***Helicobacter pullorum* sp. nov. – genotype and phenotype of a new species isolated from poultry and from human patients with gastroenteritis**

John Stanley,¹ Dennis Linton,¹ André P. Burnens,³ Floyd E. Dewhirst,⁴ Stephen L. W. On,¹ Anthony Porter,² Robert J. Owen¹ and Menelaos Costas¹

Author for correspondence: John Stanley. Tel: +44 81 200 4400 ext. 3738. Fax: +44 81 200 7874.

^{1,2} National Collection of Type Cultures¹ and Virus Reference Division², Central Public Health Laboratory, 61 Colindale Avenue, London NW9 5HT, UK

³ Swiss National Reference Laboratory for Foodborne Diseases, Institute for Veterinary Bacteriology, University of Berne, Langgass-Strasse 122, CH-3012 Berne, Switzerland

⁴ Forsyth Dental Center, Department of Molecular Genetics, 140 The Fenway, Boston, MA 02115, USA

***Campylobacter*-like organisms were isolated from the liver, duodenum and caecum of broiler and layer chickens, and from humans with gastroenteritis. They formed a unique DNA homology group and a polyphasic taxonomic analysis was made of 16 strains. Analysis of the nucleotide sequence of the 16S rRNA gene from seven of the strains identified them as belonging to a single species, within the genus *Helicobacter*. This conclusion was supported by the studies of relative DNA homology and of total protein electrophoretic patterns. The new species could be biochemically differentiated from other helicobacters and its ultrastructure in the electron microscope was typical of the genus except that the flagellum was not sheathed. We propose the name *Helicobacter pullorum* sp. nov. for this group. Like *H. fennelliae* or *H. cinaedi* it represents another non-gastric urease-negative *Helicobacter* species colonizing the lower bowel. Its isolation from the livers of chickens with vibronic hepatitis is significant. We describe a species-specific PCR assay for *H. pullorum* sp. nov. which will facilitate its identification and further studies of its epidemiology.**

Keywords: *Helicobacter pullorum* sp. nov., polyphasic taxonomy

INTRODUCTION

The rRNA homology group VI defined by Vandamme *et al.* (1991) contains the genera *Helicobacter*, *Campylobacter*, *Arcobacter* and *Wolinella* in addition to several other poorly defined groups such as '*Flexispira*'. The species of greatest current interest are *Helicobacter pylori* (Blaser, 1993), which is associated with gastroduodenal disease of man (Blaser, 1990), and *Campylobacter jejuni*, the most common bacterial pathogen causing gastroenteritis in man (Stern, 1993). *Helicobacter* species are largely associated with specific niches in the gastrointestinal tract of mammalian and avian hosts. For example, *H. pylori*, *H. mustelae* and *H. felis* were isolated from gastric mucosa, and cause gastritis in their respective hosts. Other species (for example, *H. cinaedi* and *H. fennelliae*) can be isolated from human lower-bowel tissue, and are enteric

pathogens. The genus *Helicobacter* is currently composed of 11 named species, the most recently defined being *H. canis* (Stanley *et al.*, 1993), *H. hepaticus* (Fox *et al.*, 1994) and *H. pametensis* (Dewhirst *et al.*, 1994).

Campylobacter-like organisms (CLOs) were identified in the late 1970s and early 1980s as important rectal and intestinal pathogens in homosexual men (Mishu *et al.*, 1993). Two of these three CLOs were subsequently placed in the species *Campylobacter cinaedi* (CLO-1A) and *C. fennelliae* (CLO-2) (Totten *et al.*, 1985), and ultimately designated *Helicobacter cinaedi* and *Helicobacter fennelliae* (Vandamme *et al.*, 1991). Strain CLO-3, of which there was a single example, has remained unnamed (Totten *et al.*, 1985). These studies showed that infectious intestinal pathogens are to be found also in *Helicobacter*, a genus originally established around the human gastric-adapted bacterium *Helicobacter pylori*.

This report concerns a group of CLOs which has recently been isolated in Switzerland from three sources: the caeca

Abbreviation: CLO, *Campylobacter*-like organism.

