Helicobacter canis sp. nov., a new species from dogs: an integrated study of phenotype and genotype

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(Received 17 March 1993; revised 14 May 1993; accepted 20 May 1993)

A group of *Campylobacter*-like organisms (CLOs) were isolated from the faeces of diarrhoeic or healthy dogs, constituting 4% of all CLOs from this source. Since they formed a unique DNA homology group within the genus *Helicobacter*, and exhibited distinctive phenotypic properties, they were collectively termed the HC group. A polyphasic taxonomic analysis was made of this group. The phenotype of four dog isolates and a single human isolate was unique and could be distinguished bacteriologically from other *Helicobacter*. Electron microscopic ultrastructure revealed defining characteristics of *Helicobacter*. The 16S rRNA gene of the nominated type strain NCTC 12739^T was sequenced, and its analysis delineated the group as a new species of *Helicobacter*. This conclusion was supported by relative DNA homology and whole-cell protein electrophoretic patterns. We therefore propose the name *Helicobacter canis* sp. nov. for this group. The species most closely related to *H. canis* sp. nov. were *H. cinaedi*, '*Flexispira rappini*' and *H. fennelliae*. A species-specific recombinant DNA probe was cloned from NCTC 12739^T for use in routine laboratory identification and epidemiological studies. The faecal source, bile tolerance and lack of urease activity of *H. canis* sp. nov. suggest that this new *Helicobacter* species colonizes the lower bowel rather than the stomach.

Introduction

The genus *Helicobacter* currently includes eight named species. It forms a distinct rRNA homology group within superfamily VI, which also includes the genera *Campylobacter* and *Arcobacter* (Vandamme *et al.*, 1991). The first-described member was *H. pylori*, a microaerophilic Gram-negative spiral organism (Warren & Marshall, 1983) associated with human gastritis and peptic ulcers (Blaser, 1990). Four other species have been isolated

from the gastric mucosa of animal hosts: *H. mustelae* from ferrets (Fox *et al.*, 1986, 1989; Goodwin *et al.*, 1989), *H. felis* from cats and dogs (Lee *et al.*, 1988; Paster *et al.*, 1991b), *H. nemestrinae* from macaque monkeys (Brondson *et al.*, 1991) and *H. acinonyx* from cheetahs (Eaton *et al.*, 1991, 1993). All except *H. nemestrinae* have been causally associated with gastritis of their animal host. Three species of *Helicobacter* appear to colonize the lower bowel of their host, rather than the gastric mucosa. These are *H. muridarum*, which was isolated from the intestinal mucosa of rodents (Lee *et al.*, 1992), and *H. cinaedi* and *H. fennelliae*, which were isolated from humans (Fennell *et al.*, 1984; Totten *et al.*, 1985; Vandamme *et al.*, 1991).

A single isolate identified in the same studies as *H. cinaedi* and *H. fennelliae* could be differentiated from them on the basis of total DNA homology, and was termed 'Campylobacter-like organism' (CLO) 3 (Fennell *et al.*, 1984; Totten *et al.*, 1985). Vandamme *et al.* (1991) placed strain 'CLO-3' in homology group III of rRNA

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Abbreviations: CLO, Campylobacter-like organism; HC, Helicobacter, canine (group).

The GenBank accession number for the 16S rRNA gene sequence of NCTC 12739^T is L13464. The accession numbers for reference organisms included in the 16S rRNA phylogenetic analysis are given in Table 1.

superfamily VI, which includes *Helicobacter* species, but no further isolates have been reported and no species designation has been made. A species originally named as '*Flexispira rappini*' (Bryner, 1987) may also be a true *Helicobacter*, according to rRNA cistron similarity (Vandamme *et al.*, 1991) and 16S rRNA gene sequencing (Lee *et al.*, 1992). The phylogenetic analysis of 16S rRNA gene sequences suggests that several potential new species of *Helicobacter* exist in avian and mammalian hosts (Paster *et al.*, 1991a).

Special isolation media and conditions have been employed to study carriage of CLOs in domestic pet animals (Burnens *et al.*, 1992; Burnens & Nicolet, 1992). One thousand dogs were screened in this way and fifteen strains, 4% of the total CLOs isolated, constituted a unique DNA homology group exhibiting weak homology with *H. fennelliae* and *H. cinaedi* only. The strains were unable to reduce nitrate and were resistant to polymyxin B. Since the latter phenotype distinguishes *Helicobacter* spp. from *Campylobacter* spp. (Burnens & Nicolet, 1993), they were provisionally termed the HC (*Helicobacter*, canine) group. In a parallel analysis of children with infectious gastroenteritis, one isolate was recovered which exhibited the phenotypic and DNA homology relationships of the HC group (Burnens *et al.*, 1993).

Here we present a polyphasic taxonomic study of representative strains (four dog, one human) which establishes the HC group to be a new species of *Helicobacter*, for which we propose the name *Helicobacter canis* sp. nov.

