MacConkey	24	7	1	1	ND	ND	ND
Growth on media containing							
1% (w/v) bile	ND	ND	ND	ND	27	10	0
0,04% (w/v) TTC	17	0	0	0	ND	ND	ND
0,1% sodium selenite	ND	39	0	ND	ND	ND	ND
Growth at							
37°C (mO ₂)	24	39	16	1	33	17	42
37°C (O ₂)	0	0	0	0	0	0	0
37°C (AnO ₂)	0	0	0	0	1	9	0
42°C (mO ₂)	24	39	16	1	33	15	42
25°C (mO ₂)	0	0	0	0	0	0	0
Resistant to							
nalidixic acid	14	3	0	1	1	1	0
cephalotin	24	0	0	1	13	14	41

a ND, not done; mO₂ microaerobically plus H₂; O₂, aerobically; AnO₂, anaerobically; TSI, Triple Sugar Iron Agar; TTC, 2,3,5-triphenyltetrazolium chloride

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508 Table 3. Phenotypic characteristics of the 30 isolates of *Anaerobiospirillum* spp.

Phenotypic tests	<i>A. succiniciproducens</i>	<i>A. thomasi</i>	Unidentified <i>Anaerobiospirillum</i> ^b
	14	15	1
Positive reaction to ^a			
α -galactosidase	0	0	1
β -galactosidase	14	0	1
α -glucosidase	14	0	1
β -N-acetyl-glucosaminidase	14	15	1
leucine arylamidase	9	0	0
Acid from			
D-mannitol	0	0	0
D-(-)-fructose	14	0	1
adonitol	0	15	1
D-(+)-raffinose	12	0	1

^aAPI enzymes, rapid ID 32 A (BioMérieux); ^bIsolate number 78/9/02

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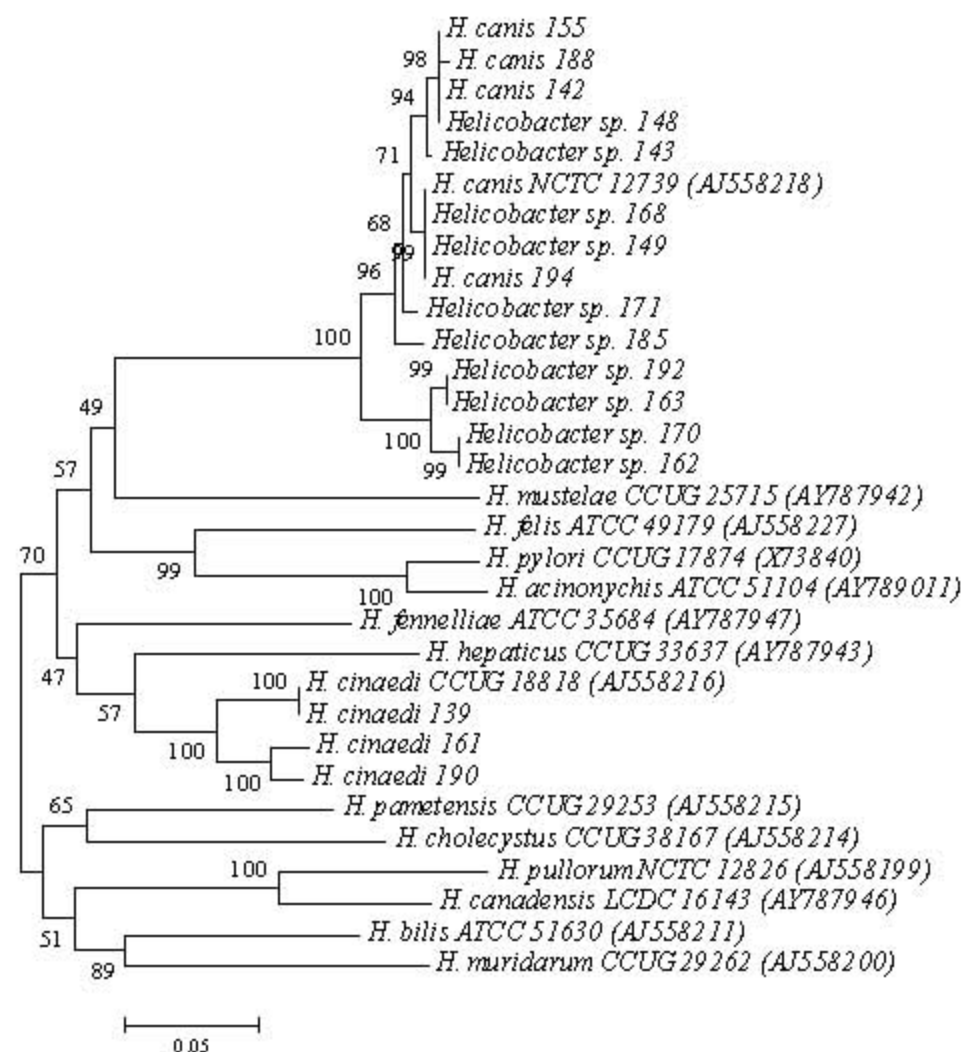
Table 4. Distribution of MICs for 24 *C. jejuni*, 38 *C. upsaliensis* and 16 *C. helveticus* isolated from dogs and cats