Table 1. Bacterial strains

Bacterium	Strain no.*	Source	GenBank no.†
<i>Helicobacter</i>			
<i>H. pylori</i>	NCTC 11638 (R)	Human	M88157
<i>H. pylori</i>	NCTC 11637 ^T	Human	—
<i>H. felis</i>	NCTC 12436 ^T	Cat	M37642
<i>H. mustelae</i>	NCTC 12435	Ferret	M35048
<i>H. acinonyx</i>	NCTC 12686 ^T	Cheetah	M88148
<i>H. cinaedi</i>	NCTC 12423 ^T	Human	M88150
<i>H. fennelliae</i>	NCTC 11612 ^T	Human	M88154
<i>H. nemestrinae</i>	NCTC 12491 ^T	Monkey	X67854
<i>H. muridarum</i>	NCTC 12714 ^T	Rat	M80205
<i>H. canis</i>	NCTC 12739 ^T	Dog	L13464
<i>H. hepaticus</i> ‡	ATCC 51448 ^T	Mouse	U07574
<i>H. pametensis</i> ‡	CCUG 29255 ^T	Tern	M88147
<i>H. sp.</i> Bird-B‡	CCUG 29256 (R)	Tern	M88139
<i>H. sp.</i> Bird-C‡	CCUG 29261 (R)	Sparrow	M88144
'Chicken CLO' group	NCTC 12824 ^T	Chicken	L36141
(<i>H. pullorum</i> sp. nov.)	NCTC 12825	Chicken	L36142
	NCTC 12826	Human	L36143
	NCTC 12827	Human	L36144
	UB121	Chicken	—
	UB122	Chicken	—
	UB151	Chicken	L36145
	UB155	Chicken	—
	UB1439	Chicken	—
	UB4910	Chicken	—
	UB4914	Chicken	—
	UB5381	Chicken	—
	UB3166	Human	L36146
	UB3659	Human	L36147
	UB4371	Chicken	—
	UB4375	Chicken	—
<i>Campylobacter</i>			
<i>C. fetus</i> subsp. <i>fetus</i>	NCTC 10842 ^T	Sheep	L04314
<i>C. coli</i>	NCTC 11366 ^T	Pig	L04312
<i>C. jejuni</i> subsp. <i>jejuni</i>	NCTC 11351 ^T	Cattle	L04315
<i>C. lari</i>	NCTC 11352 ^T	Gull	L04316
<i>C. upsaliensis</i>	NCTC 11541 ^T	Dog	L14628
<i>C. hyointestinalis</i>	NCTC 11608 ^T	Pig	M65010
<i>C. mucosalis</i>	NCTC 11000 ^T	Pig	L06978
<i>C. sputorum</i> subsp. <i>sputorum</i>	NCTC 11528 ^T	Human	—
<i>C. concisus</i>	NCTC 11485 ^T	Human	L04322
<i>C. curvus</i>	NCTC 11649 ^T	Human	L04313
<i>C. rectus</i>	NCTC 11489 ^T	Human	L04317
<i>C. helveticus</i>	NCTC 12470 ^T	Cat	U03022
<i>Arcobacter</i>			
<i>A. cryaerophilus</i>	NCTC 11885 ^T	Cattle	L14624
<i>A. butzleri</i>	NCTC 12400 ^T	Human	L14626
<i>A. skirrowii</i>	NCTC 12713 ^T	Sheep	L14625
<i>A. nitrofigilis</i>	NCTC 12251 ^T	Plant	L14627
'CLO-3'	NCTC 12462	Human	M88151
	(= CCUG 14564)		
' <i>Flexispira rappini</i> '	NCTC 12461 ^T	Human	M88137
<i>Wolinella succinogenes</i>	NCTC 11488 ^T	Cattle	M88159
[<i>Bacteroides</i>] <i>ureolyticus</i> §	NCTC 10941 ^T	Human	L04321
<i>Salmonella typhimurium</i>	NCTC 12416 ^T , subsp. 1	—	—

* A superscript T indicates a type strain; (R) indicates a reference strain.

† 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 strain numbers in Fig. 3.

‡ Strains included in the 16S rRNA gene sequence analysis only.

§ Generically misnamed. This species is not a true *Bacteroides* but belongs in rRNA superfamily VI.

of asymptomatic poultry (broiler chickens), the livers and intestinal contents of laying hens showing pathological lesions suggestive of vibronic hepatitis, and finally faecal samples taken from various human patients suffering from gastroenteritis (A. P. Burnens, unpublished results). These isolates could be differentiated from other *Campylobacter*s, and demonstrated to be a homology group by a dot-blot genomic DNA hybridization method (Burnens & Nicolet, 1992).

We present a polyphasic taxonomic analysis of 16 of these CLO strains (12 from chicken, 4 from humans). We have established the group to be a previously undescribed species of *Helicobacter* associated with poultry, which is also an infective intestinal pathogen of man. We propose the name *Helicobacter pullorum* sp. nov. for this new species.

METHODS

Bacterial strains and culture conditions. Bacterial strains are listed in Table 1. The 16 new CLO strains which are the subject of this study were provisionally referred to as the 'chicken-CLO' group, although four isolates were from humans. *Helicobacter* spp., *Campylobacter* spp., *Arcobacter*, *Wolinella* and *Bacteroides* spp. were cultured microaerobically or anaerobically as previously described (Stanley *et al.*, 1993).

Phenotypic characterization, protein electrophoresis and electron microscopy. 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 (DNase) was determined by the method of Lior & Patel (1987). Growth was tested on nutrient agar (NA) containing 0.05% (w/v) safranin (BDH), on NA containing 0.032% (w/v) methyl orange (BDH) and on 4.0% (w/v) NaCl medium (cf. On & Holmes, 1991a). The colonial morphology of the isolates was recorded after 3 d microaerobic incubation on 5% (v/v) horse blood agar at 37 °C. Tests were performed in duplicate 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 by Costas (1992).

Nucleic acid techniques. Genomic DNA preparation, DNA-DNA slot-blot hybridization and analysis by laser densitometry were as previously described (Stanley *et al.*, 1992). The parameters governing the DNA homology studies have been discussed previously (Boivin *et al.*, 1985; Lee & McGee, 1989). Determination of mol% G + C content was by the thermal denaturation method (Owen & Pitcher, 1985).

Polymerase chain reaction (PCR) amplification of a 1500 bp fragment of the 16S rRNA gene of NCTC 12824^T and six other strains was done as described previously (Stanley *et al.*, 1993). The amplicon was purified by the method of Kuskawa *et al.* (1990) and nucleotide sequencing was carried out by the method of Embley (1991). Standard wedge-shaped sequencing gels (Pharmacia LKB Macrophor apparatus) were fixed in aqueous methanol/acetic acid/glycerol (10%, 5%, 2%, v/v), dried at 80 °C and autoradiographed on Kodak XAR5 film.

Phylogenetic analysis of 16S rRNA gene sequence and GenBank accession numbers. The 16S rRNA gene sequences for NCTC 12824^T and six other strains were 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, and 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). The 16S rRNA gene sequences for NCTC 12824^T, NCTC 12825, NCTC 12826, NCTC 12827, UB151, UB3166 and UB3659 have been deposited with GenBank; the accession numbers are given in Table 1, which also shows the accession numbers for reference strains included in the phylogenetic analysis.