Methods

Bacterial strains and culture conditions. Bacterial strains are listed in Table 1. *Helicobacter* spp., *Campylobacter* spp., '*Flexispira rappini*', *Arcobacter cryaerophila* and *Arcobacter butzleri* were cultured on 5% (v/v) horse blood agar plates in a Variable Atmosphere Incubator (Don Whitley Scientific). This maintained a microaerobic atmosphere of 5% O₂/5% CO₂/2% H₂/88% N₂ at 37 °C. *Arcobacter nitrofigilis* was grown microaerobically in a gas jar at 25 °C. *Wolinella succinogenes* and [*Bacteroides*] *ureolyticus* were grown anaerobically on modified brain heart infusion agar (Malnick *et al.*, 1990) at 37 °C in an anaerobic cabinet (Don Whitley Scientific).

Phenotypic characterization, electron microscopy and protein electrophoresis. The phenotype of the isolates was determined by employing previously described methods and media (Holmes *et al.*, 1992; On & Holmes, 1991a, b, 1992). Additional tests were performed as follows. Production of extracellular deoxyribonuclease (DNAase) was determined by the method of Lior & Patel (1987). Growth on 1.5% bile medium [5% blood agar to which 1.5% desiccated ox-bile (Oxoid) had been added prior to sterilization by autoclaving], and MacConkey agar (formulation CM-7, Oxoid) were determined by using inoculation and incubation methods previously described (On & Holmes, 1991a, b). The colonial morphology of the isolates was recorded after 3 d microaerobic incubation on 5% horse blood agar at 37 °C. All tests were performed in triplicate, on separate occasions, and with freshly prepared media. Electron microscopy was carried out as previously

described (Stanley *et al.*, 1992). Protein samples were prepared, run on SDS-PAGE gels, dried and analysed as described previously (Costas, 1992).

Nucleic acid techniques. Genomic DNA preparation, Southern blots and hybridization, DNA-DNA slot-blot hybridization and its analysis by laser densitometry, and cloning of a species-specific DNA probe were done as previously described (Stanley *et al.*, 1992). The mol% G + C content was determined by the method of Owen & Pitcher (1985).

Polymerase chain reaction (PCR) amplification of a 1500 bp fragment of the 16S rRNA gene of NCTC 12739^T was done as follows. Fifty nanograms of genomic DNA was subjected to PCR in a final reaction mixture (100 µl) containing: 10 mM-Tris/HCl (pH 8.3); 200 µM-dNTPs; 0.4 µM of each primer (forward primer 5'-AAGAGTT-TGATCCTGGCTCAG-3' and reverse primer 5'-GGTTACCTTGTT-ACGACTT-3'); 1.5 mM-MgCl₂; 50 mM-KCl; 0.1 mg gelatine ml⁻¹ and 2.5 units *Taq* DNA polymerase (Boehringer Mannheim). Samples were overlaid with 100 µl mineral oil and subjected to 35 cycles of amplification on a PCR Heating Block (Hybaid) with the following cycling conditions: denaturation, 93 °C, 1 min; annealing, 55 °C, 1 min; and extension, 72 °C, 3 min. The initial cycle, prior to the addition of *Taq* polymerase, included 5 min denaturation at 95 °C. The final cycle continued primer extension for 8 min. After removal of mineral oil, the amplification product was purified by the method of Kuskawa *et al.* (1990) and nucleotide sequencing of the amplified gene was carried out following the method of Embley (1991). Products were analysed on standard wedge-shaped sequencing gels (Pharmacia LKB Macrophor apparatus). Gels were fixed in aqueous methanol/acetic acid/glycerol (10%:5%:2%, by vol.), dried at 80 °C and autoradiographed on Kodak XAR5 film.

Phylogenetic analysis of 16S rRNA gene sequence information. The 16S rRNA gene sequence for NCTC 12739^T was entered into RNA, a program for analysis of 16S rRNA data written in Microsoft QuickBASIC for use on IBM-PC compatible computers, and aligned as previously described (Paster & Dewhirst, 1988). The database contains approximately 50 *Helicobacter*, *Wolinella*, *Arcobacter* and *Campylobacter* sequences and 300 sequences for other eubacteria. Similarity matrices were constructed from aligned sequences by using only those base positions for which 90% of the strains had data. The similarity matrices were corrected for multiple base changes by the method of Jukes & Cantor (1969). Phylogenetic trees were constructed by the neighbour-joining method (Saitou & Nei, 1987).

Results

Phenotypic characterization

In total, 34 phenotypic tests were performed on five strains of the HC group in order to arrive at a defining phenotype. The following six tests were reproducibly positive for these strains: production of cytochrome oxidase, growth at 37 °C, growth at 42 °C, growth on media containing 5-fluorouracil (100 mg l⁻¹), and growth on media containing either 1% or 1.5% bile. All strains failed to produce urease or catalase (although two strains, NCTC 12741 and NCTC 12743, exhibited a weak catalase reaction). They did not produce DNAase, or hydrogen sulphide from triple sugar iron (TSI) medium. Neither nitrate nor selenite was reduced. Hippurate was not hydrolysed. Growth was not found

