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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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Key words: *Campylobacter*, *Helicobacter*, *Anaerobiospirillum*, co-infection,
diagnostic, antibiotic resistance

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27 **Abstract**

28 In order to study the occurrence and co-infection of different species of
29 *Campylobacter*, enteric *Helicobacter* and *Anaerobiospirillum* in dogs and cats
30 and define a possible association between these microorganisms and
31 gastrointestinal disorders, 190 dogs and 84 cats, either healthy or with
32 diarrhea, were sampled between 2002 and 2003. Thirty-three *C. upsaliensis*,
33 17 *C. jejuni*, 2 *C. helveticus*, 1 *C. lari* isolates from dogs and 14 *C. helveticus*,
34 7 *C. jejuni*, 6 *C. upsaliensis* isolates from cats were identified using species-
35 specific PCR and phenotypic tests. Whole cell protein profile analysis,
36 phenotypic tests, PCR-RFLP of *gyrB* and a phylogenetic study of partial
37 *groEL* and 16S rRNA sequences were used to identify 37 *H. bilis*, 22 *H. canis*
38 and 14 *H. cinaedi* in dogs and 12 *H. canis*, 5 *H. bilis* and 2 *H. cinaedi* in cats.
39 Whole cell protein profile analysis, phenotypic tests and species-specific PCR
40 of 16S rRNA were used to identify 14 *A. succiniciproducens*, 12 *A. thomasii*
41 isolates and one unidentified *Anaerobiospirillum* sp. isolate in dogs and 3 *A.*
42 *thomasii* isolates in cats. Fifty-two animals (19%) were positive for the
43 isolation of more than one genus. No significant statistical correlation was
44 found between any isolates of *Campylobacter*, *Helicobacter* or
45 *Anaerobiospirillum* spp. or the various co-infection rates, and the presence of
46 diarrhea in either dogs or cats. *Campylobacter* isolates were also tested for
47 antibiotic resistance using the agar dilution method.

48 **Introduction**

49 *Campylobacter* species, in particular *Campylobacter jejuni* and
50 *Campylobacter coli*, are considered to be the most frequent bacterial cause of
51 human enteritis whereas their role as enteric pathogens in dogs and cats is
52 much less evident (Burnens et al., 1992; Sandberg et al., 2002; Engvall et al.,
53 2003; Koene et al., 2004; Wieland et al., 2005). In addition, enteric
54 *Helicobacter* spp. have been isolated also from normal and diarrhoeic dogs
55 and cats (Stanley et al., 1993; Kiehlbauch et al., 1995; Foley et al., 1999;
56 Kipar et al., 2001) as well as from the liver of a dog with hepatitis (Fox et al.,
57 1996) but their prevalence and role as pathogens in these animals have not
58 been well-established.

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

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

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

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

75 **Materials and methods**

76 **Sampling and isolation.** Between 2002 and 2003, 274 samples of fresh
77 faeces from pet dogs and cats were collected by the owners and delivered to
78 private veterinary clinics located in province of Bologna. The study included
79 52 healthy dogs, 21 healthy cats as well as 138 dogs and 63 cats with
80 clinically diagnosed diarrhea. A maximum of one pet per household was
81 included in the study. The anamnesis revealed that all the animals had been
82 vaccinated against canine or feline *Parvovirus*, were negative for enteric
83 parasites and had not been treated with antibiotics for two months before
84 sampling. Moreover, all the animals in the study suffering from diarrhea had
85 been tested for *Salmonella* by direct plating faeces in different selective media
86 and also by enrichment methods, resulting negative. The faeces were
87 transported to the laboratory in BBL-Cary and Blair Transport Medium
88 (Becton, Dickinson and Co., Sparks, MD 21152, USA) and processed within
89 12 h after collection. Approximately 5g of faeces were homogenized in 5 ml
90 of sterile saline and ten microlitres of each sample were streaked directly by a
91 loop onto three different selective media: Blaser-Wang's agar plate (Oxoid
92 LTD, Basingstoke, Hampshire, UK), Skirrow's agar plate (Oxoid) and
93 modified Charcoal Cefoperazone Deoxycholate Agar (Oxoid). The plates
94 were incubated in a jar at 37°C±1 under a microaerobic atmosphere with
95 hydrogen obtained by the gas replacement method with anaerobic gas mixture
96 (H₂ 10%, CO₂ 10%, N₂ 80%) (Bolton et al., 1992) and examined daily for
97 growth for up to one week. Colonies of Gram negative, curved, spiral or
98 fusiform rod organisms were subcultivated on Difco-Brucella Agar (Becton,