RESULTS

Biochemical characterization

Thirty-two phenotypic tests were performed on the 16 'chicken CLO' strains, resulting in a phenotypic profile which was distinct from the other species within rRNA superfamily VI. All the strains examined produced cytochrome oxidase, reduced nitrate, and grew at both 37 °C and 42 °C under microaerobic conditions. All were resistant to cephalothin (blood agar, BA, base, 32 mg l⁻¹), cefoperazone (BA base, 64 mg l⁻¹) and methyl orange (0.32%). None of the strains produced alkaline phosphatase, DNase or urease. Neither hippurate nor indoxyl acetate was hydrolysed. Growth was not observed at room temperature (18–22 °C), 25 °C, or 30 °C under microaerobic conditions, nor aerobically at 37 °C. There was no growth on 0.1% trimethylamine N-oxide (TMAO) medium under anaerobic conditions. Strains were unable to grow on buffered charcoal-yeast or MacConkey media. The strains did not demonstrate tolerance to: 1% glycine; 2%, 3.5% or 4.0% NaCl; 0.02% or 0.05% safranin; 0.001% sodium arsenite; or metronidazole (4 mg l⁻¹, in BA or NA bases). Most (14/16) strains produced catalase, tolerated 1% bile and were resistant to carbenicillin (32 mg l⁻¹, in BA base). Six strains tolerated 0.1% potassium permanganate and one was resistant to nalidixic acid (32 mg ml⁻¹). The colonies of all 16 strains on 5% horse blood agar were pinpoint-sized, watery, translucent and α -haemolytic. No pigments were produced. Table 2 lists phenotypic characteristics useful in distinguishing the new group from other species of *Helicobacter*.

Whole-cell protein analysis

One-dimensional SDS-PAGE was carried out in order to analyse whole-cell protein content; about 40 discrete bands were observed. The 16 'chicken CLO' strains were compared with reference or type-strains representing eight *Helicobacter* species, '*Flexispira rappini*', *Wolinella succinogenes*, and 'CLO-3'. The protein patterns of the new group were homogeneous, other than heterogeneity of several prominent bands in the 39–50 kDa range. After removal of these bands for theoretical reasons previously discussed (Costas, 1992), numerical analysis was performed on the patterns using a correlation coefficient and

Table 2. Main differential characteristics of *H. pullorum* sp. nov. and related helicobacters

Abbreviations: +, positive; —, negative; M, monopolar; B, bipolar; Po, polar; Pe, peritrichous; S, sensitive; (S), most strains sensitive (75–92%); R, resistant; I, intermediate; ND, not done; NK, not known; V, variable test (35–50% of strains positive). Source of data: this study and Eaton *et al.* (1993).

Test/characteristic	<i>H. pullorum</i> sp. nov.	<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>H. CLO-3</i>	' <i>Flexispira rappini</i> '
Periplasmic fibres	—	—	—	—	—	—	+	—	+	—	NK	+
Flagella:												
Distribution	M	B	M, B	M, B	Po	Pe	B	Po	B	Po	NK	B
Number	1	2	1–2	1–2	4–8	4–8	10–14	4–8	14–20	2–5	NK	10–20
Sheath	—	+	+	+	+	+	+	+	+	+	NK	+
Oxidase production	16/16†	+	+	+	+	+	+	+	+	+	+	+
Catalase production	14/16	—	+	+	+	+	+	+	+	+	+	+
Urease (rapid) production	0/16	—	—	—	+	+	+	+	+	+	—	+
Nitrate reduction	16/16	—	+	—	—	+	—	—	+	—	—	—
Indoxyl acetate hydrolysis	0/16	+	—	+	—	+	+	—	—	—	—	—
Alkaline phosphatase production	0/16	+	—	+	+	+	+	+	+	+	+	—
Growth at:												
42 °C	16/16	+	—	—	—	+	—	+	+	—	+	+
25 °C	0/16	—	—	—	—	—	—	—	—	—	—	—
Tolerance to:												
1% Bile	13/16	+	V	—	—	—	—	—	—	—	—	—
Safranin 'O'	0/16	—	—	+	—	—	—	—	—	ND	—	—
Methyl orange	16/16	—	+	V	—	—	—	—	—	—	—	—
Sensitivity to:												
Nalidixic acid	15/16	S	S	S	R	S	R	R	R	R	R	R
Cephalothin	0/16	(S)	I	S	S	R	R	S	S	S	R	R
Cefoperazone	0/16	(S)	(S)	(S)	S	R	S	S	S	S	R	S
G + C content (mol %)	34–35	48	36–38	35	35–37	36	34	24	43	30	47	34

† Number of positive strains/number tested.

UPGMA clustering. The resulting rooted dendrogram is shown in Fig. 1. The 'chicken CLO' strains formed a distinct cluster at the 84% similarity level. The nearest neighbour to the group was the type strain of *W. succinogenes*, the banding pattern of which was only 55% similar. The most closely related species of *Helicobacter* was *H. felis*, the banding pattern of which exhibited 52% similarity. Campylobacters are not included in the dendrogram (Fig. 1), since they were well separated and since 16S rRNA sequence data (see below) indicated that the 'chicken CLOs' were not members of this genus.

Ultrastructure

Cells were gently curved rods, 3–4 µm in length and 0.3–0.5 µm in width. Flagella were single and unipolar; flagellar sheaths were not detected. An electron micrograph of strain NCTC 12827 is shown in Fig. 2.

DNA base composition

The DNA base compositions of NCTC 12824^T, NCTC 12826, UB122 and UB3166 were determined as 34.5, 35.1, 33.8 and 34.8 mol% G + C respectively.