Antimicrobials	Species	Number of <i>Campylobacter</i> spp. isolates with MIC of (μg·ml ⁻¹) ^a :																%R ^b
		<0,015	0,015	0,03	0,06	0,12	0,25	0,5	1	2	4	8	16	32	64	128	256	
erythromycin	<i>C. jejuni</i>							1	7	10	6							0
	<i>C. upsaliensis</i>								7	21	10							0
	<i>C. helveticus</i>								2	8	3	1	2					19
chloramphenicol	<i>C. jejuni</i>									2	16	2	3	1				4
	<i>C. upsaliensis</i>			1							18	18	1					0
	<i>C. helveticus</i>								8	6	2							0
gentamicin	<i>C. jejuni</i>							16	8									0
	<i>C. upsaliensis</i>					26	12											0
	<i>C. helveticus</i>	1		1	5	8	1											0
ampicillin	<i>C. jejuni</i>								4	5	7	3	2		3			12
	<i>C. upsaliensis</i>						2	10	8	3	4	4	4	3				8
	<i>C. helveticus</i>				1			1	4	9	1							0
tetracycline	<i>C. jejuni</i>				1	9	1	3			1		3		4	1	1	37
	<i>C. upsaliensis</i>				1	12	21	4										0
	<i>C. helveticus</i>				1	10	3	1	1									0
nalidixic acid	<i>C. jejuni</i>									7	2		1	3	5	4	2	62
	<i>C. upsaliensis</i>								1	17	17				2	1		8
	<i>C. helveticus</i>								4	11					1			6
ciprofloxacin	<i>C. jejuni</i>					1	8		1			5	5	3	1			58
	<i>C. upsaliensis</i>				1	11	21	2				1	2					8
	<i>C. helveticus</i>		4	1	6	4						1						6
enrofloxacin	<i>C. jejuni</i>				1	8		1		7	3	4						58
	<i>C. upsaliensis</i>			1	29	5				1	1	1						8
	<i>C. helveticus</i>			12	3					1								6