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

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

106 **Genus identification.** The isolates underwent genus specific PCRs for
107 *Campylobacter* (Linton et al. 1996), *Helicobacter* (Bohr et al. 2002), and
108 *Anaerobiospirillum* for which we developed a new PCR primer set. Forward
109 primer anasF437 5'-ACGTTACCCACAGAAGAAG-3' and reverse primer
110 anasR937 5'-CCTACGATGTCAAGGTCAG-3', designed from regions of
111 the 16S rRNA gene of *A. succiniciproducens* ATCC 29305^T (Accession
112 number U96412) and *A. thomasii* DSM 11806^T (Accession number
113 AJ420985), were used to amplify a 500 bp specific fragment. The specificity
114 of anasF437 and anasR937 primers was confirmed by comparing the
115 sequences of the purified amplicons of four different positive isolates with
116 deposited 16S rRNA sequences in non-redundant databases using BLASTN
117 (<http://www.ncbi.nlm.nih.gov/BLAST/>). The sequence similarity among all
118 four isolates and the sequences of *Anaerobiospirillum* deposited was always
119 >92%.

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

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

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

144 **Identification of *Anaerobiospirillum* species.** The isolates were first
145 identified by whole cell protein profile analysis in 1D SDS PAGE (Zanoni et
146 al., 2007). Moreover, phenotypic identification (see Table 3) was carried out
147 by employing enzymatic reactions using rapid ID 32 A (BioMérieux SA,
148 69280, Marcy l'etoile, France) and carbohydrate fermentation tests as
149 described by Malnick et al. (1990). The isolates were also identified as *A.*

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

156 **16S rRNA gene sequence analysis.** The nearly complete 16S rRNA gene of
157 *Helicobacter* and *Anaerobiospirillum* isolates was amplified by using
158 universal primers p27f (5'-AGAGTTTGATCCTGGCTCAG-3') and p1492r
159 (5'-TACGGCTACCTTGTTACGACT-5') and the PCR-amplified template
160 was sequenced by primer walking strategy (Primm s.r.l., Milan, Italy). All
161 sequences were compared directly with the NCBI GenBank nonredundant
162 nucleotide database using MEGABLAST
163 (<http://www.ncbi.nlm.nih.gov/BLAST/>). Subsequently, the nucleotide
164 sequences were aligned in BioEdit
165 (<http://www.mbio.ncsu.edu/BioEdit/bioedit.html>) with reference sequences
166 obtained from GenBank using ClustalW and the alignment was corrected
167 visually removing IVS regions. A phylogenetic tree was constructed in
168 MEGA3 (<http://www.megasoftware.net/>) using the neighbour-joining method.
169 Data were corrected for multiple base changes using the method of Jukes and
170 Cantor (Jukes and Cantor, 1969), and bootstrap analysis was performed with
171 1000 resembled data sets.

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

176 Standards Institute (CLSI, formerly NCCLS; NCCLS, 2002). Since not all
177 strains of *C. upsaliensis* and *C. helveticus* grew on Muller Hinton Agar
178 supplement with 5% sheep blood, the original method was modified as
179 described by Zanoni et al. (2007) for *Helicobacter pullorum*. The following
180 breakpoints for resistance were used: erythromycin $\geq 8 \mu\text{g}\cdot\text{ml}^{-1}$;
181 chloramphenicol $\geq 32 \mu\text{g}\cdot\text{ml}^{-1}$; gentamicin $\geq 16 \mu\text{g}\cdot\text{ml}^{-1}$; ampicillin ≥ 32
182 $\mu\text{g}\cdot\text{ml}^{-1}$; tetracycline $\geq 16 \mu\text{g}\cdot\text{ml}^{-1}$; nalidixic acid $\geq 32 \mu\text{g}\cdot\text{ml}^{-1}$; ciprofloxacin \geq
183 $4 \mu\text{g}\cdot\text{ml}^{-1}$; enrofloxacin $\geq 4 \mu\text{g}\cdot\text{ml}^{-1}$.