DNA–DNA hybridization

The overall genetic relatedness between the chicken CLO strains and the type strains and other reference strains of the species of rRNA superfamily VI (Vandamme *et al.*, 1991) was determined by slot-blot hybridization (Boivin *et al.*, 1985; Lee & McGee, 1989). When DNA of NCTC 12824^T was employed as a probe, the homology was below the limit of detection with DNA of *Salmonella typhimurium* (negative control), *Wolinella succinogenes*, *Bacteroides ureolyticus*, *Arcobacter nitrofigilis*, *Campylobacter belveticus*, *C. upsaliensis*, *C. coli*, *C. jejuni*, *C. lari*, *C. hyointestinalis*, *C. fetus*, *C. mucosalis*, *C. concisus*, *C. sputorum*, *C. curvus*, *C. rectus*, '*Flexispira rappini*', *Helicobacter pylori*, *H. acinonyx*, *H. felis*, *H. fennelliae*, *H. mustelae*, *H. cinaedi*, *H. canis*, *H. nemestrinae* or *H. muridarum*.

Sequence of the 16S rRNA gene and phylogenetic analysis of *H. pullorum* sp. nov.

For NCTC 12824^T and the six other 'chicken CLO' strains for which GenBank accession numbers are provided in Table 1 the nucleotide sequence of the 16S rRNA gene was determined as described in Methods. Com-

Fig. 1. Dendrogram of numerical analysis of the total protein pattern of 16 'chicken CLO' (*H. pullorum* sp. nov.) isolates and the type or reference strains of other helicobacters and closely related species.

Fig. 2. Electron micrograph of single cell of 'chicken CLO' (*H. pullorum*) NCTC 12827 isolated from an HIV-positive human patient. Note the unipolar flagellum lacking a sheath. Bar, 1 µm.

parison of these sequences with other eubacterial 16S rRNA gene sequences in our database indicated that the strains should be excluded from *Campylobacter* and placed within the genus *Helicobacter*. The data for the seven strains were consistent with their belonging to a single species, since only minor sequence variation (1–8 out of 1398 nucleotides) was found in the 16S rRNA genes. On this basis we provisionally designated the 'chicken CLO' group as a new species of *Helicobacter*, *H. pullorum* sp. nov. Results of comparison of the 16S rRNA sequences with those for 27 strains representing *Helicobacter*, *Wolinella*,

Arcobacter and *Campylobacter* species are shown in a matrix (Table 3). Comparisons were made at approximately 1400 base positions, for which data existed for over 90% of the strains (Table 3). The lower half of the matrix is expressed as percentage difference, corrected for multiple base changes by the method of Jukes & Cantor (1969). NCTC 12824^T and NCTC 12826 (the most divergent sequences in the new species) were most closely related to another recently described helicobacter, *H. pametensis* (97.3–97.4% similarity), to *Helicobacter* strain CLO-3 (95.7–96.1% similarity), *H. canis* (96.2–96.8% similarity), '*Flexispira rappini*' (95.9–96.4% similarity) and *H. cinaedi* (95.5–96.1% similarity). The phylogenetic tree obtained by the neighbour-joining method is shown in Fig. 3. *H. pullorum* sp. nov. formed a distinct phylogenetic lineage within *Helicobacter*.

A species-specific PCR assay for *H. pullorum* sp. nov.

The 16S rRNA gene sequences of the seven strains were analysed in order to determine regions which would serve as species-specific priming sites for PCR amplification. A candidate forward primer was identified from nt 818–839, viz. 5' ATG AAT GCT AGT TGT TGT CAG 3'. A candidate reverse primer was identified from nt 1265–1247, viz. 5' GAT TGG CTC CAC TTC ACA 3'. The predicted PCR amplicon was 447 bp. Experiments were performed which tested the ability of the primers to amplify such a fragment from the 16 strains of *H. pullorum* sp. nov., and from the type strains of the known species of