Table 1. *Bacterial strains*

Bacterium	Strain no.	Reference/comment	Source	Genbank no.*
<i>Helicobacter</i>				
<i>H. pylori</i>	NCTC 11638	Reference strain	Human	M88157
<i>H. pylori</i>	NCTC 11637	Type strain	Human	—
<i>H. felis</i>	NCTC 12436	Type strain	Cat	M37642
<i>H. felis</i>	DS3	Paster <i>et al.</i> (1991)	Dog	M37643
<i>H. mustelae</i>	NCTC 12435	Type strain	Ferret	M35048
<i>H. acinonyx</i>	NCTC 12686	Type strain	Cheetah	M88148
<i>H. cinaedi</i>	NCTC 12423	Type strain	Human	M88150
<i>H. fennelliae</i>	NCTC 11612	Type strain	Human	M88154
<i>H. nemestrinae</i>	NCTC 12491	Type strain	Monkey	—
<i>H. muridarum</i>	NCTC 12714	Type strain	Rat	M80205
HC group	NCTC 12739	Type strain	Dog	L13464
(<i>H. canis</i> sp. nov.)	NCTC 12741	—	Dog	—
	NCTC 12743	—	Dog	—
	CCUG 19561	—	Dog	—
	NCTC 12740	—	Human	—
<i>Campylobacter</i>				
<i>C. fetus</i> subsp. <i>fetus</i>	NCTC 10842	Type strain	Sheep	L04314
<i>C. fetus</i> subsp. <i>venerealis</i>	NCTC 10354	Type strain	Cattle	—
<i>C. coli</i>	NCTC 11366	Type strain	Pig	L04312
<i>C. jejuni</i> subsp. <i>jejuni</i>	NCTC 11351	Type strain	Cattle	L04315
<i>C. jejuni</i> subsp. <i>doylei</i>	NCTC 11951	Type strain	Human	L14630
<i>C. lari</i>	NCTC 11352	Type strain	Gull	L04316
<i>C. upsaliensis</i>	NCTC 11541	Type strain	Dog	L14628
<i>C. hyointestinalis</i>	NCTC 11608	Type strain	Pig	M65010
<i>C. mucosalis</i>	NCTC 11000	Type strain	Pig	L06978
<i>C. sputorum</i> subsp. <i>faecalis</i>	NCTC 11415	Reference strain	Sheep	—
<i>C. sputorum</i> subsp. <i>bubulus</i>	ATCC 33491	Smibert (1980)	Cattle	L04319
<i>C. sputorum</i> subsp. <i>bubulus</i>	NCTC 11367	Type strain	Cattle	—
<i>C. sputorum</i> subsp. <i>sputorum</i>	NCTC 11528	Type strain	Human	—
<i>C. concisus</i>	NCTC 11485	Type strain	Human	L04322
<i>C. curvus</i>	NCTC 11649	Type strain	Human	L04313
<i>C. rectus</i>	NCTC 11489	Type strain	Human	L04317
<i>C. helveticus</i>	NCTC 12470	Type strain	Cat	—
<i>Arcobacter</i>				
<i>A. cryaerophilus</i>	NCTC 11885	Type strain	Cattle	L14624
<i>A. butzleri</i>	NCTC 12400	Type strain	Human	L14626
<i>A. skirrowii</i>	NCTC 12713	Type strain	Sheep	L14625
<i>A. nitrofigilis</i>	NCTC 12251	Type strain	Plant	L14627
'CLO-3'	NCTC 12462	(= CCUG 14564)	Human	M88151
' <i>Flexispira rappini</i> '	NCTC 12461	Type strain	Human	M88137
<i>Wolinella succinogenes</i>	NCTC 11488	Type strain	Cattle	M88159
[<i>Bacteroides</i>] <i>gracilis</i> †	NCTC 12738	Type strain	Human	L04320
[<i>Bacteroides</i>] <i>ureolyticus</i> †	NCTC 10941	Type strain	Human	L04321
<i>Escherichia coli</i>	FM15	Dowling <i>et al.</i> (1987)	—	—
<i>Salmonella typhimurium</i>	NCTC 12416	Type strain, subsp. 1	—	—

* 16S rRNA sequences for these strains are available for electronic retrieval from GenBank under the indicated accession numbers. The sequences for reference strains deposited in Genbank by F. E. Dewhirst were determined for the strains listed in this Table, but the strains were obtained from different culture collections as indicated by the accession numbers in Fig. 3.

† Generically misnamed. These species are not true *Bacteroides* but belong in rRNA superfamily VI.

under microaerobic conditions at room temperature (18–22 °C), at 25 °C or 30 °C, or under aerobic conditions. There was no growth on 0.1% trimethylamine *N*-oxide (TMAO) medium under anaerobic conditions. There was no growth on nutrient agar, on *Campylobacter* charcoal-desoxycholate (CCD, unsupplemented Preston

base) agar, on minimal agar, on starch or on MacConkey agar media. None of the strains were tolerant to safranin 'O' (0.02%), potassium permanganate (0.1%), sodium arsenite (0.001%), or to 2% and 3.5% (w/v) NaCl. Tolerance to triphenyltetrazolium chloride (TTC) was not found to be a reliable test for characterizing this

Table 2. Principal and differential characteristics of *H. canis* and related helicobacters

Abbreviations: +, positive; -, negative; W, weak positive; M, monopolar; B, bipolar; Po, polar; Pe, peritrichous; S, sensitive; R, resistant; I, intermediate; ND, not done; V, variable test. 5/5, 0/5, etc., number of positive strains/number tested. Source of data, this study and Eaton *et al.* (1993).