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

189 **Results**

190 **Isolation.** A total of 131 out of 274 animals sampled (51% of the dogs and
191 41.6% of the cats) were positive for at least one genus considered in our study.
192 The isolation rates of the different species are shown in Table 1.
193 *Campylobacter* spp. were isolated from 53 dogs and from 27 cats,
194 *Helicobacter* spp. from 65 dogs and 18 cats and *Anaerobiospirillum* spp. from
195 27 dogs and 3 cats. From the canine samples, the highest isolation rate was for
196 *H. bilis* (19.5%) followed by *C. upsaliensis* (17.3%), *H. canis* (11%), *C. jejuni*
197 (8.9%), *H. cinaedi* (7.9%), *A. succiniciproducens* (7.4%) and *A. thomasi*
198 (6.3%). In cats, the highest isolation rate was for *C. helveticus* (16.7%)
199 followed by *H. canis* (14.3%), *C. jejuni* (8.3%), *C. upsaliensis* (7.2%), *H. bilis*
200 (5.9%), *A. thomasi* (3.6%) and *H. cinaedi* (2.4%). An unidentified
201 *Anaerobiospirillum* sp. was isolated from a dog. Fifty-two animals (19%)

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

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

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

228 CCUG 18818 cluster and (iii) a group including ten isolates which did not
229 cluster with any of the reference strains. Visual analysis of the protein profiles
230 of these ten isolates showed a high level of similarity with *H. canis*, except for
231 two dense low-molecular weight bands absent in the *H. canis* reference strain
232 (data not shown). A phylogenetic tree based on the partial *groEL* sequences of
233 the isolates referring to the group mentioned above, along with randomly
234 selected 4 putative *H. canis* and 3 putative *H. cinaedi* isolates, is presented in
235 Figure 1a. Six out of the ten isolates were closely related with the four
236 putative and reference *H. canis* strains, while four isolates (isolate numbers
237 192, 163, 170, 162) clustered together in a distinct clade separated from the
238 other *H. canis* strains with high bootstrap value (100). The sequence similarity
239 between these two distinct clusters was 94% and within the ten sequences
240 ranged from 93,1% to 100%. A similar divergence in nucleotide sequences
241 was observed also for *H. cinaedi* strains. This divergence was not supported
242 by the phylogenetic tree based on deduced partial GroEL protein sequences
243 (Figure 1b), which showed a high similarity within all the *H. canis* or *H.*
244 *cinaedi* sequences. MEGABLAST analysis of partial 16S rRNA sequences
245 indicated that there was a very high degree of sequence similarity among
246 isolates 192, 163, 170 and 162 and *H. canis*. Phylogenetic analysis showed a
247 robust clade including *H. canis* strains (data not shown). For all four 16S
248 rRNA gene sequences, the similarity with the type strain of *H. canis* (strain
249 NCTC 12739) was >99%. Analysis of the partial *groEL* and 16S rRNA
250 sequences demonstrated that these ten isolates were *H. canis* and supported
251 the whole cell protein profile results.

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

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

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

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

287 **Nucleotide sequence accession numbers.** Partial sequence of 16S rRNA
288 gene of the *H. canis* isolates 162, 163, 170, 192 and the *Anaerobiospirillum*
289 isolate 78/9/02 were submitted to GenBank with the follow accession
290 numbers: EF569185, EU144017, EU144018, EU144019 and EF428122.