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

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

	<i>H.pu</i>	<i>H.py</i>	<i>H.py</i>	<i>H.ne</i>	<i>H.ac</i>	<i>H.fl</i>	<i>H.s3</i>	<i>H.fn</i>	<i>H.mr</i>	<i>H.he</i>	<i>H.ca</i>	<i>H.ci</i>	<i>F.ra</i>	<i>H.pa</i>	<i>H.sc</i>	<i>H.sB</i>	<i>H.ms</i>	<i>W.su</i>	<i>A.cr</i>	<i>A.sk</i>	<i>A.bu</i>	<i>C.re</i>	<i>C.co</i>	<i>C.fe</i>	<i>C.ap</i>	<i>C.he</i>	<i>C.la</i>	<i>C.je</i>	<i>C.co</i>
<i>H. pullorum</i> 12824*	–	99.4	94.7	94.1	94.1	94.3	96.1	95.7	95.0	95.8	96.2	95.5	95.9	97.3	96.1	95.9	95.4	92.8	86.0	85.7	86.8	85.6	87.2	86.0	87.1	86.8	87.2	86.8	86.6
<i>H. pullorum</i> 12826	0.6	–	94.8	94.4	94.2	94.0	95.7	95.4	95.6	96.2	96.8	96.1	96.4	97.4	96.3	96.2	95.5	93.0	85.8	85.5	86.5	85.8	87.4	86.2	87.4	87.1	87.3	86.9	86.8
<i>H. pylori</i>	5.5	5.4	–	98.2	97.4	95.4	93.8	92.9	93.1	93.3	93.8	92.7	93.1	94.4	94.1	93.7	93.8	90.8	84.6	84.2	85.4	84.0	85.0	85.0	85.9	85.4	85.4	85.3	85.1
<i>H. nemestrinae</i>	6.2	5.9	1.8	–	96.6	95.5	93.3	92.5	92.9	93.3	93.7	93.1	93.3	94.4	94.3	93.9	93.9	90.9	84.9	84.4	85.6	84.7	85.9	85.6	86.4	85.8	85.8	85.5	85.3
<i>H. acinonyx</i>	6.1	6.0	2.7	3.5	–	96.4	93.1	92.2	92.4	93.1	93.3	92.4	92.5	94.0	93.4	93.5	93.5	90.4	84.2	84.0	85.2	83.5	84.5	84.4	85.7	85.0	85.0	84.9	84.7
<i>H. felis</i>	6.0	6.2	4.7	4.6	3.6	–	93.3	93.0	92.6	93.2	93.2	92.6	92.6	93.9	93.2	93.4	93.5	90.2	83.7	83.5	84.4	83.1	84.6	84.5	86.0	85.0	85.1	84.7	84.3
<i>H. sp. CLO-3</i>	4.0	4.4	6.4	7.0	7.3	7.1	–	94.6	94.3	95.1	96.0	95.3	95.5	95.1	95.4	95.1	95.3	92.3	85.6	85.2	86.3	85.1	86.5	85.8	85.9	85.7	86.4	85.8	85.8
<i>H. fennelliae</i>	4.4	4.8	7.5	7.9	8.3	7.3	5.6	–	95.0	95.3	95.3	95.8	95.3	95.4	94.6	94.5	94.3	92.3	84.4	84.2	85.5	85.3	86.8	86.0	86.2	85.8	86.6	86.2	86.5
<i>H. muridarum</i>	5.1	4.5	7.3	7.4	8.0	7.8	5.9	5.2	–	97.8	96.5	95.9	96.2	95.7	96.3	96.1	95.7	93.3	85.0	84.7	85.9	84.8	86.2	85.4	86.3	86.2	86.0	85.7	85.7
<i>H. hepaticus</i>	4.3	3.9	7.0	7.0	7.2	7.1	5.0	4.8	2.3	–	97.3	96.9	97.4	96.3	96.4	96.4	96.3	92.6	84.8	84.5	85.7	85.3	86.7	85.8	86.0	86.4	86.3	85.9	86.1
<i>H. canis</i>	3.9	3.3	6.5	6.6	7.1	7.1	4.1	4.8	3.6	2.8	–	97.8	98.1	96.5	96.9	96.9	96.4	93.3	85.5	85.1	86.0	85.7	87.1	86.4	86.9	87.1	87.0	86.7	87.0
<i>H. cinaedi</i>	4.6	4.0	7.6	7.2	8.0	7.8	4.9	4.4	4.2	3.1	2.2	–	98.8	95.5	95.6	95.4	95.1	92.8	85.0	84.7	85.5	85.7	87.0	86.2	86.7	86.2	86.4	86.0	86.3
' <i>F. rappini</i> '	4.2	3.7	7.2	7.0	7.9	7.8	4.6	4.9	3.9	2.6	2.0	1.2	–	96.5	96.4	95.9	95.8	93.0	85.1	84.8	85.6	85.9	87.2	86.6	86.9	87.0	87.0	86.7	87.0
<i>H. pametensis</i>	2.8	2.6	5.8	5.8	6.3	6.4	5.1	4.7	4.4	3.8	3.6	4.6	3.6	–	98.1	97.9	97.1	94.1	86.1	85.7	86.5	85.9	87.4	86.4	87.3	87.1	87.5	87.2	87.0
<i>H. sp. Bird-C</i>	4.1	3.8	6.2	5.9	6.9	7.1	4.7	5.7	3.7	3.6	3.1	4.5	3.6	2.0	–	98.3	97.9	94.5	85.6	85.2	86.3	85.8	86.6	86.4	87.4	87.2	87.5	87.1	86.7
<i>H. sp. Bird-B</i>	4.2	3.9	6.6	6.3	6.8	6.9	5.0	5.7	4.0	3.6	3.2	4.7	4.2	2.1	1.7	–	98.6	94.1	86.2	86.0	86.9	85.1	86.4	86.1	87.0	86.8	87.1	86.8	86.5
<i>H. mustelae</i>	4.8	4.6	6.4	6.3	6.8	6.8	4.9	6.0	4.4	3.8	3.7	5.0	4.3	3.0	2.1	1.4	–	93.7	86.1	85.7	86.5	84.8	85.8	85.6	86.5	86.3	86.9	86.5	86.2
<i>W. succinogenes</i>	7.6	7.4	9.8	9.7	10.3	10.5	8.2	8.1	7.0	7.8	7.0	7.5	7.3	6.2	5.7	6.2	6.6	–	85.8	85.5	86.2	86.2	86.3	85.8	86.0	85.9	86.6	86.3	86.3
<i>A. eryaerophilus</i>	15.5	15.8	17.3	16.8	17.7	18.4	16.0	17.5	16.7	17.0	16.1	16.7	16.6	15.4	16.0	15.3	15.4	15.8	–	98.9	97.5	86.0	87.5	86.2	86.0	86.4	87.1	87.4	86.8
<i>A. skirrowii</i>	15.9	16.2	17.7	17.5	18.0	18.7	16.5	17.7	17.1	17.4	16.6	17.2	17.0	15.9	16.5	15.5	15.9	16.1	1.1	–	97.2	85.6	87.6	86.1	85.9	86.1	86.8	87.2	86.7
<i>A. butzleri</i>	14.6	14.8	16.2	16.0	16.5	17.5	15.1	16.1	15.7	15.9	15.6	16.2	16.0	14.9	15.1	14.4	14.9	15.2	2.6	2.8	–	86.5	87.9	86.3	86.4	86.3	86.6	86.9	86.4
<i>C. rectus</i>	16.0	15.7	18.0	17.1	18.6	19.2	16.6	16.4	17.0	16.4	15.9	15.8	15.6	15.6	16.1	16.6	17.0	15.3	15.5	16.0	14.9	–	95.9	93.8	91.7	91.6	92.4	92.7	93.4
<i>C. concisus</i>	14.1	13.8	16.8	15.6	17.3	17.3	14.9	14.5	15.3	14.6	14.1	14.3	14.0	13.8	14.7	15.0	15.7	15.2	13.6	13.5	13.1	4.2	–	95.4	92.2	92.0	93.8	93.5	94.2
<i>C. fetus</i>	15.5	15.3	16.7	16.0	17.5	17.3	15.8	15.4	16.2	15.7	15.0	15.2	14.8	15.1	15.0	15.4	16.0	15.7	15.3	15.4	15.2	6.5	4.8	–	93.1	93.1	94.0	94.3	95.3
<i>C. upsaliensis</i>	14.2	13.8	15.6	14.9	15.9	15.5	15.6	15.3	15.2	15.5	14.5	14.7	14.4	13.9	13.8	14.3	14.9	15.5	15.4	15.7	15.0	8.8	8.2	7.3	–	98.2	95.5	96.1	95.1
<i>C. helveticus</i>	14.5	14.2	16.3	15.7	16.8	16.8	15.9	15.7	15.2	15.0	14.1	15.2	14.2	14.1	14.0	14.6	15.1	15.6	15.0	15.4	15.2	8.9	8.4	7.3	1.8	–	96.6	97.2	96.2
<i>C. lari</i>	14.1	13.9	16.2	15.7	16.8	16.6	15.0	14.8	15.5	15.1	14.2	15.1	14.2	13.6	13.7	14.1	14.4	14.7	14.2	14.5	14.8	8.0	6.5	6.3	4.6	3.5	–	98.6	97.6
<i>C. jejuni</i>	14.5	14.4	16.3	16.1	16.8	17.1	15.8	15.2	15.8	15.6	14.7	15.5	14.7	14.1	14.1	14.5	14.9	15.1	13.8	14.0	14.4	7.7	6.8	6.0	4.0	2.9	1.4	–	98.6
<i>C. coli</i>	14.7	14.6	16.6	16.4	17.1	17.7	15.8	14.9	15.8	15.3	14.3	15.1	14.3	14.3	14.7	14.9	15.2	15.2	14.5	14.7	15.0	6.9	6.1	4.8	5.1	3.9	2.5	1.4	–