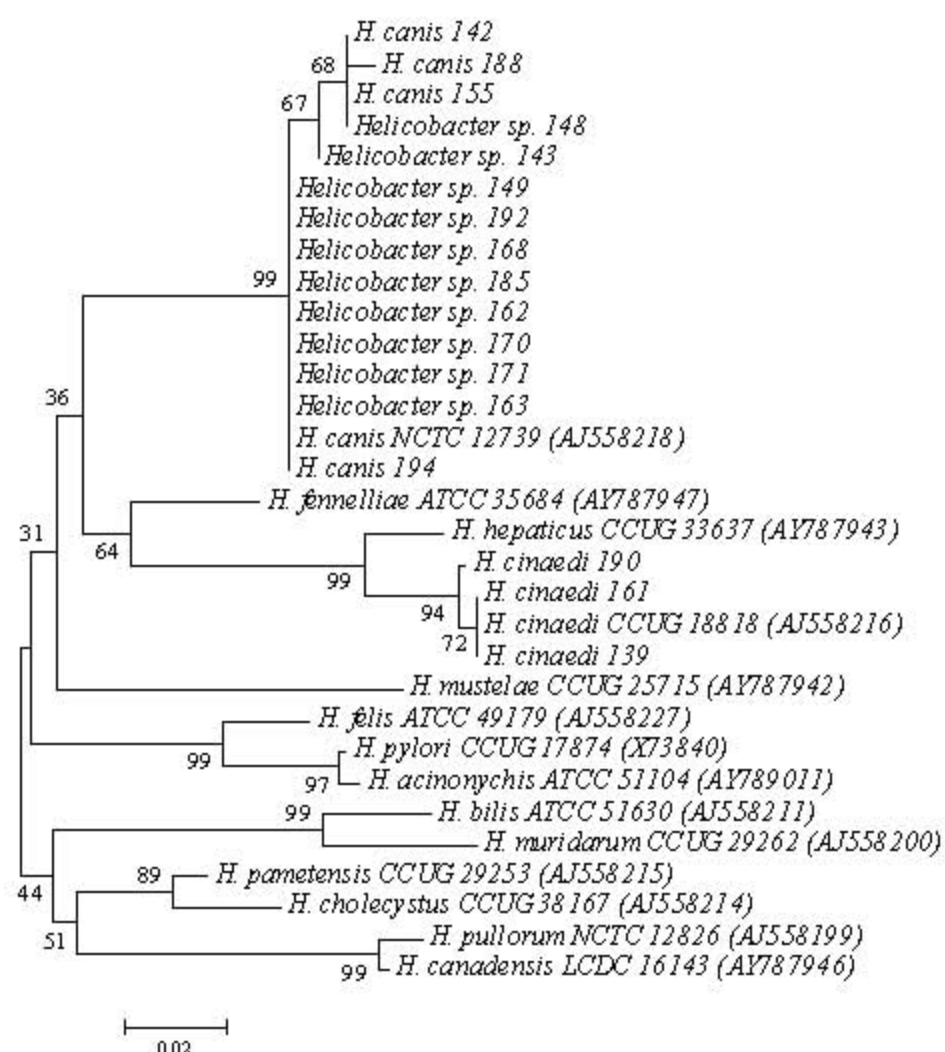
^a Resistant isolates are represented in bold; ^b %R, total resistance rates

Figure 1. a- A phylogenetic tree obtained from the analysis of partial *groEL* nucleotide sequences of 17 urease-negative *Helicobacter* spp. isolates and reference *Helicobacter* spp. strains available in GenBank; b- A phylogenetic tree obtained from the analysis of partial GroEL deduced amino acid sequences of 17 urease-negative *Helicobacter* spp. isolates and reference *Helicobacter* spp. strains.

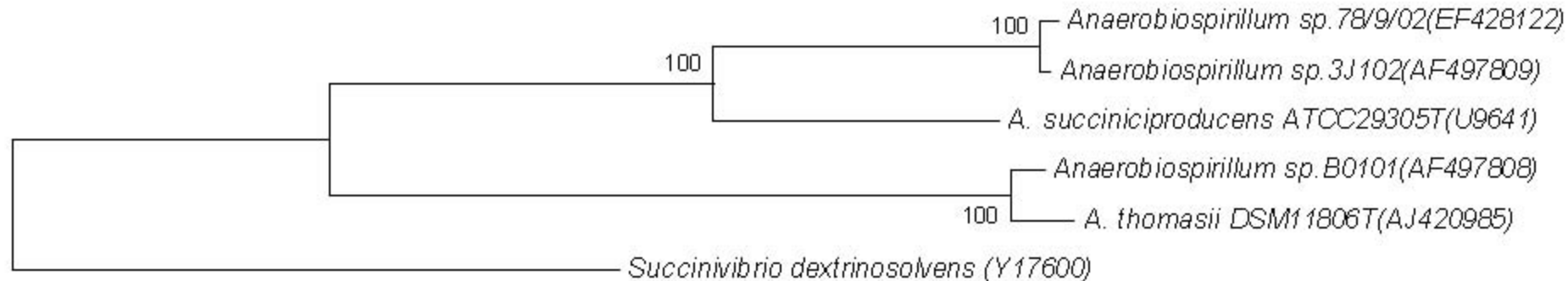
Figure 2. A phylogenetic tree obtained from the analysis of partial 16S rRNA sequences of 78/9/02 *Anaerobiospirillum* isolate and reference *Anaerobiospirillum* spp. strains available in GenBank. *Succinivibrio dextrinosolvens* (Y17600) was used as outgroup.



A



B



0.01