291 Partial sequence of *groEL* of the *H. canis* isolates 142, 143, 148, 149, 155,
292 162, 163, 168, 170, 171, 185, 188, 192, 194 and *H. cinaedi* isolates 139, 161
293 and 190 were submitted to GenBank with the follow accession numbers:
294 EU233438, EU233439, EU233440, EU233441, EU233442, EU233443,
295 EU233444, EU233445, EU233446, EU233447, EU233448, EU233449,
296 EU233450, EU233451 and EU233452, EU233453, EU233454.

297 **Discussion and conclusion**

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

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

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

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

332 Studies regarding the prevalence of different *Anaerobiospirillum* species in
333 dogs and cats have not previously been published, even if Malnick et al.
334 (1990) reported that these bacteria may belong to the normal faecal flora of
335 these animals. We detected *A. succiniciproducens* in 7.4% of animals and *A.*
336 *thomasi* in 5.5%.

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

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

351 This identification strategy, applied to *Anaerobiospirillum* spp., also allowed
352 the detection of a strain from a dog (isolate 78/9/02) which was not
353 identifiable at the species level. In particular, the nearly complete 16S rRNA
354 gene revealed a close genetic relationship between this isolate and the strain
355 3J102 isolated by Misawa et al. (2002) from a puppy as well as its clear
356 separation from the other *Anaerobiospirillum* spp., indicating that both
357 isolates probably belong to a new taxon.

358 Moreover, the polyphasic approach has permitted the development of novel
359 genomic diagnostic tools for the identification of *Helicobacter* spp. and
360 *Anaerobiospirillum* spp.. Recently, Hannula and Hänninen (2007) showed that
361 partial *gyrB* gene sequence analysis proved to be a particularly well-suited
362 tool for phylogenetic analysis of *Helicobacter* and, in particular, for
363 distinguishing closely related species which belong to the ‘flexispira-like’
364 group. In this study, we developed a new PCR-RFLP analysis of *gyrB* in order
365 to differentiate *H. bilis* from the other members of this group.
366 Likewise, new PCR approaches were described for rapid and simple
367 identification both at the genus level of *Anaerobiospirillum* and the species
368 level of *A. thomasii*; the previously used conventional phenotypic methods for
369 this identification were difficult and time consuming.
370 Some studies showed that approximately 6% of human enteric
371 campylobacteriosis is transmitted from pets (Tenkate and Stafford, 2001) and
372 that these animals represent potential sources of the spread of antimicrobial
373 resistance due to their close contact with humans (Guardabassi et al., 2004).
374 Direct evidence of the transmission of fluoroquinolone resistant *C. jejuni*
375 between humans and pets living in the same households has also been shown
376 (Damborg et al., 2004). Currently, macrolides and fluoroquinolone are the
377 antibiotics of choice when therapeutic intervention is warranted in human
378 campylobacteriosis. Our results showed the absence of resistance to
379 erythromycin in *Campylobacter* isolates but a high percentage of *C. jejuni*
380 isolates were resistant to nalidixic acid and ciprofloxacin, with values higher
381 than those described by Sanberg et al. (2002) in Norway. These data probably
382 reflect the extensive use of fluoroquinolones in dogs and cats in Italy.
383

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386 for his kind suggestion on the identification of *Helicobacter* and for her
387 technical support.

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499 Occurrence and antibiotic susceptibility of *Helicobacter pullorum* from broiler
500 chickens and commercial laying hens in Italy. Int. J. Food Microbiol. 116(1),
501 168-173.

502 Table 1. Isolation rates (%) of *Campylobacter* spp., enteric *Helicobacter* spp. and
 503 *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. thomasii</i>	6,3	[12]	17,3	2,2	3,6	[3]	-	4,8
Unidentified	0,5	[1]	-	1,9	-	-	-	-
<i>Anaerobiospirillum</i>								

504 ^a In square bracket the number of isolates

505 Table 2. Phenotypic characteristics of the 80 isolates of *Campylobacter* spp. and 92506 *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

507

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 - <i>N</i> -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

509

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 ($\mu\text{g}\cdot\text{ml}^{-1}$) ^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	>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