Test/characteristic	<i>H. canis</i>	<i>H. cinaedi</i>	<i>H. fennelliae</i>	<i>H. pylori</i>	<i>H. mustelae</i>	<i>H. muridarum</i>	<i>H. nemestrinae</i>	<i>H. felis</i>	<i>H. acinonyx</i>	' <i>F. rappini</i> '
Periplasmic fibres	-	-	-	-	-	+	-	+	-	+
Flagella: Distribution	B	M, B	M, B	Po	Pe	B	Po	B	Po	B
Number	2	1-2	1-2	4-8	4-8	10-14	4-8	14-20	2-5	10-20
Sheath	+	+	+	+	+	+	+	+	+	+
Oxidase production	5/5	+	+	+	+	+	+	+	+	+
Catalase production	0/5	+	+	+	+	+	+	+	+	+
Urease (rapid) production	0/5	-	-	+	+	+	+	+	+	+
Nitrate reduction	0/5	+	-	-	+	-	-	+	-	-
Indoxyl acetate hydrolysis	4/5	-	+	-	+	+	-	-	-	ND
Alkaline phosphatase production	5/5	-	+	+	+	+	+	+	+	-
Growth at:										
42 °C	5/5	-	-	-	+	-	+	+	-	+
25 °C	0/5	-	-	-	-	-	-	-	-	-
Tolerance to:										
1% Bile	5/5	V	-	-	-	ND	-	ND	ND	ND
Safranin 'O'	0/5	-	+	-	-	ND	ND	ND	ND	ND
Sensitivity to:										
Nalidixic acid	S(5/5)	S	S	R	S	R	R	R	R	R
Cephalothin	S(3/5)	I	S	S	R	R	S	S	S	R
G + C content (mol %)	48	36-38	35	35-37	36	34	24	43	30	34

group. They were inhibited by the antibiotics nalidixic acid (32 mg ml⁻¹), metronidazole (4 mg ml⁻¹) and carbenicillin (32 mg ml⁻¹). All strains produced alkaline phosphatase, although NCTC 12741 exhibited a weak reaction. Strain NCTC 12743 hydrolysed indoxyl acetate, although NCTC 12739^T, NCTC 12740 and CCUG 19561 gave a weak reaction (visible after 20 min) and NCTC 12741 failed to hydrolyse this substrate. Only NCTC 12741 and NCTC 12743 were tolerant to cephalothin (32 mg l⁻¹). Colonial morphology on 5% horse blood agar was watery, translucent and invariably α -haemolytic. Colony size was pinpoint and no pigments were produced. A list of characteristics useful in discriminating the HC group from described species of *Helicobacter* and '*Flexispira rappini*' is given in Table 2.

Ultrastructure

Cells of NCTC 12739^T were typically spiral, sometimes possessing truncated ends. Flagella were single and bipolar (Fig. 1a). They were sheathed, a defining characteristic of the genus *Helicobacter*, and were connected to a basal plate at the point of insertion into the cell (Fig. 1b).

Whole-cell protein analysis

One-dimensional SDS-PAGE was carried out in order to analyse whole-cell protein content and about 40 discrete

bands were observed. Four HC group strains were compared with ten reference or type strains of the seven *Helicobacter* species, '*Flexispira rappini*', *Wolinella succinogenes* and 'CLO-3'. Visual inspection of gels indicated that the HC group was homogeneous with respect to protein pattern and substantially different to other helicobacters. Numerical analysis of total protein patterns was made by calculating the correlation coefficient, and clustering was performed using the UPGMA algorithm. A rooted dendrogram representing this analysis is shown in Fig. 2. Within the HC group the banding patterns of three of the four strains were more than 90% similar. However, NCTC 12743 differed in lacking a prominent band of 37 kDa, resulting in its clustering with the other strains at the 77% similarity level. The nearest neighbour to the HC cluster was the 'CLO-3' isolate, whose banding pattern was only 47% similar. The closest defined species were *H. cinaedi* and '*F. rappini*', whose banding patterns exhibited 45% similarity. Other *Helicobacter* species had patterns of less than 40% similarity.

DNA base composition

The DNA base compositions of NCTC 12739^T, CCUG 19561 and NCTC 12743 respectively were determined as 48.4, 48.8 and 48.2 mol% G + C.

Fig. 1. (a) Electron micrograph of a single cell of *H. canis* NCTC 12739^T with bipolar, partially sheathed flagella. Bar, 1000 nm. (b) Attachment site of flagellum. Enlargement of one end of the cell shown in (a). Partial sheathing and attachment site of the flagellum are seen. Bar, 125 nm.

DNA–DNA hybridization and cloning of specific chromosomal DNA probes

The overall genetic relatedness between the five HC strains and the species type strains and other reference strains representing rRNA superfamily VI (Vandamme *et al.*, 1991) was determined by slot-blot hybridization between genomic DNAs. Theoretical aspects and taxonomic applications of this method have been discussed (Boivin *et al.*, 1985; Lee & McGee, 1989). DNA of NCTC 12739^T was employed as a probe. No homology was detected with DNA of *Salmonella typhimurium* (negative control), with *Wolinella succinogenes*, [*Bacteroides*] *ureolyticus*, *Arcobacter cryaerophilus*, *A. butzleri*, *A. nitrofigilis*, or with any species type strain of *Campylobacter* (data not shown). Very low relative DNA homology was detected with *H. felis*, *H. nemestrinae* and *H. acinonyx* (6% each). Low homology was detected with *H. mustelae* (10%), '*Flexispira rappini*' (11%) and *H. pylori* (14%). Relative DNA homologies of 30% and 52% were detected with *H. fennelliae* and *H. cinaedi* respectively. The four other strains in the HC group

exhibited more than 70% relative homology with NCTC 12739^T, in accordance with criteria established for the phylogenetic definition of a species (Wayne *et al.*, 1987).