Campylobacter and *Helicobacter*, '*Flexispira rappini*' and *Wolinella succinogenes*. Using an annealing temperature of 60 °C, no product was observed from any strain except those belonging to *H. pullorum* sp. nov. From all the latter strains a PCR amplicon of ~ 450 bp was detected (Fig. 4).

The microaerophilic nature, cytochrome oxidase activity and general morphology of the 'chicken CLO' strains are typical of a species belonging to rRNA superfamily VI (Vandamme *et al.*, 1991). From the molecular systematic analysis of the group it was evident that it was a previously undescribed *Helicobacter* species, for which we propose the name *H. pullorum* sp. nov., to indicate its isolation from chickens. This new species is clearly distinguishable biochemically from other species of *Helicobacter* (see Table 2). It can readily be classified into the urease-negative enteric group of *Helicobacter* species (*H. cinaedi*, *H. fennelliae* and *H. canis*). The urease enzyme is considered necessary for survival in the acidic environment of the mammalian stomach, and is characteristic of the gastric helicobacters such as *H. pylori*. Its absence in non-gastric *Helicobacter* species might be explicable on the basis that the deamination of urea in non-acidic environments would yield toxic end-products. Among the enteric helicobacters *H. pullorum* can be distinguished from *H. fennelliae* and *H. canis* by its ability to reduce nitrate and inability to hydrolyse

indoxyl acetate. It differs from the *Helicobacter* strain CLO-3 by nitrate reduction, and its inability to produce alkaline phosphatase. The phenotype of *H. pullorum* sp. nov. closely resembles that of *H. cinaedi*, but can be distinguished by growth at 42 °C. It is differentiated biochemically from the recently described avian species *H. pametensis* (to which it is most closely related in terms of 16S rRNA gene sequence) by inability to produce alkaline phosphatase, and resistance to cephalothin. The absence of alkaline phosphatase production also distinguishes *H. pullorum* from the other, as yet unnamed strains designated *Helicobacter* sp. Bird-B and Bird-C in our phylogenetic database (Seymour *et al.*, 1994).

The cellular ultrastructure (Fig. 2) was consistent with assignment to *Helicobacter*, but the flagellum was found not to be sheathed, the first such case for a *Helicobacter* species (Vandamme *et al.*, 1991). The monopolar flagellum, cellular ultrastructure and lack of complex surface features resemble those found in *H. cinaedi* and *H. fennelliae* (Han *et al.*, 1989). Indeed, one or two polar flagella seem to be a common feature of the enteric, as opposed to gastric, species of *Helicobacter*. The 16 strains formed a tight cluster in the rooted dendrogram derived from SDS-PAGE protein analysis (Fig. 1). This cluster was clearly separated from other *Helicobacter* species, the nearest of which was *H. felis*. The biochemically similar species *H. cinaedi* had a quite distinct total protein pattern

Fig. 3. Phylogenetic tree for two *H. pullorum* strains and 27 reference *Helicobacter*, *Campylobacter*, *Wolinella* and *Arcobacter* species, based on 16S rRNA gene 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.

(only 45% similarity) whilst *H. fennelliae* and *H. canis*, other enteric helicobacters, were even less similar (both ~ 38% similarity).

Between the seven strains of *H. pullorum* sp. nov. whose 16S rRNA genes were sequenced, there was much less than 1% sequence divergence, consistent with membership of a single species. On the basis of these seven sequences, primers for a species-specific PCR assay could be designed to avoid regions of intraspecific variation. The resulting assay will facilitate definitive identification of the new species.

From the phylogenetic analysis the nearest neighbour of *H. pullorum* sp. nov. was *H. pametensis*, a urease-negative helicobacter isolated from avian and porcine faeces (Dewhirst *et al.*, 1994). *H. canis* and CLO-3 were related at about the 96% level. The most related urease-positive gastric species was '*Flexispira rappini*'. When the overall genetic relatedness of *H. pullorum* to the other *Helicobacter* species was assessed by DNA-DNA hybridization, no significant hybridization was observed. This concurs with the electrophoretic analysis of proteins, and the 16S rRNA gene phylogeny, in defining a new species of

Helicobacter. The mol% G+C was also typical of the genus *Helicobacter*.