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

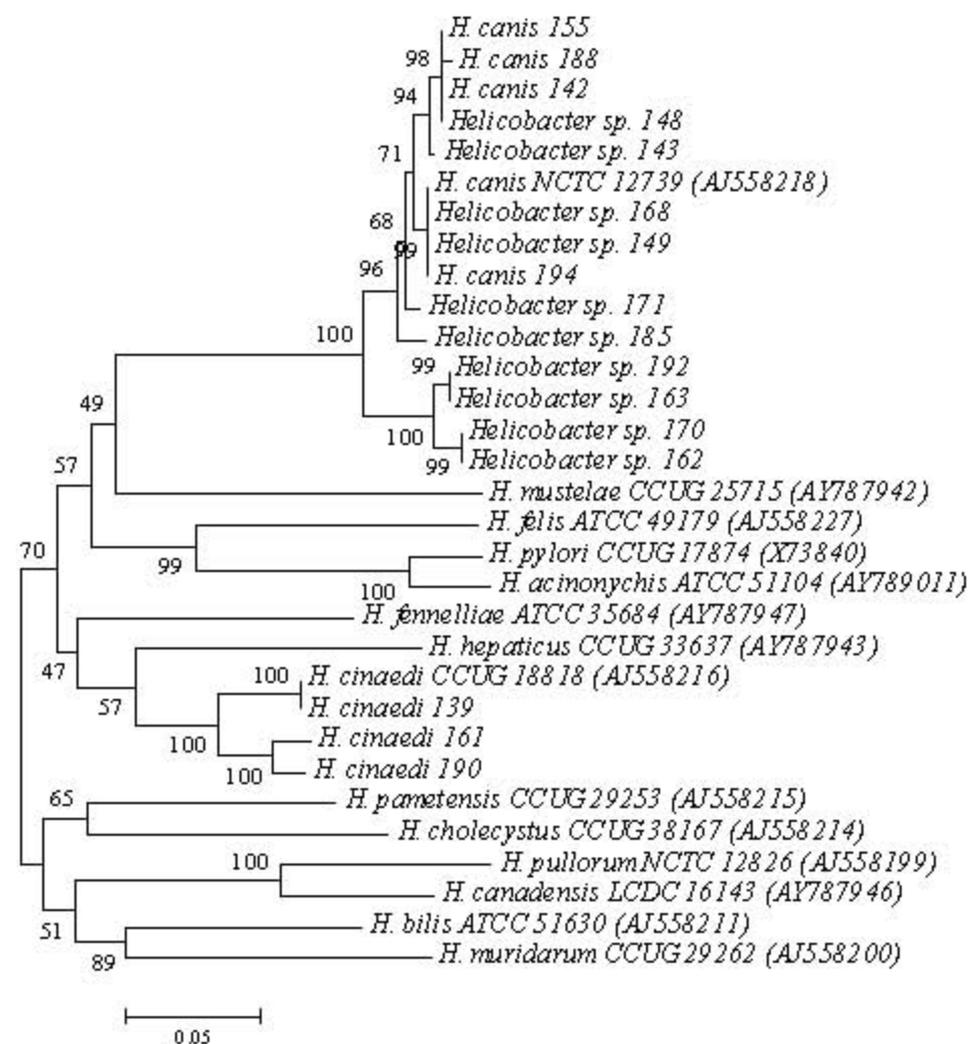
556

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

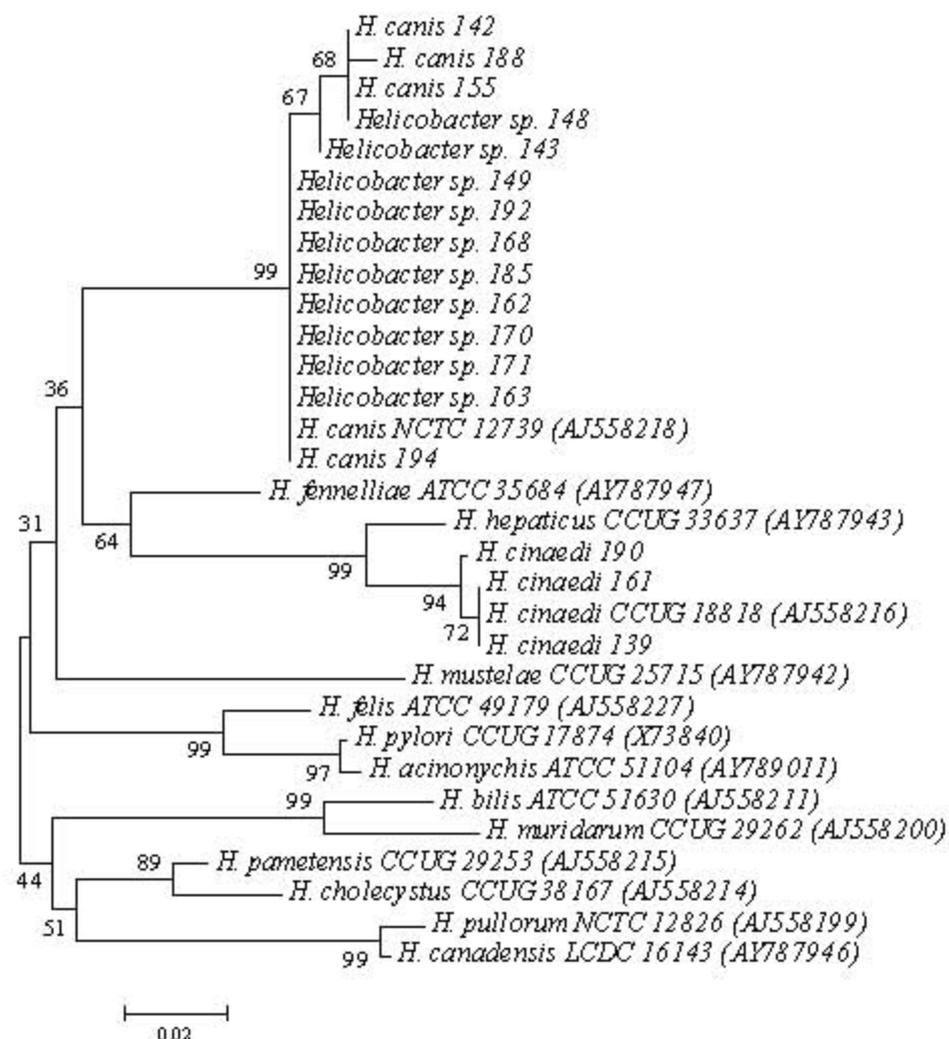