Six recombinant plasmids containing *Hind*III fragments cloned from NCTC 12739^T (sized between 0.5 and 1.5 kb) were tested for species-specific DNA homology in slot-blot hybridizations. All showed no homology with any described species of *Campylobacter*, *Wolinella*, *Arcobacter*, *Bacteroides* or *Helicobacter* (see Table 1) under high stringency (Stanley *et al.*, 1992) filter-washing conditions. pHCl, containing a cloned fragment of 1.0 kbp, was a probe suitable for the differentiation of the HC group from other helicobacters and their closest phylogenetic relatives (data not shown).

Sequence of the 16S rRNA gene and phylogenetic analysis

The nucleotide sequence of the 16S rRNA gene of NCTC 12739^T was determined by DNA sequencing of a PCR-generated 1500 bp fragment as described in

Fig. 2. Dendrogram of numerical analysis of the total protein pattern of four *H. canis* isolates, and type or reference strains of the most closely related species in rRNA superfamily VI.

Methods. Comparison of the sequence for strain NCTC 12739^T with other eubacterial 16S rRNA sequences in our database indicated that NCTC 12739^T was closely related to species in the genus *Helicobacter*. The results for comparison of the 16S rRNA sequence for strain NCTC 12739^T with those for 28 strains representing *Helicobacter*, *Wolinella*, *Arcobacter* and *Campylobacter* species are shown in Table 3. This matrix was based on comparisons at 1378 base positions. At each of these points, data existed for over 90% of the strains. The lower half of the matrix is expressed as percentage difference corrected for multiple base changes by the method of Jukes & Cantor (1969). NCTC 12739^T was most closely related to '*Flexispira rappini*' (98.2% similarity) and *H. cinaedi* (97.9% similarity).

A phylogenetic tree obtained from the corrected matrix using the neighbour joining method is shown in Fig. 3. There were three major clusters in the phylogenetic tree. The first cluster was comprised of *Campylobacter* species and two generically misnamed *Bacteroides* species. The second cluster contained the four *Arcobacter* species. The third cluster was comprised of *Helicobacter* species, '*F. rappini*' and *Wolinella succinogenes*. NCTC 12739^T formed part of a *Helicobacter* subcluster which included *H. cinaedi*, '*F. rappini*', *H. muridarum* and *H. mustelae*. *H. pylori*, *H. acinonyx* and *H. felis* formed a second *Helicobacter* subcluster. *H. fennelliae* and 'CLO-3' branched deeply within *Helicobacter* and were not part

of subclusters. NCTC 12739^T was clearly different (7.0%) from the canine isolate of *H. felis*, DS3 (Paster *et al.*, 1991*b*).

Discussion

Having examined the phenotypic characteristics and phylogenetic relationships of the HC group we consider it to constitute a previously undescribed species of *Helicobacter*, for which we propose the name *Helicobacter canis*. The phenotypic characteristics, notably microaerophily and overall biochemical inertness, were those generally associated with *Campylobacter* and *Helicobacter* (see Table 2). The presence of sheathed flagella (Fig. 1) placed it within the genus *Helicobacter* (Vandamme *et al.*, 1991). Bipolar flagella, as found in *H. canis* (Fig. 1), have also been found in *H. cinaedi* and *H. fennelliae* and their sheathing is notably similar to that of *H. canis* (Han *et al.*, 1989). The cellular ultrastructure resembled that of *H. pylori*, and we observed no complex features such as the periplasmic fibres and multiple flagella of *H. felis* (Paster *et al.*, 1991*b*) and *H. muridarum* (Lee *et al.*, 1992).

The G+C content of the nominated type strain NCTC 12739^T (48.4 mol%) was somewhat higher than that of *H. felis* (42.5 mol%), which was until now the highest reported value for the genus *Helicobacter* (Paster *et al.*, 1991*b*). Low (6–14%) relative DNA homology