It should be noted that *H. pullorum*, like *H. hepaticus* (Fox *et al.*, 1994) and '*Flexispira rappini*', resides in the intestinal tract, but has the ability to invade the liver and cause hepatitis in some hosts. This aspect of the organism deserves further study. One-quarter of the strains of *H. pullorum* in this study were isolated from faecal specimens of human patients with diarrhoeal disease; one of these was an immunocompromised individual with human immunodeficiency virus (HIV) infection. These strains clustered with the chicken strains according to all criteria. *H. pullorum* is therefore likely to be an infective enteric pathogen. Its potential as a causative agent of serious gastrointestinal disease in humans is evidenced by the case of strain NCTC 12826, which was isolated from a young woman with chronic diarrhoea of one month's duration. Since the specific route of infection was not established for these human cases, the potential of *H. pullorum* in zoonotic infection should also be made a subject of further study. The PCR identification assay described in this report can be carried out from DNA or from cells and

Fig. 4. Species-specific PCR identification of *H. pullorum* sp. nov. Genomic DNA (100 ng) of each *H. pullorum* strain or type strain (as listed in Table 1) was used as a substrate for PCR as described in Results. Tracks 1 and 14 contained the 123 bp ladder (Gibco BRL); track 2, *H. pullorum* NCTC 12824; track 3, *H. pullorum* NCTC 12825; track 4, *H. pylori*; track 5, *H. felis*; track 6, *H. mustelae*; track 7, *H. acinonyx*; track 8, *H. cinaedi*; track 9, *H. fennelliae*; track 10, *H. nemestrinae*; track 11, *H. muridarum*; track 12, *H. canis*; track 13, '*F. rappini*'; track 15, *H. pullorum* NCTC 12826; track 16, *H. pullorum* NCTC 12827; track 17, *C. hyointestinalis*; track 18, *C. fetus*; track 19, *C. helveticus*; track 20, *C. upsaliensis*; track 21, *C. lari*; track 22, *C. coli*; track 23, *C. jejuni*; track 24, *C. sputorum*; track 25, *C. mucosalis*; track 26, *C. concisus*.

will be a useful tool for screening clinical and veterinary samples for the presence of *H. pullorum*.

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

(*pullorum*, from Latin noun *pullus*, chicken; after source of first isolation)

Gram-negative, non-spore-forming, gently curved, slender, rod-shaped cells, 3–4 µm in length. Cells carry an unsheathed monopolar flagellum. Typical darting motility. Microaerophilic. Grows microaerobically at 37 °C and 42 °C. No growth under aerobic conditions or anaerobically [on TMAO medium]. Colonies pinpoint, non-pigmented, translucent and α-haemolytic on 5% horse blood agar. Produces oxidase and reduces nitrate. Most strains (14/16) produce catalase. No production of urease or alkaline phosphatase. No hydrolysis of indoxyl acetate. Resistant to cephalothin and cefoperazone. The G + C content of the genomic DNA by thermal denaturation is 34–35 mol%. A 447 bp PCR amplicon is specifically generated using the primers 5' ATG AAT GCT AGT TGT TGT CAG 3' and 5' GAT TGG CTC CAC

TTC ACA 3' and an annealing temperature of 60 °C. Isolated from broiler and layer chickens, with or without vibronic hepatitis. Also isolated from humans with gastrointestinal disease.

Formal description of the type strain

NCTC 12824^T is the type strain of *H. pullorum*. This strain conforms to the description of the species given above. Its G + C content is 34.5 mol%. It was isolated from a healthy broiler chicken in Switzerland. Three additional strains have been deposited with the National Collection of Type Cultures: NCTC 12825, NCTC 12826 and NCTC 12827.

ACKNOWLEDGEMENTS

This work was initiated with the support of a British Council Grant to J. Stanley for British–Swiss scientific cooperation and supported in part by United States Public Health Service Grants (to Floyd Dewhirst) DE 08303 and DE 10374.

REFERENCES

- Blaser, M. J. (1990). *Helicobacter pylori* and the pathogenesis of gastroduodenal inflammation. *J Infect Dis* **161**, 626–633.
- Blaser, J. (1993). *Helicobacter pylori*: microbiology of a 'slow' bacterial infection. *Trends Microbiol* **1**, 255–260.
- Boivin, M. F., Morris, V. L., Lee-Chan, E. C. M. & Murray, R. G. E. (1985). Deoxyribonucleic acid relatedness between selected members of the genus *Aquaspirillum* by slot blot hybridization: *Aquaspirillum serpens* (Mueller 1786) Hylemon, Wells, Krieg and Jannasch 1973 emended to include *Aquaspirillum bengal* as a subjective synonym. *Int J Syst Bacteriol* **35**, 512–517.
- Burnens, A. P. & Nicolet, J. (1992). Detection of *Campylobacter upsaliensis* in diarrheic dogs and cats, using a selective medium with cefoperazone. *Am J Vet Res* **53**, 48–51.
- Costas, M. (1992). Classification, identification and typing of bacteria by the analysis of their one-dimensional polyacrylamide gel electrophoresis protein patterns. In *Advances in Electrophoresis*, pp. 351–408. Edited by A. Chambrach, M. J. Dunn & B. J. Radola. Weinheim: VCH Verlagsgesellschaft.
- Dewhirst, F. E., Seymour, C., Fraser, G. J., Paster, B. J. & Fox, J. G. (1994). Phylogeny of *Helicobacter* isolates from bird and swine feces and description of *Helicobacter pametensis* sp. nov. *Int J Syst Bacteriol* **44**, 553–560.
- Eaton, K. A., Dewhirst, F. E., Radin, M. J., Fox, J. G., Paster, B. J., Krakowka, S. & Morgan, D. R. (1993). *Helicobacter acinonyx* sp. nov., isolated from cheetahs with gastritis. *Int J Syst Bacteriol* **43**, 99–106.
- Embley, T. M. (1991). The linear PCR reaction: a simple and robust method for sequencing amplified rRNA genes. *Lett Appl Microbiol* **13**, 171–174.
- Fox, J. G., Dewhirst, F. E., Tully, J. G., Paster, B. J., Yan, L., Taylor, N. S., Collins, M. J., Jr, Gorelick, P. L. & Ward, J. M. (1994). *Helicobacter hepaticus* sp. nov., a microaerophilic bacterium isolated from the livers and intestinal mucosal scrapings from mice. *J Clin Microbiol* **32**, 1238–1245.
- Han, Y.-H., Smibert, R. M. & Krieg, N. R. (1989). Occurrence of sheathed flagella in *Campylobacter cinaedi* and *Campylobacter fennelliae*. *Int J Syst Bacteriol* **39**, 488–490.
- Holmes, B., On, S. L. W., Costas, M. & Ganner, M. (1992). Some new applications of probabilistic identification. In *Proceedings of the*