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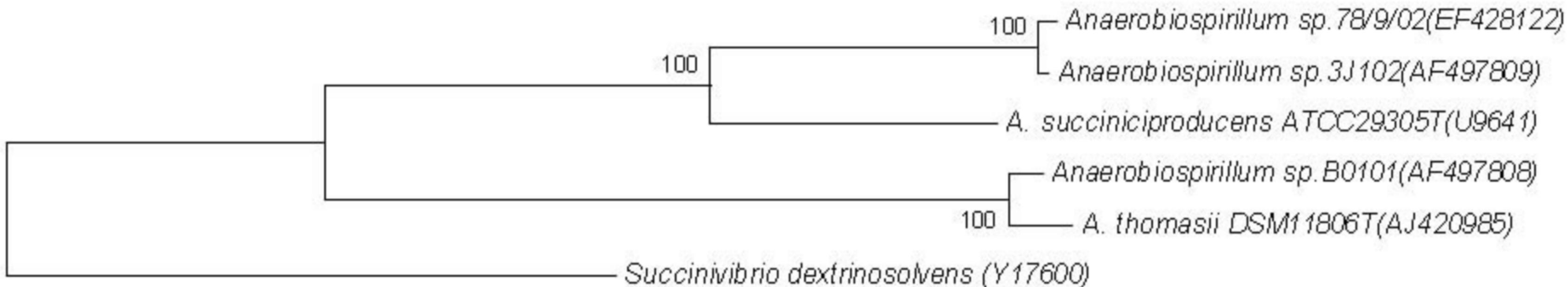
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A



B



0.01