Table 3. Similarity matrix based on 16S rRNA sequence comparisons

Organism	Percentage similarity and percentage compared with:																												
	Hca	Hci	Fra	Hmr	Hms	CLO	Hpy	Hac	Hf1	Hf2	Hfe	Wsu	Acr	Ask	Abu	Ani	Bur	Cre	Bgr	Csb	Ccu	Cco	Cmu	Cff	Chy	Cup	Cci	Cje	Cla
Hca	—	97.9	98.2	96.5	96.5	96.1	94.0	93.5	93.6	93.3	95.9	93.6	85.8	85.5	86.4	85.3	86.3	85.9	85.2	88.2	86.2	87.1	86.4	86.9	86.2	87.4	87.3	87.0	87.5
Hci	2.1	—	98.8	95.9	95.2	95.5	92.8	92.5	92.8	92.6	96.1	93.2	85.4	85.0	86.0	84.6	86.2	86.1	85.5	88.2	86.2	87.1	86.4	86.9	86.3	87.2	86.8	86.5	86.9
Fra	1.9	1.2	—	96.2	95.9	95.7	93.3	92.7	92.9	92.7	95.6	93.4	85.5	85.1	86.1	84.7	86.5	86.3	85.6	88.3	86.4	87.3	86.7	87.2	86.6	87.5	87.5	87.1	87.6
Hmr	3.6	4.2	3.9	—	95.7	94.4	93.1	92.7	92.9	92.9	95.4	93.6	85.3	85.1	86.4	85.0	85.0	85.0	84.3	86.6	85.3	86.3	85.6	86.0	85.5	86.7	86.1	86.1	86.5
Hms	3.6	5.0	4.3	4.4	—	95.3	93.9	93.6	93.7	93.3	94.7	94.1	86.4	86.1	87.0	85.5	86.0	85.0	84.7	87.3	85.8	86.0	85.7	86.2	85.9	87.0	86.7	87.0	87.5
CLO	4.0	4.7	4.4	5.8	4.8	—	94.0	93.3	93.6	93.3	95.2	92.7	85.9	85.6	86.9	85.9	85.3	85.5	84.8	87.2	86.1	86.9	86.3	86.4	85.8	86.5	86.3	86.4	87.0
Hpy	6.3	7.6	7.0	7.2	6.4	6.2	—	97.3	95.4	95.3	93.3	91.1	84.6	84.3	85.6	83.6	84.3	84.3	84.1	85.6	85.1	85.3	85.1	85.5	84.9	86.2	85.5	85.8	85.9
Hac	6.8	7.9	7.7	7.7	6.7	7.0	2.7	—	96.6	96.3	92.8	90.7	84.4	84.2	85.4	83.7	84.1	84.0	83.3	85.1	84.8	84.9	84.8	85.1	84.3	86.2	85.3	85.5	85.7
Hf1	6.7	7.5	7.4	7.4	6.6	6.7	4.7	3.5	—	99.4	93.5	90.7	83.8	83.7	84.7	83.8	83.9	83.4	83.1	85.0	84.2	84.8	85.1	85.1	84.1	86.3	84.8	85.3	85.8
Hf2	7.0	7.8	7.6	7.5	7.0	7.0	4.9	3.8	0.6	—	93.1	90.7	83.9	83.8	84.8	83.7	84.1	83.5	83.2	85.0	84.2	84.9	85.1	85.2	84.3	86.5	84.9	85.4	85.9
Hfe	4.3	4.0	4.5	4.8	5.5	4.9	7.0	7.6	6.8	7.2	—	92.9	84.9	84.8	86.1	85.3	85.7	86.0	85.4	86.4	86.6	87.2	86.7	86.8	86.2	86.7	87.1	86.8	87.2
Wsu	6.7	7.1	6.9	6.7	6.2	7.7	9.5	10.0	10.0	9.9	7.5	—	86.0	85.7	86.5	85.5	86.0	86.5	85.7	86.3	86.9	86.3	86.0	86.4	85.7	86.3	86.6	86.7	87.1
Acr	15.7	16.3	16.2	16.3	15.0	15.6	17.2	17.5	18.2	18.1	16.9	15.5	—	99.0	97.6	94.6	86.7	86.0	86.5	87.0	86.3	87.6	87.1	86.6	86.8	86.0	86.8	87.4	87.2
Ask	16.1	16.7	16.6	16.6	15.4	16.0	17.6	17.7	18.4	18.3	17.0	15.8	1.0	—	97.3	94.6	86.5	85.6	86.0	87.0	86.3	87.6	87.1	86.4	86.5	85.8	86.6	87.2	86.9
Abu	15.0	15.5	15.4	15.1	14.2	14.4	15.9	16.3	17.1	17.0	15.3	14.9	2.4	2.7	—	95.0	86.2	86.6	86.8	86.8	87.0	88.1	86.9	86.7	86.6	86.5	86.5	87.0	86.7
Ani	16.4	17.2	16.8	16.1	15.7	18.5	18.4	18.3	18.3	16.3	16.1	5.7	5.7	5.2	—	86.1	85.7	85.5	85.7	86.4	87.4	86.5	86.1	86.3	86.0	86.6	87.2	87.1	
Bur	15.1	15.2	14.9	16.7	15.5	16.3	17.6	17.8	18.2	17.9	15.8	15.5	14.6	14.9	15.3	15.4	—	92.0	91.7	91.9	90.8	91.9	92.3	93.0	93.1	91.3	93.7	93.3	92.9
Cre	15.6	15.4	15.1	16.7	16.7	16.2	17.6	18.0	18.7	18.7	15.5	14.9	15.4	16.0	14.7	15.9	8.4	—	95.7	95.0	96.5	95.6	94.1	93.9	93.0	91.8	93.3	92.6	92.3
Bgr	16.5	16.1	15.9	17.6	17.1	17.0	17.8	18.8	19.1	19.0	16.2	15.9	14.9	15.6	14.5	16.1	8.8	4.5	—	94.0	94.5	94.9	93.6	93.9	93.0	90.9	92.1	91.6	91.3
Csb	12.9	12.9	12.8	14.8	13.9	14.0	16.0	16.6	16.7	16.8	15.0	15.2	14.3	14.3	14.5	15.8	8.6	5.1	6.2	—	95.1	94.9	93.4	93.3	92.5	91.6	93.0	92.4	92.0
Ccu	15.2	15.3	15.0	16.3	15.7	15.3	16.6	16.9	17.8	17.8	14.8	14.4	15.1	15.2	14.3	15.0	9.8	3.6	5.7	5.0	—	97.0	94.7	94.3	93.4	92.0	93.4	92.7	92.2
Cco	14.1	14.1	13.9	15.1	15.5	14.4	16.4	16.9	17.0	16.9	14.0	15.1	13.5	13.5	12.9	13.8	8.6	4.5	5.3	5.3	3.1	—	96.2	95.5	94.6	92.1	94.2	93.5	93.8
Cmu	14.9	15.1	14.7	15.9	15.8	15.1	16.6	17.0	16.6	16.7	14.7	15.4	14.1	14.2	14.4	14.8	8.1	6.2	6.7	6.9	5.4	3.9	—	96.1	94.7	93.0	94.4	93.9	93.7
Cff	14.4	14.4	14.0	15.5	15.3	15.0	16.1	16.6	16.6	16.4	14.5	15.0	14.8	15.0	14.6	15.3	7.4	6.4	6.3	7.0	5.9	4.6	4.0	—	98.1	93.2	95.8	94.7	94.4
Chy	15.2	15.2	14.8	16.1	15.6	15.8	16.8	17.7	17.9	17.7	15.3	15.8	14.6	14.8	14.7	15.1	7.3	7.3	7.3	7.9	6.9	5.6	5.5	1.9	—	93.0	96.0	94.9	94.6
Cup	13.9	14.0	13.7	14.7	14.3	14.9	15.2	15.3	15.1	14.8	14.6	15.2	15.5	15.7	14.9	15.5	9.3	8.7	9.7	9.0	8.4	8.3	7.3	7.1	7.3	—	95.2	96.2	95.7
Cci	13.9	14.5	13.7	15.4	14.6	15.1	16.1	16.4	16.9	16.8	14.2	14.7	14.5	14.7	14.9	14.7	6.6	7.0	8.4	7.4	6.9	6.0	5.8	4.4	4.1	5.0	—	98.5	97.6
Cje	14.3	14.9	14.1	15.3	14.3	15.1	15.8	16.1	16.4	16.2	14.5	14.6	13.8	14.0	14.3	14.1	7.1	7.8	8.9	8.0	7.7	6.7	6.4	5.5	5.3	3.9	1.5	—	98.6
Cla	13.7	14.4	13.6	14.9	13.7	14.2	15.6	15.9	15.8	15.7	14.0	14.1	14.1	14.4	14.6	14.1	7.4	8.2	9.2	8.4	8.2	6.5	6.6	5.8	5.6	4.4	2.4	1.4	—