- Conference on Taxonomy and Automated Identification of Bacteria, pp. 6–8. Edited by J. Schindler. Prague: Czechoslovak Society for Microbiology.
- Jukes, T. H. & Cantor, C. R. (1969).** Evolution of protein molecules. In *Mammalian Protein Metabolism*, pp. 21–132. Edited by H. N. Munro. New York: Academic Press.
- Kusukawa, N., Uemori, T., Asada, K. & Kato, I. (1990).** Rapid and reliable protocol for direct sequencing of material amplified by the polymerase chain reaction. *Biotechniques* **9**, 66–72.
- Lee, A. S. G. & McGee, J. O. (1989).** The signal intensity on Southern blots developed by nonisotopic methods is linear with time and quantity of DNA. *Nucleic Acids Res* **17**, 2364.
- Lior, H. & Patel, A. (1987).** Improved toluidine blue-DNA agar for detection of DNA hydrolysis by campylobacters. *J Clin Microbiol* **25**, 2030–2031.
- Mishu, B., Patton, C. M. & Tauxe, R. V. (1993).** Clinical and epidemiological features of non-*jejuni*, non-*coli* *Campylobacter* species. In *Campylobacter jejuni: Current Status and Future Trends*, pp. 31–41. Edited by I. Nachamkin, M. J. Blaser & L. S. Tompkins. Washington, DC: American Society for Microbiology.
- On, S. L. W. & Holmes, B. (1991a).** Effect of inoculum size on the phenotypic characterization of *Campylobacter* spp. *J Clin Microbiol* **29**, 923–926.
- On, S. L. W. & Holmes, B. (1991b).** Reproducibility of tolerance tests that are useful in the identification of campylobacteria. *J Clin Microbiol* **29**, 1785–1788.
- On, S. L. W. & Holmes, B. (1992).** Assessment of enzyme detection tests useful in identification of campylobacteria. *J Clin Microbiol* **30**, 746–749.
- Owen, R. J. & Pitcher, D. G. (1985).** Chemical methods for estimating DNA base compositions and levels of DNA–DNA hybridization. In *Chemical Methods in Bacterial Systematics*, pp. 67–93. Edited by M. Goodfellow & D. E. Minnikin. London: Academic Press.
- Paster, B. J. & Dewhirst, F. E. (1988).** Phylogeny of Campylobacters, Wolinellas, *Bacteroides gracilis*, and *Bacteroides ureolyticus* by 16S ribosomal ribonucleic acid sequencing. *Int J Syst Bacteriol* **38**, 56–62.
- Saitou, N. & Nei, M. (1987).** The neighbour-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406–425.
- Seymour, C., Lewis, R. J., Kim, M., Gagnon, D. F., Fox, J. G., Dewhirst, F. E. & Paster, B. J. (1994).** Isolation of *Helicobacter* strains from wild bird and swine feces. *Appl Environ Microbiol* **60**, 1025–1028.
- Stanley, J., Burnens, A. P., Linton, D., On, S. L. W., Costas, M. & Owen, R. J. (1992).** *Campylobacter helveticus* sp. nov., a new thermophilic species from domestic animals: characterization and cloning of a species-specific DNA probe. *J Gen Microbiol* **138**, 2293–2303.
- Stanley, J., Linton, D., Burnens, A. P., Dewhirst, F. E., Owen, R. J., Porter, A., On, S. L. W. & Costas, M. (1993).** *Helicobacter canis* sp. nov., a new species from dogs: an integrated study of phenotype and genotype. *J Gen Microbiol* **139**, 2495–2504.
- Stern, N. J. (1993).** Reservoirs for *Campylobacter jejuni* and approaches for intervention in poultry. In *Campylobacter jejuni: Current Status and Future Trends*, pp. 49–60. Edited by I. Nachamkin, M. J. Blaser & L. S. Tompkins. Washington, DC: American Society for Microbiology.
- Totten, P. A., Fennell, C. L., Tenover, F. C., Wezenberg, J. M., Perine, P. L., Stamm, W. E. & Holmes, K. K. (1985).** *Campylobacter cinaedi* (sp. nov.) and *Campylobacter fennelliae* (sp. nov.): two new *Campylobacter* species associated with enteric disease in homosexual men. *J Infect Dis* **151**, 131–139.
- Vandamme, P., Falsen, E., Rossau, R., Hoste, B., Segers, P., Tytgat, R. & De Ley, J. (1991).** Revision of *Campylobacter*, *Helicobacter*, and *Wolinella* taxonomy: emendation of generic descriptions and proposal of *Arcobacter* gen. nov. *Int J Syst Bacteriol* **41**, 88–103.

Received 9 May 1994; revised 1 August 1994; accepted 3 August 1994.