Abbreviations: Hca, *H. canis*; Hci, *H. cinaedi*; Fra, '*F. rappini*'; Hmr, *H. muridarum*; Hms, *H. mustelae*; CLO, *Helicobacter* sp. 'CLO-3'; Hpy, *H. pylori*; Hac, *H. acinonyx*; Hf1, *H. felis* CS1; Hf2, *H. felis* DS3; Hfe, *H. fennelliae*; Wsu, *W. succinogenes*; Acr, *A. cryaerophilus*; Ask, *A. skirrowii*; Abu, *A. butzleri*; Ani, *A. nitrofigilis*; Bur, [*B.*] *ureolyticus*; Cre, *C. rectus*; Bgr, [*B.*] *gracilis*; Csb, *C. sputorum* subsp. *bubulus*; Ccu, *C. curvus*; Cco, *C. concisus*; Cmu, *C. mucosalis*; Cff, *C. fetus* subsp. *fetus*; Chy, *C. hyointestinalis*; Cup, *C. upsaliensis*; Cci, *C. coli*; Cje, *C. jejuni* subsp. *jejuni*; Cla, *C. lari*.

Numbers above the diagonal are uncorrected percentages of similarity. Numbers below the diagonal are percentages of difference corrected for multiple base changes by the method of Jukes and Cantor (1969).

was detected with most *Helicobacter* species, but values of 52% and 30% were detected with *H. cinaedi* and *H. fennelliae* respectively. It should be noted however that the mol % G + C content of these species is quite different from *H. canis* (Table 2). The nucleotide sequence of its 16S rRNA gene identified NCTC 12739^T as representative of a unique taxon within *Helicobacter*, whose closest phylogenetic relatives were '*Flexispira rappini*' (1.9% sequence divergence), *H. cinaedi* (2.1% sequence divergence), *H. muridarum* and *H. mustelae* (Table 3, Fig. 3). In an analysis of electrophoretic protein patterns (Fig. 2) *H. canis* formed a homogeneous cluster ($\geq 77\%$ level of similarity) separated from other *Helicobacter* species. This cluster was linked to the nearest species, *H. cinaedi* and '*F. rappini*', at a 45% level of similarity. There are notable correspondences in the dendrograms derived from 16S rRNA gene sequence and from electrophoresis of total proteins (compare Figs 2 and 3) in the placement of *H. canis* and its nearest neighbours. Therefore there is agreement between relative genomic

DNA homology, 16S rRNA gene sequencing, cellular ultrastructure and electrophoretic protein pattern that this taxon is a novel species of *Helicobacter*.

H. canis could be bacteriologically distinguished from other *Helicobacter* species since it was simultaneously catalase and urease negative, and exhibited marked tolerance to bile. Its distinguishing phenotypic characteristic with respect to the most related species, *H. cinaedi*, was the inability to reduce nitrate. We compared *H. canis* with 'CLO-3' because of the historical and biological association of that strain with *H. cinaedi* and *H. fennelliae*, the closest relatives of the HC group in our original dot-blot hybridizations. *H. canis* exhibited 4.0% 16S *rrn* gene sequence divergence from 'CLO-3' (Table 3) and 40% DNA homology with it (data not shown); these values are well beyond the limits for species separation. *H. canis* could also be distinguished from 'CLO-3' by the analysis of whole-cell protein profiles (Fig. 2). Finally, *H. canis* was sensitive to nalidixic acid, failed to produce catalase and was able to tolerate 1%

Fig. 3. Phylogenetic tree for *H. canis* and 28 reference *Helicobacter*, *Wolinella*, *Arcobacter* and *Campylobacter* species based on 16S rRNA sequence similarity. The scale bar represents 5% difference in nucleotide sequence as determined by measuring the lengths of the horizontal lines connecting any two species. See footnote to Table 1 for an explanation of strain numbers.

bile, whilst the 'CLO-3' isolate exhibited the converse results for these phenotypic tests. Results presented here (Table 3, Fig. 3) indicate that 'CLO-3' and '*F. rappini*' are clearly helicobacters.

Species-specific probes are particularly useful since the bacteriological (biochemical) differentiation of *Helicobacter* and *Campylobacter* species can be difficult and laborious. The probe pHCl provides a means of rapid identification of *H. canis* among CLOs from diverse sources. This is important for studies of its prevalence, and potential routes of zoonotic transmission (see below) for this new species.

There was no direct association with diarrhoeal disease among the dogs from which the HC strains were isolated (A. P. Burnens, unpublished results). One strain among those examined here was from a boy with diarrhoea (Burnens *et al.*, 1993). *H. cinaedi* and *H. fennelliae* were initially isolated from rectal swabs of homosexual men (Totten *et al.*, 1985), a group in which they were separately shown to cause bacteraemias (Cimolai *et al.*, 1987; Ng *et al.*, 1987; Pasternak *et al.*, 1984). *H. cinaedi* has also been isolated from the blood and faeces of children and adult females (Tee *et al.*, 1987; Vandamme *et al.*, 1990). It has been suggested that this species may have a reservoir in hamsters (Gebhart *et al.*, 1989), and

evidence has been provided that its zoonotic transmission to newborn human babies can result in sepsis and meningitis (Orlicek *et al.*, 1993). The isolation of a single strain of *H. canis* from a human indicates that this species can colonize the human gastrointestinal tract. Further studies will be required to establish whether the prevalence of *H. canis* in domestic dogs constitutes a potential reservoir for zoonotic transmission to man. The isolation of *H. canis* from faeces, its bile tolerance and its lack of urease activity, suggest that it is an intestinal, rather than a gastric-colonizing organism. The identification of another non-gastric *Helicobacter* (in addition to *H. cinaedi*, *H. fennelliae* and *H. muridarum*) supports the concept that *Helicobacter* species might be grouped according to the specific tissues and environments with which they are associated throughout the gastrointestinal tract of animals and man. Furthermore, all the enteric helicobacters seem to be characterized by one or two polar flagella, in contrast to gastric helicobacters, which have multiple flagella (Table 2).

Formal description of *H. canis* sp. nov.

(*canis*, Latin noun, dog; after source of first isolation)
Gram-negative, non-spore-forming, helically curved and

slender rod-shaped cells; typically $0.25 \times 4 \mu\text{m}$. Cells have one to two spiral turns, and carry single bipolar sheathed flagella. Exhibits darting motility in hanging-drop preparations of broth cultures. Colonies pinpoint, non-pigmented, translucent and α -haemolytic after 48 h on blood agar. Microaerophilic. No growth under aerobic or anaerobic conditions. No growth at 25°C , but growth at 37°C and 42°C (thermotolerant). Produces oxidase. Does not produce catalase or urease. Glucose not fermented. Hydrogen sulphide not produced in triple sugar iron medium. Neither nitrate nor selenite is reduced. Hippurate is not hydrolysed. Tolerant to 1.5% bile, but not to safranin 'O'. Resistant to polymyxin B and sensitive to nalidixic acid. The G+C content of genomic DNA by thermal denaturation is 48 mol%. Isolated from faeces of diarrhoeic or healthy domestic dogs. One strain has been isolated from human faeces. Pathogenicity uncharacterized.

Formal description of the type strain

NCTC 12739^T is the type strain of *H. canis*. This strain conforms to the description of the species given above. The G+C content of NCTC 12739^T is 48 mol%. The strain was isolated in Switzerland from a domestic dog with diarrhoeal illness. Three additional strains of *H. canis* have been deposited with the National Collection of Type Cultures: NCTC 12740, NCTC 12741 and NCTC 12743.

This paper was initiated with the support of a British Council grant to John Stanley for scientific cooperation between the UK and Switzerland. We are grateful to Richard Birtles and Nick Saunders for their assistance with the set-up of PCR-based sequencing, and to Pauline Borman for assistance with culture and characterization of *Helicobacter* isolates.

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